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Cancer Stem Cells

The Cutting Edge

Edited by Stanley Shostak

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Notice

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Meet the editor

For fifty years, I have studied the evolution of growth's integration with form. Hydras' ability to move excess cells into buds was my model for cancer's ability to support metastasis (e.g., Vegetative reproduction by budding in Hydra: A perspective on tumors. Perspectives in Biology and Medicine, 20:545–68; 1977; "*Hydra and cancer: Immortality and budding*", pp. 275-86 in C.J. Dawe, J.C. Harshbarger, S. Kondo, T. Sugimura, and S. Takayama, eds., Phyletic Approaches to Cancer. Tokyo: Sci. Soc. 1981). I have concentrated on the origins of stem cells (Symbiogenetic origins of cnidarian cnidocysts. Symbiosis, 19:1–29; 1995 [with V. Kolluri]; "*Speculation on the Evolution of Stem Cells*", Breast Disease, 29:3–13; 2007–8) and have developed my ideas further in books (*Evolution of Death: Why We Are Living Longer*. Albany: SUNY Press; 2006; Becoming Immortal: Combining Cloning and Stem-Cell Therapy. Albany: SUNY Press; 2002; Evolution of Sameness and Difference: Perspectives on the Human Genome Project. Amsterdam: Harwood Academic Publishers, 1999; Death of Life: The Legacy of Molecular Biology. London: Macmillan, 1998). I am also the editor of Cancer Stem Cells Theory and Practice (Rijeka, Croatia: InTech; 2011).

Contents

Preface

To see a world in a grain of sand,
And a heaven in a wild flower,
Hold infinity in the palm of your hand,
And eternity in an hour.
William Blake — Auguries of Innocence

The in vitro cultivation of mouse embryonic stem cells in 1981 conjured visions of repairing tissues, restoring organs, regenerating limbs, and renewing organisms. Plated embryonic stem cells also rekindled interest in cancer stem cells. They inspired a world of novel ideas for preventing, detecting, and diagnosing cancers, and stirred up infinite notions for advancing therapeutic practices and improving prognostic outlooks. Similarities among normal and cancer stem cells then shaped the groundbreaking cancer stem cell theory, revolutionized concepts of malignancy, recurrence, and resistance, and launched massive research offensives against stem cells in the war on cancer.

How much broader are visions of cancer stem cells today, and how well has the offensive fared against stem cell-based malignancies? Answers to these questions are found in *Cancer Stem Cells - The Cutting Edge* (the present volume) and in *Cancer Stem Cells: Theories and Practice* (the previous volume [Rijeka, Croatia: InTech; 2011]). Both volumes consist of chapters approved by this editor reviewing contributions to research inspired by the concept of cancer stem cells and offering interim assessments of progress in the war against cancers' stem cells.

Cancer Stem Cells - The Cutting Edge contains twenty-seven chapters by world-class researchers and clinicians working at university laboratories and hospitals around the world and one chapter by a retired biologist with a long-term interest in cancer stem cells (this editor). These chapters are grouped at the editor's discretion in seven vaguely independent but largely overlapping parts intended to proceed from the general to the specific (from cancer stem cells and theory to competing models and stem cells in a specific cancer, namely glioma), from the laboratory to the clinic (signaling pathways and markers to new perspectives on targeted therapies), and from the practical problems confronting researchers (techniques and technical details) to the esoteric evolution of cancer (a biologist's speculations).

Finally, as a kindness to readers unfamiliar with the argot of cancer researchers, the editor has assembled and appended a glossary of acronyms and abbreviations used in the chapters. This glossary is not authorized by the authors and is intended solely as an aid to readers.

The chapters assembled in Part 1, Cancer Stem Cells and Cancer Stem Cell Theory, loosely share the concept of cancer stem cells epitomized in the cancer stem cell theory by unique, undifferentiated, small populations of slowly dividing, self-renewing cells spreading and supporting cancers. The chapters cover the parameters of stem cells in theory, their variables under laboratory conditions, and the extension cancer stem cell research to clinical practice.

Chapter 1, "Stem Cells and Cancer Stem Cells" by Lucinei Roberto Oliveira and Alfredo Ribeiro-Silva defines stem cells generally as "clonal precursors of more identical stem cells" and cancer stem cells (CSCs) specifically as stem cells at the beginning of "tumour progression, metastasis and recurrence after therapy." The authors focus on methods of isolation and purification and cells bearing specific markers, for example, those expressed in human tumors (listed in an especially accessible table), and signaling pathways controlling "stem cell fate decisions." Contradictions are allowed to surface (e.g., "However … putative CSCs were [also] found with a CD24-/CD44+ phenotype"). But the take-home message is unambiguous: "[P]atients with tumours expressing high levels of the molecules associated with CSCs tend to have a poorer clinical outcome than patients with tumours that express low levels of these markers."

Chapter 2, "Clinical significance of putative cancer stem cells in residual cancer cells after chemoradiotherapy for rectal cancer" by Koji Tanaka, Yasuhiro Inoue, Yuji Toiyama, Keiichi Uchida, Chikao Miki, and Masato Kusunoki takes up the critical problem of whether treatment failure is in part "due to the resistance of CSCs to chemotherapy or radiotherapy". Specifically, is the "amount of residual cancer cells after CRT [chemo- radio-therapy] … predictive of disease recurrence and survival"? The authors seek answers in the results of a brilliant and exacting real time polymerase chain reaction examination of cDNA from microscopically isolated colorectal tumor cells following CRT. Ultimately, "it seems plausible that CRT may cause an imbalance between two distinct populations [putative CSCs and non-CSCs] within the tumor." Indeed, "residual cancer following CRT may contain more CSCs than primary tumors before CRT." Similar results are obtained in vitro and with immuno-staining of sections. Moreover, "[p]atients who developed distant metastatic recurrence (e.g. liver, lung) had … significantly higher [concentrations of stem cells] post-CRT … compared with those patients without recurrence." The authors conclude, "[t]his may indicate that the proportion of … CSCs in primary, non-treatment tumor might be predictive for less treatment efficacy, more chance of disease recurrence, and poor prognosis of CRC patients."

Chapter 3, "Cancer Stem Cells in Solid Organ Malignancies: Mechanisms of Treatment Resistance and Strategies for Therapeutic Targeting" by Marcus M. Monroe, Crystal J.

Hessman, Daniel R. Clayburgh, Emily J. Bubbers, and Melissa H. Wong portrays cancer stem cell theory in broad terms: "tumors are composed of a small population of cells possessing the characteristics of self-renewal and pluripotency, and thus [tumors have] the ability to initiate or support tumor growth, as well as their differentiated progeny which lose these abilities with increasing differentiation". But cancers contain more than stem cells: "tumors are [also] composed of a heterogeneous population of cells with various levels of cellular differentiation and morphologic features." In their beautifully illustrated chapter, including a strikingly well-structured table listing targets, drugs, trial numbers, phase, and cancer types, the authors champion heterogeneity as the cause underlying the "frequent failure of standard cytotoxic therapies to provide a lasting cancer-free survival."

The authors candidly acknowledge their "incomplete understanding of the functional aspects of these CSC markers/molecules. In many cases these antigens are used because they have been shown to conveniently mark a population of cells that happen to have stem-like properties rather than because their expression is intrinsically tied to CSC functionality." But the authors also highlight ongoing "[p]reclinical studies suggest[ing] that the combination of CSC-specific and broad cytotoxic therapy holds the best chance for disease eradication."

In Chapter 4, "Cancer Initiating Cells in Head and Neck Squamous Cell Carcinoma," Jesús Paramio describes efforts employing a "genetically engineered mouse model" to sort out the "cellular and functional heterogeneity" among cancer initiating cells (CICs aka CSCs) in head and neck squamous cell carcinoma (HNSCC). The challenge of boiling this complex carcinoma down to basics is complicated by "variation … evident within individual tumors (high intratumoral heterogeneity), as cells display different functional properties and express miscellaneous markers." Paramio begins by reviewing ways of identifying putative CICs with different markers and with stromal or niche properties before coming to the chapter's major agenda, namely, using "transgenic or conditionally targeted gene technologies to explore the effects of oncogenes and tumour suppressors in different cellular contexts … through [the] three sequential steps … [of] initiation, progression and conversion." Ultimately, with due qualifications, Paramio ends on a cautiously hopeful note: "[T]he available mouse models, and cell lines derived from them, will turn into essential tools in uncovering the cellular origins of cancer and the impact of specific mutations on tumorigenesis."

In Chapter 5, "Stem Cell Growth as a Model of Carcinogenesis," Steve Poser, Joseph Alisky, Kuei-Fang Chung, Monika Ehrhart-Bornstein, Stefan Bornstein, and Andreas Androutsellis-Theotokis "explore … using normal somatic stem cells as models to study the properties of cancer stem cells." The authors concentrate on "the kind of hierarchical organization that is considered to be a hallmark of the cancer stem cell hypothesis … [as] opposed to a clonal or stochastic model where there is a certain probability that any cell will develop mutations that allow for it to show unregulated proliferation and the ability to generate new tumors." Instead of seeing these models as antithetical, the authors ask, "how much each [model] contribute[s] to the

distribution of cells in any given cancer type". In the case of an acute myelogenous leukemia driven by epogen (a synthetic form of erythropoietin) "the cause and effect relationship between trophic factor and stimulation of malignant cells is … iron clad." Hence, the "cancer stem cell hypothesis will likely guide the thinking that brings about important future breakthroughs in cancer treatments."

Chapter 6, "Cancer Stem Cells in Multiple Myeloma" by Sabino Ciavarella, Annalisa Milano, Annalisa Savonarola, Oronzo Brunetti, Franco Dammacco, and Franco Silvestris rounds up the usual CSC suspects, "suggesting that CBCs [clonotypic B cells] could represent an ongoing differentiating population … in MM [multiple myeloma] and … play a pivotal role in MM pathogenesis. These cells may originate in [the] peripheral lymphoid system and, once having undergone key oncogenic mutations, migrate toward the BM [bone marrow] to complete their differentiation into malignant plasma cells". Moreover, "human malignant plasma cells possess low colony-forming capacity and only rare cells within tumor clone[s] give rise to colonies in vitro". Other results also "supported the derivation of the malignant plasma cell from a post-germinal cellular element."

But data compiled by the authors "emphasize the ability of phenotypically mature tumor cells by themselves to recapitulate MM in vivo" and come close to overturning cancer stem cell theory in the case of MM. Conspicuously, "in the initial phases of most chronic lymphoproliferative disorders neoplastic cells do not resemble early hematopoietic stem cells, but rather cellular elements in late maturation phases." Indeed, "the clinical uselessness of Rituximab … [demonstrated in] preclinical results are in line with early studies suggesting that all clonal malignant plasma cells are highly capable of self-renewal in vitro as well as initiating MM growth in vivo". The authors conclude, however, that "discrepancies in defining the exact phenotype of MM CSCs, but also conflicting results on their functional properties have contributed to generate skepticism in the scientific community regarding the real existence of stem cells in this disease." And instead of rejecting cancer stem cell theory, the authors conclude that "improvements in discriminating both advantages and limitations of the specific assays used to assess the phenotype and the functional properties of MM CSCs are necessary, so that they can provide important knowledge in defining their actual role in MM biology."

Part 2, Competing Models of Cancers' Stem Cells, brings together chapters illustrating a variety of models tucked under the umbrella of the stem cell theory. Some models take into account specific subsets of proliferating CSCs driving turmorigenesis; other models consider transitions and transformations producing cancer cells with increased malignancy; still other models incorporate effects of a cancer's microenvironment, niche, and angiogenesis on genetic stability and cellular behavior.

Chapter 7, "Systems and Network Understanding of Cancer Stem Cells" by Asfar S. Azmi, Ramzi M. Mohammad, Sanjeev Banerjee, Zhiwei Wang, Bin Bao, and Fazlul H. Sarkar begins with the admonition that the study of "[c]ancers that contain a hierarchy

of epigenetically distinct populations of tumorigenic and non-tumorigenic cells will only achieve its promise if we carefully distinguish between cancers that follow a cancer stem cell model and those that do not." The authors present evidence that the difference in the malignant potential of cells with the same genotypic alteration "is not as striking as it has been initially envisioned." Thus, the issue of cancer initiation and promotion comes around to "epigenetic abnormalities" and "a gene expression signature reminiscent of ES cells". In light of this heterogeneity and ambiguity, "applying therapy that is ineffectively targeting the CSC population … [would not only] be unsuccessful in curing the patient but would also promote malignant features including rapid expansion, increased invasion, and further stimulates [*sic.*] heterogeneity directly after therapy." Different kinds of stem cells would seem to demand individual attention.

In Chapter 8, "What Do We Know about Cancer Stem Cells? Utilizing Colon Cancer as an Example" Heinz-Joseph Lenz tests the CSC theory of colon cancer with the same rigor that led to identifying CICs supporting hematological malignancies. The critical issue is whether the bulk of an engrafted heterogeneous cancer transferred by CICs is supported and spread by a distinct "colongenic subset" of CSCs. Arguing that "differentiation always reproduces the heterogeneity of the parental tumor" Lenz concludes that "CSC are the only tumor initiating cells … [and that] cancer treatment will not be successful unless this population of cells can be completely eradicated." Accordingly, the chapter continues with a massive effort to hunt down a "specific signaling pathway exclusively used by CSC" and not by normal cells or their stem cells. But, in the end, Lenz's quarry eludes his grasp and "remains unknown."

Chapter 9, "Epithelial–Mesenchymal Transformation and Cancer Stem Cells" by Ouyang Gaoliang is a *tour de force* of issues raised by epithelial-mesenchymal transformations [EMTs]. The EMT program enables "cancer cells to disseminate from a primary tumour and … [assert] their self-renewal capability to … [generate] the critical tumour mass required for progression from micro- to macro-metastases." Specifically, the EMT induces "signaling pathways, such as TGF-β, Wnt, Notch and Hedgehog (Hh), along with other tumour microenvironmental cues … [that convert] well-differentiated epithelial cells … into motile mesenchymal cells via the activation of … transcription factors". Furthermore, the author identifies "[s]imilarities between [a] developmental and [an] oncogenic EMT [that] have led to the identification of common contributing pathways [in embryogenesis and tumorigenesis], suggesting that the reactivation of developmental pathways in cancers contributes to tumor progression."

Chapter 10, "Cancer Stem Cell Niche: Role of Mesenchymal Stem Cells in Tumor Microenvironment" by Kanya Honoki, Hiromasa Fujii, and Toshifumi Tsujiuchi reads like a thriller tracing the exploits of mesenchymal stem cell (MSC) "as a co-conspirator of tumor development." The other "co-conspirator" is the CSC, and the plot thickens around their interactions in building and maintaining a vascular niche in the tumor's microenvironment. The authors concentrate on MSCs' role in laying down tumor-

associated fibroblasts (TAFs) or myofibroblasts, contributing to angiogenesis, modulating the immune system, and shaping tumor architecture with the help of paracrine secretions, chemoattractants, inflammatory factors, chemokines, and interleukins. Ultimately, the authors' conspiracy theory is not proven but rests on the success of "therapeutic approaches that disrupt communication between tumor cells and stromal cells."

Chapter 11, "Transformation of Mesenchymal Stem Cells" by Nedime Serakinci, Rikke Christensen, and Umut Fahrioglu provides "an in depth discussion on genetic stability and neoplastic transformation" in the human mesenchymal stem cell line (hMSC-telo1) immortalized by retrovirus carrying the hTERT telomerase gene. Surprisingly, "exogenously administrated hMSCs can be recruited to the stroma of developing tumors when systemically infused in animal models for glioma, colon carcinoma, ovarian carcinoma, Karposi's sarcoma and melanoma." That discovery led the authors to the inspired proposal for using hMSCs as a "cell-based delivery vehicle for the site-specific release of therapeutic proteins." Happily, "[t]his strategy has been shown to work by the observation of specific homing of intravenously administered hMSCs, engineered to produce interferon"! However, "the key questions [*sic.*] that still needs to be addressed is; how safe is using hMSC for targeted cancer therapy?"

Part 3, Glioma Stem Cells, sets aside concepts, theories, and models in favor of a close look specifically at cancers' stem cells in glioma. And close inspection proves its worth by turning up so many surprises: glioma cells' behavior is sensitive to their microenvironmental setting; the cells are unexpectedly flexible, and their biomarkers and signaling pathways offer a variety of excellent targets for therapy.

Chapter 12, "Brain Tumor Stem Cells and Anti-Angiogenetic Therapy" by Katsuya Saito, Yoshida Kazunari, and Masahiro Toda plunges into the "vascular niche" where the "glioma stem cell's characteristics, such as self-renewal and differentiation … [regulate] signals from the surrounding niche … [and are] modulated … through the secretion of VEGF [vascular endothelial growth factor]." After paying their respects to the late Judah Folkman's pioneering work, the authors study five members of the VEGF family, the placental growth factor (PlGF), their receptors (VEGFRs) and co-receptors. But the most exciting and challenging discussion surrounds therapeutics. Some of the conclusions are cautionary: "the function of VEGFR-1 differs with the stages of development, physiologic and pathologic conditions, and cell types, which expresses the receptor." And some of the news is mixed: "anti-angiogenic therapy enhances glioma and glioma stem cells' sensitivity for cytotoxic chemotherapy and radiotherapy" but at the cost of "potential side effects" to say nothing of devastating and increased resistance with prolonged treatment. On the other hand, clinical data also "support the hypothesis that antiangiogenic therapy may transiently 'normalize' the dilated, abnormally permeable tumor vasculature."

Chapter 13, "Targeting Glioma Stem Cells: Path Leading to Cure" by Ke Sai and Zhong-ping Chen is more than a review of therapies for glioma stem cells (aka brain

tumor stem cells [BTSC]). The chapter is a thorough if disappointing assessment of therapeutic possibilities and problems. Unexpectedly, "[b]oth CD133-positive and CD133-negative glioma cells in individual GBMs [glioblastomas multiforme] exhibit self-renewal in vitro and initiate highly aggressive tumours in vivo. Notably, CD133-negative glioma cells can even give rise to CD133-postive cells … Moreover, CD133 expression has been proposed as an indicator of bioenergetic stress rather than … obligatorily related with BTSC phenotype in human gliomas." As depressing as are most of the reported results, "BTSCs are [still thought to be] promising therapeutic targets".

Chapter 14, "Glioma Stem Cells" by Ryan Y. Kim, Ali Mahta, and Santosh Kesari provides a close look at cell-extrinsic and cell-intrinsic signals that contribute "to the cancer cells' extensive proliferation within their perivascular niche." The chapter reviews "highly discriminating targets and tumor biomarkers of therapeutic [consequence] … epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), RAS/Raf/MAPK & AKT/PI3K pathways, Sonic hedgehog (Shh), and PTEN." Happily, the authors conclude that, "targeted molecular therapies hold great promise and will become an important component of combined treatment approaches in our effort to fight cancer."

Hong-Yan Qin, Luo-An Fu, and Hua Han, the authors of Chapter 15, "Regulation of glioma Stem Cells by the Notch Signaling Pathway: Mechanisms and Therapeutic Implications" pioneered "the application of Notch signaling as a therapeutic target of glioma, emphasizing its potential advantages and disadvantages." The chapter reviews the history of CSC research in gliomas, an assortment of possibilities for "targeted" therapies, and the signaling literature before focusing on the Notch signaling pathway in the hope and expectation that it "should be regulated within a subtle and strict molecular network." The objective is to identify "downstream molecules of Notch signaling [that] are abnormally regulated in different grades of glioma" and can be countered without provoking unwanted side effects. For example, exhausting "GSCs by inhibiting Notch signaling seems … [an] exciting option." At the moment, intriguing networks "need further investigation."

Part 4, Signaling Pathways and Markers for Cancer Stem Cells, focuses on cancers' stem cells' molecular qualities shared with normal stem cells and unique to cancer stem cells. Hopefully, molecular biologissts will identify points of vulnerability in cancers' stem cells with the potential for targeted therapies that will not damage normal stem cells.

In Chapter 16, "The Hedgehog Signaling Network and the Development of Gastric Cancer," Jessica M. Donnelly, JeanMarie Houghton, and Yana Zavros focus on the hedgehog signaling pathway and its role in the development of gastric cancer, specifically in response to *Helicobacter pylori* infection. This is a fascinating multifaceted story in which the Hedgehog signaling network is a regulatory mechanism within the bone marrow-mesencyhmal stem cell (BM-MSC) compartment during the induction of

gastric cancer. The authors' "laboratory is responsible for discovering that in the mammalian system, Shh [sonic hedgehog] secretion from parietal cells is acid- and hormonally-regulated … [while] regulating the differentiation of cell lineages and controlling gastric physiological function". Moreover, results "suggest that BM-MSCs, harboring mutations, are recruited to the sites of inflammation and drive cancer progression through the elevated production of Shh protein that may subsequently induce proliferation of the cancer stem cells."

In Chapter 17, "Differentiation of Cancer Stem Cells," Taro Yamashita, Masao Honda, and Shuichi Kaneko "summarize current knowledge on the differentiation of cancer stem cells and discuss the utility and limitation of differentiation therapy to eliminate cancer." The authors confront the problem that, "cancer stem cells can only be defined experimentally and their self-renewal ability is generally evaluated by the capacity of serially transplanted cells in immunodeficient mice." In order to escape this operational *cul de sac*, the authors' look at pathways responsible for self-renewal, such as Wnt/b-catenin, Hedgehog, and Notch signaling, and at pathways responsible for differentiation, namely bone morphogenic protein and oncostatin M signaling. But difficulties only increase. Ironically, "the induction of differentiation programs in cancer stem cells may result in cell proliferation of the tumor". What is more, "cancer stem cells may acquire resistance against differentiation therapy by additional epigenetic changes during the differentiation treatment." Clearly more work is warranted by this promising but difficult approach.

Chapter 18, "Carbohydrate Antigens as Cancer-Initiating Cell Markers" by Wei-Ming Lin, U. Karsten, S. Boletz, R-C. Cheng, and Y. Cao raises the question, what makes cancer initiating cells (CICs) different? Remarkably, tracing the antigenic mix on putative CICs demonstrates the presence of blood group antigens CD173, CD174, and especially CD 176 "which is almost absent on normal and benign adult human tissues." The blood group carbohydrate antigens are all the more intriguing, since they would "contribute to adhesion, cell aggregation, invasion, and metastasis" of the CIC. The authors thus make their case for glycosylation as a diagnostic marker and these carbohydrate antigens as "potential targets for antibody-mediated diagnosis and therapy" as well as vaccination.

Chapter 19, "Influence of Culture Environment and *Mollicutes* Contaminations on CD133 Modulation in Cancer Stem Cells" by Elisabetta Mariotti, Peppino Mirabelli, Francesca D'Alessio, Marica Gemei, Rosa Di Noto, Giuliana Fortunato, and Luigi Del Vecchio examines "how environmental factors, culture conditions and mycoplasma infections can play a relevant role in CD133 modulation." Of special concern is the "influence on CD133 expression by [colon carcinoma's] CSCs by *Mycoplasma hyorhinis* contamination." Surprisingly, modulation is reversible in decontaminated cultures indicating that, "a tighter control of microenvironmental variables combined with prevention measures, detection methods and eradication of *Mollicutes* infections, will be an integral part of the basic panel of CSC manipulation techniques."

Part 5, Cancer Stem Cells in Targeted Therapeutics: New Perspectives, is an extension of Part 4. There, the chapters examined vulnerabilities; here, the chapters probe these vulnerabilities for therapeutic applications. The chapters consider an enormous range of anti-cancer strategies, problems, and prospects. Fortunately, some of the strategies turn out to be effective.

Chapter 20, "Therapeutic Strategies Targeting Cancer Stem Cells" by Atique U. Ahmed, Bart Thaci, Derek A. Wainwright, Mahua Dey, and Maciej S. Lesniak focuses on a central issue of stem cell biology: the basis of CSCs resemblance to "the normal stem or progenitor cells of their tumor's derivative tissue." Thus, they acknowledge "the importance of combining phenotypic markers with functional markers as a signature to identify tissue specific CSCs." Otherwise, CSCs will continue to elude detection and be "responsible for the unlimited growth of a tumor … [as well as] the maintenance of the minimal residual disease and constitutive recurrences following therapy and metastasis." Moreover, "CSCs may resist standard anti-cancer therapies via a combination of molecular mechanisms associated with normal stem cell biology." Are tumors "capable of evading conventional chemo- and/or radiotherapy due to their inherent stem cell characteristics"? The odds may be against them, but stakes are high, and these researchers continue to bet on pharmacological targeting, immunotherapy, genetic targeting, miRNA, and oncolytic viruses.

Chapter 21, "Latest Therapeutic Approaches Based on Cancer Stem Cells" by Jun Dou, Jing Wang, and Ning Gu offers a cornucopia of therapeutic possibilities beginning with oncolytic adeno- and reoviruses aimed at CD44, aldehyde dehydrogenase, and telomerase on top of cytotoxic drug-conjugated antibodies aimed at surface markers. Neovascularization in CSCs' survival niche (i.e., the vascular niche or pre-metastatic niche) is attacked by anti-VEGF monoclonal antibodies and poly-ADP-ribose polymerase inhibitors interfering with the recruitment of bone marrow derived stem cells. Tumor vascularization, the transformation of tumor cells into endothelial cells, the activities of cancer-associated fibroblasts, and the expression of tumor specific extracellular matrix in cancers' microenvironment suggest other targets for blocking and radio-labeled antibodies, gene therapy, and combined immuno- chemotherapies. Other prospective therapies target signal pathways (hedgehog proteins, notch and its ligands, and components of the Wnt/β-catenin pathway) with siRNA and anti-inflammatory drugs, while specific miRNAs could block self-renewal and reverse the epithelial-to-mesenchymal transition of CSCs. Targeted therapies also employ RNA interference with siRNAs against onco- and other genes associated with a tumor's plasticity such as Oct4. The authors review the advantages of using 3D cultures to survey the scope of CSCs' traits in vitro and to explore prospects for antitumor immunity resulting from the use of dendritic cell vaccination designed to elicit antigen-specific T cell responses against CSCs through induced interferon-γ production.

Chapter 22, "Potential Application of Natural Dietary Components to Target Cancer Stem Cells" by Yanyan Li, Steven J. Schwartz, and Duxin Sun does exactly what it sets

out to do: "reviews current attempts to target CSCs with bioactive dietary components, with a special emphasis on … [the authors'] work." The authors target three pathways, Wnt/β-catenin, hedghog, and Notch, and examine the chemopreventive properties in vivo and in vitro of sulforaphane, an isothiocyanates "converted from glucoraphanin, the principal glucosinolate in broccoli and broccoli sprouts," curcumin, a "polyphenol present in the Indian spice turmeric … and usually used in preparation of mustard and curry," epigallocatechin-3-gallate (EGCG) "the most abundant catechin in green tea," and quercetin, "a ubiquitous plant polyphenol, naturally occurring in most edible fruits and vegetables, with the high levels being found in apples, cranberries, and blueberries." The impressive results obtained with a wide range of tumors will surely whet the readers' appetite for "new anticancer … phytochemical compounds".

Chapter 23, "Towards New Anticancer Strategies by Targeting Cancer Stem Cells with Phytochemical Compounds" by Sharif Tanveer, Emhemmed Fathi, and Fuhrmann Guy reviews relevant signal pathways, transcription factors, and co-regulators including Oct4 transcriptional networks as targets for phytochemical compounds "with chemopreventive properties [able to] hinder the (re)appearance of a cancer by targeting CSCs". The authors concentrate on curcumin that "can restore the chemosensitization in drug-resistant cancer cells [by down-regulating the expression of transporter proteins]". Curcumin is also "a very potent inducer of apoptosis and interferes with both the intrinsic and extrinsic proapoptotic signaling pathways … [thereby] killing a wide variety of cancer cells." What is more, "curcumin has been shown to prevent angiogenesis and cancer cell invasion, thanks to its antiproliferative and proapoptotic activity", and "curcumin can reverse DNA hypermethylation and … [may] reactivate methylation-silenced tumor suppressor genes". Other phytochemicals, such as the polyphenolic flavonoids have "dual effects … as antioxidants and prooxidants … [and are] therefore [capable of] acting as double-edged swords by targeting the redox regulatory system." Thus, the authors "target the Achilles' heel of CSCs, in particular those harboring a selective sensitivity to oxidative stress and/or … weakly differentiated Oct-4 expressing cancers."

Part 6, Techniques and Technical Details, contains chapters presenting the practical problems of doing the laboratory research behind clinical practice. These chapters support and augment each other, confront difficulties of cancer research and present the means employed to overcome these difficulties.

Chapter 24 "Isolation of Liver Cancer Stem Like Cells by Hoechst3342 or Rhodamine123 Efflux" by Weihui Liu, Nan You, and Kefeng Dou looks at the specifics for isolating stem cells from the heterogeneous MHCC97 cell line of hepatocarcinoma cells (HCCs) by Hoechst 3342 (Hoe) and Rhodamin 123 (Roe) efflux through fluorescence activated cell sorting (FACS). After exhaustive experimentation, the authors conclude that Hoe's toxicity is a serious drawback for cellular research, which, added to Hoe's ability to "disrupt DNA replication during cell division … [and its potential] mutagenic and carcinogenic [activity]" argues against its use in

experimental protocols. Roe, on the other hand, is not toxic even at high doses, and "Rho^{low} cells had … high proliferative ability and high expression of CD133." Rho might thus be the supravital dye of choice for FACS.

Chapter 25, "Large-Scale Production of Human Glioblastoma-Derived Cancer Stem Cell Tissue in Suspension Bioreactors to Facilitate the Development of Novel Oncolytic Therapeutics" by Krishna Panchalingam, Wendy Paramchuk, Parvinder Hothi, Nameeta Shah, Leroy Hood, Greg Foltz, and Leo A. Behie, faces a straightforward problem and solves it admirably. The problem is that "the development of novel oncolytic therapeutics to target CSCs is currently hindered by the scarcity of CSCs". The solution was to develop suspension bioreactors to provide "a continuous and reproducible supply of tissue for genomic analysis and high-throughput drug screening" and for "the development of novel drug treatments."

This chapter tells the story of building suspension bioreactors for glioblastoma multiforme cancer stem cells (GBM-CSCs). The authors begin with a critique of the CSC model, but experimental results strengthen the authors' confidence in the reality of GBM-CSCs, although "histopathological diagnosis of brain tumours is very subjective and may be unable to identify tumour variability between patients … [while] the variability seen between GBM-cell lines might explain the differential response of tumour patients to treatment modalities." Consequently, "[i]n order to generate a clinically-relevant number of patient-specific GBM-CSCs, it is necessary to optimize the in vitro culture environment, such that the basic genetic nature of the GBM-CSCs does not significantly change." Thus, their medium "support[s] the long-term expansion of human NPCs [neural precursor cells]" and their apparatus, engineered to exacting requirements for "(1) liquid shear, (2) inoculation density, (3) feeding strategy, (4) medium composition and, (5) hypoxia" provides optimal "(1) oxygen supply, and (2) hydrodynamic shear in the liquid medium" for up-scaling production in computer-controlled suspension bioreactors. The brilliantly illustrated results are nothing less than spectacular.

Chapter 26, "Cancer Stem Cells: The Role of the Environment and Methods to Identify Them" by Giuseppe Pirozzi, begins with an edifying narrative of the history of cancer stem cell theory and the development of cancers, from stem cells' signaling pathways, and membrane micro-domains to their myriad niches (e.g., stromal, vascular, extra-medullary, and endosteal). Pirozzi proceeds to methods for detecting, isolating, and characterizing cancer stem cells "highlighting the imperative to delineate more specific markers or to use combinatorial markers and methodologies," and he does not spare the specifics or the critique, including challenges to the "assertion that CSCs are necessarily a rare phenomenon."

In Chapter 27, "Modulation of Multidrug Resistance on the Same Single Cancer Cell in a Microfluidic Chip: Intended for Cancer Stem Cell Research," XiuJun Li, Yuchun Chen, and Paul C.H. Li tackle one of cancer's most intractable problems: resistance to chemotherapy traced to a MDR [multidrug resistance] "class of genes … [that] actively

transports drugs out of the cancer cells … [thereby causing] the intracellular drug concentration to be lower than the drug's efficacy threshold". The authors designed and employed a "microfluidic single-cell analysis approach for the study of multidrug resistance modulation by real-time monitoring of drug efflux in MDR cancer cells". Thankfully, the chapter devotes sufficient attention to the methodology, including several figures with micrographs of living cells in the apparatus to reassure the reader that the virtual magic of technology is quite real.

Results with flow cytometry with a fluorescence-activated cell sorter (FACS) requiring considerably more than one cell (10,000 cells) are compared with results with different-single-cell analysis (DISCA) employing a treated and a control cell, and same-single-cell analysis (SASCA) using the same cell as both control and treated. The object was to test the efflux effect of putative MDR modulator compounds: "verapamil (VER) … [known to] inhibit the MDR efflux and result in more drug retention" and "IQ, an ingredient of the traditional Chinese herb licorice … [reported to have an] anti-tumor effect on human gastric cancer … prostate cancer … and hepatoma." Briefly, "IQ was ruled out as a MDR modulator candidate" by the results of SASCA. But the authors did not stop there. They proceeded to monitor drug accumulation, instead of drug efflux and hence "evaluate the MDR modulations or MDR reversal effect of various drug candidates." Hopefully, their modified SASCA (SASCA-A) method will have "clinical use as a companion diagnostics method, e.g. to check the MDR profile and drug responses so as to identify personalized drugs before patient treatment starts."

Part 7, A Biologist's View, looks at cancer stem cells and cancers throughout the animal kingdom. The question is, can cancer stem cells and cancers be traced to proterozoic origins and followed through evolutionary scenarios of adaptations to contemporary malignancies?

"Evolution of Cancer Stem Cells," Chapter 28 by Stanley Shostak (editor and contributor) suggests that an evolutionary perspective will help make sense of cancers' stem cells. But the problems of tracing cancer's evolution are massive: Most animals do not have cancers unless induced, and "spotty spectra of mutations all but obliterate the trail of cancers' genomic phylogeny." Consequently, cancers' stem cells' evolution is "pursued … the old-fashioned way, by following Charles Darwin's lead and asking … what 'kinds of modification' would produce cancers' stem cells?"

Some possible routes in this pursuit lead nowhere. For example, "tracing cancers' stem cells' origins to a rudimentary stem cell" fails because cancers' stem cells do not conform to criteria for a single cell type. Other possible routes point to cancer stem cells originating in particular cell populations, but like all evolutionary speculation, this one cannot be corroborated by experiment. Even though, in the "six categories [of stem and non-stem cell populations fostering cancers], cancer and normal cells have more in common with each other than they have with cells in other categories … [one can do no more than suggest] that each of these cancer and normal cell pairs arose in a

common cell-population ancestor and adopted their normal and malignant phenotypes by competition."

Viewed through an evolutionary prism, cancers, like all other parts of organisms, are manipulated by evolution, and some of our cancers seem to have even been pushed beyond our period of greatest reproductive activity. Likewise, some ancient cancers may have evolved an equilibrium with normal tissue, and, following their lead, highly malignant, presumably recently evolving cancers may yet be manipulated therapeutically into less malignant states.

In sum, a fascinating convergence emerges in *Cancer Stem Cells* between the many facets of cancer stem cells seen "in a grain of sand" with Blakean perspective and the plodding, evolving malignant cell populations seen with Darwinian perspective. All the qualities of cancers' stem cells that inspired the research documented here point toward a comprehensible if complex understanding of cancers' stem cells' interactions with the environment, with organismic systems, with surrounding normal tissues, vascular and stromal niches, with epigenetic transmutations, signal pathways, nuclear transcription factors, and, indeed, with an individual's unique genes. At the same time, competition and selection of cell populations in adult tissues and the parenchyma of organs figure in explanations of cancers' stem cells' proliferation, invasiveness, and destructiveness.

Each chapter of *Cancer Stem Cells* focuses on a distinct property of cancers' stem cells. The chapters thus examine precise roles of cancer stem cells in cancers' initiation, promotion, progress, metastasis, recurrence, and resistance with emphasis on methods of cellular isolation, cell markers employed to identify cancer stem cells, signal pathways controlling stem-cell fate (hedgehog proteins, notch and its ligands, and components of the Wnt/β-catenin pathway), roles played by epigenetic modification, by aberrant substrates, and runaway nuclear transcription factors in preventing differentiation or apoptosis. And the chapters do not shy away from the details and difficulties of research. Discussions span specifics for identifying antigens, immortalization, the design and application of techniques for growing cancer cells in vitro, for measuring efflux in microfluidic chips, and for solving problems arising from fluid dynamics in bioreactors and contaminating mycoplasm.

Clues for prevention and treatment are sought in bioactive dietary components and in viruses as agents in transgenic or conditionally targeted gene technologies. Research on differentiation and molecular therapies, especially components of combined therapies, takes aim at cancer stem cells, epigenetic abnormalities, and gene expression signatures. A host of agents and interventions are found useful: RNA interference, oncolytic adeno- and reoviruses, cytotoxic drug-conjugated antibodies, pharmacological targeting, immunotherapy, monoclonal antibodies, inhibitors targeting cancers' stem cells' signal pathways involved in cell division, differentiation, cell death, reversing the epithelial-to-mesenchymal transition, and preventing malignant transformations. Dendritic cell vaccination is also discussed as a device for

eliciting an antigen-specific T-cell response. And strategies are outlined for using anti-inflammatory drugs and anti-angiogenic therapies aimed at the cancer's vascular niche and the recruitment of bone marrow derived cells to the cancer's stroma.

Ultimately, *Cancer Stem Cells* documents the enormous contribution to contemporary understanding of cancers made by researchers on the trail of cancers' stem cells. All the problems laid at the feet of cancers' stem cells in the past may have to be distributed over a larger terrain including non-stem cells, and different types of cancers' stem cells may have to be pursued through different paradigms, but wherever they do occur and of whatever type, cancers' stem cells continue to threaten recovery through their potential for recurrence with increased resistance to chemo- radio-therapy. Indeed, cancer stem cells may be present in proportionately greater numbers in residual cancers following chemo- radiotherapy than in primary cancers. *Cancer Stem Cells*, thus, bears witness to these threats but also provides credible clues for coping with them: for detecting, isolating, and characterizing cancers' stem cells early, rapidly, and unambiguously, and designing programs for comprehensive prevention, early diagnosis, efficacious therapy, and improving prognosis.

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Part 1

Cancer Stem Cells and Cancer Stem Cell Theory

1

Stem Cells and Cancer Stem Cells

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1. Introduction

The different organs in human organism are constituted by tissues with mature and specialized cells and its specific stem cells. Stem cells represent only a minuscule fraction of cells that constitute each tissue but they are the only cells with self-renewal capacity. The organ-specific stem cells have specific properties that maintain tissue integrity and are defined mainly by their capacity to undergo self-renewal, as well as differentiation into mature cell types that comprise each organ (Shipitsin & Polyak, 2008).

The malignant neoplasias are believed to result from sequential mutations that can occur as a consequence of progressive genetic instability and/or environmental factors. An accordant observation in several investigations has been the association between deregulation of stem cells and carcinogenesis, because there are regulatory mechanisms of self-renewal in normal stem cells that also frequently regulate oncogenesis. In consequence, experimental and clinical evidences that have recently been accumulated support the hypothesis that cancer may arise from mutations in normal stem cell populations, and that these cells would be subject to ongoing genetic and epigenetic changes that could help to establish the disease.

The cancer stem cell (CSC) hypothesis states that normal stem cells may be the cells of cancer origin, and that a specific subset of cancer cells with stem cell characteristics can give rise to a hierarchy of proliferative and progressively differentiated bulk of tumoral cells, leading to tumor initiation, progression, and recurrence. In fact, there are several investigations that recently have identified specific CSC markers showing similar expression profiles than the normal stem cells of same organ. Moreover, CSCs can be prospectively isolated based on expression of a specific molecule or combination of molecules, and have the ability to give rise to new tumors when xenografted in immunodeficient mice.

Additional confirmations that stem cells can play a role in carcinogenesis are the homologies found between normal adult stem cells and cancer cells. Besides self-renewal capacity, these characteristics include the production of differentiated cells, activation of antiapoptotic pathways, induction of angiogenesis, resistance to apoptosis and drugs (due to active telomerase expression and elevated membrane transporter activity), and the ability to migrate and propagate (Wicha et al., 2006). Notwithstanding, different from normal adult stem cells that remain constant in number, CSCs can increase as the tumors grow, and originate the progeny that can be both locally invasive and/or colonize distant sites. Therefore, the consolidation of CSCs knowledge into our current view of multistep cancer development has important implications for defining the target population for therapeutical

approach and for understanding specific events required for realization of malignant potential, and the advances in CSC knowledge can help to build further evidences for potential targeting pathways in treatment of several cancer types.

2. Stem cells

Despite wide variety of cells that can be identified in adult tissues, all cells derive from a single egg cell after fertilisation of an ovule by a spermatozoid. Egg fertilisation results in creation of totipotent stem cells, which are the precursor cells of all tissues of embryo, yolk sac, amniotic sac, allantois, and embryonic portion of placenta – chorion and others placental membranes. Approximately four days after fertilisation, these totipotent stem cells undergo several mitotic divisions to form identical cells, and after this point, they tend to lose their high proliferative potential and begin to specialise by becoming pluripotent stem cells, which then can generate most of tissues necessary for embryo formation. Subsequently, the pluripotent stem cells begin to divide, and they mature into more specialised stem cells – the progenitor cells. These progenitor cells are called multipotent stem cells. They are committed to generate specific cell groups that have distinct functions, such as haematopoietic stem cells, which produce erythrocytes, white blood cells and platelets. Furthermore, multipotent stem cells become more specialised and give rise to precursor committed cells or unipotent stem cells, which are able to differentiate into only one cell lineage.

The unipotent stem cells' function is to act as cell reservoirs for different tissues. Certain unipotent cells, such as adult hepatocytes, may even have long-term repopulating functions. Finally, from unipotent stem cells originates the nullipotent cells that are terminally differentiated and have lost their self-renewal capabilities. Therefore, stem cells show diverse degrees of plasticity or differentiation potential and can be defined as units of biological organisation that are clonal precursors of more identical stem cells; in addition, they can produce a defined set of differentiated and specialised progeny (De Miguel et al., 2009; He et al., 2009; Slack, 2008) (Figure 1).

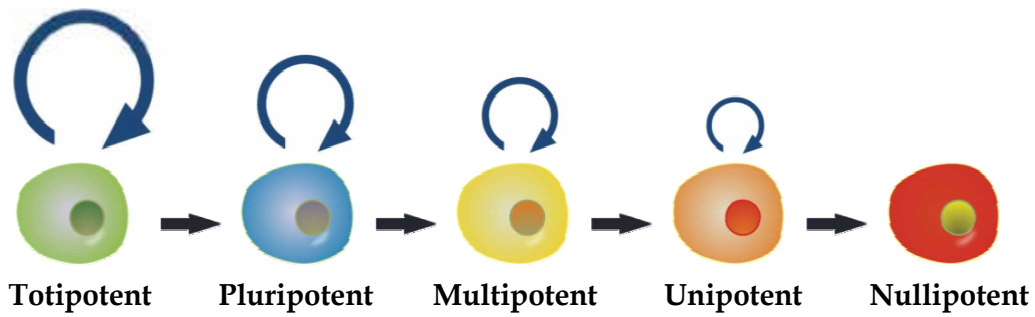

Fig. 1. Stem cells plasticity. Stem cells show diverse degrees of differentiation potential

The integrity of adult tissues is maintained by the continuous replacement of cells that regularly differentiate and die. Thus, in most adult tissues, there are pools of progenitor cells that are able to multiply and differentiate into specialised tissue of origin, while at the same time, they are able to maintain a reserve of undifferentiated cells. These adult progenitor cells are defined as adult tissue-specific stem cells or somatic stem cells.

The liver is probably the best example of a tissue with stem cells and differentiated cells because it has a remarkable regeneration capacity. Centuries ago, Greek mythology described liver regeneration through story of Prometheus, the mortal who stole the secret of fire from Zeus and introduced it to humans. Prometheus was then punished by having his liver plucked out by an eagle daily. His liver regenerated overnight, thus providing the eagle with eternal food and Prometheus with eternal torture. This phenomenon was later recognised in medicine, albeit at a slow rate, and it was probably first introduced into scientific literature in the 1800s in several German reports (Ankoma-Sey, 1999).
In modern times, the next significant scientific advance in elucidation of liver regeneration was introduced by Higgins & Anderson in 1931. They demonstrated experimentally that surgical removal of two-thirds of the rat liver (partial hepatectomy) was possible and that it resulted in regeneration of remaining lobes of liver by compensatory hyperplasia. The whole process lasted five to seven days (Higgins & Anderson, 1931).
During the 1960s, first genetic evidence of stem cells existence was detailed. The authors of these studies demonstrated that bone marrow contains a unique specific type of cell that could give rise to myeloerythroid colonies in spleen. In these experiments, genetically marked cells (random DNA breaks and translocations) were generated by sublethal irradiation of the donor bone marrow. These cells could self-renew and differentiate in spleens of conditioned transplanted host mice, indicating that the genetically marked stem cells were able to reconstitute and radioprotect mice after sublethal irradiation (Becker et al., 1963; Becker et al., 1965).
In summary, stem cells differ from other cells in the body because they have four major properties: a) they are undifferentiated and unspecialised; b) they are able to multiply for long periods while remaining undifferentiated (generally slowly cycling), such that a small number can create a large population of similar cells; c) they are capable of differentiating into specialised cells of a particular tissue (produce progeny in at least two lineages); and d) they can be serially transplanted. The combination of these properties is often referred to as "stemness" (Mikkers & Frisen, 2005).
Stem cells can divide symmetrically or asymmetrically. A symmetrical division occurs when two daughter cells share the same stem cell features, and it occurs when their numbers (stem cell pool) need to be expanded, such as during embryonic development or after tissue injury. An asymmetrical division occurs when one of the progeny remains undifferentiated, thereby replenishing the pool of stem cells, while the other daughter cell can proliferate and differentiate into specialised cells to generate new tissue mass (Figure 2).

2.1 Pluripotent stem cells

During embryonic development, the embryo originates from a single fertilised egg, also called a zygote, and it divides into extraembryonic (trophoblasts) and embryonic components (Gardner, 1983). The embryonic component is located inside the embryo. It refers to the inner cell mass of blastocysts, and is the originator of all tissues of embryo, foetus and adult organism (Brook & Gardner, 1997; Evans & Kaufman, 1981). The inner cell mass is also the source of embryonic stem (ES) cells and has the ability to give rise to all three embryonic germ layers: ectoderm (epidermal tissues and nervous system), endoderm (interior stomach lining, gastrointestinal tract, lungs), and mesoderm (muscle, bone, blood, urogenital) (Li & Xie, 2005; Thomson et al., 1998).

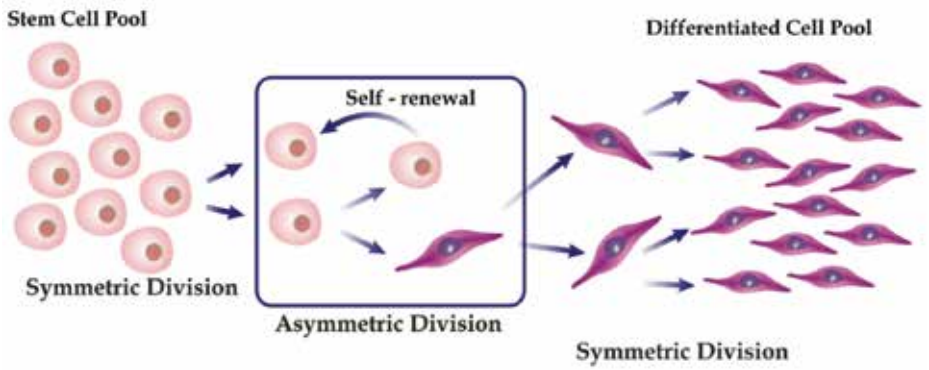

Fig. 2. Self-renewal is the fundamental characteristic of stem cells. Stem cell can be induced to undergo symmetric division when necessary and stem cells also are able to divide asymmetrically, originating one undifferentiated cell, which restores the stem cell pool, and another cell committed to differentiation

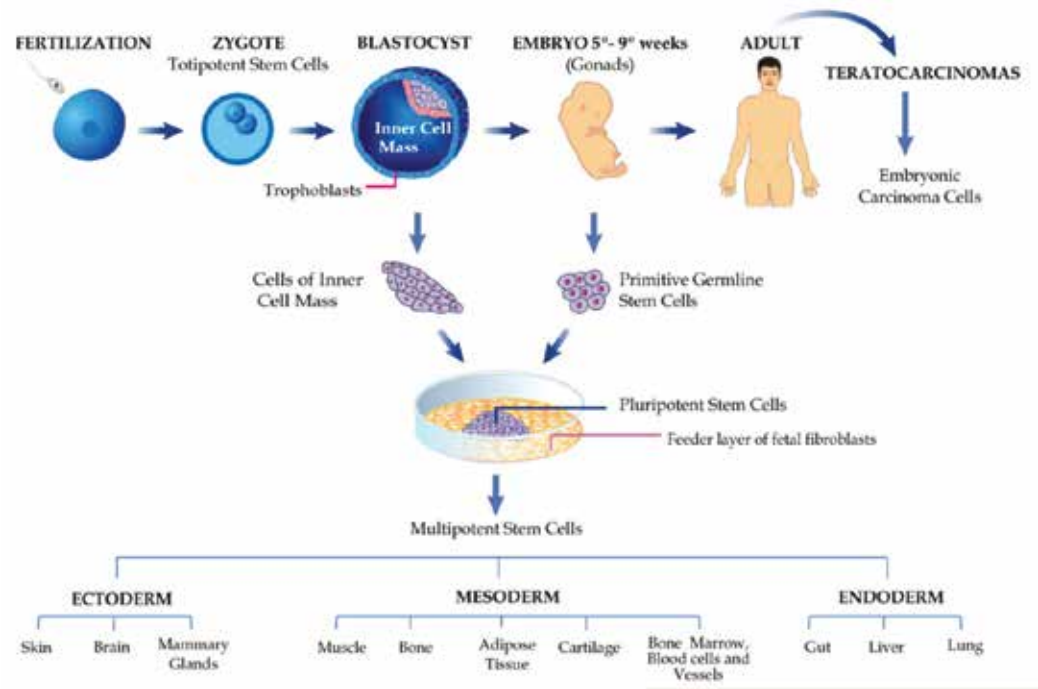

Fig. 3. The totipotent zygote is formed after fertilisation of an ovule by a spermatozoid and undergoes several mitotic divisions to form blastocyst, which is divided into extraembryonic (trophoblasts) and embryonic components (inner cell mass), from which all tissues of adult organism originate. Pluripotent stem cells can be isolated from inner cell mass or gonadal buds of embryo using a feeder layer of foetal fibroblasts, and these cells can be differentiated into cells of every lineage in human body. Stem cells restricted to one lineage (ectoderm, mesoderm or endoderm) are called multipotent stem cells

As development proceeds, the need for organogenesis arises, and over five-nine weeks post-fertilisation, the embryo proper forms germline stem cells for reproduction and somatic stem cells for organogenesis. Germline stem cells derive from gonadal buds of the embryo and are an alternative source of ES cells (Liu et al., 2004). The ES cells of the inner cell mass or the gonads are considered to be pluripotent due their ability to differentiate into cells of every lineage in the body (Anderson et al., 2001). Moreover, ES cells can undergo cell divisions without differentiation through symmetrical divisions (Fuchs & Segre, 2000) (Figure 3).

Interestingly, the term "ES cells" was introduced to distinguish pluripotent embryonic stem cells from teratocarcinoma-derived pluripotent embryonic carcinoma (EC) cells (Martin, 1981). Teratocarcinomas are malignant, multidifferentiated tumours containing a significant population of undifferentiated cells. These tumours were first described in 1970 when researchers reported that early mouse embryos grafted into adult mice produced teratocarcinomas (Solter et al., 1970; Stevens, 1970). In humans, teratocarcinomas are formed from a malignant form of primordial germ cells and usually occur in ovaries and testes. The EC cells proliferate extensively *in vitro* and remained undifferentiated even at high densities, and unlike ES cells and germline stem cells, they contain chromosomal alterations. Another characteristic of EC cells is that they have a more limited differentiation potential than ES cells *in vitro* and *in vivo* (Andrews, 1998).

The first isolated ES cells were obtained from mouse blastocysts in 1981 (Evans & Kaufman, 1981; Martin, 1981). After 17 years, James Thomson's team described the first human ES cells that were isolated (using a similar protocol as for mice) from fresh or frozen embryos obtained through *in vitro* fertilisation for reproductive purposes, which were donated by parents (Thomson et al., 1998). In same year, Shamblott & colleagues isolated pluripotent cells from human embryonic and foetal gonads. Since then, it has been possible to obtain several immortal ES cell lines from mice and humans using feeder layers of mouse foetal fibroblasts in presence of leukaemia inhibitory factor (LIF). These immortal cell lines present the same *in vivo* properties *in vitro* and grow indefinitely in laboratories under specific conditions. However, differences between mouse ES cells and human ES cells have been found, and subsequently, several lines of human ES cells has been described and added to a record that can be found on homepage http://stemcells.nih.gov/research/registry.

The criteria used to define cell lines as ES cells are the following: a) must be derived from pre-implantation embryos, b) must have prolonged proliferation in undifferentiated state, and c) must be able to differentiate into cells of the three germ layers, even after prolonged culture. In this manner, some investigators observed that ES cell lines subcutaneously injected into SCID mice could give rise to distinct tissues, such as neural epithelium (ectoderm); cartilage, bone and smooth/striated muscle (mesoderm); and gut (endoderm) tissues (Pera et al., 2000; International Stem Cell Initiative, 2007).

Human ES cells can grow as colonies, and they express certain undifferentiated stem cell markers, such as transcription factors Oct-4 (octamer-binding protein 4), Sox-2 and Nanog, as well as cell surface proteins SSEA (Stage Specific Embryonic Antigen)-3, SSEA-4, TRA (Tumour Rejection Antigen)-1-60, TRA-1-80 and alkaline phosphatase (Miguel et al., 2010). These cells have normal and stable karyotypes during continuous passaging and can be kept in their undifferentiated state for multiple cell divisions when cultured under specific conditions *in vitro* (Shamblott et al., 1998; Shamblott et al., 2001; Amit et al., 2000). On the other hand, when grown in conditioned media, ES cell lines can be induced to differentiate in tissue-specific manners or into several other tissues (embryoid bodies), which simulates

the development of a pre-implanted embryo. Moreover, human ES cell lines have been used to generate cells of different lineages, including neurons, cardiomyocytes, blood progenitors, hepatocytes, retinal precursors and β-cells of pancreatic islets (Cowan et al., 2004).

These remarkable characteristics of human ES cells have generated great interest among researchers around the world, and studies of ES cell lines have been conducted to elucidate the molecular mechanisms involved in totipotency and pluripotency of stem cells, as well as to develop methodologies of ES cell differentiation into different tissues. Future manipulation of these pathways involved in cell potency may serve as the basis for modification of adult tissue-specific stem cells into less differentiated cells, thereby increasing their ability to differentiate and proliferate. Furthermore, pluripotent stem cell lines could allow for the testing of new medications in several cell types, thereby aiding the advancement of drug development process. As a result, only drugs that are both safe and have beneficial effects in various tests on these cell lines will be forwarded to animal experimentation and human trials.

The most fascinating development in history of ES biology is the generation of ES-like cells, called "induced pluripotent stem (iPS) cells", that do not involve destruction of human embryos. The destruction of embryos has caused huge religious and ethical problems and significant public unease. Several countries (e.g., Austria, Germany, Italy, and Brazil) have introduced legislation prohibiting human embryo research. However, in 2006, Takahashi and Yamanaka demonstrated that retroviral-mediated overexpression of a set of only four pluripotent genes, Oct4, Sox2, c-Myc and Klf4 (Kruppel-like factor 4), was sufficient to reprogram murine fibroblasts to ES-like cells. The first iPS cells generated were from mice, but within months, the same group described the generation of human iPS cells (Takahashi et al., 2007; Yu et al., 2007).

2.2 Adult tissue-specific stem cells

Adult tissue-specific stem cells are indispensable components of tissue homeostasis because they support ongoing tissue regeneration by replacing cells that are lost due to natural cell death (apoptosis) or injury (Spradling et al., 2001). These cells are undifferentiated but are found in adult differentiated tissues, and most of them have self-renewal capacity throughout the entire lifetime of an organism; in addition, they can give rise to other adult tissue-specific stem cells and precursor cells that can produce mature differentiated cells by asymmetric division (Weissman et al., 2001).

Adult tissue-specific stem cells represent a small percentage of total cellularity. Previous studies have reported many kinds of adult tissue-specific stem cells, and their experimental assays have revealed different characteristics of stem cell behaviour. Adult bone marrow, for example, contains at least three distinct types of adult multipotent stem cells: haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). The HSCs are quite rare, with a frequency about of 1 in 10,000 of all bone marrow cells, and the selection of human HSCs is based on combined expression of CD34 and aldehyde dehydrogenase (ALDH) (Mirabelli et al., 2008). The MSCs are also scarce in human bone marrow aspirates (1-20 in 10.000), and they decrease in quantity with age. Human MSCs express a wide range of markers, such as CD105, CD73, CD90, CD29, HLA class-I, CD44, CD49e, CD34, CD31, CD14, CD19, and HLA class II. *In vitro,* MSCs adhere to plastic surfaces and can differentiate into bone, cartilage or fat. Finally, bone marrow-derived EPCs are a unique population of blood mononuclear cells that have a role

in postnatal neovascularisation during wound healing and tumour development. The identification of human EPCs relies on expression of VEGFR2, c-kit, or CD34.
In small intestine, there is estimated to be around 10 stem cells near the bottom of the crypt out of a total crypt population of less than 300 cells. Small intestinal stem cells are multipotent and can generating Paneth cells, mucin-producing goblet cells, columnar enterocytes and enteroendocrine cells (all four lineages) (Sancho et al., 2004). In skeletal muscle, satellite stem cells are unipotent and the major source of myogenic cells for growth and repair, and they comprise around 5% of adult muscle nuclei present within muscle fibres. The stem cell markers of these cells include M-cadherin and transcription factor Pax-7 (Goldring et al., 2002).
Kidney stem cells compose 0.8% of all cortical cells and have been isolated from the cortical interstitium. They have been shown to express Pax-2, CD133, and classical mesenchymal markers such as CD73, CD29, and CD44. *In vitro*, these cells have been shown to differentiate into epithelial and endothelial cells (Gupta & Rosenberg, 2008). In mammary glands, stem cells are bipotent, generating luminal and myoepithelial cells, and they can be identified from terminal ductal lobulo-alveolar units by expression of CD44, CK19, or epithelial surface antigen-positive (ESA) or by negative expression of common acute lymphoblastic leukaemia antigen ($CALLA^-$) (Clarke et al., 2003). In the skin, epithelial stem cells are multipotent and give rise to epidermal progenitors for tissue repair and also hair matrix progenitors, which generate the hair shaft. These stem cells can be identified by CD34 and α6 integrin expression (Blanpain et al., 2004).
In summary, there are two criteria to define functional adult tissue-specific stem cells: self-renewal capacity and multipotentiality, which is most important when investigating new adult tissue-specific stem cells populations. However, there are controversies regarding the identity and functional potency of stem cells in some organs, such as in lung and pancreas. Kim & colleagues (2005) identified bronchoalveolar stem cells, but in 2009, Rawlins & colleagues revealed that these stem cells do not contribute to alveoli lineages during normal homeostasis and regeneration. Similar controversies have been debated in endocrine pancreas, which is composed of islets of Langerhans formed by α, β, δ and PP cells. Although embryonic pancreatic duct stem cells have the plasticity to give rise to endocrine and exocrine lineages, adult pancreatic duct stem cells generate acinar cells (exocrine pancreas) but not insulin-producing β-cells (Solar et al., 2009). Therefore, the distinction between adult tissue-specific stem cells characteristics, as well as their true potential, remains unclear. Thus, these facts should lead to future investigations aiming to clarify whether there are other common features among adult tissue-specific stem cells and to define the true roles of these cells that possess a wide *in vivo* differentiation potential.

2.3 Stem cell niches

Stem cells reside in a special microenvironment termed a "niche," which varies in nature and location depending on tissue type. The concept of niche was first proposed by Schofield (1978) to describe how bone marrow-derived haematopoietic stem cells, while in proliferative state, had increased proliferative potential when compared to haematopoietic cells that reside in spleen (spleen colony-forming cells, CFU-S). Historically, the term niche is typically used to identify the location of stem cells. Currently, the definition of niche is broader and includes the cellular components of microenvironment surrounding the stem cells, in addition to the signals that are emitted by these stromal support cells *in vivo*. Furthermore, the stem cell niche can be defined as a group of cells in a specific tissue whose

aim is the maintenance of adult tissue-specific stem cell pool (Spradling et al., 2001). Therefore, the niche provides a mechanism to precisely balance the production of stem cells and progenitor cells to maintain tissue homeostasis.

Although there are specific niches for each stem cell type and these special microenvironments appear to be structurally and functionally diverse, it is possible to find common features among them. The pioneering system used to study HSCs is the bone marrow, and currently, the HSC niche is conceptually divided into three parts: osteoblastic zone, vascular zone, and zone neighbouring haematopoietic stem cells. Another example is the neural stem cell niche, which supports neurogenesis in the adult brain and can be found in both subventricular zone (SVZ) and subgranular zone (SGZ) of hippocampal region. In these zones, the endothelial cells that form blood vessels and the specialised basal lamina are essential cellular components of neural stem cell niche (Doetsch, 2003).

The niche functions as a physical anchor for stem cells by generating factors that control stem cell proliferation and fate. Calvi & colleagues (2003) demonstrated in bone marrow that osteoblastic cells located in lining of endosteal surface express N-cadherin and physically attach HSCs, thereby acting as a regulatory component with capacity to control the HSCs number. In general, other mediating adhesion molecules can anchor cells to extracellular matrixes; for example, integrins, certain types of collagen (I-V), cadherins, and β-catenin play an important role in stem cell/microenvironment interaction (Simmons, 1997). With regard to the brain, it is known that endothelial cells can attach to astrocytes, which have stem cell features and give rise to neuroblasts in the SVZ and SGZ, thereby producing signals that control the stem cell population (Doetsch, 2003).

Inside the niche, stem cells are often in the quiescent state in terms of cell cycle. This quiescent state is vital for ensuring maintenance of tissues throughout life and prevents premature extinction of the stem cell pool caused by numerous conditions of stress experienced by cells. Niches for quiescent stem cells are located in hypoxic tissue regions that are poor in vasculature. For example, in the bone marrow, quiescent HSCs are maintained in osteoblastic niche (hypoxic niche), while the HSCs and haematopoietic progenitors in highly proliferative state are found in vascular niche (oxygenic niche) (Yin & Li, 2006; Jang & Sharkis, 2007). In response to injury, a microenvironmental change in tissue might actively signal to the niche to mobilise quiescent cells, which would induce the proliferation and transition of stem cells to the vascular niche area. Furthermore, after irradiation treatment, surviving HSCs must enter the proliferative stage to produce progenitor cells that will give rise to differentiated cells. Nonetheless, HSCs tend to exit of the cell cycle once the haematopoietic cells have been compensated (Suda et al., 2005).

Signalling pathways and molecular mechanisms can control stem cell fate decisions through a delicate balance between regulatory factors. To ensure appropriate control of cellular behaviour, the intrinsic stem cell factors must be subjected to microenvironmental regulation or extrinsic factors provided by niche. Therefore, both intrinsic and extrinsic factors are required to maintain stem cell properties and to direct stem cell self-renewal and differentiation. Several signalling molecules have been shown to be involved in maintenance of stem cell niche. For example, the Wingless-related protein (Wnt) signalling pathway is important for stem cell self-renewal, but expression of Wnt pathway inhibitors, such as axin, leads to inhibition of stem cell proliferation (Nusse, 2008). Studies using gene targeting have demonstrated that the bone morphogenetic protein (BMP) signalling pathway has an important role in the suppression of Wnt signalling pathway, thereby providing balanced control between stem cell activation and self-renewal. Homeobox genes induced by Wnt

activity, such as HoxB4 and Notch, can also participate in the process of stem cell expansion. The Notch pathway is important for maintaining stem cells in an undifferentiated state. Signals mediated by transformation growth factor beta (TGF-β) and family members, including BMPs, Nodal and activins, have been implicated in the maintenance and differentiation of various types of adult tissue-specific stem cells (Watabe et al., 2008). In short, there are several growth factors that operate at different stages in the stem cell lineages, indicating the need for strict control over cell division within the stem cell niche.

For maintenance of an adequate number of stem cells within their niches and meeting the demand of differentiated cells within the surrounding tissue, it is essential that there be a strict regulation of balance between symmetric and asymmetric stem cell divisions. The niche contributes to orientation of asymmetric division, with the aim of controlling the flow and direction of committed progeny. As a result, one daughter cell is destined to become a stem cell, stays in stem cell niche, retains its self-renewal properties and receives inhibitory differentiation factors. In contrast, the other daughter cell leaves the niche to become committed to proliferate and differentiate along a determinate lineage (progeny cell), and it can receive differentiation signals that can overcome this state to eventually become a functionally mature cell.

In general, both embryonic and adult stem cells must have the capacity to grow and differentiate in response to signals emitted by their specific niche. To sustain these functions throughout the organism's life span, there are essential mechanisms that control adult tissue-specific stem cells and the nature of a possible tumour transformation (Iwasaki & Suda, 2009).

3. Stem cells and cancer

3.1 Brief historical review of stem cells and cancer

The resemblance between stem cells and cancer cells was observed a long time ago. The first study concerning hypothesis of cancer origin from a rare population of normal cells with stem cell properties was proposed almost 150 years ago (Durante, 1874; Wicha et al., 2006). At that time, Cohnheim (1875) also proposed the hypothesis that stem cells could be misplaced during embryonic development and become the source of tumours that would be formed later in life.

This subject was revived over 40 years ago when a several investigators confirmed the CSC hypothesis by demonstrating that a single tumour cell could generate heterogeneous progeny and give rise to a new tumour through studies performed in tumours derived from ascites fluid in rats and teratocarcinomas and leukaemias in mice (Bruce & Van Der Gaag, 1963; Kleinsmith & Pierce, 1964; Makino, 1956). In this vein, Park et al. (1971) observed certain myeloma tumour stem cells in mice using a primary cell culture assay, and Hamburger and Salmon (1977) corroborated the hypothesis that some cancers contain a small subpopulation of cells that are similar to normal stem cells. They observed in primary bioassays that the expansive growth of malignant lesions suggests the presence of a CSC population with stem cell properties, including indefinite proliferation.

In animal models, the ability of a small population of cells to originate a new malignant neoplasia was demonstrated in a classic experiment utilising transplantation of cells from human acute myeloid leukaemia (AML) that expressed certain cell surface markers associated with normal haematopoietic stem cells (Lapidot et al., 1994). The authors showed that these transplanted cells could initiate leukaemia in non-obese diabetic/severe combined

immunodeficient (NOD/SCID) mice, while other isolated cells could not. Since then, this assay has become the standard method for determining whether cell populations isolated from solid tumours are CSCs.
Based on ability of diverse purified populations to form leukaemia in NOD/SCID mice, various studies embarked on a search for stem-like cells in leukaemias. Bonnet and Dick (1997) recognised in AML that the injection of a small subset of leukaemic cells with a primitive haematopoietic progenitor phenotype ($CD34^+CD38^-$) resulted in leukaemias that could be serially transplanted into secondary recipients, and they also observed their ability to perpetually self-renew. Since then, putative CSCs have been isolated from many other tumour types, including brain, breast, colon, pancreas, prostate, lung, and head and neck cancer (Collins et al., 2005; Dalerba et al., 2007; Kim et al., 2005; Li et al., 2007; Prince et al., 2007).

3.2 Cancer stem cell hypothesis

The research fields of cancer and stem cell biology share common features regarding cellular proliferation properties. In humans, normal adult multipotent stem cells are usually self-renewing. This self-renewal ability allows stem cells to produce at least one progeny cell with a similar developmental capacity, and available current lines of evidence indicate that this cell population, through initial genetic or epigenetic alterations, can become the cells responsible for the development of several tumours through a progressive establishment of a CSC population.
It is widely accepted that genetic instability drives malignant transformation. The stem cell origin of cancer hypothesis considers that stem cells or other differentiated cells that have acquired self-renewal ability tend to accumulate genetic alterations and evade the strict control of their microenvironment, thereby giving rise to tumoural evolution (Shipitsin & Polyak, 2008). Thus, the CSC model suggests that tumour progression, metastasis and recurrence after therapy can be driven by a rare subgroup of tumoural cells that have the capacity to self-renew, while the bulk of the tumour does not have this capacity. Therefore, the deregulation of this self-renewal process leading to stem cell expansion may be a key event in carcinogenesis, and while self-renewal can drive tumorigenesis, the differentiation process may contribute to tumour phenotypic heterogeneity (Kakarala & Wicha, 2008; Shay & Wright, 2010).
Normal adult stem cells have relatively long telomeres compared to more differentiated somatic cells, they are usually quiescent or proliferate more slowly than their differentiated progeny, and they have increased longevity; for this reason, they are exposed to more damaging agents than more differentiated cells over time. Thus, they accumulate mutations that are then transmitted to the rapidly proliferating progeny (Dontu et al., 2003). Mutations in the DNA of normal adult stem cells appear to be the initiating events in several types of malignant tumours, and some of the strongest evidence supporting this hypothesis is that a specific group of cells can be prospectively isolated based on their peculiar features; later, these cells can be serially transplanted into immunodeficient mice (Alison et al., 2010).
If normal adult stem cells are the founding cells of several cancer types, then CSCs probably inherit many of their characteristics. The CSCs are a population of cells that are more tumourigenic than the bulk tumour population and can be defined mainly through the expression of unique properties, such as specific detoxification enzyme systems, molecular surface markers, and embryonic signalling pathways (Alison et al., 2010). The main hallmarks of CSCs are their properties of self-renewal, their ability to generate tumours from

very few cells, their slow cell division rate, their ability to give rise to phenotypically diverse progeny, and their selective resistance to radio- and chemotherapy (Reya et al., 2001).
The self-renewal and differentiation characteristics of CSCs lead to the production of all cell types in a tumour, thereby generating wide heterogeneity (Campbell & Polyak, 2007). The differentiated cells constitute the bulk of the tumour but are not usually tumourigenic due to their lack of self-renewal capacity and limited proliferation potential (Ginestier et al., 2007). However, it has been shown that the switch to carcinogenesis can occur in either the stem cells or their differentiated progeny, which sometime acquire the ability to self-renew (Dontu et al., 2003). In several tissue systems, it has been proposed that certain committed progenitor cells might become CSCs through a dedifferentiation process, which would occur by acquisition of stem cell properties (Cobaleda et al., 2007).
Further evidence indicates that stem cells can play a role in carcinogenesis. A previous study showed that there are similarities seen between normal stem cells and cancer cells. In addition to self-renewal capacity, these characteristics include activation of anti-apoptotic genes, production of more differentiated cells, induction of angiogenesis, resistance to conventional radio- and chemotherapy (e.g., due to active telomerase expression, high ALDH expression, elevated membrane transporter activity), and ability to migrate and disseminate in metastasis (Wicha et al., 2006).
Conversely, there are some important differences between these two types of cells, which also corroborates the CSC hypothesis. While normal stem cells are chromosomally stable and contain a normal diploid genome, cancer cells have a significant number of chromosomal rearrangements and are almost always characterised by aneuploidy. Moreover, cancer cells may lack cell cycle checkpoint activity that allows them to completely growth arrest. More importantly, a major difference that has been found between normal adult tissue stem cells and cancer cells is that stable telomere length is maintained in malignant cells (Shay & Wright, 2010).
Notwithstanding the evidence that has been found, the extensive characterisation of murine CSC models has not yet resulted in the identification of their human counterparts for all tumour types. More than one CSC type with a different phenotype per tumour type could be likely, which makes the search for a definitive cancer stem cell hypothesis even more difficult.

3.3 Isolation and purification of CSCs

Although the concept that cancers arise from stem cells was first proposed more than 150 years ago, it is only recently that advances in stem cell biology have allowed for more direct testing and validation of the CSC hypothesis. It is well settled that CSCs share some properties expressed by normal stem cells. Current methods for determining whether cell populations isolated from solid tumours are CSCs consist of purification of these cells from tumour samples based on the properties of normal stem cells, such as expression of specific cell surface markers of stemness (Al-Hajj et al., 2003), their ability to form spheres in culture (Dontu et al., 2003), membrane efflux activity through drug-efflux pumps (Goodell, 2002), and enzymatic activity detection of cytoprotective enzymes as aldehyde dehydrogenase 1 (ALDH1) (Nagano et al., 2007). Additionally, purified cells are then tested for the capacity to originate tumours when injected into immunodeficient mice.
The tumour initiation aspect of CSCs refers to the ability of these cells (at a reduced number) to originate malignant tumours in immunocompromised mices. The expression of some specific cell surface markers has been investigated to facilitate the identification and

purification of CSCs, and there are currently several stem cell markers that are shared by CSCs in multiple human tumour types, but this issue is best addressed in the next section of this chapter.

Hoechst 33342 membrane efflux activity is a discriminating characteristic of quiescent stem cells that is lost when these cells enter in cycle, and this activity allows the identification through flow cytometric analysis of a small stem-like cell population designated as a side population (SP). In fact, it has been hypothesised that the main characteristic of the SP is a universal stem cell phenotype (Zhou et al., 2001). Although heterogeneous, SP cells are observed in primitive retinal and cardiac cells (Bhattacharya et al., 2003; Hierlihy et al., 2002); in epidermal, neural, mammary, and haematopoietic stem cells; and also in certain embryonic stem cells (Zhou et al., 2001).

The SP cells are also associated with resistance to toxins and drugs, and this characteristic is a result of increased expression of membrane transporter proteins (ABC drug transporters), such as P-glycoproteins or BCRPs (breast cancer resistance proteins). In addition to acting as functional regulators of stem cells, they contribute to the defence against damaging agents through the elimination of xenobiotic toxins (Zhou et al., 2001). Thus, tumours might have a population of drug-resistant pluripotent cells that can survive chemo- and radiotherapy and subsequently repopulate the tumour (Charafe-Jauffret et al., 2008).

The *ALDH* gene superfamily encodes a family of $NAD(P)^+$-dependent metabolic enzymes that are involved in detoxifying a wide variety of aldehydes to their corresponding weak carboxylic acids. ALDH activity, as a CSC specific marker, was discovered recently after great investigation, especially of haematological and breast malignancies (Charafe-Jauffret et al., 2009; Ginestier et al., 2007), although it has also been implicated as a CSC marker in several others tumour types (Ma et al., 2008). In human breast cancer cell lines, high ALDH activity has been used successfully to select CSCs (Charafe-Jauffret et al., 2009).

In vivo tumourigenic xenotransplantation assays performed in immunodeficient mice (NOD/SCID) are currently the gold standard for successful CSC isolation and purification These mice have a lack of major elements of the immune system, and therefore, they do not reject human cells. Because a large amount of human tumour cells must be xenotransplanted into immunodeficient mice to originate tumours, it was initially thought that CSCs were infrequent in tumours. However, this might be because the human cells in this assay are in a foreign microenvironment, as transplantation of mouse tumour cells into other mice indicates that CSCs can be quite common in some determined cancers. This *in vivo* assay is frequently supplemented by a clonogenicity assay that assesses the ability of the cells to form spheres and determines the frequency of which these isolated cells can form colonies (neurospheres, mammospheres, or colonospheres) when they are plated at a low density under non-adherent conditions in semi-liquid medium. This technique is based on the unique property of stem cells to survive and grow in serum-free suspensions, while differentiated cells undergo anoikis and die under these conditions. The resulting spheres of cells can be then serially passaged for experiments, originating secondary and tertiary spheres with a cellular composition resembling that of primary spheres and proving their self-renewal capacity (Alison et al., 2011).

Therefore, the standard procedures for the isolation of CSCs have been similar in several investigations. Among the most used *in vivo* models is tumour cell fractionation according to cell-surface markers with stem cell characteristics, which is followed by a clonogenicity assay to verify the sphere formation capacity and their implantation into NOD-SCID mice to assess xenograft growth and cellular composition (Shipitsin & Polyak, 2008).

3.4 CSCs markers in human tumours

CSCs have been prospectively isolated on the basis of the expression of specific surface molecular markers, and recent interest in CSCs arose from experiments suggesting that cells with stem-like properties can be sorted from solid tumours based on the expression of these markers. However, there is still no apparent consensus regarding the more reliable markers associated with the identification of CSC phenotype in some particular solid tumours, such as in gastrointestinal carcinomas (Alison et al., 2010). In haematological malignancies, the consensus is that the $CD34^{+}CD38^{-}$ phenotype can identify most of the CSCs, and the accumulated evidence found in other tumour types indicates that markers such as cytoprotective enzymes, cell-adhesion molecules, and drug-efflux pumps can be associated with a CSC phenotype. The main surface markers currently associated with stem cells and CSCs include CD133, CD44, and CD24 (Al-Hajj et al., 2003; Hermann et al., 2007; O'Brien et al., 2007).

The CD133 cell surface marker, also called prominin 1 (PROM1), was discovered as a marker of normal haematopoietic stem cells and was later used to purify putative CSCs in several tumour types. In brain tumours, Singh et al. (2004) found that $CD133^{+}$ cells could successfully grow under non-attachment conditions with neurosphere-like formations, whereas CD133- cells could not. According to other studies, CD133 also has been shown to play a role in migration and asymmetrical stem cell division (Beckmann et al., 2007).

The CD44 marker is a transmembrane glycoprotein cell surface receptor for hyaluronic acid that is frequently expressed as several isoforms, and it is involved in cell adhesion, migration, and metastasis (Shipitsin et al., 2008). It has been used to identify putative CSCs in breast tumours (Shipitsin et al., 2008), as well as in other tumour types, such as prostate (Collins et al., 2005), pancreatic (Li et al., 2007), and head and neck carcinomas (Prince et al., 2007). Shipitsin et al. (2007) found that CD44+ tumoural mammary cells were associated with more invasive, proliferative, and angiogenic tumour status, thereby predicting more aggressive tumoural cell behaviour. Furthermore, there was a correlation between CD44+ tumoural cells and decreased patient survival (Shipitsin et al., 2007).

CD24 is a mucin-like adhesion molecule expressed by neutrophils, pre B lymphocytes and a large variety of solid tumours. Functionally, CD24 enhances the metastatic potential of malignant cells because it has been identified as a ligand of P-selectin, an adhesion receptor on activated endothelial cells and platelets. It also enables cancer cells to bind to platelets, and these tumour-platelet thrombi protect cells in the bloodstream and in turn facilitate tumour invasion through interactions with endothelia. Lim & Oh (2005) investigated the role of CD24 in various human epithelial neoplasias and demonstrated that intracytoplasmic CD24 expression was found to be highly associated with adenocarcinomas of the colon, stomach, gallbladder, and ovaries. Positive or negative CD24 expression also has been used in combination with other markers to identify putative CSCs in tumours, and some studies have defined the phenotype of pancreatic CSCs as CD24+/CD44+ (Li et al., 2007). However, in breast and prostate cancer, putative CSCs were found with a CD24-/CD44+ phenotype (Al-Hajj et al., 2003; Hurt et al., 2008).

These investigations suggest that diverse stem cell markers can be expressed by CSCs in different tumours, and each tumour may express a phenotypic pattern with a specific CSC marker combination. The significance of these observations in most human cancers remains to be determined. Table 1 shows the most prevalent and specific CSC phenotypes according to stem cell markers in tumours from different organs.

STEM CELL MARKER	TUMOR
CD34/CD38	Acute myeloid leukemia
CD44/CD24-/ALDH	Breast carcinoma
Side Population (SP)	Bladder carcinoma
CD133, CD44, EpCAM, ALDH	Colorectal carcinoma
CD133, Side Population (SP)	Endometrial carcinoma
CD133	Ewing's sarcoma
CD44	Gastric carcinoma
CD44, Side Population (SP), ALDH	Head and neck carcinoma
CD133, CD44, ALDH	Hepatocellular carcinoma
CD133, Side Population (SP), ALDH	Lung carcinomas (non-small cell and small cell)
CD133	Medulloblastoma, Glioma
CD133, CD44, CD24, ALDH, EpCAM	Pancreatic carcinoma
CD133, CD44, ALDH	Prostate carcinoma

Table 1. Cancer stem cell phenotypes according to stem cell markers expression in tumors of different organs.

3.5 The CSC niche

Tumours in general have a hierarchical organisation that can be dynamically regulated by the surrounding microenvironment. In adults, the niche prevents tumorigenesis through strict control of stem cell behaviour and maintenance of the balance between self-renewal and differentiation, as well as between quiescence and proliferation. Accordingly, intrinsic mutations that regulate self-renewal, including those in the Wnt, Notch and Hedgehog pathways, can lead to stem cells escaping from niche control. These mutations can initiate dysregulation of CSCs and result in tumorigenesis. Thus, a specialised microenvironment, consisting of cells, matrix proteins and growth factors, is thought to physically restrain stem cells and enable them to maintain their stemness by providing the required factors.

The CSC hypothesis suggests that CSCs reside in a supportive niche with a poor vascular supply and frequently hypoxic conditions, which would result in poor drug perfusion and therefore contribute to an ineffective chemotherapy response (Deonarain et al., 2009). Furthermore, in addition to normal adult stem cells, CSCs appear to be regulated through molecular stimuli that are supplied from the microenvironment by neighbouring connective tissue cells, mainly the fibroblast-like (mesenchymal) and endothelial cells (Alison & Islam, 2009). There is increasing evidence that disruption of epithelial homeostasis, whereby tumour cells acquire a mesenchymal phenotype, is necessary for cancer development. In colorectal cancer, for example, the promotion of Wnt signalling in CSCs requires co-stimulation by hepatocyte growth factor (HGF) secreted by stromal fibroblasts (Vermeulen et al., 2010).

It has been established that the microenvironment adjacent to blood vessels can serve as the main CSC niche that controls some aspects of CSC behaviour, and this microenvironment is also associated with the highest tumour proliferation rates (Alison & Islam, 2009). In brain

tumours, CSCs identified through expression of CD133 and nestin were observed to be concentrated in a niche close to capillaries (Calabrese et al., 2007).
According to Charafe-Jauffret et al. (2008), genetic and epigenetic mechanisms in the progenitor cells, in addition to environmental influences in the niche where these cells grow, may contribute to the cellular heterogeneity found in the malignant neoplasms. Recently, it has been suggested that the microenvironment adjacent to tumours can regulate asymmetric versus symmetric divisions (Alison & Islam, 2009).

3.6 The embryonic self-renewal pathways

Different mutations associated with cancer occur in pathways that govern stem cell maintenance, suggesting that dysregulation of normal mechanisms of stem cell functionality may also be involved in carcinogenesis. Thus, the signalling pathways that regulate normal stem cell development and proliferation can be identical to those that promote carcinogenesis, possibly through initiation of CSC proliferation (Reya et al., 2001).
The CSCs generally have or can re-acquire the self-renewal mechanisms needed for their maintenance, development and expansion. In this manner, the embryonic signalling pathways, such as Wnt, Notch, Hedgehog (Hh), Bmi-1, PTEN, and p53, are fundamental for normal stem cell development and organogenesis, and these same pathways are also involved in driving CSC activity (Takebe & Ivy, 2010).
The Wnt pathway is clearly important for the preservation and self-renewal of stem cells. Wnt signalling is known to regulate cell fate decisions and influence morphology, proliferation, differentiation, apoptosis, migration, and stem cell self-renewal (Turashvili et al., 2006). Moreover, Wnt proteins can assist in maintaining stem cells in an undifferentiated state within their niche, and defects in the Wnt pathway have been observed in breast and colon cancer carcinogenesis (Olsen et al., 2004).
In the same manner, the Hh pathway is associated with the maintenance of stem cells in several malignant neoplasms, including myeloid leukaemia (Zhao et al., 2009), multiple myeloma (Peacock et al., 2007), and colorectal cancer (Varnat et al., 2009). The Hh pathway is one of the main pathways that control stem cell fate, self-renewal, and maintenance. In human gliomas, Hh signalling represents a new therapeutic target through its essential control of the behaviour of glioma CSCs (Clement et al., 2007). Through the use of both *in vitro* culture systems and NOD/SCID mice, Liu et al. (2006) found that the Hh pathway, together with the polycomb protein Bmi-1, play important functions in regulating self-renewal of both normal and malignant human mammary stem cells. Furthermore, in agreement with Byrd & Grabel (2004), Hh signalling can target endothelial stem cells directly or stimulate blood vessel support cells to produce vascular growth factors.
Recently, the Notch pathway has attracted increased consideration because several Notch receptors and ligands are frequently overexpressed in tumours, as has been observed in breast and cervical cancers (Nickoloff et al., 2003). In a study performed in human breast cancer, the high expression of Notch intracellular domain in ductal carcinoma in situ (DCIS) has been shown to correlate with reduced disease-free survival time at five years after surgery (Farnie & Clarke, 2007). In experimental gliomas, Notch signalling activation appears to be dependent on nitric oxide (NO) released by endothelial cells of the perivascular niche, which is important for stem-like character promotion and CSC maintenance (Charles et al., 2010).
Oncogenic or tumour suppressor genes, such as *HER-2*, *PTEN* and *p53*, have also been implicated in the regulation of CSC self-renewal. These genes are usually impaired in CSCs,

leading to uncontrolled self-renewal, which in turn can generate resistant tumours in relation to current therapeutic approaches.

3.7 Targets for therapy

Cancer progression can be viewed as an evolutionary process that generates multiple novel clones, each with a specific identity. If CSCs are the origins of tumours, then these are the cells that must be specifically eliminated for effective therapy.

Currently, it is well known that several cancers are peculiarly resistant to conventional radiotherapy and chemotherapeutic drugs that typically kill the majority of cancer cells. These clinical responses may reflect the targeting of the bulk of non-stem cell population. On the other hand, there are several specific key intracellular signalling pathways implicated in CSC self-renewal and proliferation processes that appear to be promising therapeutic targets, and a wide and diverse range of advances to eliminate the CSCs in malignant neoplasms are becoming evident; however, although several seem promising, a major difficulty will be specifically targeting these cells to avoid undesirable toxicity *in vivo* (Oliveira et al., 2010).

An ideal therapeutic strategy might be to sensitise CSCs to chemo- and radiotherapy by inhibiting their stemness properties and then by promoting direct cytotoxicity. Furthermore, as previously mentioned, the CSC population is driven by embryonic signalling pathways, and the targeting of these pathways could result in increased likelihood of a successful cure. In this vein, several drugs directed toward the inhibition of embryonic signalling pathways are under development, and strategies based on targeting intracellular pathways active in CSCs, such as Wnt, Bmi-1, Hh, Twist, and Notch, have all been currently considered for therapeutic investigation.

Wnt signalling is a key pathway in cell development and has been shown to be upregulated in about 50% of cancers (Deonarain et al., 2009). Inhibition of the Wnt/β-catenin signalling pathway has been shown to be effective at blocking epidermal squamous cell carcinoma development, and a new approach to antagonise Wnt signalling involves the stabilisation of axin, thereby maintaining the β-catenin destruction complex (Huang et al., 2009). The Bmi-1 molecule has been demonstrated to have a role in lung tumorigenesis and bronchioloalveolar stem cell expansion, and Hh signalling has been shown to be critical for normal lung development, lung injury repair, and lung carcinogenesis (Peacock & Watkins, 2008). Furthermore, another study has shown that the Hh pathway can maintain a tumour stem cell compartment in multiple myeloma (Peacock et al., 2007). The development of specific Hh inhibitors, such as cyclopamine, is currently underway for breast cancer, and clinical trials utilising these chemotherapeutical agents are in the planning stages (Liu et al., 2006; Kakarala & Wicha, 2008). Similarly, aberrant Notch signalling that has been observed in several human cancers, such as human T-cell acute lymphoblastic leukaemia, cervical cancer, and breast cancer, suggesting that inhibition of Notch may represent a potential effective therapeutic target (Nickoloff et al., 2003). Telomerase inhibition also could be another effective anti-cancer therapeutic approach that would target both the proliferating CSCs as well as the bulk of the cancer cells (Shay & Wright, 2010).

The inhibition of the Epithelial-mesenchymal transition (EMT) process through transcriptional pathways, such as Snail and Twist, can slow the generation of CSCs with metastatic capacity. There has been intense investigation with regard to further therapeutic strategies based on blocking molecules at the cell surface that are implicated in invasion, migration and metastasis, such as integrins, CXCR-4, and CD44. In basal-like breast cancer,

the inhibition of Wnt signalling was shown to block stem cell self-renewal and also to repress the expression of the CDH1 repressors Slug and Twist, which in turn, block metastasis dissemination. In ovarian tumours, it was observed that $CD44^+$ cells expressing markers of pluripotent stem cells might have a selective advantage for dissemination through their adherence to the hyaluronic acid pericellular coat of adjacent mesothelial cells (Bourguignon et al., 2008).

As therapeutic resistance of CSCs can often be directly attributed to the activity of ALDH or ABC surface transporters, additional approaches based on targeting these molecules might sensitise CSCs to current standard adjuvant therapies. In addition, given that several types of cancer cells have a specific microRNA (miRNA) expression profile, the manipulation of mRNA expression levels through miRNAs is another promising strategy by which to target CSCs (Alison et al., 2010).

The stem cell status of the cells of certain cancers can be dynamically regulated by the tumour microenvironment. Like normal stem cells, CSCs depend on support from the vascular and stromal niche for survival. As the microenvironment adjacent to the blood vessels can serve as the local CSC niche, another interesting alternative that is being addressed is the targeting of the vasculature, and this strategy could destroy the niche as well as the tumour bulk (Calabrese et al., 2007).

Throughout cancer evolution, it is likely that the genetic instability initiated by several selection pressures, such as hypoxia, immune or nutritional status, may result in the selection of new phenotypically malignant clones with increased genetic and epigenetic alterations. These malignantly transformed cells can acquire a selective growth advantage over their normal cell neighbours through resistance to apoptosis or higher proliferation rates, and subsequently, a specific clone of cells will develop. Increasingly, additional tumour progression with mutations and clonal expansion may give rise to more abnormal clones. In this manner, the more advanced tumours exhibit a complex heterogeneous picture, whereas early tumours may be more homogeneous because they did not have appropriate time to develop this clonal diversity. The existence of these clones can then eventually compromise a targeted therapy against a specific CSC clone because some of the cells would tend to expand due to a mutation for selective growth or survival superiority (Alison et al., 2011). This is an important problem that must be addressed when designing therapies against CSCs.

The correct identification and targeting of signalling mechanisms that are specific for CSCs could provide an opportunity for selective targeting of these cells. In fact, there is currently a need for the development of highly specific therapies that target CSCs. Later, these therapies will need to be tested in the appropriate oncological patient population, along with the use of adequate pharmacodynamic markers. However, the use of combined targeting of different CSC pathways, together with the commonly used radio- and chemotherapy applications and other types of targeted therapies, remains to be further explored in cancer therapy.

3.8 The influence of CSCs on tumour prognosis

If CSCs are associated with carcinogenesis, it follows that their frequency in primary tumours correlates with the extent of tumour invasion and dissemination and consequently, with patient prognosis. Generally, it is believed that elevated stemness characteristics and a high proportion of CSCs in tumours are associated with a worse prognosis.

Tumoural recurrence, metastasis and survival might be determined by the behaviour of the more resistant CSC population. In most cases, patients with tumours expressing high levels

of molecules associated with CSCs have a poorer clinical outcome than patients with tumours that express low levels of thee molecules. In breast cancer, a high prevalence of CSCs was associated with higher biological and molecular heterogeneity, as well as with less differentiated tumours (Pece et al., 2010).
In brain tumours, the ability of tumour cells to propagate neurospheres in culture and high CD133 expression on these cells are regarded as independent prognostic factors that are being considered by some studies as relevant parameters associated with a reduced time of disease-free survival and overall survival. In human pancreatic cancer, in which 60% of tumours are $CD133^+$, the $CD133^+$ CSCs that simultaneously displayed CXCR4 expression were directly involved in the occurrence of metastasis after orthotopic xenografting, and remarkably, these metastases could be blocked by a small molecule inhibitor of CXCR4 (AMD3100) (Hermann et al., 2007).
Elevated ALDH expression is also associated with a poor prognosis in several tumour types, including AML, prostate cancer, breast cancer, head and neck squamous cell carcinoma, and pancreatic cancer (Charafe-Jauffret et al., 2010; Ginestier et al., 2007; Rasheed et al., 2010). Similarly, high activity levels of the ABC transporters have also been reported to be a sign of poor prognosis in patients with AML (Guo et al., 2009).
Thus, fundamentally, patients with tumours expressing high levels of the molecules associated with CSCs tend to have a poorer clinical outcome than patients with tumours that express low levels of these markers.

4. The prognostic influence of cancer stem cell immunophenotypes in oral squamous cell carcinomas

We recently investigated the presence of CSC antigens by immunostaining to identify a putative CSC immunophenotype in oral squamous cell carcinoma (OSCC) and to determine its influence on prognosis (Oliveira et al., 2011).
The initial demonstration that the tumoural cells of head and neck carcinomas have a hierarchy of development and embody a subpopulation of cells with self-renewal and differentiation capacities was reported by Prince et al. (2007), who found CSCs in low percentages and were able to characterise them through CD44 immunoexpression. However, to our knowledge, our study was the first to verify the association between prognostic factors in OSCC and conventional CSC immunophenotype markers.
The CD44 proteins are commonly found in epithelial tissues and were previously established as fundamental regulatory factors in squamous epithelium for processes such as cellular adhesion, cell-cell interaction, infiltration and metastatic dissemination (Bajorath, 2000). Our findings regarding CD44 immunoexpression in OSCC showed that $CD44^+$ tumour cells occurred at a frequency of 41.4% and were associated with basal cell morphology. Moreover, our results demonstrated that the overall survival curves presented significant differences between $CD44^+$ or $CD44^-$ immunophenotypes, as configured by an independent factor of poor prognosis in multivariate analysis (hazard ratio, 0.316 [95% confidence interval, 0.070–0.664]; $P = 0.033$).
These results were consistent with other prognostic studies, suggesting that alterations in adhesion molecules can act as either positive or negative regulators of progression and metastasis in OSCC, depending on stage when tumour is diagnosed (Wang et al., 2007; Wang et al., 2009). In agreement, Bankfalvi et al. (2002) also found that the high immunoexpression of CD44 (specifically the CD44v9 alternative splice isoform) was significantly associated with a poorer clinical outcome in OSCC.

Nevertheless, the effect of CD44 immunoexpression on OSCC prognosis still shows discordant results, and there are several candidate stem cell markers that need to be assessed. A trustworthy immunophenotypical marker that can be used to isolate the CSCs has yet not been definitively established in head and neck cancers, and the identification of reliable markers required to characterise CSCs in OSCC could certify the clinical effectiveness of future targeted therapies, possibly resulting in a more effective outcome for the patients.

5. Final considerations

There are still many aspects that remain to be discovered in the field of CSCs. Although there is much to be learned about the mechanisms that regulate normal stem cell function and how they can be used by malignant cells to propagate the disease, the careful identification of the main differences between normal adult stem cells and CSCs, as well as of their overlapping aspects, are important to discern how cancers progress and to transform the advances in CSC biology into effective therapies that could help patients in the near future. Therefore, the interaction between the expression of CSC markers and malignant behaviour need to be adequately understood as they relate to prognostic factors in several cancer types.

We believe that most human solid and haematological cancers contain a subpopulation of CSCs. Experimental and clinical evidence sustain the hypothesis that in humans, the process of tumorigenesis initiates in an adult normal stem cell, although other more committed cells, particularly in the haematopoietic system, might also be the founder cells of malignancy. Several therapeutic approaches have been shown to be promising by targeting CSCs in tumours, which is a great challenge given that these cells seem to be specifically resistant to currently available therapies.

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2

Clinical Significance of Putative Cancer Stem Cells in Residual Cancer Cells After Chemoradiotherapy for Rectal Cancer

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1. Introduction

Cancer stem cells (CSC) seem to be resistant to conventional chemo- and radiation therapies when compared with non-CSCs. Conventional cytotoxic therapies initially shrink the bulk of a tumor, but fail to eradicate it, resulting in tumor recurrence. Treatment failure may in part be due to the resistance of CSCs to chemotherapy or radiotherapy (Baumann et al., 2008; Eyler & Rich, 2008).

Drug or radiation surviving cells (residual tumor cells following treatment) have been shown to contain a higher frequency of putative CSCs in a number of human malignancies. Bao et al. demonstrated that the population of cells enriched for glioma CSCs was dramatically increased by irradiation and that radioresistant gliomas showed an increased percentage of CD133 positive cells (Bao et al., 2006). Tsuchida et al showed that anti-cancer drug treatment increases the side-population fraction (considered CSCs) in cancer cell lines (Tsuchida et al., 2008).

Over the past decades, preoperative chemoradiotherapy (CRT) has been established as one approach in the multimodal treatment of several types of gastrointestinal malignancies. In rectal cancer, preoperative CRT followed by surgery has improved sphincter preservation, local pelvic control and survival of patients with locally advanced rectal cancer (Bosset et al., 2006; Guillem et al., 2005). However, disease recurrence (especially for distant metastases) remains the major cause of mortality in these patients (Collette et al., 2007; van den Brink et al., 2004).

In rectal cancer, tumor regression grading (TRG) following CRT was determined by quantifying the proportion of residual cancer cells to the stroma of the entire tumor bed on formalin-fixed paraffin embedded (FFPE) specimens. TRG or pathologic response has been shown to predict clinical outcome (disease recurrence or patient survival) of oesophageal (Brücher et al., 2006; Chirieac et al., 2005), gastric (Patel et al., 2007; Rohatgi et al., 2006), or rectal cancer (Rödel et al., 2005) in patients after preoperative CRT followed by surgery, rather than pre-CRT clinical stage. The amount of residual cancer cells after CRT seems to be predictive of disease recurrence and survival in relation to CRT resistance.

Therefore, we hypothesized that CRT decreased or eradicated non-CSCs, which are sensitive to CRT, while increasing the percentage of putative CSCs characteristic of CRT resistance in the population of residual cancer cells. Residual cancer cells following CRT may be expected to contain a higher frequency of putative CSCs expressing stem cell markers, compared to primary, non-CRT tumor cells.
To test this hypothesis, we investigated the expression of stem cell markers in post-CRT residual cancer cells on FFPE specimens using microdissection and real-time quantitative reverse transcription polymerase chain reaction (RT-PCR).

2. Microdissection and RNA isolation from FFPE specimens

2.1 Microdissection in FFPE specimens

Tumor specimens were fixed in 10% formaldehyde solution v/v and embedded in paraffin. FFPE specimens (10 μm sections) were stained with nuclear fast red (Vector Laboratories, Inc., CA) and subsequently manually microdissected under microscope magnification (from ×5 to ×10). Residual cancer cells were isolated using a sterile blade and carefully collected with reference to hematoxylin and eosin sections, containing more than 70% cancer cells. Fibrotic tissue areas, necrotic cells, and non-neoplastic cells were identified.

2.2 RNA extraction from FFPE specimens

Microdissected samples were digested with proteinase K in lysis buffer containing Tris-HCl, EDTA, and sodium dodecyl sulfate as previously reported with minor modification (Bijwaard et al., 2001). RNA was purified using phenol and chloroform extraction. Isolated RNA was purified using ethanol precipitation. The concentration and quality of RNA was measured with UV absorbance at 260 nm and 280 nm (A260/280 ratio).

3. Expression of stem cell markers in residual cancer cells after CRT

3.1 cDNA synthesis

To reverse transcribe the fragmented mRNA from FFPE tissue materials, we used random hexamer priming, instead of oligo (dT)-based priming. cDNA was synthesized with random hexamer and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

3.2 Real-time quantitative RT-PCR

Real-time quantitative RT-PCR analysis was performed using a fluorescence-based real-time detection method (TaqMan) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). Although SYBR-Green based detection is less specific than TaqMan-based detection, we used SYBR-Green based detection to save experimental time and costs.
Primers were strictly selected or designed to be intron spanning to avoid amplification from contaminated genomic DNA. Target sequences were kept as small as possible (approximately 100 bp) to ensure the detection of fragmented and partially degraded RNA. To confirm primer specificity, a single band of expected amplicon size for each target gene was verified using gel electrophoresis on a 2% agarose gel and visualized with ethidium bromide.

Primers for CD133, CD44, OCT4, SOX2, VEGF, and beta actin were designed with primer3 software (Biology Workbench Version 3.2, San Diego Supercomputer Center, at the University of California, San Diego). Primers for EGFR were synthesized according to previously published sequences (Schneider et al., 2005). Primer sequences are shown in Table 1. PCR was performed in a final volume of 25 µl with a SYBR Green PCR Master Mix using 1 µl cDNA, and 400 nM of each primer for the respective genes. Cycling conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

gene	primer	sequence	product size
CD133	Forward	5'-GCTTTGCAATCTCCCTGTTG-3'	
	Reverse	5'-TTGATCCGGGTTCTTACCTG-3'	94bp
CD44	Forward	5'-CGGACACCATGGACAAGTTT-3'	
	Reverse	5'-CACGTGGAATACACCTGCAA-3'	115bp
OCT-4	Forward	5'-CTGGAGAAGGAGAAGCTGGA-3'	
	Reverse	5'-CAAATTGCTCGAGTTCTTTCTG-3'	79bp
SOX2	Forward	5'-CAAGATGCACAACTCGGAGA-3'	
	Reverse	5'-GCTTAGCCTCGTCGATGAAC-3'	95bp
VEGF	Forward	5'-CAGAAGGAGGAGGGCAGAA-3'	
	Reverse	5'-CTCGATTGGATGGCAGTAGC-3'	80bp
EGFR	Forward	5'-CCTATGTGCAGAGGAATTATGATCTTT-3'	
	Reverse	5'-CCACTGTGTTGAGGGCAATG-3'	88bp
Beta actin	Forward	5'-ACAGAGCCTCGCCTTTGC-3'	
	Reverse	5'-GCGGCGATATCATCATCC-3'	75bp

Table 1. Primer sequences of target genes

3.3 Relative mRNA levels of target genes

The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The Ct is inversely proportional to the amount of cDNA, i.e., a higher Ct value means that more PCR cycles are required to reach a certain level of detection.

Relative mRNA levels were determined by the standard curve method. Standard curves and line equations were generated using five-fold serially diluted solutions of cDNA from colon cancer cell line, Lovo. All standard curves were linear in the analyzed range with an acceptable correlation coefficient (R^2). Target gene expression was calculated using the standard curve.

Quantitative normalization of cDNA in each sample was performed using the expression of the beta actin gene as an internal control. Finally, mRNA levels of the target gene were presented as ratios between the genes of interest and the internal reference gene (beta actin). Real-time PCR assays were performed twice for each sample and mean values were used for calculations of mRNA levels.

4. Difference in gene expression profile of primary tumor and residual tumor following CRT

4.1 Correlations of CD133, SOX2 and OCT4 mRNA levels in pre-CRT or post-CRT tumor cells

A cell surface protein CD133, known as prominim-1, has been regarded as one of the most important markers of colorectal CSCs (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). OCT4 and SOX2 are essential transcription factors for normal pluripotent cell development and maintenance in embryonic stem (ES) cells, which are also known as reprogramming genes that induce an ES cell-like state in fibroblasts i.e., induced pluripotent stem (iPS) cells (Takahashi et al., 2007; Yamanaka, 2008).

As shown in Fig.1-1, we examined how these 'stem cell' related genes were correlated in primary non-treated tumor cells and post-CRT residual tumor cells. A positive correlation between OCT4 and SOX2 was observed in pre-CRT endoscopic tumor specimens. There was no correlation between CD133 and OCT4 or SOX2 in pre-CRT specimens. In post-CRT residual cancer on FFPE specimens (Fig. 1-2), significant positive correlations among all three stem cell markers were seen (Saigusa et al., 2009).

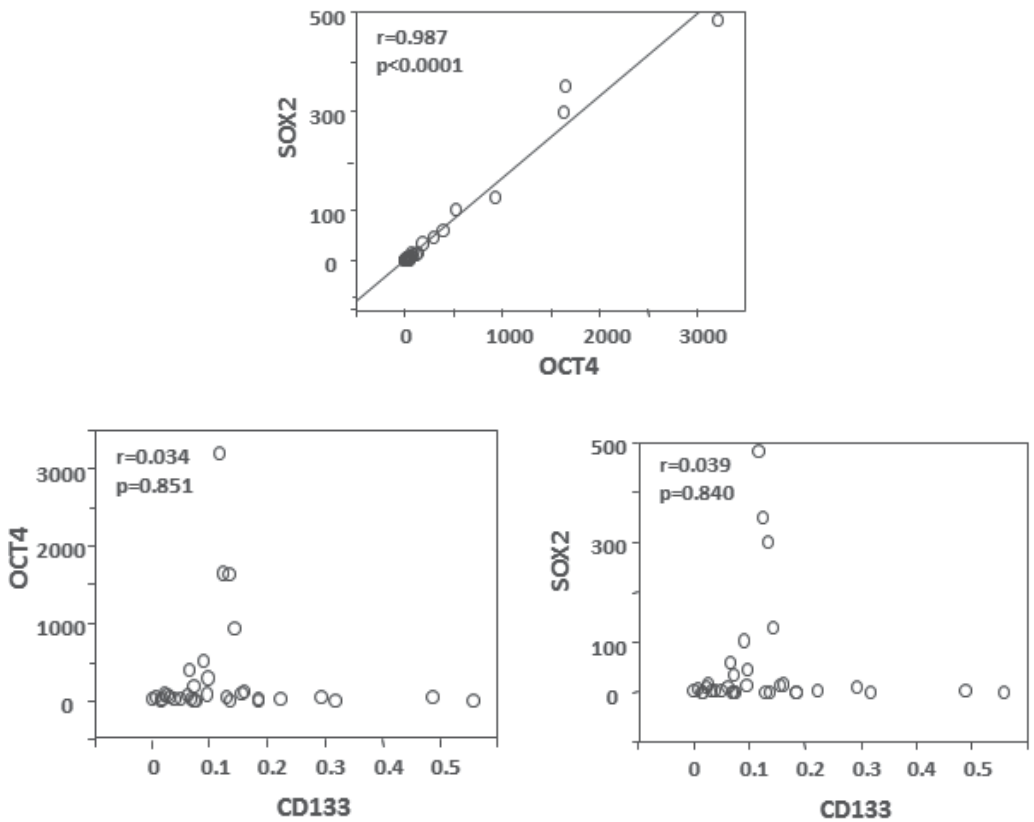

Fig. 1-1 Correlation between CD133, SOX2 and OCT4 in pre-CRT tumor biopsy specimens

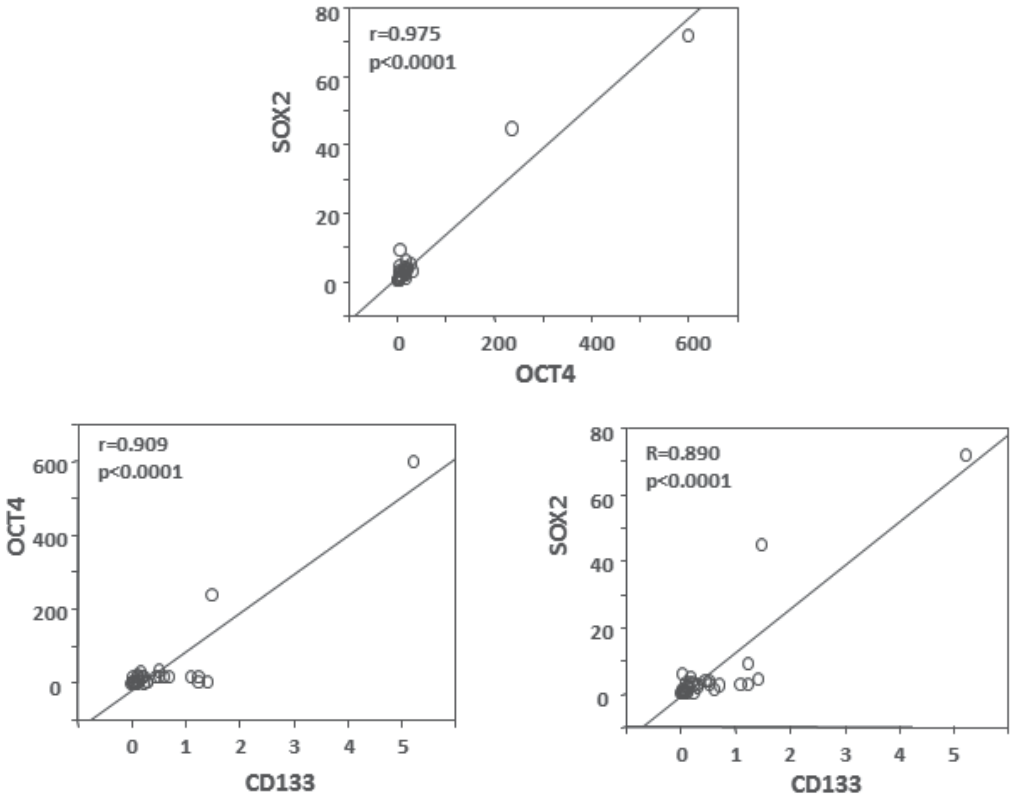

Fig. 1-2 Correlation between CD133, SOX2 and OCT4 in post-CRT residual cancer cells

Strong co-expression of OCT4 and SOX2 in both pre-and post-CRT tumor cells may indicate that these two genes have an indistinguishable relationship associated with maintenance of pluripotency in stem cells.
Also, residual cancer cells surviving CRT may enrich a population of putative CSCs expressing CD133, OCT4 and SOX2 because of the potential association between CSCs and treatment resistance.

4.2 Correlations of CD133, VEGF and EGFR mRNA levels in pre-CRT or post-CRT tumor cells

The vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) pathways are closely associated with each other, and share common downstream signaling, especially for tumor angiogenesis. Increased expression of VEGF or EGFR has been reported to be involved in tumor aggressiveness, metastasis, and poor prognosis in several types of malignancies (Galizia et al., 2004; Galizia et al., 2006). To date, anti-VEGF and anti-EGFR antibodies have become indispensible in the treatment of metastatic CRC (Chau & Cunningham, 2009). In other words, both VEGF and EGFR are important therapeutic targets in CRC.
We then examined how CD133 correlated with these therapeutic targets for CRC in primary tumor and post-CRT residual tumor cells. There were significant positive correlations between CD133 and VEGF, between CD133 and EGFR or between VEGF and EGFR in pre-

CRT tumor biopsy specimens (Fig. 2-1). However, these correlations were not observed in post-CRT FFPE specimens (Fig. 2-2; Yasuda et al., 2009).
We previously described several possibilities for explaining these findings. We believe it seems plausible that CRT may cause an imbalance between two distinct populations (putative CSCs and non-CSCs) within the tumor. A majority of tumor cells expressing VEGF and EGFR (considered as non-CSCs) may respond to CRT and then shrink or disappear. By contrast, a very small population of tumor cells expressing CD133 (considered as CSCs) may resist CRT and be left as residual cancer cells in post-CRT specimens.

4.3 CD133 and CD44 expression in pre-CRT or post-CRT tumor cells

CD44 is a transmembrane glycoprotein molecule, which is widely expressed as a cell surface hyaluronan receptor in normal epithelial, mesenchymal and hematopoietic cells. Also, CD44 has been reported as one of the important cell surface markers for isolating colorectal CSCs (Du et al., 2008; Haraguchi et al., 2008).
We examined whether the expression of potential markers (CD133 and CD44) for colorectal CSCs were changed during CRT. As shown in Fig. 3, tumoral CD133 mRNA levels were significantly increased in post-CRT resected specimens, compared with pre-CRT biopsy specimens. By contrast, tumoral CD44 mRNA levels were significantly decreased in residual cancer cells from post-CRT resected specimens (Yasuda H et al., 2009).

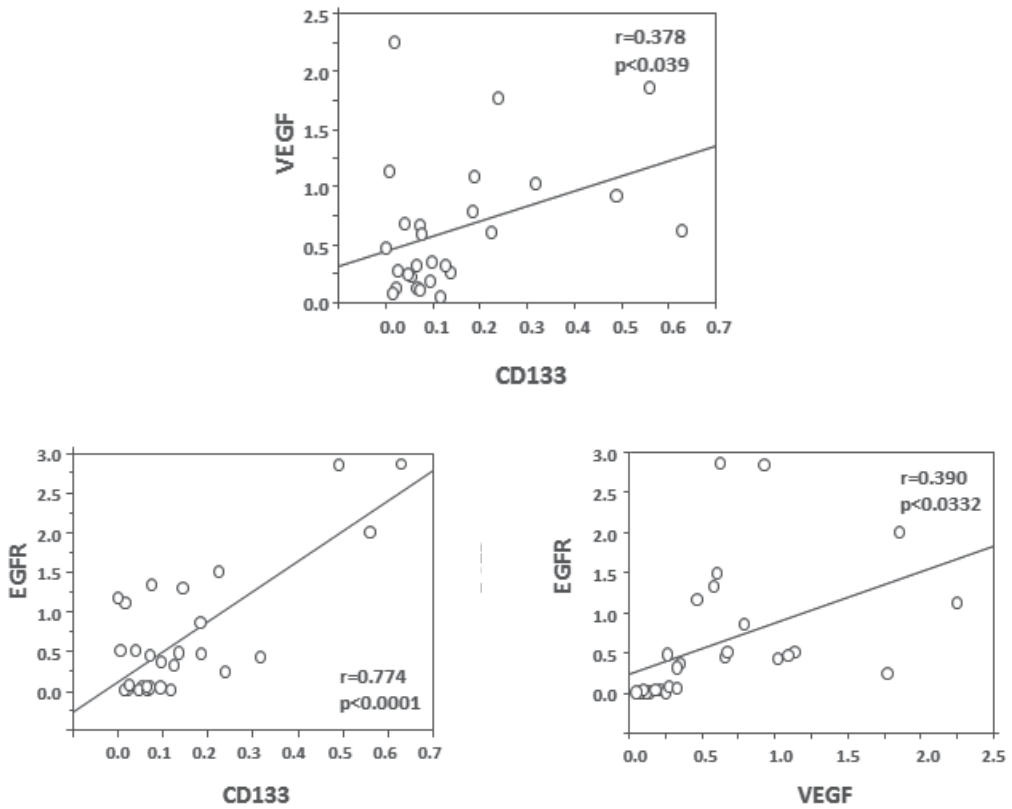

Fig. 2-1 Correlation between CD133, VEGF and EGFR in pre-CRT tumor biopsy specimens

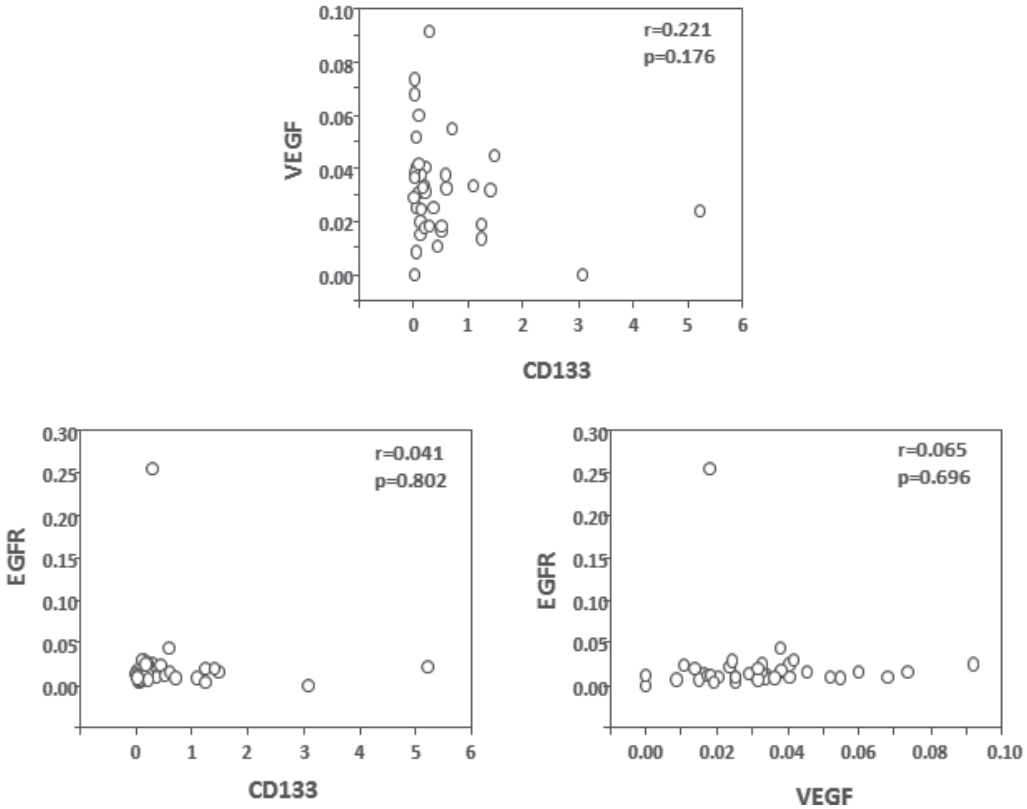

Fig. 2-2 Correlation between CD133, VEGF and EGFR in post-CRT residual cancer cells

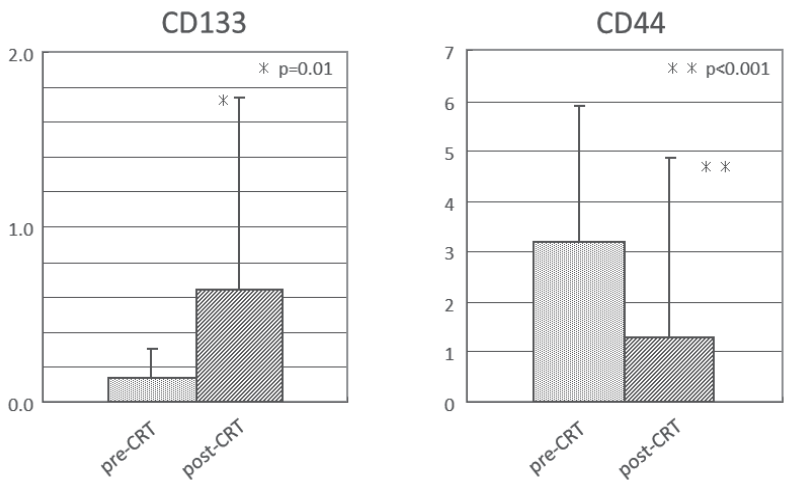

Fig. 3. CD133 and CD44 expression in paired pre-and post-CRT specimens

The comparison of potential surface markers for colorectal CSCs between pre-CRT and post-CRT tumor cells indicate that changes in expression of CD133 and CD44 during CRT were

quite opposite. We speculate that residual cancer following CRT may contain more CSCs than primary tumors before CRT. The relative proportion of CSCs may increase in residual cancer following CRT. Thus, gene expression related to CSCs may also increase in residual cancer following CRT, compared with primary tumors. In this context, CD133 seemed preferable to CD44 as the marker for colorectal CSCs.

5. Radiation surviving cells *in vitro* and residual cancer cells after CRT

5.1 CD133 and CD44 in radiation surviving HT-29 cells *in vitro*

We performed an *in vitro* experiment using human colorectal cancer cell lines to determine whether irradiation itself can induce the expression of CD133 or CD44.

Exponentially growing colorectal cells of the HT29 cell line were plated on a 10 cm dish and irradiated at a dose of 1, 2.5, and 5 Gy (CAX-150-20, Chubu medical Co. Ltd). Fourteen days later, colony formation assays were performed to evaluate cell survival after irradiation. Approximately 23%, 7%, and 5% survival fraction were found following irradiation with 1.0, 2.5, and 5.0 Gy, respectively. These surviving cells were collected for western blotting analysis. Single dose of 2.5 Gy and 5 Gy radiation increased CD133 protein levels, compared with control (Fig. 4-1). Densitometric analysis showed that CD133 was 1.4 times increased at 5 Gy radiation with respect to control. By contrast, radiation decreased CD44 protein levels regardless of radiation dose.

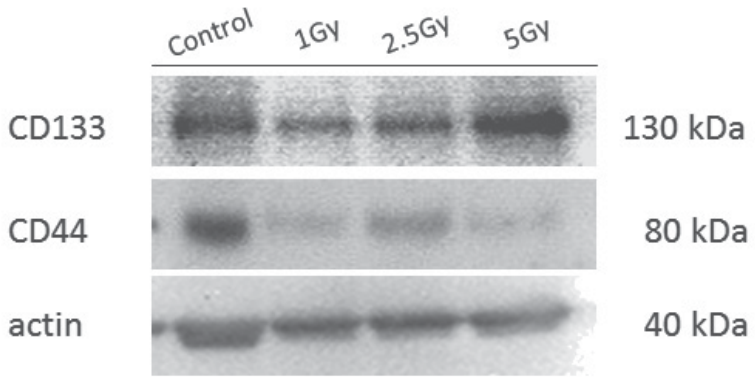

Fig. 4-1 CD133 and CD44 in radiation surviving HT-29 cells *in vitro*

CD133 increased in a radiation-dose dependent manner, despite the decreased number of radiation-surviving HT-29 cells. These *in vitro* results were consistent with CD133 mRNA levels increasing in residual cancer cells after CRT, compared with primary tumor cells before CRT. These results suggest that CRT may enrich the relative proportion of CD133 expressing CSCs within residual cancer, or that CRT may induce the expression of CD133 in tumor cells, or both.

5.2 Immunoreactive CD133 and CD44 in residual cancer cells after CRT

For immunohistochemical analysis, CD133 rabbit monoclonal antibody (Cell Signaling Technology, Inc. Boston, MA) and CD44 mouse monoclonal antibody (R&D Systems, Inc. Minneapolis, MN) were used. The primary antibody was detected using Envision reagents (Envision kit/HRP, Dako Cytomation, Denmark).

Immunoreactive CD133 and CD44 expression were observed in the minority of residual cancer cells within entire residual tumors (Fig. 4-2). There was no obvious concordance between CD133 and CD44 positivity of residual cancer cells, which may support the notion that CD133 positive and CD44 positive cells did not colocalize in colorectal cancer specimens (Du et al., 2008).

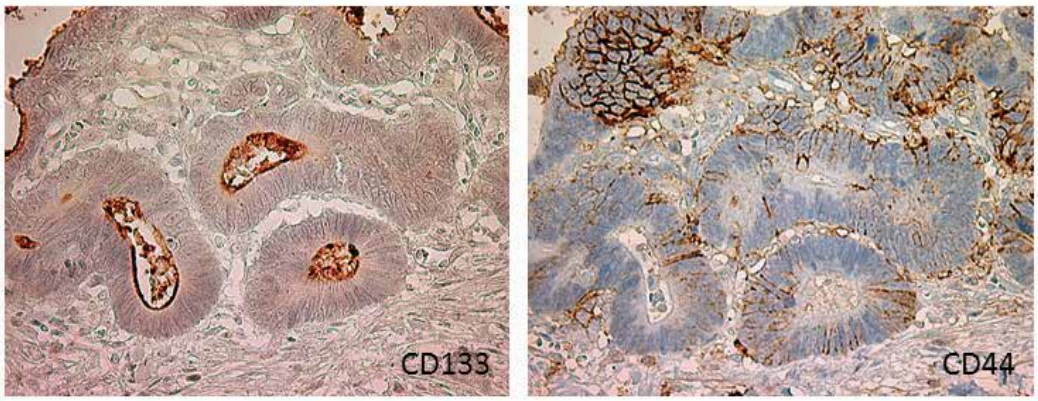

Fig. 4-2 Immunoreactive CD133 and CD44 in residual cancer cells after CRT

CD133 immunostaining in residual cancer cells after CRT showed not only apical/endoluminal membranous staining, but also cytoplasmic staining. Immervoll et al reported that apical/endoluminal membranous CD133 staining was characteristic by well-oriented, polarized and differentiated cells, while cytoplasmic CD133 staining was found in a minor population of cells (Immervoll et al., 2008). We have previously reported that residual cancer cells after CRT showed strong CD133 and moderate OCT4 and SOX2 staining, but no CK20 staining (a known epithelial marker) was observed (Saigusa et al., 2009). These lines of evidence suggest that CRT may induce dedifferentiation of cancer cells or may select putative CSCs with undifferentiated phenotype.
To determine if CRT may increase the relative proportion of CD133 expressing CSCs within residual cancer, it is necessary to compare the number of CD133 expressing tumor cells in pre-CRT endoscopic biopsy specimens and post-CRT resected specimens. This study has not yet been completed.

6. Clinical significance of CD133, OCT4, and SOX2 expression on residual cancer cells in patients with rectal cancer

6.1 Association of post-CRT CD133, CD44, OCT4, and SOX2 expression with clinicopathological variables

Thirty-three patients undergoing CRT followed by surgery were analyzed for an association between post-CRT OCT4 and SOX2 expression with clinical outcome. A total of 52 patients were analyzed for an association of post-CRT CD133 and CD44 expression with clinical outcome.
Patients who developed distant metastatic recurrence (e.g. liver, lung) had a significantly higher post-CRT CD133, OCT4, and SOX2 compared with those patients without recurrence. No such relationship was observed for post-CRT CD44.

6.2 Association of post-CRT CD133, CD44, OCT4, and SOX2 expression with patient survival

To identify the cut-off values of CD133, CD44, OCT4, and SOX2 predictive of distant metastatic recurrence, receiver operating curve (ROC) analysis was used. As shown in Fig. 5, patients with post-CRT CD133, OCT4, and SOX2 above cut-off value ('High') showed significantly worse disease free survival, compared with those with 'Low'. No such relationship was observed for post-CRT CD44.

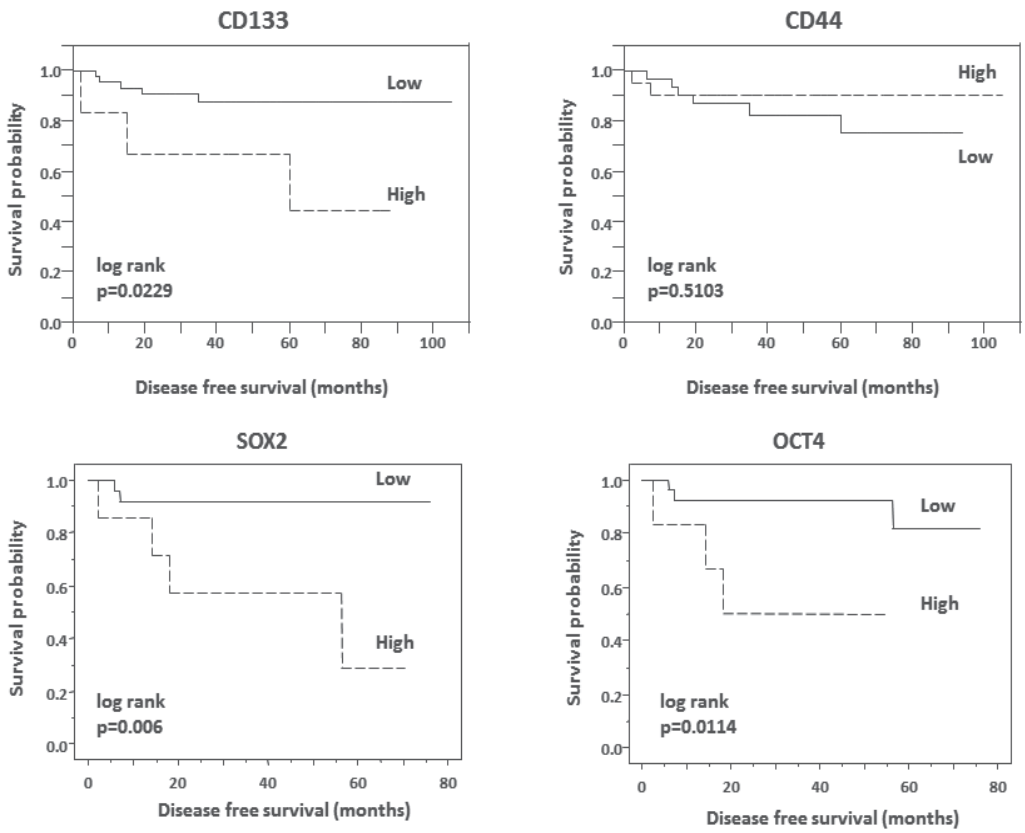

Fig. 5. Kaplan-Meier plots of disease free survival according to post-CRT CD133, CD44, SOX2, and OXT4 expression

Introduction of preoperative CRT followed by surgery (total mesorectal excision) in the management of rectal cancer significantly decreased local recurrence rate and improved patients' survival. However, the rate of distant metastatic recurrence still remains as high as 15-20% of rectal cancer treated with preoperative CRT followed by TME (Guillem et al., 2005). Identifying predictive markers for disease recurrence or poor prognosis of such patients is urgently required for appropriate treatment stratification.

Our results suggest that the expression of 'stem cell' genes such as CD133, OCT4 and SOX2 on post-CRT residual cancer cells may predict metachronous distant metastasis and poor prognosis of rectal cancer patients treated with preoperative CRT followed by surgery.

7. Clinical significance of residual cancer cells after CRT as putative colorectal CSCs

7.1 The proportion of putative CSCs in primary tumors

The presence of CSCs in primary tumors seems to be of prognostic significance for several malignancies (Liu et al., 2007; Zeppernick et al., 2008). In primary colorectal cancer, CD133 expression has also been reported to be a significant prognostic marker (Horst et al., 2008; Kojima et al., 2008). This may indicate that the proportion of CD133 expressing CSCs in primary, non-treatment tumor might be predictive for less treatment efficacy, more chance of disease recurrence, and poor prognosis of CRC patients.

In this study, the expression of CD133, CD44 SOX2, and OCT4 in pre-CRT primary tumor did not correlate with disease recurrence or survival of rectal cancer patients (data not shown). Since we had only 30 pre-CRT endoscopic tumor biopsies available, our data should be interpreted with caution. However, post-CRT, but not pre-CRT CD133, SOX2, and OCT4 has shown to be associated with metachronous distant metastasis and poor prognosis of rectal cancer patients treated with preoperative CRT followed by surgery.

7.2 The proportion of putative CSCs in post-treatment residual tumors

According to the CSC hypothesis, CRT surviving cancer cells (residual cancer cells following CRT) should contain a higher frequency of CRT-resistant colorectal CSCs, compared with primary, pre-CRT cancer cells. Our correlation results between pre-CRT or post-CRT CD133, SOX2, OCT4, VEGF, and EGFR, show that CRT may eliminate a majority of cancer cells expressing VEGF or EGFR (considered non-CSCs with CRT sensitive phenotype), and may leave a small population of cancer cells expressing CD133, OCT4, or SOX2 (considered CSCs with CRT resistant phenotype).

Although we think that residual cancer cells are not completely identical to CSCs, our results suggest that the relative proportion of putative CSCs expressing CD133, OCT4, or SOX2 may increase in post-CRT residual cancer cells in FFPE specimens, compared with pre-CRT primary tumor cells. Our findings are consistent with recent experiments (Dallas et al., 2009; Dylla et al., 2008).

7.3 CD133 and CD44 as potential markers for colorectal CSCs

Both CD133 and CD44 are of functional importance as potential cell surface markers for colorectal CSC. It still remains to be resolved if either CD133 or CD44 could be clinically important in CRT surviving cells, or if CRT can increase the expression of these markers. In pre- and post-CRT paired specimens, significant increase in tumoral CD133 and significant decrease in tumoral CD44 was observed. *In vitro*, CD133, but not CD44 was increased in radiation-resistant surviving colorectal cancer cell lines (HT29 cells).

CD133 seemed preferable to CD44 as the marker of colorectal CSCs according to the notion that CRT may increase the relative proportion of CSCs which express the potential markers of colorectal CSCs.

8. Conclusion

The proportion of CSCs in residual tumors following preoperative CRT may be more accurately predictive for less treatment efficacy, more chance of disease recurrence, and poor prognosis of rectal cancer patients than the proportion of CSCs in non-pretreatment

primary tumor. Expression of the potential markers of colorectal CSCs in microdissected residual cancer on FFPE specimens may provide useful information regarding treatment stratification and clinical management of rectal cancer patients after CRT and surgery.

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Cancer Stem Cells in Solid Organ Malignancies: Mechanisms of Treatment Resistance and Strategies for Therapeutic Targeting

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1. Introduction

It has been long recognized that tumors are composed of a heterogenous population of cells with various levels of cellular differentiation and morphologic features. Previous explanations for this phenomenon have centered around the concept of clonal evoluation, with the gradual acquisition of mutations leading to distinct tumor cell populations. While this model has validity, more recent evidence suggests that distinct tumor cell populations likely also arise from differentiation of cancer cells with stem-like properties. Termed the cancer stem cell (CSC) theory, this model posits that tumors are composed of a small population of cells possessing the characteristics of self-renewal and pluripotency, and thus the ability to initiate or support tumor growth, as well as their differentiated progeny which lose these abilities with increasing differentiation (Figure 1).

Much in the way a normal organ is supported by endogenous stem cells, the CSC theory holds that similarly-functioning cells with stem-like abilities are the driving force behind tumor initiation, progression and metastatic spread. Since they were first identified in acute myelogenous leukemia (AML) (Lapidot et al., 1994), CSCs have been indentified in a wide variety and number of malignancies, including colorectal, head and neck, pancreatic, prostate, central nervous system (CNS), lung and breast cancer.

The CSC theory has garnered a great deal of attention, in part, because it proposes a fundamental shift in the way we think about and treat cancer. Similarly to how normal tissue stem cells are resistant to traditional cytotoxic cancer therapies, CSCs have increasingly been demonstrated to be preferentially spared by such treatment. It is thought that standard chemotherapy and radiation targets the differentiated tumor cell bulk, leaving the resistance CSC behind, which can lead to recurrence even years later (Figure 2).

Along with the identification and an increasing focus on characterization of CSCs has been the search for therapies that effectively target this resistant subpopulation. While the search is still in its infancy, a number of intriguing treatment strategies have been proposed. In many cases these strategies target known resistance mechanisms employed by CSCs.

Although efficacy for these strategies has yet to be determined in phase II or III clinical trials, early preliminary evidence is encouraging. In this chapter we will discuss mechanisms of CSC treatment resistance as well as the exciting possibility of current therapeutic approaches that seek to specifically target the CSC population.

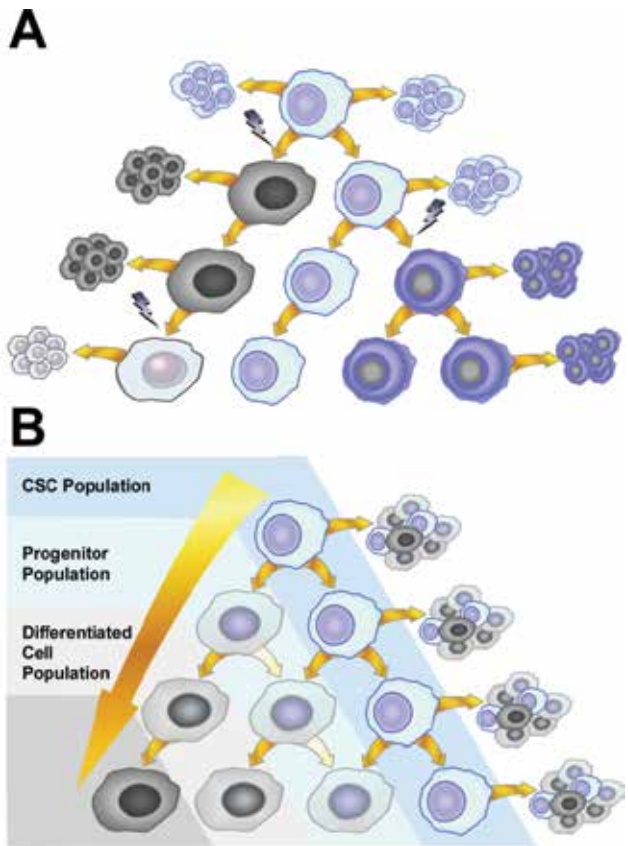

Fig. 1. Comparison of two models of tumor development and progression, (A) Traditional stochastic model of tumor progression. Each tumor cell is capable of giving rise to new tumors. Tumor heterogeneity develops from the stochastic accumulation of mutations. (B) Cancer stem cell theory. Tumor cell hierarchy with a CSC population at the hierarchical apex. Heterogeneity develops from differentiation of CSC progeny. Tumorigenic capability is lost with increasing differentiation

2. Cancer stem cells and treatment resistance

2.1 Resistance to standard cytotoxic therapy

An unfortunate number of advanced cancers recur despite an initial response to treatment. The CSC theory proposes that this phenomenon is likely due to the inability of current anti-cancer therapy to specifically target and eradicate the cells capable of "seeding" tumor growth, i.e. the CSC population. Studies in blood, brain, breast, and colon cancer have shown that identified tissue specific CSC populations exhibit decreased cell death after chemotherapy and radiation as compared to the more differentiated cancer cells (Woodward

et al., 2007). This leads to the selection of an enriched population of treatment-resistant CSCs that are capable of initiating new tumor growth (recurrence) and spread to distant organs (metastasis).

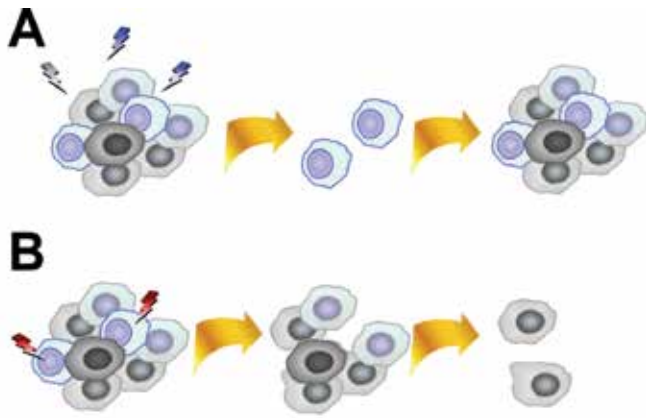

Fig. 2. Treatment Implications of CSC Theory. (A) Traditional cytotoxic therapy targets the differentiated tumor bulk, sparing the relatively more resistant CSC subpopulation (light blue cells) which can lead to tumor recurrence, (B) CSC-directed therapy kills CSC subpopulation, leading to eventual tumor eradication

These findings are supported by a number of studies using both *in vitro* methods as well as *in vivo* xenograft models. One such study by Dylla et al demonstrated that a subpopulation of highly tumorigenic colorectal cancer cells expressing the cell surface antigens $ESA^+CD44^+CD166^+$ increased by 2.2-fold following treatment with cyclophosphamide and irinotecan (Dylla et al., 2008). Serial transplantion of these chemoresistant cells gave rise to heterogeneous tumors identical to the parent tumor, demonstrating that the chemotherapy selected for a resistant population of cells able to maintain their original tumorigenic capacity. A similar effect has been described in $CD44^+$ pancreatic cancer cells following high-dose gemcitabine treatment. Interestingly, as the proportion of $CD44^+$ cells decreased in culture, tumor colonies became re-sensitized to gemcitabine treatment (Hong et al., 2009), suggesting that the progeny of the CSC do not have the drug resistant behavior. Additionally, using primary lung tumors, Bertolini et al demonstrated that a population of tumor-initiating cells expressing CD133 were enriched after treatment with platinum-based chemotherapy both *in vitro* and *in vivo*. And furthermore, on retrospective analysis of formalin-fixed tissue biopsies, tumors with increased expression of CD133 by immunohistochemistry demonstrated a shorter time to recurrence following chemotherapy than $CD133^-$ tumors (Bertolini et al., 2009), indicating that CSC enriched tumors possess a more aggressive behavior.

Similar selection of treatment-resistant CSC populations has been observed following radiotherapy in other tumor types. Glioblastomas are a uniformly lethal malignancy with a median survival of less than 12 months (Bao et al., 2006). Radiation is currently the most effective treatment for glioblastomas and can lead to significant treatment responses, although the tumor invariably recurs. Studies have shown that glioblastoma surviving radiation are enriched for $CD133^+$ cells and, as described previously, are just as efficient in recapitulating tumors in xenograft models as non-radiated $CD133^+$ cell populations (Bao et al., 2006; Eyler et al., 2008).

2.2 Mechanisms of treatment resistance in CSCs

While a comprehensive understanding of the mechanisms of CSC resistance to chemotherapy and radiation are lacking, a number of genetic and cellular adaptations that confer resistance have been observed. These include slow cell cycling kinetics, efficient DNA repair mechanisms, increased expression of multidrug-resistance transporters, protection from a specialized microenvironment and apoptotic resistance (Figure 3).

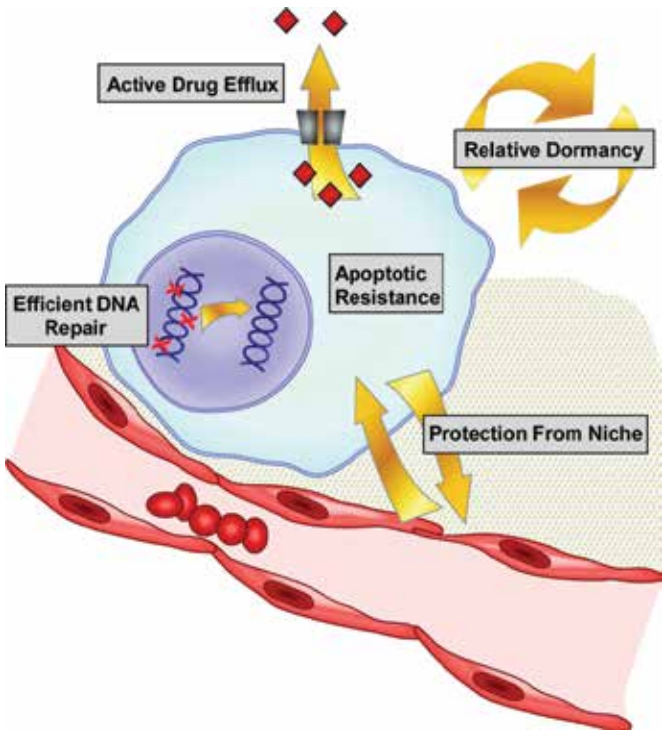

Fig. 3. Mechanisms of CSC Treatment Resistance. CSCs exhibit multiple behaviors that have been cited reasons for their resistance to current cytotoxic-based therapies. These include active drug pumps such as members of the ATP-binding cassette transporters (ABC-transporter), efficient DNA repair mechanisms, apoptotic resistance, relative dormancy due to a slowly cycling state, and protection from a specialized microenvironment (niche)

2.2.1 Slow cell cycle kinetics

Both radiation and chemotherapy target cells that are rapidly replicating and dividing. CSCs are inherently resistant to these cell cycle-dependent therapies because of their low proliferation rate. Similar to a normal stem cell, a CSC cycles significantly less often than more differentiated transit-amplifying cells. In head and neck cancer, CSCs identified by high CD44 expression displayed increased clonogenicity and spent extended time in G2, which was protective against apoptosis. Targeting G2 checkpoint proteins released the G2 blockade from these cells and made them more prone to apoptosis (Harper et al., 2010). The relative dormancy of a CSC also provides it with the opportunity to accumulate multiple mutations over time. These mutations may be passed along to the cell's progeny, creating another avenue to acquired therapeutic resistance.

2.2.2 Efficient DNA repair mechanisms

Like normal stem cells, CSCs possess a well-fortified defense system that protects against DNA damage and mutation. In a study by Eyler et al, radiation was shown to cause equal levels of damage to all cells within a tumor, but CSCs were able to repair the damage more rapidly (Eyler et al., 2008). Several mechanisms exist for detection of DNA damage as well as rapid repair. Cell cycle checkpoints, including ataxia telangiectasia mutated and checkpoint kinases (Chk1 and Chk2), are activated in response to genomic stress, halting further replication and division until the DNA damage is repaired. Chk 1/2 have been found to have higher basal and inducible activity in CSCs than in non-stem cells (Eyler et al., 2008; Morrison et al.). Inhibition of the Chk 1/2 kinases partially reverses the radioresistance of glioblastoma cells, suggesting that these checkpoints are critical to the radioresistance seen in glioblastoma (Bao et al., 2006).

The presence of certain DNA repair proteins can give cells a survival advantage as well. One specific DNA repair protein, O^6-methylguanine-DNA-methyltransferase (MGMT), has been implicated in conferring resistance to the chemotherapeutic agent temozolomide (Beier et al., 2008). Temozolomide impairs DNA replication by methylating the O^6 position of guanine, which can then be reversed by the function of MGMT. Consequently, temozolomide has little effect in tumor expressing active MGMT.

2.2.3 Multidrug transporters

CSCs exhibit a large number of drug efflux pumps that work to preserve DNA integrity by selectively removing cytotoxic chemicals, including chemotherapeutics, from the cell. Members of the ATP-binding cassette (ABC) superfamily are known to be involved in the multidrug-resistant phenotype of CSCs from many different cancers, including melanoma, lung, breast and pancreas (Bertolini et al., 2009). Of these, the ABCG2 (BCRP1) transporter appears to be exclusively expressed in stem cells and has been shown to be upregulated in multidrug resistant stem cell lines (Hong et al., 2009). ABCB1 (MDR1) has been shown to remove vinblastine and paclitaxel from stem cells, whereas ABCG2 prevents accumulation of imatinib mesylate, topotecan and methotrexate (Eyler et al., 2008). Antibodies to these protein transporters can effectively block tumor growth and increase chemosensitivity as seen in melanoma xenografts (Schatton et al., 2008). Similarly, lung cancer cells co-expressing CD133 and ABCG2 are enriched following chemotherapy, further implicating the role of ABC transporters in conferring chemoresistance and increased survival to CSCs (Bertolini et al., 2009).

In addition to an increased capacity for drug efflux, CSCs also express molecular mediators, like Aldehyde Dehyrogenase (ALDH), that are able to degrade metabolically-active byproducts of chemotherapeutic agents and render them inactive. By these means, ALDH1, 3 and 5 confer resistance to cyclophosphamide in several blood, breast and colon cancer cell lines (Dylla et al., 2008). Additionally, knockdown of ALDH1 expression in resistant colorectal CSCs has been shown to increase cell sensitivity to cyclophophamide *in vivo* (Dylla et al., 2008).

2.2.4 Wnt signaling

Expression of β-catenin, an essential component of the Wnt signaling pathway, has been shown in multiple studies to be linked to CSC survival and tumorigenesis (Taipale et al., 2001; Chen, M. S. et al., 2007; Woodward et al., 2007; Morrison et al.). Woodward et al showed that the Wnt/β-catenin pathway is also involved in CSC resistance to radiation in

mammary progenitor cells and breast cancer cell lines (Woodward et al., 2007). Irradiation of a murine mammary epithelial cell culture resulted in high levels of activated β-catenin in cells expressing stem cell antigen (Sca), while accumulation of β-catenin was not present in Sca-negative cells. Increased levels of β-catenin in irradiatiated Sca-positive cells correlated to enhanced self-renewal in mammospheres as well as upregulation of the anti-apoptotic protein Survivin. Upregulation of Survivin has also been reported in colon cancer cell lines where it seems to assist cancer cells in escaping senescene by enhanced telomerase activity (Endoh et al., 2005).

2.2.5 Specialized microenvironment (niche)

The CSC microenvironment undoubtedly influences CSC behavior. Surroundling stromal cells likely modulate CSC susceptibility to cytotoxic stress, such as radiation. Radiation-induced apoptosis depends on an oxygen-rich environment to generate free radicals capable of damaging DNA. Traditionally, CSCs were believed to preferentially reside in hypoxic microenvironments as a means of resisting radiation-induced cell death. However, CSCs are more often found next to blood vessels where they are well-oxygenated (Calabrese et al., 2007; Krishnamurthy et al.), further evidence that they require or co-opt the microenvironment for propagation and survival. Interestingly, CSCs seem to contribute to tumor angiogenesis, producing higher levels of VEGF in both normoxic and hypoxic conditions than non-CSC populations (Eyler et al., 2008). CSCs also rely upon factors secreted by the vasculature, such as leukemia inhibitory factor, brain-derived neurotrophic factor and pigment epithelial-derived factor, for normal stem cell maintenance (Eyler et al., 2008).

Similar to normal tissue stem cells, CSCs within breast and head and neck tumors have been found to exhibit increased antioxidant defenses in comparison to their non-tumorigenic progeny (Diehn et al., 2009). Lower ROS levels are the result of increased free radical scavengers that can protect the cell from radiation-induced damage and apoptosis.

Hypoxic states appear to enrich CSC populations. This effect has been described in medulloblastomas as well as endothelial-derived tumors (Blazek et al., 2007; Eyler et al., 2008). Hypoxia-induced factor (HIF)-1 may be responsible for mediating radioresistance in this situation as well as inducing the production of VEGF. Tumors derived from irradiated-CSCs are often highly vascular, indicating that HIF in radio-resistant CSCs contributes to angiogenesis and tumor growth in an irradiated environment. Furthermore, recent clinical trials have shown enhanced cell killing when antiangiogenic therapy is combined with radiation (Lee et al., 2000; Hess et al., 2001).

2.2.6 Resistance to apoptosis

CSCs may also acquire resistance to apoptosis by sustained activation of cell survival pathways or by inhibition of apoptotic pathways. Ma et al showed that cells expressing CD133 in hepatocellular carcinoma demonstrated a prolonged expression of the Akt/PKB and Bcl-2 survival pathways in response to treatment with fluorouracil and doxorubicin (Ma et al., 2008). Treatment with an Akt1-inhibitor sensitized the cells to chemo-induced apoptosis. NFkB, an anti-apoptotic transcription factor downstream of Akt, has also been implicated in the survival and progression of several cancers and may also promote EMT conversion in CSCs leading to metastasis (Sarkar et al., 2008).

Ultimately, it seems that CSCs may resist cytotoxic therapies through a combination of mechanisms that may differ among individual tumors. This emphasizes the need to develop

CSC-directed therapies to augment current anti-cancer treatments. In cancers where growth is dependent on CSCs, complete eradication of this sub-population may achieve long-term cure.

3. Therapeutic targeting of cancers cells

The frequent failure of standard cytotoxic therapies to provide a lasting cancer-free survival may be explained, in part, by the resistance of CSCs to standard chemotherapy and radiation. While traditional therapies can lead to early and often dramatic clinical responses, by failing to eradicate the tumorigenic CSC population, disease relapse can be expected. Clearly strategies that incorporate our increasing understanding of tumor cell heterogeneity with regards to treatment response are needed.

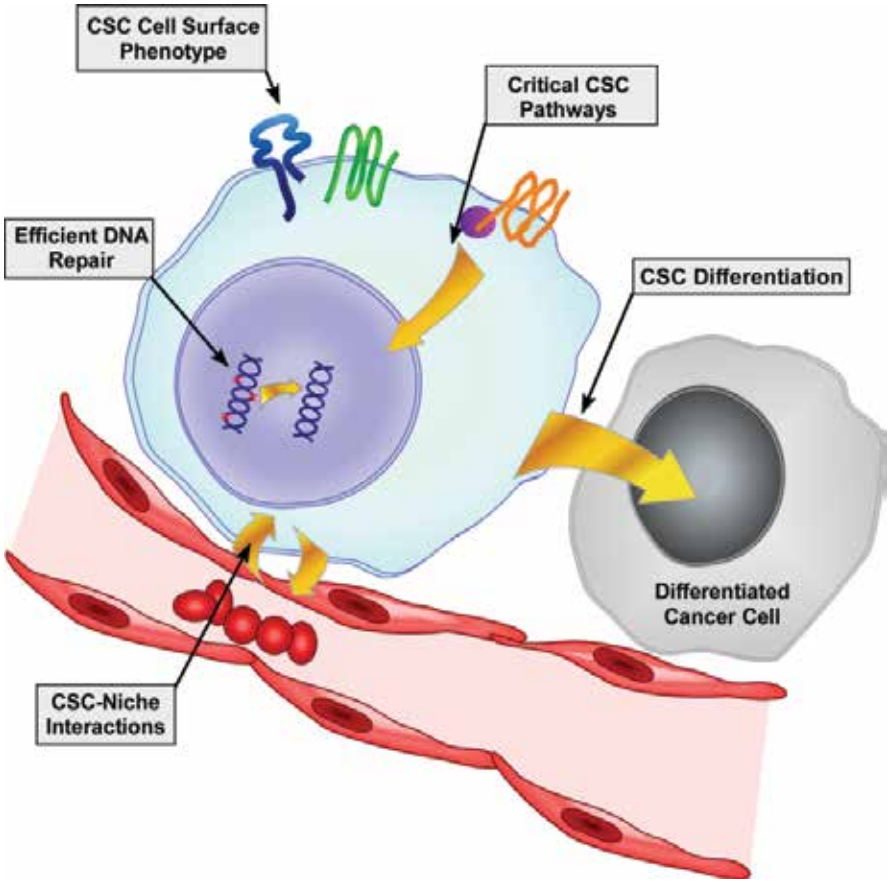

Fig. 4. Overview of Strategies for Targeting CSCs. Proposed strategies for selectively targeting CSCs include addressing their mechanisms of resistance such their efficient DNA repair mechanisms, critical survival pathways and/or specialized microenvironment. Other strategies try to take advantage of the unique cell surface phenotype that differentiates these cells from the remaining tumor bulk or force the CSC to differentiate into a more treatment-sensitive target

Given the resistance to standard cytotoxic therapy displayed by many CSCs, therapeutic targeting of this tumor cell population will likely prove to be a challenging endeavor. Several different strategies are currently being developed to selectively target CSCs. These include therapies that target the unique cell surface phenotype of CSCs and critical CSC signaling pathways as well as strategies aimed at forcing CSCs to differentiate and thereby increase their therapeutic sensitivity. Alternative strategies are aimed not at the CSC itself, but at its microenvironment (Figure 4). Most of these approaches have shown success in preclinical trials, with current early clinical phase I and II studies underway in a subset.

3.1 Targeting the cell surface phenotype

For many malignancies, distinct cell surface phenotypes have been defined which identify tumor cell populations enriched in CSCs. It is not surprising, therefore, that therapies directed against these cell surface antigens are under development. Monoclonal antibody therapy, antibody-drug conjugates and dendritic cell vaccinations are under investigation as potential methods of selectively targeting the CSC population using their cell surface phenotype (Figure 5).

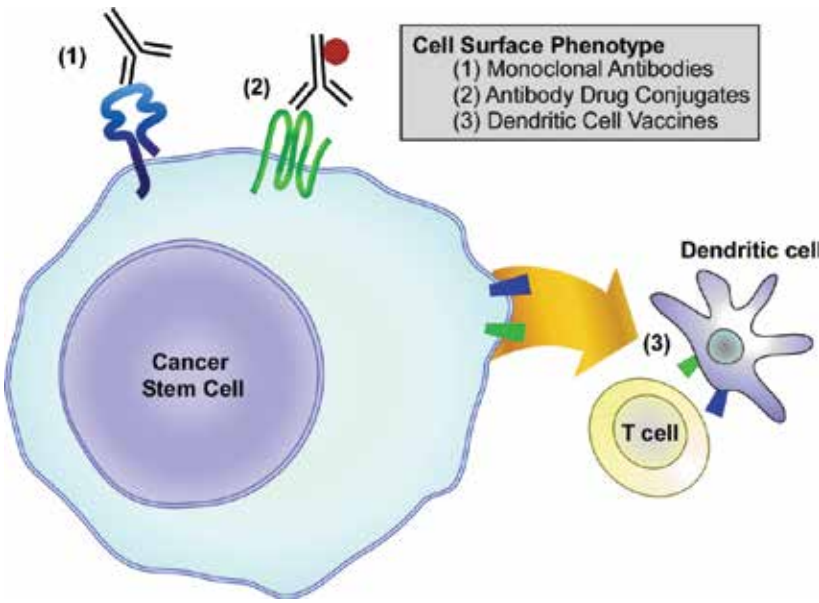

Fig. 5. Targeting the CSC Phenotype. Strategies for targeting CSC antigens include antibody-based therapy such as monoclonal antibodies and antibody-drug conjugates. Dendritic cell vaccines primed with CSC antigens are another possible targeting method under development

A wide variety of cell-surface antigens have been identified in tumor populations enriched in CSCs. Although there are some antigens that appear to mark CSCs in multiple tumor types there is also a variety of different surface antigens used to define CSCs across tumor types. In many cases, multiple cell-surface antigens have been identified that can be used to selectively enrich for a population with stem-like properties. While little is currently known about the functional role of many of these proteins, early preclinical work suggests that targeting them may have therapeutic value.

3.1.1 Antibody-based therapy

Monoclonal antibody (mAb) therapies are being used with increasing success as targeted agents in cancer therapy. They are believed to function through diverse mechanisms, including interactions with the host immune system through antibody-dependent cell cytotoxicity and complement activation, as well as the blockade of important tumor cell signaling pathways or the elimination of critical cell surface antigens (Adams et al., 2005). In addition, mAbs often display synergism when used in combination with traditional cytotoxic chemotherapy and can act as delivery vectors for more traditional cytotoxic therapies when conjugated to radioisotopes or chemotherapeutics. Increasingly, monoclonal antibodies are being shown to be a valuable addition to standard therapeutic regimens in multiple solid organ malignancies (Bonner et al., 2006; Vermorken et al., 2008; Tebbutt et al.; Ibrahim et al.).

Antibody therapies directed against CSC antigens are a logical outgrowth of the CSC theory and the increasing evidence in its support. Given the important roles in tumor development and growth displayed by tumor cells expressing CSC antigens, targeting these same antigens brings with it the hope of being able to selectively target the command center of the tumor. In preclinical testing, antibody-based therapies directed against CSC antigens has demonstrated encouraging results.

CD44: CD44 has been defined as a CSC antigen in a number of malignancies including breast, colorectal and head and neck cancer. CD44 is a large, heavily glycosylated transmembrane protein that has known functions in cell adhesion, signaling, migration and defense against reactive oxygen species (Ishimoto et al.). It undergoes complex alternative splicing resulting in functionally different isoforms with variable tissue expression. CD44 is known to interact with the CSC niche by binding to components of the extracellular matrix, most notably hyaluron as well as osteopontin, collagen and fibronectin to a lesser degree (Culty et al., 1990; Jalkanen et al., 1992; Weber et al., 1996).

Even prior to its recognition as a marker of CSCs, a variant of CD44 was recognized for its ability to promote metastatic behavior in a rat model of pancreatic cancer (Gunthert et al., 1991). Furthermore, blockade of CD44 with a mAb slowed growth of lymph node and lung metastases as well as prevented metastatic formation in this same model of pancreatic cancer, presumably through blocking of ligand interaction (Seiter et al., 1993).

Increased CD44 expression correlates with locoregional recurrence following radiation therapy for laryngeal cancer (de Jong et al.). In addition, CD44 expression has been correlated to patient prognosis in colorectal (Lugli et al.), breast (Neumeister et al., ; Zhou, L. et al.) and pancreatic cancer (Gotoda et al., 1998).

Early phase I clinical studies examined the effect of a humanized mAb to CD44v6 (Bivatuzumab) labeled with the radio isotope rhenium-186 in patients with head and neck squamous cell carcinoma (Stroomer et al., 2000; Borjesson et al., 2003). These studies demonstrated acceptable toxicity with stable disease in patients who received higher drug doses.

However, the enthusiasm for further development of CD44-based antibody therapy waned after a subsequent phase I study demonstrated unacceptable toxicity. In this dose-escalation study, bivatuzumab conjugated to the chemotherapeutic mertansine was evaluated in patients with head and neck cancer. Skin-related toxicity occurred with increasing dose and the trial had to be halted early after one patient died from toxic epidermal necrolysis (Tijink et al., 2006; Rupp et al., 2007).

CD133: CD133 is a well-recognized CSC marker in multiple malignancies, including glioblastoma, colorectal, prostate, pancreatic, ovarian and renal cancer (Hermann et al., 2007; Ricci-Vitiani et al., 2007; Baba et al., 2009). CD133 is a pentaspan transmembrane glycoprotein that localizes to cell protrusions. It has known interactions with cholesterol and is speculated to be involved in plasma membrane organization, although the exact functional properties of this molecule are not well characterized (Mizrak et al., 2008). Mutations in this gene are associated with multiple retinal diseases and it has been well-defined as a marker for hematopoetic and neural progenitor cells.

Expression of CD133 has been linked to adverse tumor behavior. CD133-positive cells have been shown to be resistant to standard chemotherapy in multiple tumor cell types, among them head and neck (Zhang, Q. et al.), pancreatic (Hermann et al., 2007), glioblastoma (Blazek et al., 2007), and colorectal cancer cells (Dallas et al., 2009). CD133 expression has also been correlated to tumor recurrence in patients with colorectal cancer treated with chemotherapy and radiation (Nagata et al.). Whether CD133 has a direct functional role or is merely a convenient marker of cells that express these abilities is, as yet, unknown.

As of yet, antibody therapy directed against CD133 has only been evaluated in limited preclinical models. Chen and colleagues demonstrated that CD133 targeting with a mAb could inhibit proliferation of colorectal cancer cell in vitro (Chen, W. et al.). Damek-Poprawa and colleagues conjugated a genetically modified cytotoxin from Aggregatibacter actinomycetemcomitans to an anti-human CD133 mAb and demonstrated its ability to selectively target $CD133^+$ head and neck cancer cells in vitro (Damek-Poprawa et al.).

The potential for success of anti-CD133 antibody therapy has been recently questioned by the discovery that CD133 expression may not be as tightly linked to CSC function as previously suggested. Chen and colleagues elegantly demonstrated that the CD133 negative cell population in neuroblastoma, a tumor in which CD133 is well-characterized as a CSC antigen, harbors a subset of cells with tumor-initiating capability (Chen, R. et al.). Clearly, a more in-depth knowledge of the correlation between cell-surface phenotype and functional activity is needed if we are to be successful in selectively targeting the CSC population.

ALCAM: Activated leukocyte cell adhesion molecule (ALCAM), also known as CD166, has been characterized as a stem cell niche marker in the colon (Levin et al.) and as a CSC marker in colorectal (Dalerba et al., 2007) and prostate cancer (Rajasekhar et al.). CD166 is a member of a subfamily of immunoglobulin receptors with five extracellular immunoglobulin-like domains, a transmembrane section and a short cytoplasmic tail (Weidle et al.). It is involved in homotypic interactions as well as heterotypic interactions with CD6.

Altered CD166 function, levels of expression and subcellular localization are all suspected to play a role in tumor biology. Functional polymorphisms of the CD166 gene that confer increased transcriptional activity have been correlated to an increased risk of the development of breast cancer (Zhou, P. et al.). Furthermore, overexpression of CD166 as compared to surrounding normal tissue has been demonstrated in papillary and medullary thyroid cancer (Micciche et al.). In pancreatic cancer, overexpression of CD166 has been associated with shorted disease-free and overall survival (Kahlert et al., 2009). However, in gastric cancer, decreased CD166 expression through microRNA and siRNA ALCAM silencing has been shown to increase cellular proliferation (Jin, Z. et al.). The conflicting reports on the role of over- or under-expression of CD166 in multiple cancers highlights our incomplete understanding of the functional role of this molecule. It is quite possible that the function of CD166 differs by malignancy type.

Rather than over- or under-expression, altered cellular localization of CD166 has been correlated with disease progression in multiple tumor types. In colorectal (Lugli et al.) head and neck (Sawhney et al., 2009), ovarian (Mezzanzanica et al., 2008) and breast cancer (Burkhardt et al., 2006) loss of membranous staining has been associated with disease progression. In many cases, loss of membranous CD166 expression (or an increase in cytoplasmic CD166) is associated with loss of cell-cell adhesion and the acquisition of a metastatic phenotype (Mezzanzanica et al., 2008). Some have speculated that CD166 functions as a sensor of cell density, which may help to explain the fact that it is strongly expressed on CSCs.

In preclinical studies, selective targeting of CD166 with a recombinant single-chain antibody inhibited breast cancer invasion *in vitro* and colorectal tumor growth in a nude mouse xenograft model (Wiiger et al.). CD166 internalization as a means for intracellular drug delivery has also been studied *in vitro*. Piazza and colleagues demonstrated that the human single-chain antibody fragment I/F8 selectively targets CD166 and induces internalization of the antibody-CD166 complex. They then developed an immunotoxin from the conjugation of I/F8 to the ribosome inhibiting protein saporin and demonstrated the ability of their antibody fragment to deliver the toxin intracellularly and selectively kill CD166 expressing cells (Piazza et al., 2005). A similar strategy targeting CD166 for intracellular delivery of liposomal drugs has been shown to have some efficacy in vitro in select prostate cancer cells (Roth et al., 2007). Given the loss of cell surface CD166 expression that has been shown to occur with disease progression in many malignancies, targeted therapy directed at extracellular CD166 epitopes may prove to have limited therapeutic efficacy in vivo.

3.1.2 Dendritic cell vaccines

In addition to antibody-based therapy, cancer stem cell antigens are also being targeted through dendritic cell vaccines. In a rat model of glioblastoma, dendritic cell vaccination using CSC antigens produced T-cell responses against CSCs but not those primed with daughter cells. Furthermore, survival was prolonged in animals receiving CSC dendritic cell vaccines as compared with non-CSC tumor cell vaccination (Xu et al., 2009). Current phase I trials are underway in patients with glioblastoma using dendritic cell vaccines primed with mRNA or whole cell lysates from CD133 positive tumor cells.

3.2 Targeting cancer stem cell signaling pathways

3.2.1 Targeting notch signaling

The Notch signaling pathway is a highly conserved pathway in multicellular organisms. There are four different Notch receptors (Notch 1-4) that are single pass transmembrane proteins with large extracellular and small intracellular domains. The two most well-characterized notch ligands are Delta-like and Jagged, which are also single pass transmembrane proteins. Upon ligand binding, the extracellular portion of Notch is cleaved by a metalloprotease called TACE (Tumor Necrosis Factor Alpha Converting Enzyme) and the ligand and notch extracellular domain are then endocytosed by the ligand-expressing cell. Subsequently, γ-secretase cleaves the intracellular notch domain, releasing it move to the nucleus and regulate gene expression (Harrison et al.). Strategies to target Notch signaling in cancer have focused on multiple points in this pathway, including γ-secretase inhibition and antibody therapy directed against notch ligands as well as the notch receptor (Figure 6).

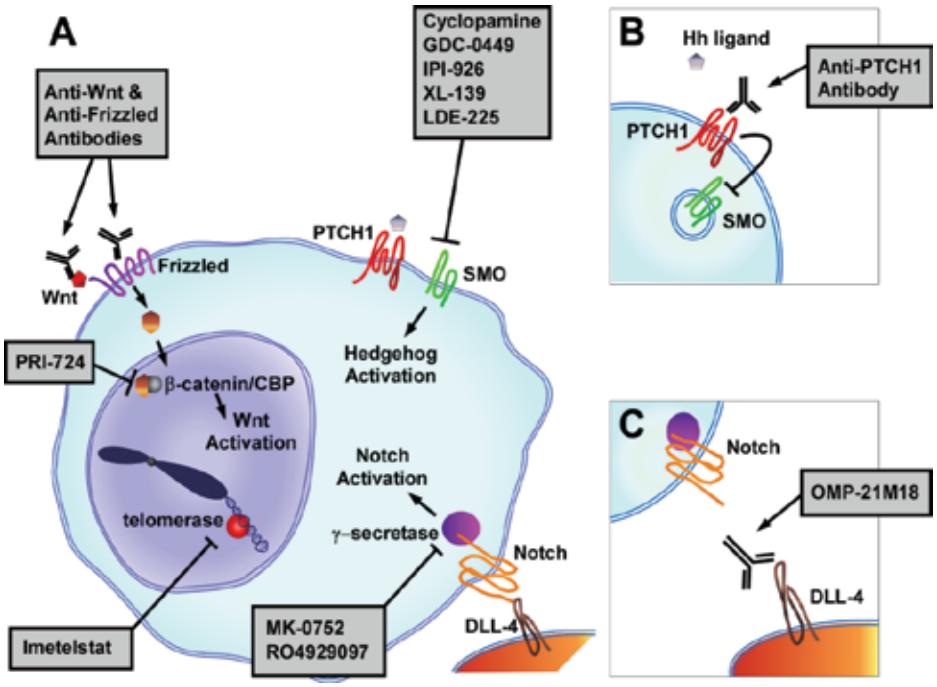

Fig. 6. Targeting critical CSC pathways

The Notch pathway is known to regulate cell fate and renewal, particularly during embryonic development. Notch signaling in cancer has been demonstrated to be a particularly important in regulating the function of the CSC tumor population. For instance, in lung adenocarcinoma CSCs, Notch signaling is important for key stem-like properties. Sullivan and colleagues demonstrated that lung adenocarcinoma CSCs identified by high Aldehyde Dehydrogenase activity had elevated expression of Notch pathway transcripts. Furthermore, when the notch pathway was targeted either through γ-secretase inhibition or expression of a shRNA against Notch3, decreased tumor cell proliferation and clonogenicity were noted (Sullivan et al.).

In glioblastoma xenograft models, γ-secretase inhibition has been shown to deplete $CD133^+$ CSCs and prolong survival (Fan et al.). In addition, when combined with temozolomide therapy, γ-secretase inhibition blocked tumor progression in 50% of mice with established xenografts (Gilbert et al.). Preclinical activity of γ-secretase inhibition has also been demonstrated in colorectal (Akiyoshi et al., 2008), breast (Han et al., 2009; Rasul et al., 2009), ovarian (Wang, M. et al.) and lung cancer (Konishi et al., 2007). These encouraging preclinical results have paved the way for currently ongoing Phase I studies. RO4929097, MK-0752 and PF-03084014 are three different γ-secretase inhibitors currently being evaluated in phase I oncology trials.

Gamma-secretase inhibition is a relatively non-specific method of decreasing Notch signaling. Although Phase I data is not yet available for us to understand treatment toxicity in human patients, early preclinical evidence suggests that dual inhibition of Notch1 and 2 through γ-secretase inhibitition may lead to intestinal morbidity through depletion of crypt-based progenitor cells (Riccio et al., 2008). In an effort to more selectively target individual Notch receptors (Notch1-4), antibodies selectively targeting Notch1 and Notch2 have been

developed. In xenograft tumor models, Notch1 blockade inhibits tumor growth through inhibition of both cancer cell growth and angiogiogenesis (Wu et al.). Based upon these encouraging early results, there may be a role for the selective targeting of individual Notch receptors as a way to minimize therapeutic morbidity.

As well as notch receptor blockade and γ-secretase inhibition, blocking notch receptor ligands is an alternative strategy to abolish notch signaling. Preclinical studies in colorectal tumor xenograft models have demonstrated the efficacy of anti-DLL4 antibodies in inhibiting tumor growth, particularly in combination therapy with irinotecan (Fischer et al.). Interestingly, in contrast to cetuximab therapy, anti-DLL4 showed efficacy in both KRAS wild-type and mutant tumors. Preclinical efficacy of anti-DLL4 therapy has also been noted in pancreatic cancer (Oishi et al.), Ewing's sarcoma (Schadler et al.). DLL4 blockade appears to work through similar mechanisms to notch receptor blockade in that it reduces CSC frequency and tumor cell growth (Hoey et al., 2009) as well as inhibits angiogenesis (Ridgway et al., 2006).

Phase I clinical studies are currently underway to evaluate the safety of a humanized mAb targeting the N-terminal epitope of DLL4 (OMP-21M18) in combination with other chemotherapeutics in colorectal, lung and pancreatic cancer. The potential for significant toxicity with anti-DLL4 therapy will be carefully evaluated given that chronic DLL4 blockade has been demonstrated to induce hepatic toxicity and, in a dose-dependent manner, lead to the development of subcutaneous vascular neoplasms in rats (Yan et al.).

3.2.2 Targeting hedgehog signaling

The hedgehog (Hh) signaling pathway is a key developmental pathway that regulates animal morphogenesis. In cells receiving Hh signaling, pathway activity is controlled at multiple levels. In the absence of Hh, Patched1 (PTCH1), a transmembrane receptor, suppresses the activity of Smoothened (Wiiger et al.) by preventing its cell surface localization. In the presence of Hh ligand, the Hh pathway is activated by PTCH1 relieving its inhibition of SMO. SMO localizes to the cell surface and initiates a signaling cascade that activates the glioma-associated (Gli) family of zinc finger transcription factors (Evangelista et al., 2006).

Dysregulation of the Hh signaling pathway has been noted in multiple types of cancer, the prototype of which is basal cell carcinoma. Inactivating mutations of PTCH1 are noted to be the cause of Gorlin syndrome, a disease characterized by the development of multiple basal cell carcinomas (BCCs) and keratocystic odontogenic tumors with increased susceptibility to the development of medullblastoma and rhabdomyosarcoma. In addition, most sporadic BCCs have been demonstrated to have inactivating PTCH1 mutations (Caro et al.). Increased Hh pathway expression has also been documented in a large number of malignancies, among them medulloblastoma (Raffel et al., 1997; Taylor et al., 2002), head and neck (Schneider et al.), pancreatic (Walter et al.) and breast cancer (ten Haaf et al., 2009).

It has been speculated that the hedgehog pathway may promote key tumor behaviors by acting predominantly on CSCs (Evangelista et al., 2006). The Hh pathway is well-known to regulate tissue growth and regeneration through its effects on normal tissue stem cells (Bhardwaj et al., 2001; Machold et al., 2003; Ahn et al., 2005; Palma et al., 2005; Plaisant et al.). Shin and colleagues have shown that in response to injury, sonic hedgehog protein expression is upregulated in bladder epithelial stem cells. This in turn elicits increased Wnt expression in the adjacent stroma, with resultant epithelial and stromal cell proliferation and restoration of urothelial function (Shin et al.).

Accumulating evidence suggests that CSCs may rely on Hh signaling in a similar manner. In gastric cancer cells, inhibition of Hh signaling selectively reduced proliferation and increased susceptibility to chemotherapy in the CSC subpopulation (Song et al.). In CD133-positive glioma CSCs, treatment with the Hh inhibitor cyclopamine increased sensitivity to temozolomide therapy. Interestingly, the combination of cyclopamine with a γ-secretase inhibitor provided an even greater increase in CD133-positive cytotoxicity with temozolomide, indicating a potential role for the simultaneous inhibition of multiple CSC signaling pathways.

Current strategies to target the Hh pathway rely mainly on SMO targeting. The naturally-occuring plant teratogen cyclopamine and subsequent synthetic derivates were first demonstrated to inhibit aberrant Hh pathway activation due to oncogenic SMO and PTCH mutations through inhibition of SMO over a decade ago (Taipale et al., 2000). Since that time the efficacy of SMO inhibition has been demonstrated in preclinical models of glioblastoma, small cell lung, gastric, pancreatic and prostate cancer (Evangelista et al., 2006). GDC0449, an oral small molecule inhibitor of SMO, is the furthest along in clinical development. Other small molecule inhibitors of SMO under development include LDE-225, BMS-833923, IPI-926 and PF-04449913 (Figure 6).

Phase I clinical trial data for GDC0449 in patients with advanced and/or metastatic solid organ malignancies has recently been reported (Lorusso et al.). An acceptable side-effect profile was observed. Furthermore, a clinical response was seen in 19 of 33 patients with basal cell carcinoma and in 1 patient with medulloblastoma, both of which are tumors known to be driven by PTCH1 and SMO mutations. Phase II trials in multiple malignancy types are now underway with this compound as well as other phase I studies evaluating other small molecule SMO inhibitors. Interestingly, the well-characterized antifungal Itraconazole was recently demonstrated to inhibit Hh pathway activation and cancer growth through SMO inhibition (Kim et al.). Undoubtedly, future studies will likely evaluate the efficacy of this relatively well studied and well-tolerated agent in a cancer setting.

Future strategies to overcome tumor dependence on Hh signaling will likely incorporate downstream targeting of Hh pathway components. Because current therapeutic approaches predominately target the transmembrane protein SMO, they would not be expected to abrogate Hh pathway activation due to overexpression of molecules further downstream in the signaling cascade. This is becomes a valid consideration given that overexpression of Gli, a downstream effector of the Hh pathway, has been documented in some tumor types, including esophageal and colorectal cancer (Rizvi et al., ; Mazumdar et al.). In addition, the acquisition of SMO mutations may interefere with the ability to target this protein. In fact, in medulloblastoma, the acquisition of a SMO mutation that disrupts the ability of GDC0449 to bind SMO has been demonstrated to confer resistance to this method of targeted Hh pathway inhibition (Yauch et al., 2009). Several small molecule anatagonists of downstream Hh pathway effectors have been discovered (Hyman et al., 2009; Mazumdar et al.) and may provide a basis for the development of future therapeutics that more comprehensively target the Hh pathway.

3.2.3 Targeting Wnt signaling

The Wnt highly conserved signaling pathway that plays a key role in maintenance of the stem cell population, proliferation, differentiation and apoptosis (de Sousa et al.). In the canonical Wnt pathway, signaling is mediated primarily through β-catenin. In the absence of Wnt ligands, β-catenin is phosphorylated which primes it for ubiquitination by a

destruction complex composed, in part, by the tumor suppressor protein APC. After ubiquitination Wnt undergoes proteosomal degredation. Upon Wnt ligand binding to the Wnt receptor Frizzled (FZD), β-catenin is no longer degraded due to dissolution of the destruction complex. β-catenin translocates to the nucleus where along with coactivators CBP and p300 it activates the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors, leading to expression of Wnt target genes.

Upregulation of Wnt signaling is a common finding in cancer (Deonarain et al., 2009). A prime example of this is colorectal cancer, in which frequent activating mutations of β-catenin or inactivating mutations of APC lead to constitutive Wnt pathway activation. Interestingly, Wnt signaling appears to be particularly important in CSC function. In colorectal cancer, higher Wnt pathway activation is noted in the cancer stem cell population adjacent to the tumor stroma as compared with the bulk of tumor cells. Furthermore, high Wnt signaling has been shown to functionally define the CSC compartment *in vitro* and *in vivo* (Vermeulen et al.). Wnt signaling has also been demonstrated to play a key role in the regulation of cancer stem cells in lung cancer (Teng et al.) and glioblastoma (Jin, X. et al.).

Therapies are under development that target multiple points in the Wnt signaling pathway, from antibodies directed against Wnt ligands and their receptor Frizzled to small molecules such as PRI-724 that inhibit the β-catenin/CBP transcription activating complex (Figure 6). Of these, PRI-724 is the furthest along in clinical development, with phase I clinical trials currently ongoing.

3.3 Telomerase inhibition

Telomerase is an enzyme that adds repeating sequences of TTAGGG to the 3′ ends of DNA strands, thereby preventing loss of important DNA from chromosome ends. Telomerase activity has been implicated in the limitless self-renewal potential of CSCs, making it an attractive target for inhibition. In preclinical models, telomerase inhibition depletes the CSC tumor cell subpopulation in breast and pancreas (Joseph et al.), neural (Castelo-Branco et al.) and prostate (Marian et al.) cancer cell lines. Imetelstat (GRN163L), a synthetic oligonucleotide that targets the template region of telomerase is currently being evaluated in Phase I clinical trials.

3.4 Targeting CSC DNA repair mechanisms

Efficient DNA repair has been identified as one mechanism by which the CSC tumor subpopulation is more resistant to standard DNA-damaging therapy (Bao et al., 2006). In glioblastoma, $CD133^+$ CSCs display increased DNA damage checkpoint response to radiation and have more efficient DNA repair. Furthermore, the radioresistance of these CSCs can be reversed with inhibition of the checkpoint kinases Chk1 and Chk2 (Ropolo et al., 2009). Of the many compounds that have been identified to inhibit Chk1 and Chk2 (Garrett et al.), Ly2606368, a Chk1 inhibitor, is currently being evaluated alone and in combination with cisplatin in phase I clinical trials.

3.5 Targeting CSCs through differentiation therapy

Rather than targeting drugs to specific features of CSCs, an alternative strategy may be to make CSCs more responsive to existing chemotherapeutic agents. This may be accomplished by promoting differentiation of CSCs from their resistant, stem cell state to more responsive differentiated cells. This has already been shown to be an effective strategy

in some model systems. In chronic myeloid leukemia, primitive, quiescent CSCs are resistant to imatinib, an inhibitor of the BCR-ABL fusion kinase. However, treatment with several days of G-CSF stimulates differentiation of these CSCs, increasing sensitivity to imatinib (Jorgensen et al., 2006). Similarly, $CD133^+$ CSCs in glioblastoma are very drug resistant. Treatment with bone morphogenic proteins (BMP), particularly BMP4, effectively initiates differentiation of glioblastoma cells, thereby reducing $CD133^+$ cells, clonogenic ability, and cell proliferation in mouse xenografts (Piccirillo et al., 2006). This therapy may also make these glioblastoma cells more sensistive to other drugs, increasing the efficacy of chemotherapy for this tumor (Figure 7).

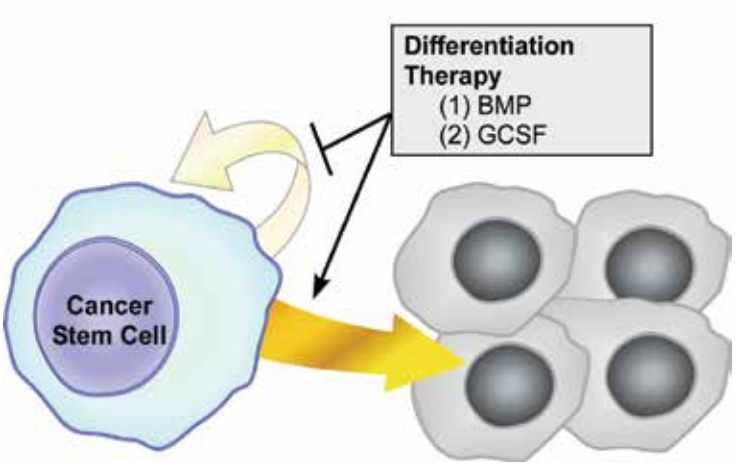

Fig. 7. Differentiation therapy. Strategies forcing CSC differentiation may increase therapeutic efficacy of traditional cytotoxic therapy

3.6 Targeting the CSC microenvironment

Rather than targeting the CSC directly, attempts to disrupt the CSC's specialized microenvironment may prove an alternative strategy for eradicating the CSC population. Much in the way normal adult tissue stem cells require a specialized microenvironment, or niche, to maintain a balance between self-renewal and differentiation, increasing evidence suggests that CSC behavior relies on similar microenvironmental cues.

Components of this specialized microenvironment include both non-tumor cells such as fibroblasts, myoepithelial cells, osteoblasts, leukocytes and endothelial cells, as well as the extracellular matrix proteins and signaling molecules they produce. The composition of the niche varies by tumor type; for example, hematopoetic stem cells reside in an osteoblastic niche (Zhang, J. et al., 2003; Arai et al., 2004), while epithelial stem cells reside in a niche composed of fibroblasts and myoepithelial cells (Ohlstein et al., 2004).

Evidence that CSCs require a similar microenvironment is mounting. Leukemic CSCs preferentially home to the niche and enjoy a growth advantage once there (Kawaguchi et al., 2001), while both glioblastoma (Charles et al.) and HNSCC (Krishnamurthy et al.) CSC's reside in a perivascular niche that is critical for their survival. The CSC-niche interaction functions to support and maintain CSCs through a variety of interactions and signaling cascades, and it has been suggested that maintaining the CSC-niche interaction is the primary role of many known CSC surface markers such as CD44 (Marhaba et al., 2008).

Targeting these physical interactions as well as niche-CSC signaling pathways hold therapeutic promise (Figure 8).

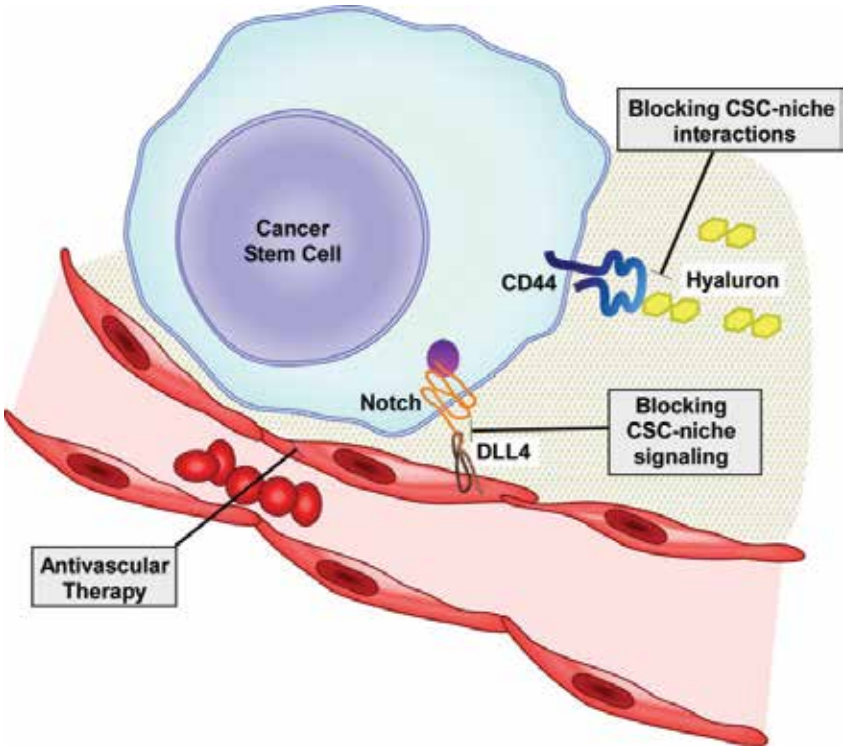

Fig. 8. Several differing strategies are currently under evaluation for targeting the CSC niche. Targeted anti-vascular therapy and anti-Notch strategies highlight the prominent role of the endothelial cell in the niche. Other therapies seek to target physical interactions between CSC proteins and non-cellular niche components, such as therapies that disrupt the interaction of CD44 with components of the extracellular matrix

This interaction appears to function both ways, as glioblastoma CSCs may create and maintain their vascular niche by differentiating into endothelial cells (Ricci-Vitiani et al., 2007). Furthermore, there is evidence that some tumors may induce creation of a niche-like environment prior to the arrival of tumor cells in metastatic spread, likely via secretion of tumor-derived growth factors (Kaplan et al., 2005). Thus inhibition of the CSC-niche interaction may be a useful strategy for elimination of CSCs. In mouse glioblastoma xenografts, inhibition of CSC-derived endothelial differentiation led to tumor reduction, likely via inhibition of the CSC niche (Ricci-Vitiani et al., 2007). These findings may help to explain the efficacy of anti-vascular therapies, such as VEGF inhibition, in select cancers.
Anti-CD44 therapy may be another strategy to disrupt the CSC niche. CD44 is well-known to interact with components of the extracellular matrix such as hyaluron, osteopontin, fibronectin and collagen. There is increasing evidence that disruption of this interaction may impact CSC survival. Current strategies using antibody therapy directed against CD44, hyaluron-chemotheraeputic conjugates, and even miR-34a are under development (Wang, S. J. et al., 2006; Li et al.; Liu et al.).

Target	Drug	Trial #	Phase	Cancer types
Hedgehog Pathway	GDC-0449 (SMO and/or PTCH1 inhibitor)	NCT01088815	II	Pancreatic
		NCT00980343	II	Brain & CNS
		NCT00957229	II	BCC
		NCT00959647	II	BCC, CRC, Ovarian
		NCT00982592	II	Gastric, Esophageal
		NCT01267955	II	Chondrosarcoma
		NCT00887159	II	Lung
		NCT00961896	II	BCC
	LDE-225 (SMO inhibitor)	NCT01125800	I	Pediatric solid malignancies
		NCT01208831	I	Solid malignancies
		NCT00880308	I	Solid malignancies
		NCT01033019	II	BCC
	BMS-833923 (XL139) (SMO inhibitor)	NCT00670189	I	Solid malignancies
		NCT00909402	I	Gastric, Esophageal
		NCT00927875	I	Small cell lung cancer
	IPI-926 (SMO inhibitor)	NCT00761696	I	Solid malignancies
		NCT01130142	I, II	Pancreatic
		NCT01310816	II	Chondrosarcoma
	PF-04449913 (SMO inhibitor)	NCT01286467	I	Solid malignancies
Notch Pathway	MK-0752 (γ-secretase inhibitor)	NCT00645333	I, II	Breast
		NCT00106145		Breast
	RO4929097 (γ-secretase inhibitor)	NCT01071564	I	Breast
		NCT01192763	I	Pancreatic
		NCT01193868	II	Lung
	PF-03084014 (γ-secretase inhibitor)	NCT00878189	I	Solid Malignancy & Leukemia
	OMP-21M18 (anti-DLL4 mAb)	NCT01189942	I	CRC
		NCT01189929	I	Pancreatic
		NCT01189968	I	Lung
Telomerase	Imetelstat (GRN163L)	NCT01137968	II	Lung
Dendritic Cell Vaccines	Dendritic cell vaccine to CD133+ CSC mRNA	NCT00846456	I, II	Glioblastoma
		NCT00890032	I	Brain & CNS
	Dendritic cell vaccine to whole CD133+ CSC lysate	NCT01171469	I	Brain & CNS
Wnt Pathway	PRI-724	NCT01302405	I, II	CRC, Pancreatic
	Resveratrol	NCT00256334	I, II	CRC

Table 1. Current clinical studies evaluating CSC-directed therapies in solid organ malignancies. BCC = Basal cell carcinoma, CRC = Colorectal carcinoma

4. Conclusions

The CSC theory, aside from the contribution to our understanding of tumor biology, has potential far-reaching clinical implications. Early preclinical success, while certainly encouraging, has yet to be confirmed in clinical studies. For many of the therapeutic strategies discussed, phase I and II clinical studies are currently ongoing and will additional evidence as to the safety and efficacy of these therapies in the near future (Table 1).

In order to specifically target CSCs while sparing somatic stems cells, it will be critical to identify unique molecules and dysregulated pathways in the CSC population when compared to the somatic stem cell population. Our understanding of the differential regulation of CSCs and normal tissue stem cells is yet in its infancy and clearly needs further exploration.

Our ability to consistently and reliably identify tumor cell populations with CSC functionality needs to be improved. It is becoming increasingly apparent that currently identified CSC antigens are insufficient to detect all cells harboring CSC functions (Chen, R. et al.). This may be due to plasticity in the CSC compartment with cells gaining and losing CSC functions in response to environmental signals. It may also be due to the possibility that current surface antigens simply are not selective enough, or the combination of surface antigens not fully refined. Part of this problem stems from our incomplete understanding of the functional aspects of these CSC markers/molecules. In many cases these antigens are used because they have been shown to conveniently mark a population of cells that happen to have stem-like properties rather than because their expression is intrinsically tied to CSC functionality.

Furthermore, if we wish to target these CSC antigens, our understanding of their expression patterns in normal tissues needs to be elucidated. A prime example of this is the case of anti-CD44v6 therapy discussed previously. Clinical trials for a CD44-drug conjugate were halted early due to excessive skin toxicity and a patient death that occurred because of targeting of CD44v6 expressed in the basal layer of the skin. A more comprehensive knowledge of CSC antigen expression patterns may help better predict and subsequently avoid treatment-related toxicity.

Preclinical studies suggest that the combination of CSC-specific and broad cytotoxic therapy holds the best chance for disease eradication. In many preclinical examples, CSC-targeted therapy appears to increase the sensitivity of the CSC subpopulation to traditional cytotoxic therapies. Furthermore, it is possible that differentiated tumor cells may provide feedback and support to their undifferentiated CSC counterparts and that removal of this population may impact CSC survival as well. As therapeutic strategies are developed to target the CSC subpopulation, consideration will need to given to appropriate combinations with traditional cytotoxic therapies.

While there are clearly many obstacles to overcome, CSC-directed therapy has the ability to revolutionize cancer treatment. By focusing on tumor subpopulation heterogeneity in treatment response and tumorigenic potential, we will undoubtedly uncover novel therapeutic targets that would have remained otherwise undiscovered. Although CSC theory is yet in its infancy, the success of early preclinical studies brings hope that it may carry with it improved treatments.

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4

Cancer Initiating Cells in Head and Neck Squamous Cell Carcinoma

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1. Introduction

The clonal genetic model of cancer defined it as a proliferative disease originating from mutated tumour cells. However, not all the cells in a tumour have the same characteristics and only particular cells have the capability of originating it. Cancer stem cells (CSCs) and cancer initiating cells are the main responsible of this property and are considered, due to their characteristics, the actual target for therapy. Moreover, recent evidences indicated that these cancer initiating cells are also responsible for the metastatic behaviour of tumours. Data obtained in human patients and in mouse models have revealed that CSCs are characterized, at the molecular level, by the expression of certain specific markers, a complex circuitry of transcriptional and epigenetic regulation, and, in solid tumours, by high predisposition to undergo the so called epithelial mesenchymal transition.
Head and neck squamous cell carcinoma is one of the most prevalent type of malignancy worldwide. The mortality due to HNSCC is mainly caused by local recurrence and local metastasis to cervical lymph node, and occasionally by distant organ metastasis. The primary tumours are characterized by cellular and functional heterogeneity. Numerous evidences have indicated the presence of cancer initiating cells or cancer stem cells in this type of cancer. Here the current evidences of such cell population in the context of putative markers, transcriptional and posttranscriptional regulation, in vivo genetically engineered mouse models and possible therapeutic approaches will be reviewed.

2. Relevance of head and neck cancers

The term Head and neck cancer comprises several epithelial-derived tumors arising in the oral cavity, pharynx, larynx and nasal cavity. The most common type of head and neck cancer, including oral cancer, is squamous-cell carcinoma (HNSCC). Head and neck cancers represent the sixth most common cancer worldwide, with roughly half million new cases each year, and its incidence is still increasing in several geographic areas and its trend is now affecting younger individuals (Leemans et al., 2011). Surgery and/or radiation therapy remain the gold standards for treatment of cancers of the lip and oral cavity. Recent evidence suggests that addition of chemotherapy may provide a survival advantage over radiation therapy alone in advanced stages of this disease (Bardet et al., 2011; Calais et al., 1999; Calais et al., 2004). Despite progress in surgery, radiation, and chemotherapy, the 5-year survival rate for oral cancer has not improved significantly over the past several

decades and remains at about 50–55% (Leemans et al., 2011). As for other types of cancer, the main risk factors are alcohol and/or tobacco use (up to 100 times higher for both) (Neville and Day, 2002); another important risk factor is HPV infection, which has been detected in around 20% of all cases and 50% of oropharynx cases (Leemans et al., 2011). An important aspect of these diseases is the significantly increased risk of developing subsequent second primary or recurrent tumor which shows similar genetic alterations as the original tumor (Braakhuis et al., 2006; Tabor et al., 2002; Tabor et al., 2001). This has led to the field cancerization concept (Slaughter et al., 1953), which reflects that premalignant clones are present at the surgical margins without macroscopic signs of disease that may allow the development of secondary tumors close to the areas where primary tumors arose (Bradley et al., 2007; Tabor et al., 2002; Tabor et al., 2001). The development of distant metastases, loco-regional recurrence or a second primary tumor cause in many circumstances the failure of the treatment, making essential an accurate analysis of the origin and spread of the disease.

As for other types of cancer, HNSCCs show marked heterogeneity affecting cellular morphology, proliferative and metastatic index, genetic lesions and therapeutic response. Such variability can occur between tumors arising in the same organ (intertumoral heterogeneity) identifying distinct tumor subtypes, which are characterized by their molecular profile, morphology and expression of specific markers. However, variation is also evident within individual tumors (intratumoral heterogeneity), as cells display different functional properties and express miscellaneous markers. Such heterogeneity within a single tumor is usually a reflection of the hierarchical organization within it. In addition, the intratumoral heterogeneity is an indirect indication of the existence of a subpopulation of self-renewing cells that can generate the full repertoire of tumor cells.

3. Cancer initiating vs. cancer stem cells

For many researchers in basic and translational Oncology the concept “cell of origin” is mixed and used interchangeably with the cancer stem cell (CSC) notion. It is important to note these two concepts are not necessarily identical (Visvader, 2011). The cell of origin refers to the normal cell that acquires the first cancer-promoting mutation(s), whereas, the CSC is applied to the cells in the tumor that exclusively sustains the malignant growth. In other words, each concept refers to cancer-initiating cells and cancer-propagating cells, respectively, two cell populations that substantially differ in their phenotype. However, both cell types are also related because the nature of mutations acquired by the cell of origin may dictate whether a cancer follows a CSC archetype. The possible reasons for such confusing concepts come form the usual way in which CSCs are tested, by injecting or implanting into immunocompromised mice, assuming the minimal number of cells required to develop a tumor. In this case both CSCs and initiating cells are the same, but this does not indicate that the original tumor initiating cell in the primary tumor was a CSC. Also the two current models of tumor evolution and intratumoral heterogeneity source can contribute to this confusing aspect: the “cancer stem cell” (CSC) and the “stochastic” or “clonal evolution” models. In the first one, the process of tumor growth follows a pattern highly similar to that of normal tissues relying on the presence of stem cells, and implicate that most tumor cells do not actually contribute to tumor perpetuation, and the heterogeneity is due to aberrant differentiation processes from these cancer stem cells. On the other hand, in the “clonal evolution model” the self-renewal and tumor maintenance are

mediated by the majority of tumor cells, and the differentiation, intraclonal genetic and epigenetic variation determine tumor heterogeneity. Whether which of these models is the correct one remains to be elucidated. However, the presence of multiple subclones in a single tumor and the identification of putative CSC in a very high proportion (20%) of the tumors strongly suggest that the real situation will be a combination of both. Accordingly, tumors that evolve through the CSC model, progress on acquiring additional mutations that resemble the clonal model. In this regard in, hematological malignancies it has been recently reported a tremendous genomic heterogeneity that evolve as clonal branches (Anderson et al., 2011; Notta et al., 2011)

4. Relevant signaling pathways in HNSCC

As in other solid tumors, the neoplastic process in HNSCC begins with the normal epithelium progressing through hyperplasia to dysplasia to carcinoma in situ and invasive carcinoma by a multistep process of genetic and epigenetic changes. The earliest manifestations of these lesions are leukoplakia or erythroplakia (white or red patches observed in the epithelium). The finding that only up to 20% of oral leukoplakias progress to malignancy (Braakhuis et al., 2004) indicates that it is possible to treat the disease at this premalignant stage.

As in other cancer types, HNSCC arises through the accumulation of genetic and epigenetic changes in genes to acquire a characteristic phenotype that includes limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, ability to evade apoptosis, invasion and metastasis, and angiogenesis (Hanahan and Weinberg, 2000; Negrini et al., 2010). To acquire these properties HNSCC cells need to overcome multiple cellular brakes such as those imposed by cell cycle machinery and in particular the p53 and Rb-dependent pathways. Somatic mutations in TP53 are found in 60–80% of HNSCC cases (Smeets et al., 2011). Moreover, inactivation of p53, either by knock down, by expression of dominant-negative mutant p53R172H or by expression of the HPV16 oncoprotein E6, conferred extended lifespan on oral keratinocytes (Smeets et al., 2011). Similarly, the $p16^{INK4A}$–cyclin D–CDK4(6)–RB axis is inactive in most HNSCC. In these cases, the inactivation is attributable to mutation or methylation in combination with chromosome loss or, in most cases, by homozygous deletion of CDKN2A (coding for $p16^{INK4A}$) and amplification of Cyclin D1 locus (occurring in 80% of HNSCC cases). In addition, high-risk human papillomavirus (HPV) 16 and 18 infections are also frequent in certain HNSCC (Leemans et al., 2011). In these HPV-associated HNSCC, two oncogenes expressed by the HPVs, E7 and E6 target the p53 and the entire Rb family (pRb, p107 and p130) to degradation (zur Hausen, 2002).

Among the different signaling pathways acting in HNSCC, EGFR seems to be crucial. Upon binding to its ligands, this receptor triggers activating signals predominantly through the Ras–MAPK and PI3K–PTEN–AKT pathways, and may also concur to activate Stat3-dependent transcription. EGFR is overexpressed in many cases of HNSCC (Grandis and Tweardy, 1993; Hama et al., 2009; Ozanne et al., 1986). This results in the clinical use of EGFR-specific antibodies in combination with radiotherapy that showed increased efficacy to treat HNSCC patients (Bonner et al., 2006). As mentioned, EGFR activation proceeds though the PI3K–PTEN–AKT signaling pathway. This is also of extreme relevance in HNSCC. Both, activating mutations in PIK3CA as well as inactivating mutations of PTEN, have been found in HNSCC. In addition, PIK3CA gains are very frequent in this type of

tumors. All these alterations lead to AKT activation. Although the possible presence of activating mutations in Akt genes (AKT1, 2 and 3) and gene amplifications remains to be confirmed, overall the wide majority of HNSCC display active Akt signaling (Segrelles et al., 2006). This induces not only resistance to apoptosis, but also facilitates an angiogenic switch (Segrelles et al., 2004) and cell proliferation (Paramio et al., 1999). This raised the possibility of using anti Akt therapies in HNSCC (Moral and Paramio, 2008).

5. Identification of putative CSC in HNSCC

Based on cell surface markers, dye efflux, in vivo tumorigenicity in immunocompromised mice, slow-cell cycle progression, upregulation of stemness-related genes, resistance to therapy and clonogenic proliferation assays, it has been possible to demonstrate the presence of tumor-initiating cells exhibiting stem cell properties in tumors of breast, colon, brain and melanoma. All these approaches have been also used to identify the putative ideal cancer stem cell marker, which should impart all the acquired hallmarks of self-sufficiency in growth signals, anchorage-independent growth, apoptotic/drug resistance, invasiveness, metastatic potential and impinge a high self-renewal capacity. However, it is worth noting that certain controversies in the experimental systems and the assays followed in these characterizations precluded a broad applicability for the cancer stem cell theory in all solid tumors. Nonetheless, several cell markers have been used to identify and characterize the putative CSCs in HNSCC.

5.1 CD44

CD44 is a broadly distributed polymorphic cell surface glycoprotein that mediates important processes such as wound healing and tumor metastasis (Naor et al., 2008; Orian-Rousseau, 2010). Based on its involvement in breast cancer stem cells, several studies have analyzed the relevance of CD44 in HNSCCs. In primary HNSCC CD44 expression is confined within the basal epithelial cells and is co-expressed with the stem cell-related gene BMI-1. The CD44+ cells usually found to be less than 10%, could self-renew indefinitely and produce CD44− progeny, and could generate the original tumor heterogeneity after serial transplants in NOD/SCID mice (Prince and Ailles, 2008; Prince et al., 2007). Importantly, the binding of CD44 to extracellular molecules can drive the activation of PI3K/Akt (Ghatak et al., 2005; Gilg et al., 2008; Misra et al., 2005) and MAP kinase and Ras signaling pathways (Cheng et al., 2006). However, the CD44+ fraction is probably not a pure population of CSCs and, unlike breast CSC, the use of CD44 and CD24 does not appear to further enrich the CSC population (Prince and Ailles, 2008; Prince et al., 2007). It is worth mentioning that CD44 is actually a family of several proteins that differ in the extracellular domain by insertion of variable regions through alternative splicing (Ponta et al., 2003). In particular two isoforms CD44s and CD44 v6 are widely expressed in oral epithelia (Mack and Gires, 2008). However, these proteins were abundantly expressed in carcinomas and also in normal tissue (Mack and Gires, 2008) thus indicating that the value of CD44 as a marker for a small CSC population in HNSCC needs to be reconsidered.

5.2 CD133

This is a 5-transmembrane glycoprotein, also known as Prominin-1 identified in a subpopulation of human hematopoietic cells, and has been used for the identification and isolation of putative CSCs stem cells from different human tumors such as brain, prostate,

colon, liver, pancreas and hematological tumors (Mizrak et al., 2008). Of note, CD133 expression doe not solely identify these CSC; for instance, in a large series of prostate tumors, CD133 is expressed in combination with CD44+ and $\alpha 2\beta 1^{hi}$ in approximately 0.1% of cells, irrespective of their grade or metastatic state. These cells were capable of self-renewal, proliferation, and multi-lineage differentiation in vitro to recapitulate the original tumour phenotype, consistent with CSC properties (Collins et al., 2005). CD133 expression has been found in a small fraction (1-2%) of HNSCC tumors and cell lines (Zhang et al., 2010). Interestingly this subpopulation of CD133+ cells possess distinct properties characteristic of CSCs, including higher potential for clonogenicity, increased expression of specific stem cell markers, invasion and in vivo tumorigenicity as well as increased chemoresistance as compared with their CD133− counterparts (Zhang et al., 2010). The possibility that CD133 is a bona fide CSC for HNSCC however remains to be elucidated in wide number of tumor samples.

5.3 Emmprin

CD147, also known as EMMPRIN (extracellular matrix metalloproteinase-inducer) TCSF (tumor cell-derived collagenase stimulatory factor) and Basigin, is a transmembrane glycoprotein that is upregulated during the early stage of premalignant lesions and remains stable during invasive and metastatic progression (Vigneswaran et al., 2006). EMMPRIN/ CD147 is a major candidate mediating anchorage-independent growth, angiogenesis, drug resistance, hypoxic survival and invasion, all of which are essential molecular events of carcinogenesis (Vigneswaran et al., 2006). Moreover, its ability to stimulate the production of hyaluronan by tumor cells, which in turn promotes cell survival and anchorage-independent growth via activation of Akt, Erk and FAK (Marieb et al., 2004), and the fact that it is a CD44 binding patner may indicate that the combination of CD147/CD44 positive cells may help to define a potential subpopulation of CSCs in HNSCC (Richard and Pillai, 2010).

5.4 The side population (SP)

The existence of a cell population that exclude the dye Hoechst 33342 was described in bone marrow stem cells (Goodell et al., 1997). Subsequently SP cells have also been found in a number of other stem cell populations, including putative CSCs for neuroblastoma, glioblastoma, breast, cancer and ovarian cancer. Using the same approach Harper et al., (Harper et al., 2007) identified a small cell population in HNSCC cell lines. More recently Zhang et al., (Zhang et al., 2009) characterized this SP in cell lines and clinical samples of HNSCC; they observed that SP+ cells give rise to SP+ and SP-, whereas SP- only produced SP- cells. They studied their clonogenic and tumorigenic properties and suggested that these cells posses tumor stem cell phenotypes. Moreover, they also observed that these cells expressed higher amounts of Bmi1, NSpc1, CD44 and Oct4 together with several members of the ABC tansporter family (ABCG2 and ABCB1), thus further reinforcing their suggestion. However, although SP+ were clearly more tumorigenic than SP- cells, 10^4 cells are required, a number enough high to exclude that a small subpopulation is the actual responsible for tumorigenesis potential.

5.5 ALDH1

The aldehyde dehydrogenase (ALDH) families of enzymes are cytosolic isoenzymes that are responsible for oxidizing intracellular aldehydes and contributing to the oxidation of retinol

to retinoic acid in early stem cell differentiation (Yoshida, 1992). ALDH1 is expressed at early stages during HNSCC progression and studies have shown that ALDH1 is a CSCs marker and that its presence strongly correlates with tumor malignancy as well as self-renewal properties of stem cells in different tumors, including breast cancer, hepatoma, colon cancer, and lung cancer. Accordingly, ALDH1 cells were isolated from HNSCC patients (Chen et al., 2009). These cells showed the ability to form tumorspheres, displayed increased migratory properties and demonstrated higher abilities to induce tumor growth (Chen et al., 2009). In addition, the expression of ALDH1 correlates with stage and grade of tumors and inversely related to poor outcome in HNSCC patients, probably due to an increased predisposition to undergo epthelial-mesenchymal transition and increased Snail1 expression (Chen et al., 2009). Of note, these capacities were more evident in CD44+CD24-ALDH1+ cells, suggesting that this CD44+CD24-ALDH1+ cell population is likely a CSC in human HNSCC.

5.6 The niche

Both stem cell maintenance and tumor growth are supported by the stroma. This is not a casual event, as in both cases the intricate interrelationship appears to be essential. It is worth mentioning that such stroma is composed, in the case of tumors, by a plethora of different cells such as fibroblasts and different inflammatory cells. Although there is almost no data regarding the niche of oral stem cells, there is mounting evidence that stem cells in other stratified epithelia, such epidermis, are supported at least in part by extensive cross talk between these cells and their environment (Blanpain and Fuchs, 2009; Fuchs, 2009; Moore and Lemischka, 2006). Whether there is a unique microenvironment surrounding these progenitors remains to be determined (Moore and Lemischka, 2006). Interestingly, cancer stem cells derived from epidermal tumours may exist independently of the classic skin stem cell niche, yet also have stem cell properties, including multi-lineage differentiation (Ambler and Maatta, 2009). A relative quiescent state is an important feature distinguishing adult stem cells. Such quiescence is, in part, directed by external cues expressed in the presumptive microenvironment, the niche, although the cellular and molecular nature of the niche remains obscure in most SC systems. In turn, this may lead to specific restrictive proliferation in the stem cell population (Lopez et al., 2009; Lorz et al., 2010). Similarly, tumor maintenanace and progression is modulated in part by the extracellular matrix and the cells components of the stroma such as fibroblasts (Bhowmick and Moses, 2005; Bhowmick et al., 2004b; Kalluri and Zeisberg, 2006), and/or inflammatory cells (Andreu et al., 2010; Coussens et al., 1999; Coussens et al., 2000; Kerkela and Saarialho-Kere, 2003; Spadaro and Forni, 2004; van Kempen et al., 2003). Recently, it has been suggested that these cells may affect the tumor growth by specific signaling pathways such as TGFβ (Bhowmick et al., 2004a; Cheng et al., 2005b), HGF/cMet axis (Daly et al., 2008; Grugan et al., 2010; Knowles et al., 2009), SDF-1 (Daly et al., 2008; Kojima et al., 2010; Orimo et al., 2005), and that the induction of senescence in the stromal cells (Krtolica et al., 2001; Liu and Hornsby, 2007; Parrinello et al., 2005; Yang et al., 2006) or the specific inactivation of certain pathways such LKB1 (Katajisto et al., 2008) or PTEN (Trimboli et al., 2009) in these cells may effectively influence tumor progression. It becomes obvious that such crosstalk may also influence CSCs. However, with the clear exception of colon cancer (Borovski et al., 2011; de Sousa et al., 2011; Vermeulen et al., 2010), the possible effect that the niche can exert on this cell population remains unknown.

6. Genomics of HNSCC CSC

6.1 Gene expression studies

The sequentiation of the human genome and the development of high throughput technologies, in particular gene-expression profiling, have provided the opportunity to describe biological features, including pathologies, in a quantitative manner. Accordingly, there is a astonishing number of genomic studies in cancer, and also the number in HNSCC is huge (Braakhuis et al., 2010; Hunter et al., 2005; Molinolo et al., 2009). Subsequently, genomics studies of CSCs purified on the basis of cell surface marker have been characterized for various tumor types including leukemia, glioblastoma, sarcoma, breast, colorectal, lung, pancreatic and prostate cancer. In an early study using quantitative RT-PCR analysis performed in CD44+ cells from HNSCC demonstrated an increased expression of the stem cell regulator Bmi1 (Prince et al., 2007). Later on, using microarray studies on CD44+CD24-ALDH1+ vs CD44+CD24-ALDH1- permitted the identification of 1082 genes differentially expressed (Chen et al., 2009). Remarkably among the upregulated genes there are stemness genes (OCT4, NANOG, SOX2, KLF4, BMI-1, and NESTIN), Cell cycle regulators (CCNA2, GTSE1, MAD2L1, MCM7, RAD21), transcriptional modulators (FOSL1, HMGB1, MBNL1, POU5F1, TPX2), modulators of epithelial mesenchymal transition (DKK1, SNAI1, SNAI2, TWIST1) and other signaling proteins belonging to mTOR, Cytokine and ABC transporter families or pathways (Chen et al., 2009). Importantly the upregulated genes included CD147 (Chen et al., 2009). It is therefore conceivable that this genomic study may have identified a CD147+CD44+CD24-ALDH1+ population with enriched CSC properties.

6.2 MicroRNA studies

miRNAs are a class of endogenous non-coding RNAs that function as important regulatory molecules by negatively regulating gene and protein expression. They have been implicated to control a variety of cellular, physiological, and developmental processes and the aberrant expression of miRNAs is connected to human diseases such as cancer acting as a tumor suppressors or oncogenes (Brown and Naldini, 2009; Croce, 2008; Croce, 2009; Esteller, 2008; Garzon et al., 2009; Garzon et al., 2010; Inui et al., 2010; Ryan et al., 2010). In addition, miRNAs are important factors in stem cell function and the expression levels of certain miRNAs in stem cells are different from other normal tissues, thus implying that they may have a unique role in stem cell regulation (Alvarez-Garcia and Miska, 2005; Cheng et al., 2005a; Croce and Calin, 2005; Gangaraju and Lin, 2009; Hammond and Sharpless, 2008; Sartipy et al., 2009; Wang et al., 2009; Zhang et al., 2006). The obvious parallelism indicates that miRNAs may exert specific roles in self-renewal, proliferation, and differentiation of cancer cells including CSCs. In agreement, recent studies have shown involvement of several miRNAs in the regulation of CSCs. For example, the miR-200 family inhibits CSC functions in breast and pancreatic cancer. Functional inhibitory roles have been suggested for miR 125b, miR 183 and miR-34, whereas miR 30 and miR-17-19b may play essential roles in maintaining the stem-like features of cancer cells (Croce and Calin, 2005; Inui et al., 2010; Wang et al., 2009). Since there are a relatively large number of studies describing the altered expression of miRNAs in HNSCC (Liu et al., 2009; Tran et al., 2010), it is likely that in the near future the link between miRNAs and CSCs in HNSCC will find further experimental support.

7. Mouse models of HNSCC

The mouse models have proven indispensable in addressing the cellular origin of cancers. Two primary approaches have been used to tackle this question in HNSCC. One is based on the induction of squamous malignancies by the application of carcinogens to the skin, the so called two stage carcinogenesis protocol. The second is a more refined system using transgenic or conditionally targeted gene technologies to explore the effects of oncogenes and tumour suppressors in different cellular contexts.

7.1 The two-stage model of mouse skin chemical carcinogenesis

This system has proved of use in the understanding of the development of squamous tumors from a molecular point of view (DiGiovanni, 1992; Slaga et al., 1995; Yuspa, 1998). In this model, tumors progress through three sequential steps termed initiation, progression and conversion. Initiation is an irreversible and inheritable change that does not lead to phenotypic alterations; this can be achieve through the use of dimethylbenzanthracene (DMBA), which frequently induces mutations in the Ha-Ras oncogene (Quintanilla et al., 1986; Zhang et al., 1998). Promotion refers to the selection and expansion of the initiated population, giving rise to papillomas; this step is typically induced by the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Some of these papillomas proceed through the conversion phase, forming malignant squamous cell carcinomas (DiGiovanni, 1992; Slaga et al., 1995; Yuspa, 1998). Interestingly, this model displays some parallelisms with certain human tumors, including head and neck cancer (Amornphimoltham et al., 2008; Amornphimoltham et al., 2004; Segrelles et al., 2006). Such parallelism stands for more than a similar pathology or histology. In this system there are two main players that are also essential actors in the HNSCC scenario: Akt (Segrelles et al., 2006; Segrelles et al., 2002 Molinolo et al., 2009) and Stat3 (Kim et al., 2007; Sano et al., 2008). In agreement, deregulated Akt activity in transgenic mice leads to heightened sensitivity to this experimental carcinogenesis protocol (Segrelles et al., 2007). Accordingly tumors generated through injection of Akt-transfected, papilloma derived PB keratinocytes display many molecular alterations similar to those found in human HNSCC (Segrelles et al., 2006). The two stage chemical carcinogenesis model has allowed the study of the possible role of adult stem cells in cancer development (Blanpain and Fuchs, 2006; Blanpain and Fuchs, 2009; Owens and Watt, 2003), thus allowing to important aspects of CSC. However, there might be intrinsic differences between the epidermal stem cells and oral stem cells. Of note these studies have allowed to propose mTOR as a suitable molecule for the treatment of HNSCC targeting possibly also CSCs (Amornphimoltham et al., 2008; Amornphimoltham et al., 2005; Castilho et al., 2009; Molinolo et al., 2007).

7.2 Genetically modified mice

The evolution of gene manipulation techniques in mouse has allow to express or ablate virtually any gene, including oncogenes and tumour suppressors, in a target tissue. The technical refinement also permits to generate such modification only in a small subsets of cells in a tissue in a time controlled manner, thus recapitulating what really happens in human sporadic tumors (Jonkers and Berns, 2002; Meuwissen et al., 2001). A number of mouse models have tried to recapitulate human HNSCC. The first report of a genetically engineered mouse model was the targeted expression of cyclin D1 transgene to the oral-esophageal epithelium (L2-cyclinD1 transgenic mice) (Nakagawa et al., 1997). These mice

exhibited dysplasia in the tongue, esophagus, and forestomach by 16 months of age but did not develop tumors (Nakagawa et al., 1997) unless they were crossbred with p53 heterozygous mice, which resulted in invasive oroesophageal cancer development in L2-cyclinD1/p53+/- mice (Opitz et al., 2002). More recently, using inducible systems, it has been reported that the activation of an oncogenic K-rasG12D allele in the oral cavity of the mouse induces oral tumor formation in mice, but these lesions were classified as benign squamous papillomas that progress to squamous malignancy upon activation a point mutated p53 gene (Caulin et al., 2007). In contrast, transgenic mice expressing the K-ras G12D oncogene under the control of tet-regulated responsive elements in the basal layer of stratified epithelia developed multiple lesions ranging from hyperplasias, papillomas, and dysplasias to metastatic carcinomas in the skin and oral mucosa. (Vitale-Cross et al., 2004). Finally, the TGFβRII deletion in combination with activation of either K-ras or H-ras in mouse head-and-neck epithelia caused HNSCC (Lu et al., 2006), in contrast with the targeted ablation of this gene in all stratified epithelia, which results in anal and genital SCCs (Guasch et al., 2007), indicating that progression to cancers occurs rapidly when the TGFβRII null epithelial tissues are exposed to activated oncogenes and/or loss of additional tumor suppressors. Nonetheless, although in many circumstances the transgene expression or gene ablation affect the stem cell compartment no careful analysis of this cell population or the possible involvement of CSCs have been addressed. Similar to the above mentioned models a transgenic mouse model expressing a constitutive active Akt1 kinase has been generated. These mice develop spontaneous tumors in various organs and display increased sensitivity to two stage skin carcinogenesis protocols (Segrelles et al., 2007). Remarkably, these mice also display altered development of several ectodermal organs and disturbed homeostasis of the epidermal stem cells (Segrelles et al., 2008). In oral tissues, the lesions that these mice develop rarely progress to overt tumors due to induction of premature senescence (Moral et al., 2009a). Remarkably genomic analyses in these tissues and in primary cells have been used to validate the upregulation of the Kruppel-like factor 4 (Klf4/Gklf/Ezf) as a potential biomarker for HNSCC (Moral et al., 2009b), in agreement with the elevated Klf4 levels found during the early stages of oral squamous-cell carcinomas development (Foster et al., 1999) and the induction of squamous epithelial dysplasia by the ectopic Klf4 expression in transgenic mice (Foster et al., 2005). Importantly, the ablation of the tumor suppressor Trp53 in the same cells that express the active Akt kinase (or the ablation of PTEN and p53 genes in these cells) allowed progression of the tumors to squamous cell carcinomas that also showed locoregional and distant metastasis, which can be followed by imaging in vivo, and display alterations in most of the relevant signaling pathways found in human HNSCC (Moral et al., 2009a). Interestingly, both in the tumors in vivo and in cell lines derived form these mice, the expression of putative CSC markers (CD44, CD133) was noticed along with other stem cell markers (CD34, K15 and ΔNp63) (Moral et al., 2009a), thus suggesting that this model can be highly valuable for the experimental analysis and characterization of CSCs in HNSCC.

8. Conclusion

Overall, although the way leading to the identification of the potential CSCs in HNSCC is still long, the achieved findings so far are very promising. Identification of the cell of origin in these tumors may allow to the genetic analyses of the lesions involved in tumor initiation and progression, thus becoming a unique platform for the identification of early disease

biomarkers. In addition, it may have important implications for new preventive therapeutic approaches to suppress or reverse the initial phase of disease. On the other hand, the identification of CSCs will have a tremendous relevance in the therapy of these tumors. In this regard, the available mouse models, and cell lines derived from them, will turn into essential tools in uncovering the cellular origins of cancer and the impact of specific mutations on tumorigenesis. However, a major disadvantage coming form the inherent different systems, human and mouse, makes essential exhaustive validation. Since this is particularly achievable using functional and comparative genomics, this potential problem can also become a benefit as may allow to the identification of genetic or epigenetic changes common in both systems that may permit the identification of suitable targets of potential therapeutic benefit.

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5

Stem Cell Growth as a Model of Carcinogenesis

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1. Introduction

Cancers of the central nervous system are typically some of the most challenging of malignancies to treat and often have poor clinical outcomes. Even under the most aggressive of therapies, tumors such as glioblastoma recur at a high frequency. This was long thought to be due in part to the inability of delivering effective therapeutics to the site in combination with the inherent difficulties of surgical intervention in this challenging environment. With the discovery of a specific population of cell that could drive cell expansion in acute mylogenous leukemia (Lapidot et al., 1994) came a fundamental change in the thinking about the therapeutic approach necessary to effectively address certain cancers. Instead of the need to eliminate every cell in a tumor, a distinct group with identifiable properties could be the target. This would deprive the tumor of its proliferative engine, making the remaining more differentiated, and presumably less invasive, tissue dealt with more easily. Evidence has emerged over the past few years suggesting these cancer stem cells (CSCs) might be at the heart of numerous other non-hematological malignancies including breast, prostate, pancreatic, lung, and ovarian tumors (Al-Hajj et al., 2003; Eramo et al., 2008; Collins et al., 2005; Zhang et al., 2008). This concept has great potential implications for treating central nervous system (CNS) neoplasms like glioblastomas and medulloblastomas, which demonstate the kind of hierarchical organization that is considered to be a hallmark of the cancer stem cell hypothesis [Reviewed in (Gupta et al., 2009)]. In this model, tumor heterogeneity arises from distinct cell subpopulations, only some of which are capable of regenerating the tumor as demonstrated through serial transplantations experiments performed in immunocompromised mice. This is opposed to a

clonal or stochastic model where there is a certain probability that any cell will develop mutations that allow for it to show unregulated proliferation and the ability to generate new tumors (Nowell, 1976). There is ongoing debate about how much each of these models contribute to the distribution of cells in any given cancer type. This can partially be attributed to the fact that stem cells can undergo both symmetric and asymmetric divisions, making them clonal and hierarchical in their contribution to the growth of a tumor. For example, clonal evolution may drive the progression of the cancer by producing secondary CSCs with additional mutations that impart further growth advantages (e.g. hypoxia- and chemotherapeutic-resistance), confounding interpretation of the cellular origin of the malignancy. There are a number of recent reviews on the topic of CSCs reflecting the growing interest in understanding in how these cells operate (Frank et al., 2010; LaBarge, 2010; Morrison et al., 2011; O'Brien et al., 2010; Takebe and Ivy, 2010; Shackleton et al., 2009). Still, there remain significant challenges that lie ahead in translating any understanding about this unique cell population into effective therapies. Principal among these are the ability to identify the CSC itself by a set of unique validated biomarkers, developing strategies for addressing their cell biology, whether it be targeting the intracellular signal transduction pathways, or the microenvironment they establish that regulate their self-renewal and survival in a way that minimizes effects on normal stem and non-stem cell populations. Over the course of this chapter, we will look at how our understanding of mechanisms that regulate the proliferation, differentiation and survival of non-transformed neural stem cells has impacted the thinking of how tumors arise and resist current therapeutic approaches in the context of the cancer stem cell hypothesis.
Throughout this Review, the term "stem cell" will refer to non-cancerous stem cells; when we refer to stem cells in or derived from cancerous tissue, we will use the term "cancer stem cell", abbreviated as "CSC".

2. Identification of cancer stem cells

For the cancer stem cell hypothesis to hold, the CSC has to be a distinct and identifiable entity within a progressing cancer. A number of biomarkers have been proposed to label CSCs of many different sources [reviewed in (Frank et al., 2010)] and one that has drawn considerable interest is CD133/prominin1. It is found on different solid tumor types, and in the context of the CNS, is co-expressed with the neural stem cell marker nestin in medulloblastomas, ependydomas, oligodendrogliomas and gliolblastomas (Singh et al., 2004; Calabrese et al., 2007). CD133 expression is linked to the activity of the transcription factor HIF-1 α in glioma cells grown under low oxygen conditions (Platet et al., 2007). The growth of these cells specifically is enhanced during hypoxic exposure (Soeda et al., 2009), a notion consistent with them having a selective advantage, particularly under the stressful conditions present during the rapid expansion phase of a tumor when neovascularization is just beginning, and the tumor is deriving its sustenance from the existing niche environment (Pouyssegur et al., 2006; Keith and Simon, 2007; Louis et al., 2007). However, while selection of CD133 positive cells followed by serial diluted xenotransplantation can recapitulate tumor formation, it was only observed in a subset of glioblastoma tumors. This finding highlights a challenge to the CSC hypothesis, that a CSC population may not only be specific to particular forms of cancer, but that markers may change during the evolution of a tumor from early neoplasm to metastasis. As a consequence, multiple markers will be

needed to provide for an effective screen. Other markers, including CD24, A2B5 and the chemokine receptor CXCR4, are increased in CD133-positive glioma cells grown under hypoxic conditions (Soeda et al., 2009). It is not clear how these relate to the tumorogenic potential of the cells, but do have interesting characteristics such as the role of CXCR4 in controlling the migration of gliomas (Ehtesham et al., 2004).

Hairy and Enhancer of Split 3 (Hes3) may provide an alternative biomarker for CSCs. It is a transcription factor that, like other members of its family (e.g. Hes1 and Hes5), is regulated by activation of the transmembrane Notch receptor. However, Hes3 is an indirect target of Notch signaling (Lobe, 1997; Hirata et al., 2001; Hatakeyama et al., 2004), and has been shown to identify neural stem cells in established cultures from the fetal and adult mammalian brain (Androutsellis-Theotokis et al., 2006; Androutsellis-Theotokis et al., 2009). It also identifies endogenous neural stem cells in the brain and spinal cord of fetal and adult rodents, and the adult human and non-human primate brain (Androutsellis-Theotokis et al., 2010). In addition, Hes3 identifies a subpopulation of cells in biopsies from glioblastoma patients. Hes3 co-localizes with prominin (Androutsellis-Theotokis et al., 2010), suggesting that, like prominin, it marks the CSC population in these brain tumors.

Given the above observations as well as the fact that Hes3 is regulated by signaling pathways that are of critical importance for survival and proliferation of normal stem cells, raises the intriguing possibility that analogous signal transduction mechanisms may control cancer neural stem cells as well. Consequently, the study of normal stem cells can identify core mechanisms that regulate the expansion of cancer stem cells, in an experimental system that is free of the confounding mutations present in cancerous tissue or transformed cell lines. Below, we will discuss the signaling pathway that regulates Hes3 and stem cell survival, and we will review recent literature that shows how this pathway is relevant to both normal and cancer stem cells.

3. Intracellular signaling in stem cells

A number of links have been made between stem cell growth and known oncogenic pathways. This is highlighted by the observation that loss of the tumor suppressor p53 results in greater numbers of neural stem cells in the subventricular zone (Meletis et al., 2006). In fact, many of the genes that are upregulated in putative CSCs are those that define a primitive cell population, such as Oct4, Nanog, Sox2, and Myc (Glinsky, 2008; Stevenson et al., 2009). Furthermore, it is generally recognized that CSCs and normal stem cells share a number of properties, including self-renewal and differentiation. These similarities provide a strong rationale for examining the underlying mechanisms of proliferation in non-transformed population to gain novel insight into the causes of the uncontrolled growth of CSC-driven malignancies.

The developmentally conserved Notch signaling pathway plays many roles in pattern formation, expansion and differentiation processes during embryonic and adult life (Artavanis-Tsakonas et al., 1999), being "context-dependent", to serve different roles in different cells or the same cells at different developmental stages (Louvi and Artavanis-Tsakonas, 2006). For example, in the vertebrate central nervous system, it inhibits the cascade of events required for the formation of neurons and promotes the differentiation of glia (Haddon et al., 1998; Morrison et al., 2000; Tanigaki et al., 2001; Justice and Jan, 2002; Stump et al., 2002; Kamakura et al., 2004; Taylor et al., 2007).

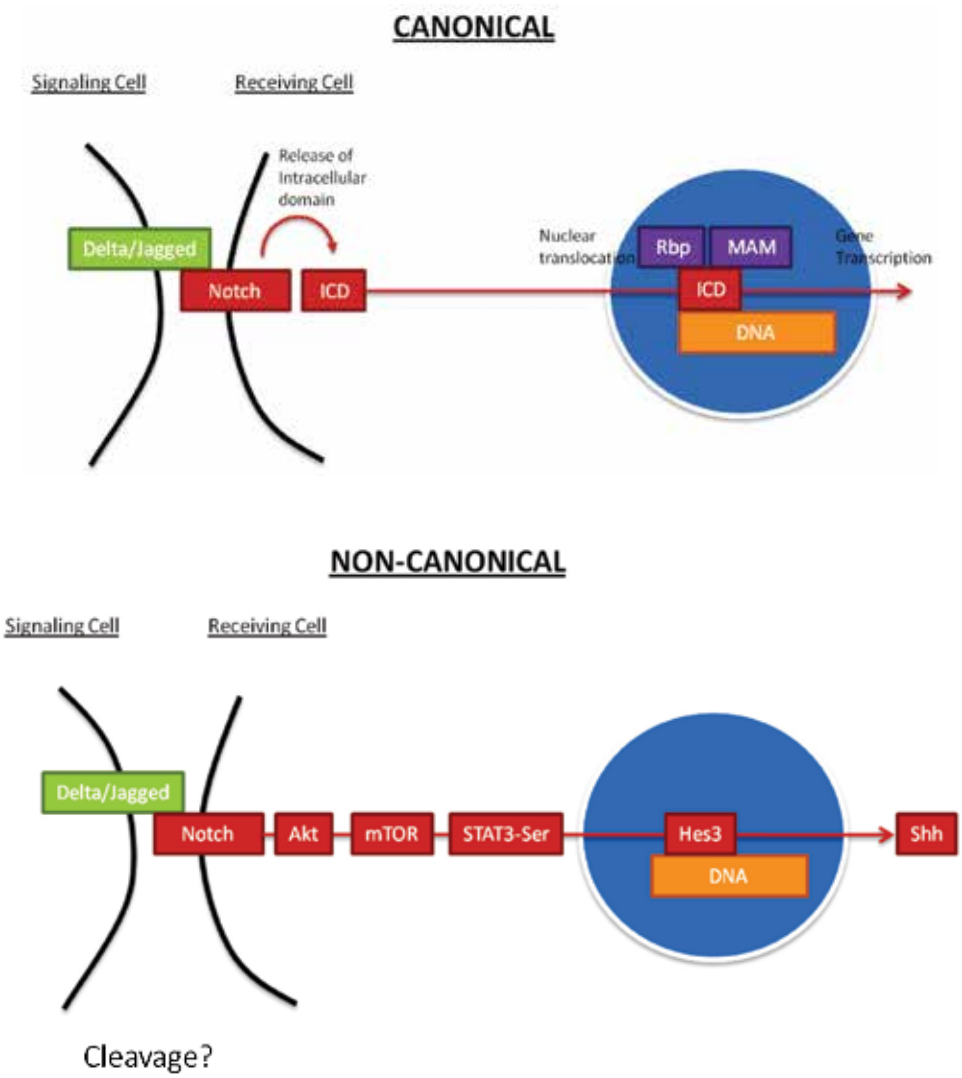

Fig. 1. Canonical vs. non-canonical Notch signaling. Engagement of the Notch receptor by its ligand leads to context dependent activation of Notch signaling. In the canonical pathway, the Notch intracellular domain (ICD) is released by γ-secretase. It then translocate to the nucleus where it complexes with factor such as Rbpsuh (Rbp) and Mastermind (MAM) to directly regulate gene expression. An alternative, non-canonical pathway involves Notch activation of transcription by an indirect mechanism through by sequential phosphorylation of kinase and subsequent increase in the transcription of Hes3. Hes3, in turn, regulates expression of sonic hedgehog (Shh), a mitogen for neural stem cells

Notch ligands and receptors are membrane-bound, so activation relies on cell-to-cell contact (Figure 1). Notch encodes a trans-membrane receptor that is cleaved on activation to release an intracellular domain that is directly involved in transcriptional control and regulates cell fate following association with Recombinant binding protein suppressor of hairless (Rbpsuh) and Mastermind (Heitzler and Simpson, 1991; Ruohola et al., 1991; Greenwald and

Rubin, 1992; Spana and Doe, 1996; Artavanis-Tsakonas et al., 1999). Many of the cellular consequences of Notch activation are mediated by the achate-scute family of bHLH transcription factors (Kidd et al., 1986; Campos-Ortega and Jan, 1991; Chiba, 2006). Together, these components constitutes the canonical Notch signaling pathway.

In Drosophila, Notch function is regulated by the inhibitor Numb (Uemura et al., 1989; Rhyu et al., 1994; Frise et al., 1996). In mice there are two homologues of Drosophila Numb, called Numb and Numb-like, which are required for the maintenance of the sub-ventricular zone and ependymal cells in the post-natal brain (Kuo et al., 2006). This suggests a continuing role for Notch in the maintenance of primitive cells associated with the ventricular surface of the brain. In addition to its function in precursor/stem cell survival, Notch inhibits neurite outgrowth in mature neurons (Sestan et al., 1999).

Soluble forms of Notch ligands increase the survival of cultured neural stem cells within hours (Androutsellis-Theotokis et al., 2006). The survival response in neural stem cells is so rapid that it suggests the involvement of second messengers that had not previously been associated with the Notch receptor. Indeed, Notch receptor activation lead to increased phosphorylation of Akt within minutes, a hallmark of growth and cancer pathways. This was followed by phosphorylation of the mammalian target of rapamycin (mTOR), another staple of cancer signaling (Cantley, 2002). Inhibition of Notch, Akt and mTOR activity resulted in reduced survival of cultured neural stem cells. These findings identified a novel branch of the Notch signaling pathway (refered to from this point as non-canonical Notch signaling) and showed that normal stem cells utilize survival signals which play central roles in cancer biology. This pathway might be a manifestation of the classical role of Notch, regulation of cell fate through lateral inhibition as originally demonstrated in Drosophila (Cabrera, 1990; Simpson, 1990). To better understand how to exploit Notch signaling for potentially therapeutic applications; it is critical to determine the signal transduction steps required for each in the appropriate cellular context.

The family of Signal Transducers and Actvators of Transcription (STAT) proteins are phosphorylated following membrane receptor activation and they mediate multiple cellular responses in the cytoplasm and nucleus of cells (Levy and Darnell, 2002). A member of the family, STAT3, has two phosphorylation sites: a tyrosine phosphorylation site at amino acid position 705 (Tyr), and a serine phosphorylation site at amino acid position 727 (Ser) and plays an important role in promoting the survival of many cell types.

STAT3-Tyr phosphorylation is a critical mediator of survival in many cell types, including transformed cell lines and cancer cells (Kiuchi et al., 1999; Levy and Darnell, 2002). STAT3-Ser phosphorylation, is of minor importance to the survival of many established transformed cell lines. Accordingly, many studies have measured increased STAT3-Tyr phosphorylation levels in many cancers (Kiuchi et al., 1999; Levy and Darnell, 2002), whereas the levels of STAT3-Ser in tumors have been less studied and somewhat unclear in the literature.

As a consequence of stem cells having the ability to both self-renew and differentiate, they interpret signals in ways different from most other cell types. Neural stem cells, for example, respond to STAT3-Tyr phosphorylation not simply by increasing their survival, but by differentiating towards the astrocyte fate (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Song and Ghosh, 2004). In neural stem cells, under conditions that support their “stemness”, phosphorylation of STAT3-Tyr is low or absent but phosphorylation of STAT3-Ser plays a major role in survival (Androutsellis-Theotokis et al., 2006). A possible interpretation is that neural stem cells simply cannot use the tyrosine phosphorylation on STAT3 for survival, and are left with only the serine site to drive survival. The serine

phosphorylation is of minor importance to most cell types (which rely mostly on tyrosine phosphorylation for survival), but of critical importance to neural stem cells (which do not have the luxury of using the tyrosine site for survival). Thus, it seems that STAT3-Tyr is required for neural stem cell differentiation, whereas STAT3-Ser phosphorylation is required for self renewal and survival. These distinct signal transduction requirements may be exploited as specific targets for the manipulation of neural stem cells and tumor cells (Figure 2).

The non-canonical Notch pathway described previously involves the fast (within approximately 20 minutes) phosphorylation of mTOR. mTOR phosphorylation has is documented to lead to STAT3 phosphorylation on serine 727 (Levy and Darnell, 2002). Indeed, inhibition of mTOR in neural stem cell cultures also inhibits STAT3-Ser phosphorylation and dramatically compromises survival (Rajan et al., 2003). These results place STAT3-Ser phosphorylation in the non-canonical Notch pathway and provide a signaling point that distinguishes stem cells from most cell types in the body in terms of their survival requirements.

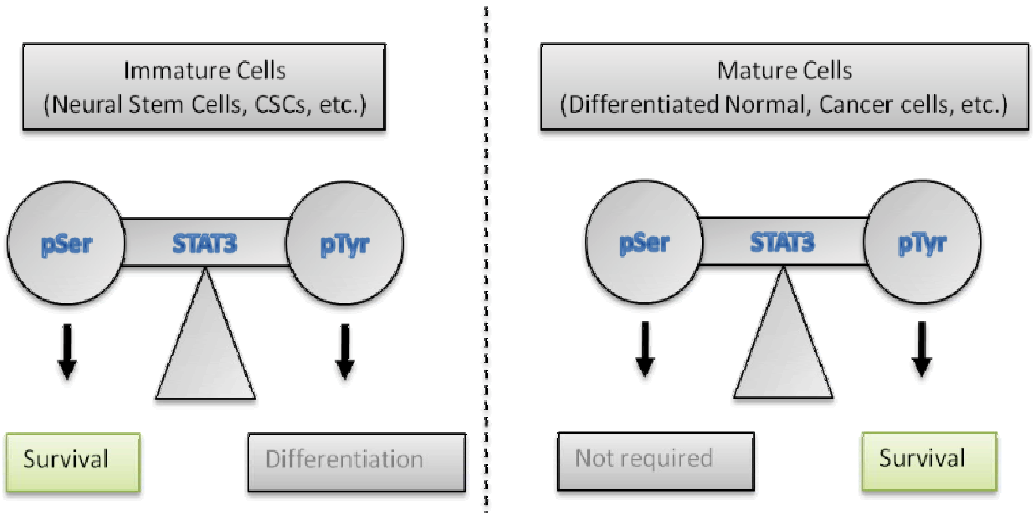

Fig. 2. Stem cell – specific use of STAT3. In neural stem cell cultures, the two phopshorylation sites on STAT3 integrate distinct signaling cascades and mediate different cellular responses. Tyrosine phosphorylation induces their differentiation through the activation of downstream genes, whereas serine phosphorylation (e.g. by the non-canonical Notch pathway) leads to Hes3 transcription, sonic hedgehog expression and increased survival. Distinct signals from multiple plasma membrane receptors and cytoplasmic kinases induce the phosphorylation of STAT3 on the tyrosine 705 or serine 727 residues. JAK2 is the direct kinase for the tyrosine site, whereas mTOR is required for phosphorylation on the serine. In neural stem cells, tyrosine phosphorylation leads to differentiation whereas serine phosphorylation maintains their self-renewal properties and promotes their survival in culture. In contrast, cancer cells rely mostly on the tyrosine site for survival, whereas the serine site is considered auxiliary. Such differences in the signal transduction requirements between cancer cells and stem cells may be exploited to specifically target the stem cell compartment in cancer as well as regenerative medicine applications

Components of the non-canonical Notch pathway are regulated by multiple other pathways in the cytoplasm, providing additional ideas for manipulation in the context of CSC biology. For example, kinases including JAK and p38 negatively regulate this pathway and inhibitors of these two kinases, predictably, promote neural stem cell survival. JAK in particular can be activated by cytokines such as CNTF, LIF or cardiotrophin and directly phosphorylates STAT3. In addition, it phosphorylates p38 (Cohen, 1996), which has a distinct role in mediating the activation of the nuclear localized mitogen- and stress-activated protein kinases that are required for the stress-induced activation of CREB and ATF1 (Arthur and Cohen, 2000; Wiggin et al., 2002). JAK and p38 inhibition, therefore, limit the survival/proliferation functions of the Notch/Akt pathway (Androutsellis-Theotokis et al., 2006).

The phosphorylation events on Akt, STAT3 and mTOR occur and diminish within an hour of Notch activation, but they initiate changes in survival that last for days. The mediators of these survival effects are unknown at present, but likely involve changes in gene expression. The Hes and Hey genes encode a family of bHLH transcription factors that are direct targets of Notch-mediated transcription (Kageyama et al., 2007). Although the function of the Hes/Hey genes remains largely unknown, transgenic animal studies showed their importance for proper brain development (Hatakeyama et al., 2004). The mRNA for one member of this family, Hes3, is elevated within one hour by Notch ligands in neural stem cells (Androutsellis-Theotokis et al., 2006). Pharmacological experiments show that Hes3 mRNA is elevated only in conditions that lead to serine phosphorylation of STAT3 and that JAK -dependent tyrosine phosphorylation is a negative regulator of Hes3 levels. These data suggest that Hes3 is a candidate mediator of the long-term effects of the initial phosphorylation events. Experiments showed that Hes3 elevates expression the protein Shh. Shh is both a morphogen and a mitogen for neural stem cells (Ericson et al., 1995; Rowitch et al., 1999). Transfection of neural stem cells with Hes3 led to long lasting (several days) elevation in Shh levels, providing yet another mechanism to translate short term effects to changes that last for days.

The non-canonical Notch signaling pathway presents a time-ordered mechanism that controls both the survival and growth of stem cells. This pathway is not confined to neural stem cells, but at least parts of it apply to many other stem cell and progenitor systems. In human embryonic stem (ES) cells, STAT3 serine phosphorylation and inhibition of tyrosine phosphorylation correlate with survival (Daheron et al., 2004; Androutsellis-Theotokis et al., 2006).

Recent studies demonstrate that p38 inhibition is essential for the in vitro propagation of adult myocardiocytes (Engel et al., 2005) and we have shown that p38 inhibitors and other treatments described above promote the expansion of fetal pancreatic precursors expressing c-peptide (the pro-insulin protein) and somatostatin. Many of these signals are likely to affect properties of multiple cell types. Activin A, for example, a member of the TGF-β superfamily of proteins (Mason et al., 1985; Shi and Massague, 2003) inhibits the proliferation of neuroblastoma cells and the angiogenic properties of vascular endothelial cells (Panopoulou et al., 2005). Pigment epithelium-derived factor (PEDF) is a factor secreted by several cell types including endothelial cells (Aparicio et al., 2005) and acts as an inhibitor of angiogenesis (Dawson et al., 1999), a trophic factor for various neurons (Steele et al., 1993), and an activator of neural stem cells in the adult brain (Ramirez-Castillejo et al., 2006). Similarly, STAT3 mediates many functions in stem cells and endothelial cells. These results provide a general model for in vitro expansion of embryonic, fetal and adult stem cells.

The core principles of the non-canonical Notch pathway that regulate stem cell number also regulate CSCs. In a prostate cancer cell line, where an equilibrium between CSCs and more differentiated cancer cells is established, manipulation of STAT3-Ser through transfection with mutant constructs selectively affects the CSC component (Qin et al., 2008). These findings provide an example of how studying normal stem cell signaling requirements results in novel therapeutic strategies to target CSCs.

Therapeutics are in various clinical stages of development for modulation of both Notch and Shh signaling (Clinicaltrials.gov). Broad spectrum inhibitors of γ-secreatase, such as MK0752, are in phase I clinical trials in the treatment of refractory or recurring CNS cancers. Also, a number of antibody-based therapies directed against specific isoforms and the Notch receptor and its ligands are in pre-clinical development. In the case of the hedgehog (Hh) of molecules, GDC-0449, LDE225 and BMS-833923 are in clinical trials for treating refractory medulloblastomas in children. Their mechanism of action is not to antagonize the interaction of Hh with their Patched receptors, but prevent the ability of the co-receptor Smoothened to be recruited to a complex necessary for transduction of ligand binding to intracellular signal transduction cascades and gene expression. This highlights an important avenue for achieving greater specificity and potentially limiting the side effect profile of any inhibitor by targeting the interactions between signaling components that are responsible for the aberrant growth driving tumor formation while leaving the interactions with other signaling components intact.

4. The balance between self-renewal and differentiation

We discussed examples of how targeting the specific pro-survival requirements of stem cells can help to kill the CSC population of a tumor. Another possible mechanism for halting the growth of a CSC-driven cancer is to induce the differentiation of the stem cell population, therefore depriving the tumor of its growth engine. This approach has been successfully applied in both hematopoetic and non-hematopoetic cancers. For example, BMP4 treatment impedes the growth of glioblastomas by pushing them to an astroglial cell fate in vitro and in vivo (Piccirillo et al., 2006). The canonical Notch pathway is largely associated with glial differentiation (Furukawa et al., 2000; Hojo et al., 2000; Chambers et al., 2001; Scheer et al., 2001; Tanigaki et al., 2001; Ge et al., 2002; Taylor et al., 2007). In this case, a complex containing Hes1, JAK2 and Tyr705-phosphorylated STAT3 has been identified after Notch1 transfection (Kamakura et al., 2004). As we have discussed above, Notch can also activate a non-canonical pathway triggered by PI3K/Akt activation that requires Ser727 phosphorylation of STAT3. These findings suggest that the site of STAT3 phosphorylation determines whether stem cell renewal or differentiation is triggered. Additionally, treatment of xenografts with the γ-secretase inhibitor GSI-18 resulted in reduced proliferation, increased apoptosis in the nestin positive cell population, and elevated neuronal differentiation (Fan et al., 2006).

The idea that a dynamic tension exists between these pathways is also supported by evidence that Akt activation inhibits canonical Notch signaling, reducing the transcription of classic Notch targets including Hes1 and Deltex in T cells (Calzavara et al., 2007). There is no information on the effects of Notch signaling on cell proliferation in these experiments but a similar outcome has been observed in HEK cells where Akt inhibition promotes Notch-1 intracellular domain- and RbpSuh-mediated canonical Notch activity (Baek et al., 2007). These results are also consistent with a role for canonical Notch signaling in differentiation rather than self renewal.

The protein Numb inhibits canonical Notch signaling and controls asymmetrical cell fate decisions in Drosophila (Roegiers and Jan, 2004). In mice, Numb and Numblike also inhibit canonical Notch signaling (Guo et al., 1996; Spana and Doe, 1996). Deletion of both genes in Nestin$^+$ precursor cells in the developing SVZ induces loss of the immature precursor cells and leads to premature neuronal differentiation (Kuo et al., 2006). The Nestin$^+$ cells that escape Numb/Numblike deletion eventually regenerate the SVZ (Kuo et al., 2006). These results further suggest that the canonical Notch pathway opposes precursor cell renewal in the developing SVZ.

Numb/Numblike activity may not always lead to self-renewal and regeneration, as Numb prevents ubiquitination of the p53 protein, increasing the activity of this tumour suppressor (Colaluca et al., 2008). Elevated p53 activity is strongly associated with reduced proliferation and increased cell death in adult neural stem cells (Meletis et al., 2006). However, a nucleolar protein that negatively regulates p53, nucleostemin, is specifically expressed in stem cells but not in transit amplifying progenitors (Tsai and McKay, 2002; Tsai and McKay, 2005). Nucleostemin is a component of the signals required for cell growth in pluripotent cells of the blastocyst and in somatic stem cells, including NSCs (Tsai and McKay, 2002; Tsai and McKay, 2005; Beekman et al., 2006). In amphibian systems where de-differentiation leads to tissue regeneration, the early up-regulation of nucleostemin is consistent with an early stem-cell specific switch in the control of the p53 system (Maki et al., 2007).

These results showing a concerted switch in signaling when NSCs differentiate offer an unusual opportunity to define how interactions between pathways leads to biologically meaningful outcomes. Here we illustrate this point with interactions between Notch, Akt, mTOR, STAT3, Shh and p53. As we acquire improved control over NSCs and other stem cells, we suggest the predictive power of these signaling models will increase with important implications for our understanding of self-renewal and cancer.

5. Stem cell regulation by controlling protein localization

Regulating processes such as membrane trafficking, cytokeletal re-organization, and protein shuffling provide an alternative approach for controlling stem cell proliferation and survival. Treatment with the entertoxin protein cholera toxin, which has long been known to inhibit the growth of numerous cancer cell lines (Coffino et al., 1975; Cho-Chung et al., 1983; Pessina et al., 1989; Viallet et al., 1990; Allam et al., 1997; Pessina et al., 1998), interferes with membrane trafficking by binding to GM1 gangliosides on a subset of lipid rafts on cell membranes (Sahyoun and Cuatrecasas, 1975). This results in increased recycling of the Tie2 receptor and nuclear shuttling of Hes3 in cultures of fetal and adult neural stem cells. Nuclear Hes3 following cholera toxin treatment correlates with the proliferative state of neural stem cells (Androutsellis-Theotokis et al., 2010). In contrast, cultured neural stem cells that are induced to differentiate by mitogen removal quickly lose nuclear Hes3 expression while retaining cytoplasmic Hes3 pools, before they fully lose Hes3 expression altogether. Like self renewing neural stem cells, cancer stem cells in glioblastoma biopsies also show nuclear Hes3 staining (along with a distinct cytoplasmic pool, co-localized in prominin+ particles) (Androutsellis-Theotokis et al., 2010). These findings raise the possibility that Hes3 localization is a common mechanism by which both normal and cancer neural stem cells regulate their expansion.

As discussed previously, driving CSCs to differentiate is a viable mechanism for inhibiting tumor growth. Interestingly, cholera toxin can sometimes induce differentiation of malignant cells as has been observed with cell lines from lymphoma, glioblastoma, medulloblastoma, and melanoma, and appears to involve disrupting tyrosine kinase-dependent mechanisms by which neurotrophic factors stimulate malignant stem cell proliferation (O'Keefe and Cuatrecasas, 1974; Houghton et al., 1982; Olsson and Breitman, 1982; Dufay et al., 1994; Shaw et al., 2002; Li et al., 2007; Xu et al., 2009). It is intriguing to think of cellular responses to cholera toxin as a distinguishing factor between stem cells and more mature cells.

6. Stem cells and the vasculature

The similarities between normal and cancer stem cells extend beyond the intracellular signals they use in common. Both cell populations associate tightly with the vasculature, a central component of the stem cell niche, and are affected by factors such as cell-cell cell-extra cellular matrix interactions, as well as soluble ligands produced by cells comprising blood vessels. An important signal is vascular endothelial growth factor (VEGF), a major activator of angiogenesis in both embryos and tumors (Coultas et al., 2005; Jain et al., 2006). The VEGFR-2 receptor (Flk1/KDR) plays a role in adult angiogenesis and VEGFR-1 and VEGFR-2 are upregulated in the injured brain (Beck et al., 2002). Indeed, VEGF directly promotes the self-renewal of stem/progenitor cells in vitro (Maurer et al., 2003; Schanzer et al., 2004; Meng et al., 2006) and in vivo (Jin et al., 2002; Schanzer et al., 2004). Notch signals are downstream of VEGF and vascular growth is inhibited by Notch signaling (Siekmann et al., 2008). During normal development of the retina, the tip-cell state is favored in endothelial cells that lack Notch signaling (Hellstrom et al., 2007). Similar results have been obtained in developing fish embryos that lack Delta-like ligand 4 (Dll4) or Rbpsuh (Siekmann and Lawson, 2007). In a reverse experiment, Dll4 up-regulation inhibited the proliferation of endothelial cells in culture (Williams et al., 2006). Taken together, these results establish that Notch signaling regulates vascular development. Consistent with this notion and in the context of cancer, two recent papers indicate that inhibition of Notch signaling disrupts the vascular supply causing tumors to shrink (Noguera-Troise et al., 2006; Ridgway et al., 2006). These studies suggest that the therapeutic benefit of Dll4 inhibition is achieved by generating vessels that are poorly perfused. However, the persistence of this effect will determine the utility of Dll4 as a cancer therapeutic. Another consideration is the dual effects of Dll4 on blood vessels and neural stem cells which pose a challenge for dissociating the effects of treatments between the vasculature and the stem cell compartment.

Angiopoietin 2, the soluble ligand of the Tie2 receptor was found to have a similar effect as Dll4 on neural stem cells, both in vitro and in vivo, but the opposite effect on blood vessels (Androutsellis-Theotokis et al., 2009). In fact, Angiopoietin 2 has been known to be a potent pro-angiogenic factor. These results established Angiopoietin 2 as a pro-angiogenic soluble factor that increases the number of neural stem cells in vitro and in vivo.

When Dll4 and Angiopoietin 2 were mixed into a cocktail that also contained insulin and a JAK inhibitor (all of which are known to increase neural stem cell numbers), the effects on the vasculature were significantly reduced. These results demonstrate the potential to separately regulate neural stem cells and blood vessels through combinations of pharmacological treatments, a concept that has important implications in addressing the growth of tumor tissue containing a cancer stem cell compartment.

Targeting the neurovascular niche is at the center of many anti-cancer and pro-regenerative therapeutic approaches, as it contains blood vessels that often feed tumors and stem cells that may be stimulated to proliferate and replenish damaged tissue. This tight association between blood vessels and stem cells is complicating efforts to specifically affect vessels or stem cells in the context of degenerative disease or cancer. For example, in the case of regenerative therapy, one can envision treatments that increase the numbers of stem cells in the tissue without affecting the vasculature. In the case of chemotherapeutics, one may aim at a reduction of both the cancer stem cell compartment and inhibition of angiogenesis.
Advances in stem cell biology suggest possible treatment strategies to specifically target cell sub-populations in the normal and neoplastic tissue. Notch signaling pathway activation by Delta4 increases the number of endogenous neural stem cells in the brain, and at the same time reduces vascular coverage. Tie2 activation by Angiopoietin2 has a similar effect on endogenous neural stem cells but the opposite effect on blood vessels. VEGF, a secreted cytokine, promotes angiogenesis as well as neural stem cells survival and neurogenic potential. Combination treatments that include Delta4, Angiopoietin2, insulin, and a JAK inhibitor maximize the effects on endogenous neural stem cell increases but significantly reduce the angiogenic effects. VEGF inhibition and Dll4 inhibition are currently being exploited in cancer research in an effort to disrupt the blood supply to a tumor. VEGF inhibition reduces tumor vascularization by opposing angiogenesis. Dll4 inhibition decreases blood flow to the tumor by enhancing non-productive angiogenesis, i.e. the formation of new blood vessels from existing ones that are not able to efficiently carry blood to the tissue. VEGF and Dll4 also promote the survival of neural stem cells. As a result, VEGF inhibition and Dll4 inhibition may also reduce the number of stem cells in a tumor, and this effect may be partly responsible for the anti-cancer functions of these treatments (Figure 3).

7. Implications and conclusions

Current therapeutic approaches for cancer are based on the decades old concept of targeting the proliferative state of the cells in a tumor. This strategy is effective at killing those cells that proliferate fast and which therefore comprise the bulk of the tumor. As a result, many tumors can be shrunk in size. However, certain tumors contain a resistant cell population, the cancer stem cell, which is often spared and can regenerate the disease as they are able to produce more CSCs (self renewal) along with more differentiated cells which will make up the bulk of a tumor (potential). In cancer, regeneration manifests itself as both recurrence and metastasis (regeneration in a new location).
Accumulating knowledge suggests that CSC resistance to therapy is partly due to the fact that they operate variations of normal proliferative and survival signaling pathways. These cells can be isolated and cultured in order to study their signal transduction requirements. However, CSCs contain vast numbers of mutations and exhibit great genomic instability, making it difficult to work with. The heterogeneity of the mutations found in CSCs from different patient samples further hinders drawing general conclusions. It is becoming appreciated that many of the properties of CSCs are also found in primary non-tumor stem cells. For example, neural stem cells use similar signaling pathways as their cancerous counterparts (e.g. from brain tumors). This distinguishes normal and cancerous stem cells from more differentiated tumor cells. Primary neural stem cells from non-cancerous tissue, therefore, are a valuable model to study the signaling requirements of CSCs from brain tumors, as they avoid the problem of heterogeneous mutations and genomic instability.

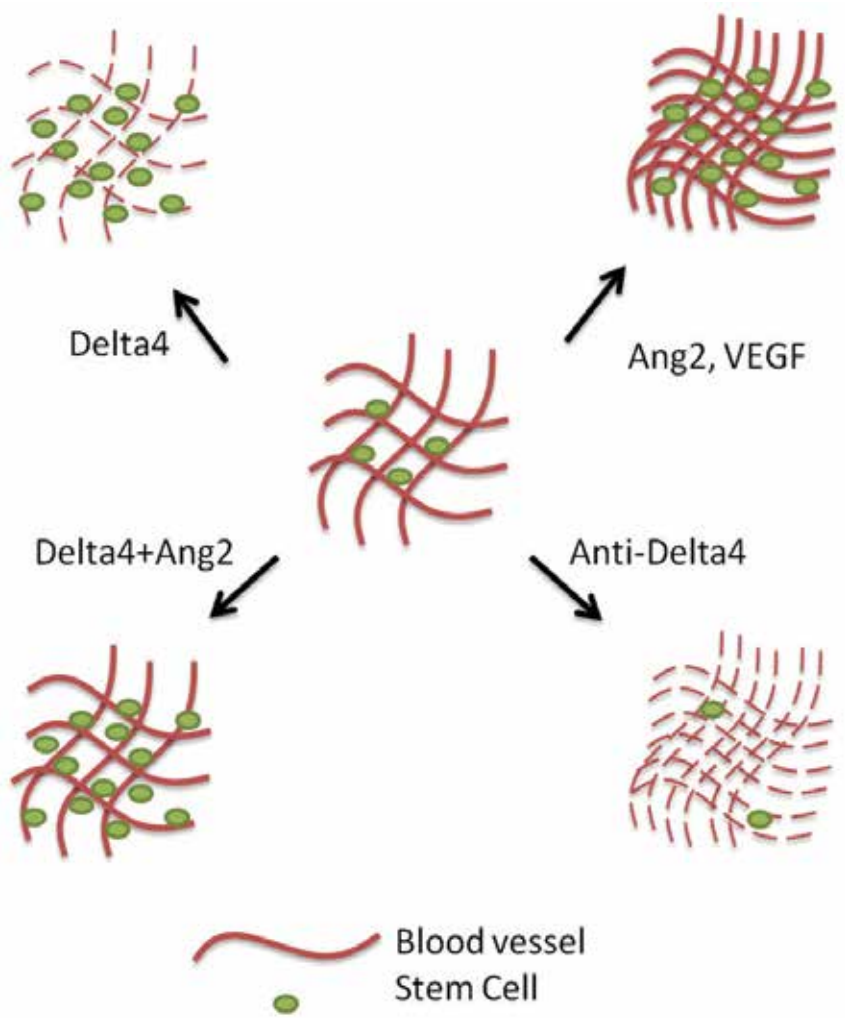

Fig. 3. Distinct modulation of stem cell and vascular biology. Combinations of pro- and anti-angiogenic factors that also influence neural stem cell numbers offer the potential to distinctly regulate the vasculature and the endogenous neural stem cell population in tissues to best address anti-cancer or regenerative medicine applications

Understanding these pathways not only provides us with novel targets for chemotherapeutics, but allows us to consider the impact current and future therapies may have on underlying malignancies. For example, VEGF is being used on a trial basis for treatment of peripheral vascular disease, chronic skin ulcers and coronary artery disease by stimulating growth of new blood vessels, but VEGF also can stimulate proliferation of malignant glioblastoma stem cells (Huang et al., 2010; Kim et al., 2004; Lekas et al., 2004; Bao et al., 2009). Trophic factors in the central nervous system such as nerve growth factor, brain-derived neurotrophic factor and others have long been investigated as a potential cure for devastating neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Aron and Klein, 2010; Rangasamy et al., 2010). There are many technical obstacles

to perfecting a workable therapeutic regimen for trophic factors in the central nervous system, such as achieving adequate delivery to large numbers of target neurons over a large absolute space within a human brain (Alisky and Davidson, 2000). However, once these technical issues are resolved, those contemplating clinical use of neurotrophic factors will have to consider their potential to stimulate cancer cells. Receptors for neurotrophic factors, and growth stimulated by these factors, can be demonstrated for a diversity of malignancies that run the gamut from solid organ to epithelial, lymphoid and hematopoietic (Dudas et al., 2011; Pearse et al., 2005; Thiele et al., 2009). Stimulation of tyrosine kinase receptors by neurotrophins is probably the molecular basis by which trophic factors proliferate malignant stem cells (Dudas et al., 2011; Zhang et al., 2010).

Evidence that a trophic factor can produce malignancy by stimulation of malignant stem cells is the occurrence of a clinically significant cancer that did not exist until a trophic factor was given, that regressed when the factor was withdrawn, and that demonstrated trophic-factor dependent growth in vitro. This was indeed true for a patient with epogen-driven acute myelogenous leukemia (Bunworasate et al., 2001), but to the best of our knowledge, this is the only case history where the cause and effect relationship between trophic factor and stimulation of malignant cells is so iron clad. Epogen is a synthetic form of erythropoietin, a polypeptide hormone produced mainly in the kidneys which stimulates production of red blood cells from erythroblast precursors in the bone marrow. Recombinant erythropoietin is frequently employed for treatment of anemia from chronic kidney disease, bone marrow failure and cytotoxic cancer chemotherapy (Spivak et al., 2009). Thus, a systemic search is needed to find cases and compile a registry for epidemiological and clinical data, and then a tumor bank could be set up, dedicated to store issue samples for further investigation, especially for isolating and purifying malignant stem cell populations from more differentiated neoplastic cells. We would in essence be seeking to extend Koch's postulates of infectious disease (proving a particular pathogen causes a specific disease) to the realm of oncology, by proving trophic factor stimulated malignant stem cells are the cause of malignancy (Garcion et al., 2009).

The cancer stem cell hypothesis will likely guide the thinking that brings about important future breakthroughs in cancer treatments. The wealth of information generated from ongoing studies of somatic stem cell biology will provide critical insight into how uni- and multimodal therapeutic approaches are applied to maximize the benefits to patients while minimize side effects. These include the establishment of extensive gene expression profiles that allow for more precise identification of CSC populations and detailed signal transduction analyses like those described in this review that define novel pharmacological targets. This understanding will also have important consequences that shape how endogenous stem cells and exogenous trophic factors are utilized as the field of regenerative medicine continues to grow.

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6

Cancer Stem Cells in Multiple Myeloma

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1. Introduction

Over the last few years, the hypothesis that rare stem cell-like cells are responsible for tumor initiation and maintenance, namely the cancer stem cell (CSC) theory, has remarkably changed the way to approach cancer biology. The scientific relevance of this theory is based, in fact, not only on the existence of hierarchically organized, self-renewing malignant progenitor cells within each tumor, but also on the assumption that their drug resistance may account for the high frequency of relapse that renders incurable most tumors despite their treatment with specific cytotoxic drugs. The view that CSCs may descend from normal stem cells finds its historical background in studies on acute myeloid leukemia where the leukemic clone is organized according to a defined hierarchy in which the majority of tumor cells rapidly proliferate, while quiescent "leukemic stem cells" resembling early hematopoietic progenitors remain as a potential reservoir of cancer propagation due to their chemoresistance to specific cytotoxic protocols.

Recently, however, a large number of studies have turned the CSC theory into more than an idea, since clear experimental evidence has demonstrated the existence of CSCs in a number of tumors. Indeed, brain (Singh et al., 2004), intestinal (O'Brien et al., 2007) and breast cancers (Al-Hajj et al., 2003) have been shown to arise from rare cells, organized in a precise hierarchy resembling that of stem cells, which are capable of generating a differentiated progeny by repetitive asymmetric divisions. In most of these tumors, however, the earliest event driving the malignant transformation remains elusive and the original cell is far from being defined, since both histological and cell-surface marker profiles of the bulk tumor cells do not necessarily resemble those of the lineage-related stem cell. These observations, moreover, have been interpreted to suggest that CSCs could derive either from normal stem cells or from committed progenitor cells that have acquired the ability to self-renew due to specific genetic mutations (Visvader, 2011).

Similar controversies have arisen with hematological malignancies of both myeloid and lymphoid origin, including multiple myeloma (MM). In fact, differently from acute leukemia, in the initial phases of most chronic lymphoproliferative disorders, neoplastic cells do not resemble early hematopoietic stem cells, but rather cellular elements in late maturation phases, as clearly demonstrated in chronic myeloid leukemia (Jamieson et al., 2004). In MM, malignant plasma cells are characterized by unique patterns of immunoglobulin (Ig) and surface antigen expression, thus suggesting that the tumor bulk is exclusively composed of terminally differentiated cells with high proliferation potential.

However, the evidence that the majority of MM cells are quiescent and that, similarly to their normal counterpart, they lack long-lasting proliferation potential both in vitro and in vivo (Dewinko et al., 1981) implies that tumor growth is actually dependent on a more restricted subset of clonogenic and self-renewing lymphoid cells. Thus, based on the observation that both blood and bone marrow (BM) from MM patients contain lymphoid elements with a hypervariable region Ig gene repertoire identical to that of bulk tumor cells, it has been hypothesized that part of the hierarchical model of the lymphoid lineage maturation may recur also in MM (Pilarski & Jensen, 1992; Billadeau et al., 1993). The functional heterogeneity of tumor cells in MM provides the rationale for exploring the coexistence of bulk plasma cells and putative tumor stem cells resembling both phenotype and biological characteristics of B lymphocytes, consistent with the similarity observed between hematopoietic progenitors and tumor cells in leukemia. To date, several studies have highlighted the existence of putative myeloma CSCs that share with normal adult stem cells a number of molecular and functional aspects, including their resistance to conventional treatments (Agarwal & Matsui, 2010). Therefore, although issues such as CSC origin, phenotype and potential pathogenetic role in MM are still being debated, investigating MM CSCs may provide new molecular targets for treatment of this still incurable disease.

2. Implication of the CSC theory in MM pathogenesis: from "clonotypic B cells" to putative MM CSCs

Major clinical manifestations of MM, including anemia, skeletal impairment, hypercalcemia and renal failure, are related to the progressive accumulation of malignant plasma cells within BM. Moreover, similarly to normal plasma cells, malignant plasma cells produce Ig as the monoclonal component commonly detectable in serum and urine of MM patients. But as early pathogenetic events, including key oncogenic mutations, occur during B cell maturation, MM is regarded as a B cell malignancy rather than a plasma cell neoplasm.

Normal plasma cells derive from "post-germinal center B cells" (PGBCs) that have completed a program of somatic hypermutations following the Ig heavy chain (IgH) switching in lymph nodes, and undergone the subsequent maturation in circulating "memory B cells" that migrate to BM, where the stromal microenvironment drives their terminal differentiation at both morphological and functional level (Johnson et al., 2005). In this context, a recent model of myelomagenesis (Anderson & Carrasco, 2011) postulates that malignant plasma cells derive from B cells of the lymph node post-germinal center, since they display an identical pattern of hypermutated Ig genes within their clone. It is thus conceivable that a PGBC likely undergoes a first oncogenic mutation, giving rise to a clone of premalignant B cells expressing identical Ig genes and capable of migrating to BM. Therefore, the asymptomatic phase of MM development, namely monoclonal gammopathy of undetermined significance (MGUS), may be sustained by the emergence within BM of only a limited number of malignant plasma cell clones producing monoclonal Ig. Subsequent changes within marrow milieu, including induction of angiogenesis, development of cytokine-based paracrine signalling loops, and acquisition of additional genetic mutations by tumor cells, are thought to promote further growth of malignant plasma cells, thus definitely mediating the switch from MGUS to MM.

Early investigations revealed that peripheral blood of both MGUS and MM patients contains a large fraction of circulating B cells at a late stage of differentiation, as proven by their

minor CD19 and CD20 expression (Jensen et al., 1991). Although morphologically heterogeneous, these cells display identical Ig gene rearrangements in support of their monoclonal derivation and, of note, expressed the same type of either kappa or lambda light chain mRNA of BM plasma cells (Jensen et al., 1992). In parallel studies, these "clonotypic B cells" (CBCs) were found to even express typical plasma cell antigens, such as the plasma cell membrane glycoprotein-1 (PCA-1) and CD38, variable levels of CD11b β2-integrin, CXCR4 and CXCR5, pivotal molecules for the homing to BM, while lacking CD34 as typical marker of the hematopoietic stem cell subset (Jensen et al., 1992). Subsequent investigations, moreover, have demonstrated that the number of CBCs is variable among patients with an average value of 65% of peripheral B cells, although they equally express identical IgH rearrangements as parental BM myeloma cells (Bergsagel et al., 1995), that has been confirmed by gene sequencing analyses (Berenson et al., 1995; Szczepec et al., 1998). Thus, in order to identify the actual differentiation state of circulating CBCs, a number of studies were aimed at assessing the CD45 expression level, since normal B cells usually lose CD45 antigen as their maturation progresses. CBCs display different patterns of CD45 expression, depending on their location, ranging from higher levels when they reside in proximity to secondary lymphoid organs to minor expression in either BM or peripheral blood (Jensen et al., 1992). Interestingly, in a single previous report, clonal circulating B cells from MM patients were found to resemble the memory B cell phenotype (Rasmussen et al., 2004), thus suggesting that CBCs could represent an ongoing differentiating population (from PGBCs to plasmablasts) in MM, and that they may play a pivotal role in MM pathogenesis. These cells may originate in peripheral lymphoid system and, once having undergone key oncogenic mutations, migrate toward the BM to complete their differentiation into malignant plasma cells (Pilarski & Jensen, 1992).

In spite of their malignant nature, myeloma cells seem to resemble their normal counterpart at both phenotypic and functional level. Examination of BM biopsies from MM patients commonly reveals the infiltration of cells expressing the typical mature plasma cell marker CD138. They appear relatively quiescent (Kyle & Rajkumar, 2004), and, similarly to their terminally differentiated normal counterpart, display low proliferative index (Robillard et al., 2005). Other early experiments using murine MM cells showed that only a small proportion of malignant plasma cells are able to form single cell-derived colonies either in vitro or in vivo (Bergsagel & Valeriote, 1968; Canc Res; Park et al., 1971), thus emphasizing that both clonogenic and proliferation potential are restricted to a small fraction of neoplastic cells. Subsequent observations underscored that, similarly to murine MM, human malignant plasma cells possess low colony-forming capacity and only rare cells within tumor clone give rise to colonies in vitro (Hamburger & Salmon, 1977).

Evidence of a functional heterogeneity in MM is also supported by other clinical observations. Today, treatment of MM with steroids, alkylating drugs, immunomodulatory agents, proteasome inhibitors and stem cell transplantation (SCT) procedures induces improvement of median survival of patients but does not provide potential for cure, due to the high rate of MM relapse after treatment. Taken together, these observations suggest that only a small fraction of tumor cells possess the capability of initiating tumor growth and, similar to stem cell behavior within normal tissues, this small fraction is able to rebuild and maintain the original MM clone after therapy.

CBCs obtained from peripheral blood of patients with MM have been shown to successfully engraft into immunocompromised mice and induce major clinical features of human MM, such as osteolytic lesions and the production of a serum monoclonal component (Pilarski et

al., 2000). These cells, moreover, possess slight sensitivity to current anti-MM agents. It has also been demonstrated that the majority of monoclonal B cells from patients with MM show high levels of P-glycoprotein which is involved in the multi-drug resistance, and that they increase its expression after chemotherapy (Pilarski & Belch, 1994). High levels of circulating CBCs have been found during transient post-treatment remissions, and patients undergoing disease progression or relapse showed larger fractions of CBCs than those responding to treatment as well as untreated patients (Bergsagel et al., 1995). Furthermore, given the high occurrence of disease relapse after auto-SCT, it is conceivable that even high-dose chemotherapy regimens used during conventional SCT procedures is not enough to eliminate CBCs or, alternatively, that CBCs are being collected during leukapheresis and, once transplanted to patient, are able to drive the tumor relapse.
Taken together, these observations support a new model of MM pathogenesis in which CBCs, as clonogenic, self-renewing, and drug resistant cells belonging to the original malignant cell clone, may also represent the malignant stem cell population in MM (Fig. 1).

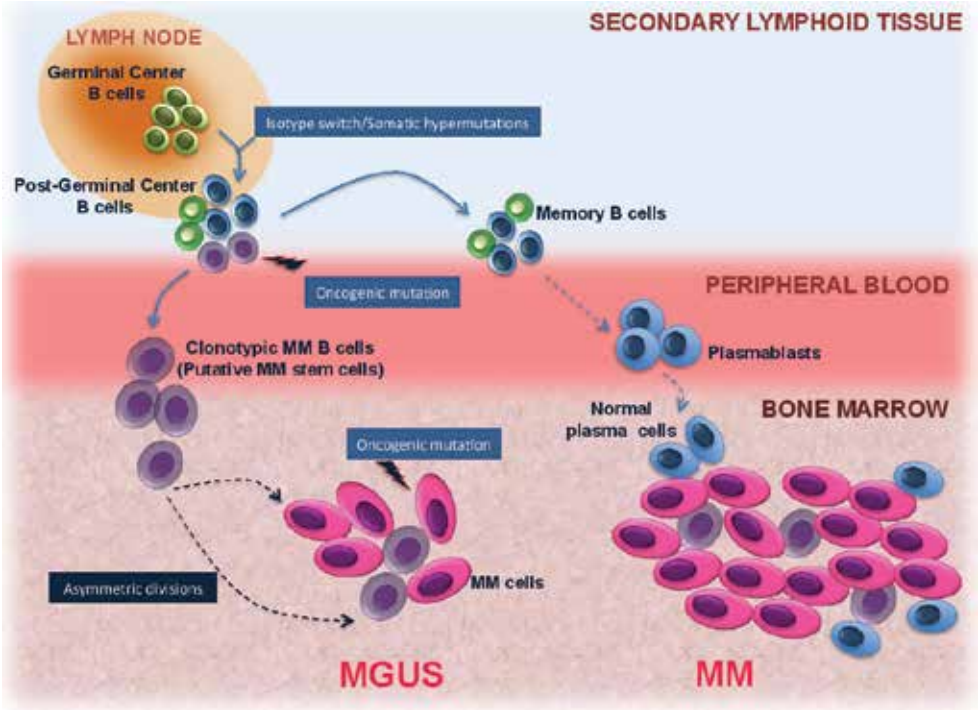

Fig. 1. Cancer stem cells in a proposed model of MM pathogenesis

In the germinal center of lymph nodes, healthy B lymphocytes physiologically undergo antigen selection and give rise to different clones of post-germinal B cells that have successfully completed sequential rounds of somatic hypermutations and Ig gene recombination. Each post-germinal B cell clone may produce memory B cells possessing consistent pattern of hypermutations within their own clone. These cells progress along their differentiation course and migrate, as plasmablasts, to BM where the stromal environment induces their terminal maturation into functional plasma cells capable of secreting Ig. A first oncogenic mutation may hit a post germinal center B cells, giving rise to

a clone of pre-malignant circulating B lymphocytes expressing identical Ig genes (clonotypic MM B cells). These cells, however, may retain their stem cell properties (self-renew and differentiation) and, once migrated to BM, divide by asymmetric divisions that can generate malignant plasma cells and a reservoir of MM stem cell fraction (MGUS). Presumably, the malignant plasma cell population may acquire additional mutations and expand by symmetric divisions. Other BM factors, including neoangiogenesis and formation of a variety of paracrine signalling loops with stromal BM components, may further support MM cell growth, whereas a little proportion of clonotypic MM stem cells with tumor-initiation capacity, persist within tumor mass.

2.1 Biological properties of MM CSCs: searching for the putative myeloma stem cell phenotype

In a number of both hematologic and solid malignancies, including acute myeloid leukemia and brain tumors, identification of CSCs was accomplished by searching the lineage-specific antigens of respective progenitors (Bonnet & Dick, 1997; Singh et al., 2004), and by their capacity to engraft into murine immunodeficient mice and recapitulate the disease. Similarly, based on the accredited theory that bulk malignant myeloma cells arise from asymmetric divisions of rare elements belonging to the B cell lineage, it has been prospectively assumed that the MM CSC population should lack CD38 and CD138 antigens, and display high self-renewal and myelomagenic potential both in vitro and in vivo.

In the first animal model engrafted with primary human myeloma cells, BM specimens from MM patients were directly injected in fetal bone chips subcutaneously implanted in SCID mice (Yaccoby et al., 1998). This model resembled several features of human MM, including the occurrence of the circulating monoclonal component, hypercalcemia and the typical MM-associated bone disease. Subsequent studies revealed that the tumor bulk is the effect of the growth of plasma cells expressing CD38, while lacking CD45 and showing high self-renewing capacity as proven by their ability to engraft into the secondary SCID-hu recipient. On the contrary, no proliferation was ascribed to the cell fraction containing $CD19^+$ B cells (Yaccoby et al., 1999). These data, however, were in apparent contrast with the knowledge that, at least in vitro, the clonogenic frequency of primary malignant cells was as low as 0.1% (Hamburger & Salmon, 1977), although it is conceivable that microenvironmental factors within the SCID-hu bone chips might support the growth of $CD38^+$ plasma cells. On the other hand, peripheral blood-derived $CD19^+$ clonotypic B cells from a patient with progressive MM were shown to successfully engraft into immunodeficient mice and produce tumor bulks mostly composed by $CD19^+/CD138^+$ malignant cells (Pilarski et al., 2002). These findings were in agreement with the previous observation showing that NOD/SCID mice are good recipients for the engraftment of circulating B cells from MM patients developing myeloma masses predominantly composed of $CD38^+$ and $CD19^+$ cells (Pilarski et al., 2000). The latter studies also emphasized that clonotypic B cells, rather than $CD138^+$ malignant plasma cells, are directly involved in tumor formation, although the proof that clonogenic B cells have self-renewal potential is still missing, since no serial transplantation has been performed in secondary animal hosts.

Based on these findings, initial studies comparing the ability of highly $CD138^+$ myeloma plasma cells with $CD138^-$ cells to form colonies in vitro revealed that cells lacking typical plasma cell markers showed higher clonogenic frequency and easy undergoing to serial passages in vitro. Accordingly, $CD138^+$ cells were unable to engraft into NOD/SCID mice

when intravenously injected, whereas cells with B cell phenotype effectively engrafted in mice and produced tumor burdens of mature $CD138^+$ plasma cells secreting the monoclonal Ig. In the same study, moreover, single cell-derived colonies capable of in vivo myelomagenesis were shown to contain variable amounts of cells expressing CD45, CD19, CD20 and CD27 as typical surface antigens of the memory B cells (Matsui et al., 2004). The concept that cells expressing markers of memory B cells share exclusive properties with normal stem cells further supported the derivation of the malignant plasma cell from a post-germinal cellular element. Consistently, although unable to undergo multilineage differentiation due to their committed state, memory B cells physiologically maintain the capacity to self-renew even during their maturation, thus ensuring the immune memory as a crucial requirement of adaptive immunity. Other independent groups, previously involved in exploring the role of B cells in MM development, showed that the gene repertoire encoding the monoclonal Ig component mostly occurred in B cells from both BM and peripheral blood rather than in mature plasma cells (Billadeau et al., 1993; Bakkus et al., 1994), albeit such a clonogenic B cell population can rarely be detected in primary bioptic specimens (0.1% of all tumor cells) (Chen & Epstein, 1996; Rasmussen, 2001).

In line with this view, blood and BM specimens from MM patients were shown to contain a $CD138^-/CD19^+/CD20^+/CD27^+$ cell fraction with higher clonogenic capacity as compared to the $CD138^+$ cell counterpart. More interestingly, this memory B cell-like subpopulation was found to share identical Ig gene rearrangement as the malignant plasma cells and, unlike these, effectively engraft into NOD/SCID mice that develop plasmacytomas mostly composed of $CD138^+$ cells clonally related to the injected cells by similar Ig light chain restriction (Matsui, 2004). In addition, small subsets of $CD138^-/CD19^+/CD20^+/CD27^+$ cells harvested from BM of the mice were successfully injected in secondary recipients that in turn developed MM (Matsui et al., 2008). These findings, in agreement with previous evidence of circulating memory B cells in MM patients (Rasmussen et al., 2004), suggest that the $CD138^-/CD19^+/CD20^+/CD27^+$ B cell subset may represent the putative CSC clone in MM (Clarke et al., 2006).

Despite these convincing data, many questions regarding the actual phenotype of the CSCs in MM remain unanswered, and new observations make this issue rather controversial. At present, several in vivo reproductions of MM have successfully utilized immunodeficient mice injected with $CD138^+$ malignant plasma cells (Yaccoby & Epstein, 1999). Although empiric, these observations emphasize the ability of phenotypically mature tumor cells by themselves to recapitulate MM in vivo. In addition, further studies underscore the absence of MM cells clonal derivation from respective B cell precursors, since $CD19^+$ cells from different patients were shown to express no tumor-specific genetic mutations (McSweeney et al., 1996). Finally, malignant plasma cells display high plasticity and are capable, after long-term interaction with other components of the BM microenvironment, to reprogram their gene array as well as to turn back to a more immature phenotype including low levels of CD19 but lacking both CD38 and CD138 molecules. In addition, these cells resemble the plasmablastic morphology, thus supporting a "de-differentiation" theory (Yaccoby, 2005) rather than the existence of a subset of CSCs within the MM bulk during early phase of its development. Fig. 2 shows the detection of putative CSCs in a representative bone biopsy from a patient with MM. As can be seen, in the context of a myeloma bone lesion including CD138 stained cells (black arrows), we found a minimal number of cells with typical plasmablast morphology and lacking the CD138 antigen that infiltrate a tumor mass mostly composed by $CD138^+$ plasma cells.

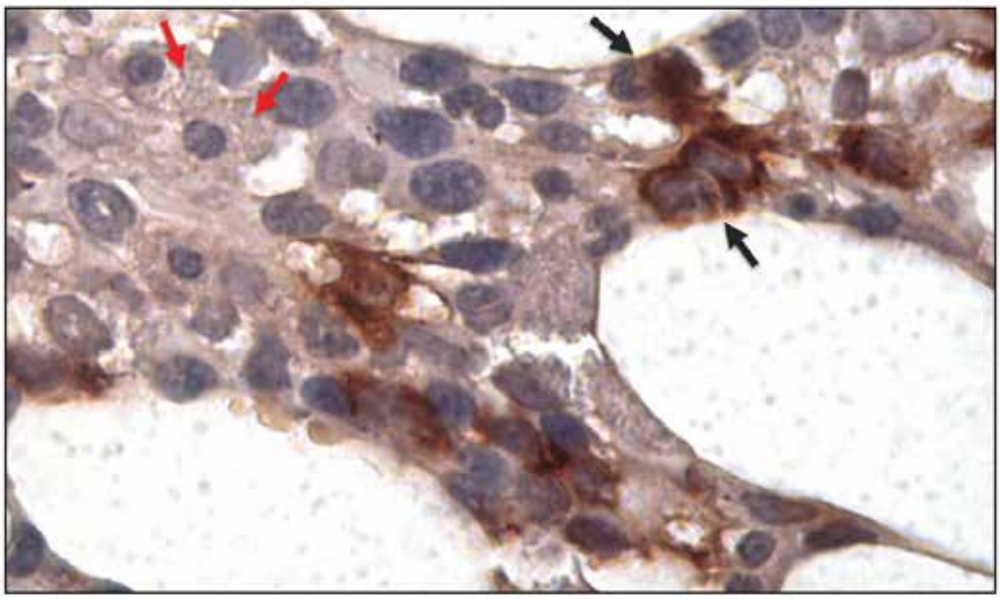

Fig. 2. Representative immunohistochemical detection of CD138+ malignant plasma cells within MM bone marrow biopsy

MM bone marrow section showing high infiltration of cells with mature plasma cell morphology and intense positivity to CD138 staining (black arrows) that stand close to cellular elements with plasmablastic appearance lacking CD138 (red arrows).

2.1.1 Functional properties of MM CSCs

The property to undergo asymmetric divisions and give rise to more differentiated cells retaining high proliferative behavior is not the unique functional hallmark that MM CSCs share with their normal progenitor cells. In fact, similarly to stem cells of either leukemia or brain tumors (Lapidot et al., 1994; Singh et al., 2004), putative MM-initiating cells are resistant to toxic chemical injury, thus accounting for the common risk of disease relapse following conventional treatments. Long-term proliferating CD138- cells, rather than both their CD138+ counterpart and myeloma cell lines, were shown to be slightly influenced in their survival and clonogenic capacities by exposure to common anti-MM agents, such as Dexamethasone, Lenalidomide and Cyclophosphamide (Matsui et al., 2008). Similarly, investigation in our laboratory demonstrated that primary CD138- malignant plasma cells incubated with different concentration of the proteasome inhibitor Bortezomib reach higher survival rates as compared with the control CD138+ U-266 myeloma cell line (Fig. 3).

Cells were cultured for 48 hours with increasing concentrations of Bortezomib and evaluated by MTT assay for their viability. CD138- MM cells show higher survival rates (red curve) as compared with CD 138+ U-266 cells (blue line).

Both normal stem cells and cancer cells have been intensively investigated for their drug resistance (Fletcher JI et al., 2010). A few biological processes are commonly claimed to explain their scarce sensitivity to most cytotoxic agents and are typical of some tumors, including MM. When long-term cultured, in fact, putative MM CSCs predominantly stand in the G0-G1 cell cycle phase (Matsui et al., 2008), thus explaining why they appear poorly

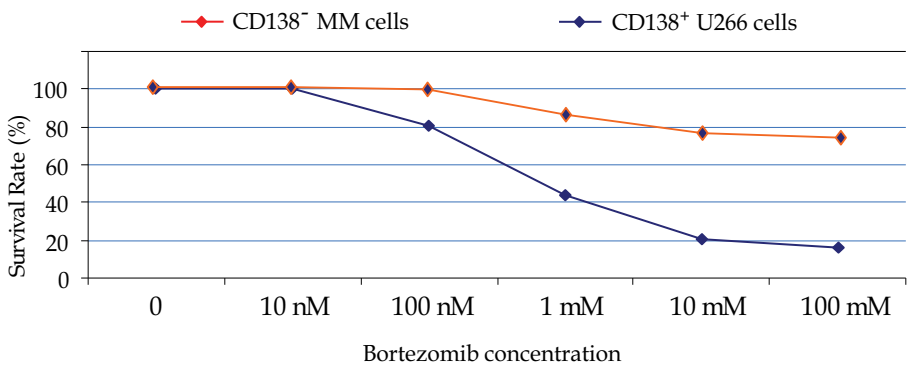

Fig. 3. Bortezomib responsiveness of CD138⁻ MM cells and CD138⁺ U-266 MM cells

responsive to DNA-damaging compounds, such as alkylating agents. However, the drug resistance in MM CSCs seems to be mediated by additional mechanisms, such as the over-expression of the ATP-binding cassette family of drug transporters (ABCG2/BCRP), as well as the high levels of intracellular detoxification enzymes such as the aldehyde dehydrogenase (ALDH) (Matsui et al., 2008). Interestingly, each of these properties is currently used for cytometric assessment of CSCs for distinguishing the putative stem cell fraction within normal (Goodell et al., 1996) and tumor tissues (Sussman et al., 2007). In MM, as well as in other tumors, the capacity to export the nuclear dye Hoechst 33342, that identifies the so-called "side population" (SP), was recently evaluated on several CD138⁺ plasma cell lines resulting in the detection of a small proportion of SP cells lacking CD138 molecules within each cell line. These cells, moreover, expressed high levels of ALDH, emphasizing that the putative drug resistant myeloma stem cells exhibit a more immature phenotype than CD138⁺ bulk malignant plasma cells (Matsui et al., 2008). On the other hand, MM cell lines stained by Hoechst 33342 showed to mostly contain distinct SP subsets that, however, were variably expressing CD138. Interestingly, these SP fractions were found to lack CD19, CD20 and CD27 surface molecules, thus arguing against previous findings that emphasized the memory B cell phenotype of MM CSCs (Jakubikova et al., 2011).

2.1.2 Molecular aspects of MM CSC biology

Given their high clonogenicity, self-renewal and drug resistance, MM CSCs have been suspected to share exclusive pathways and molecular signals with normal adult stem cells. Wnt, Notch and Hedgehog (Hh), the most conserved developmental pathways in humans, are involved in normal stem cell self-renewing and differentiation following injury and, although dormant in most human tissues, they are aberrantly triggered in a variety of human cancers (Taipale et al., 2001; Ruiz I Altaba et al., 2002). In particular, other than being involved in cancer development (Duman-Scheel et al., 2002), Hh plays a pivotal role in CSC biology of different malignancies, including breast cancer, chronic myeloid leukemia and MM (Liu et al., 2006; Zhao et al., 2009; Agarwal et al., 2010). The surface receptor patched (PTCH), a transmembrane molecule, provides the starting point of Hh signalling cascade, whereas three different ligands, namely Sonic, Indian and Desert, bind PTCH in mammalians. Once bound, PTCH de-represses a seven-transmembrane smoothened (SMO) protein which, in turn, regulates the activity of three GLI proteins that enhance the transcription of several cell cycle regulator genes (Duman-Scheel et al., 2002). Mutations of PTCH or SMO can thus

enhance the pathway activity and underlay the development of significant percentages of basal cell carcinomas and medulloblastomas (Gailani et al., 1996; Raffel et al., 1997). Interestingly, both human myeloma cell lines and primary MM cell preparations overexpress some Hh signalling components, while CD138- MM stem cells from established tumor cell lines show higher levels of Sonic-Hh (SHh) signalling activity with respect to their differentiated counterpart (Peacock et al., 2007). Moreover, treatment with Sonic ligand results in a relevant expansion of MM CSC subsets and improves their clonogenic capacity by inhibiting the cell differentiation. By contrast, inhibition of SHh pathway in MM CSCs reduces their attitude to form colonies in vitro, thus suggesting their progressive differentiation. These data imply that a constitutive deregulation of SHh may contribute to the maintenance of undifferentiated CSC clones in MM, whereas other findings revealed that stromal cells, in the context of MM microenvironment, produce Hh ligands and may strongly impact on the survival of MM CSCs within their niches (Dierks et al., 2007).

MM CSCs exhibit other molecular aspects typical of normal stem cells. MM CSCs from both in vitro established MM cell lines and patients have been shown to possess high telomerase activity (Brennan et al., 2010). CD138- cells from commercial cell lines, in fact, showed higher levels of telomerase activation than their differentiated counterpart, thus emphasizing the crucial role of telomerase activity in regulating several biologic processes, such as cellular senescence and apoptosis in normal adult progenitor cells as well as in growth and maintenance of most human cancers (Shay & Bacchetti, 1997). MM CSCs have also been described to overexpress mRNAs of OCT4, NANOG and SOX2, the major regulators of self-renewal and pluripotency of embryonic stem cells (Yu et al., 2009). In particular, OCT4 drives the major transcriptional networks during the embryonic development and, at the same time, the formation of pluripotent stem cells in the mammalian embryos (Nichols J, et al. 1998). In this context, we have detected OCT4 in CD138- cells from the BM of patients with MM. As seen in Fig. 4, this transcription factor was dramatically overexpressed in these cells and, thus, it was interpreted as a marker of their stemness.

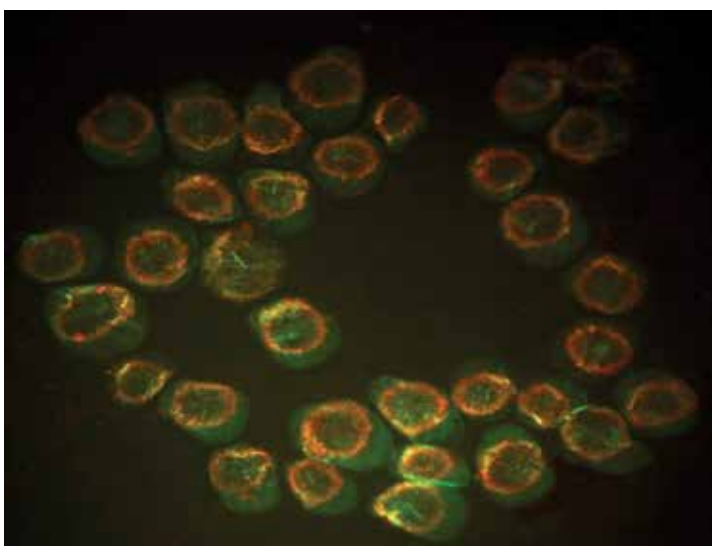

Fig. 4. Immunofluorescence analysis of OCT4 expression in MM stem cells

Fluorescence microscopy image showing a colony of putative MM stem cells stained for both cytoskeletal actin and OCT4 by falloidin (green) and anti-OCT4 PE-conjugated antibody (red), respectively. The typical ovoid shape of these cells is marked by the homogeneous distribution of cytoskeletal actin, whereas all nuclei are strongly positive for OCT4. The OCT4 signal is particularly intense at the nuclear periphery and originates red rings along nuclear contours.

Telomerase activity in MM CSCs, but not in bulk $CD138^+$ plasma cell fraction, was described to parallel the expression of embryonic stem cell genes as Notch and Hh (Brennan et al., 2010). Inhibition of telomerase activity significantly strengthens its role in biology of MM CSCs, since long-term treatment with Imetelstat, a specific competitor of telomerase reverse transcriptase, dramatically reduces both the colony-forming ability of MM CSCs and their engraftment into immunodeficient mice. On the other hand, short-term telomerase inhibition fails to induce telomere shortening in MM CSCs, but relays with a relevant down-regulation of OCT4, NANOG and SOX2 expression, resulting in parallel decrease of their clonogenicity. Therefore, it is becoming clear that specific genes and transcriptional factors driving normal stem cell fate may also affect the functions of MM CSCs and, at the same time, provide potential therapeutic targets in MM.

2.2 Targeting MM CSCs: Implication in MM treatment

For many years, the alkylator-based treatment schedules have represented the most used options for MM, due to their ability to induce prompt responses and symptom improvement (Oken et al., 1997). However, the median survival of patients treated with conventional drugs rarely exceeds 3 years and the disease commonly relapses or shortly progresses after treatment. On the other hand, either autologous or allogeneic hematopoietic stem cell transplantations induce extension of the disease-free survival, but not of the overall survival, in a small group of patients (Bensinger, 2002; Koreth et al., 2007),while a high rate of mortality may occur in allogenically transplanted patients. Based on their effectiveness in inducing significant prolongation of disease-free survival, novel classes of agents, including immunomodulatory compounds, such as Thalidomide and Lenalidomide, the proteasome inhibitor Bortezomib, and new alkylating molecules such as Bendamustin (Cheson, 2010; Lonial et al., 2011), have recently entered into the spectrum of anti-MM treatments, although they also fail to provide long-lasting remissions and their real improvement of overall survival is still to be accurately assessed.

These clinical observations further support the "CSC hypothesis" in MM, suggesting that tumor burden may be actually composed of a biologically heterogeneous cell population containing two subsets of cancer cells with different drug responsiveness, namely: i) terminally differentiated plasma cells with short doubling time and chemotherapy sensitivity; ii) quiescent stem cells with intrinsic drug resistance and the ability to self-renew that drive both disease relapse and progression. In this view, the development of strategies aimed at selectively eradicating the stem cell-like cell population offers a real perspective to achieve long-term remissions in MM. Thus, these novel strategies include: i) targeting specific pathways of CSCs, such as SHh pathway; ii) inhibition of the telomerase activity; and iii) induction of CSC differentiation (Tab. 1).

2.2.1 Targeting developmental signalling pathways

Given the exclusive role of SHh pathway in the pathobiology of MM CSCs, development of specific inhibitors of SHh signalling components, such as PTCH, SMO and GLI proteins, has

been strongly focused on by researchers over the past few years. Cyclopamine, a plant-derived alkaloid with natural antagonism against SMO, was the first anti-SHh drug successfully tested to inhibit growth of some lung, SNC and prostate cancer models, both in vitro and in vivo (Vestergaard et al., 2006; Bar et al., 2007; Mimeault et al., 2010). In line with the CSC theory, these studies describe the contribution of SHh to the initiation and expansion of aberrant cell subsets of poorly differentiated, clonogenic progenitors underlying renewal and growth of the respective tumor burdens. An interesting study, performed on both MM cell lines and patients' BM specimens, revealed the occurrence of MM tumor stem cells expressing higher levels of SHh pathway components than their more differentiated counterpart. In addition, this study showed that inhibition of SHh by cyclopamine selectively impairs the function of MM CSCs in terms of clonogenic potential, while enhancing their tendency to differentiate (Peacock et al., 2007). Thus, cyclopamine may provide a useful therapeutic approach in humans and may be primarily considered as a helpful tool to verify the CSC hypothesis in MM.

Therapeutic agent	Mechanism of action	Biologic effect	*References*
Cyclopamine	Hh pathway antagonism	Apoptosis	*Peacock et al., 2007*
GRN163L (Imetelstat)	Telomerase inhibition	Telomere shortening Cell cycle arrest Apoptosis	*Shammas et al., 2008* *Brennan et al., 2010*
Rosiglitazone Retinoic acid	PPARγ binding RAR binding	Cell cycle arrest Cell differentiation	*Huang et al., 2009*
Rituximab	CD20 targeting	Growth inhibition Apoptosis	*Matsui et al., 2008*

Abbreviations: Hh: Hedgehog; PPARγ: Peroxisome proliferator-activated receptor gamma; RAR: Retinoic acid receptor

Tab. 1. List of potential therapeutic agents targeting CSCs in MM

At present, the first trial aimed at defining the anti-cancer effects of SHh inhibition in humans included GDC-0449, a small-molecule inhibitor with a mechanism of action similar to cyclopamine, that showed a significant therapeutic effect in 55% of 33 patients with basal cell carcinoma (BCC) (Von Hoff et al., 2009). These data have been recently confirmed in subsequent studies indicating that the drug exerts a considerable though variable anti-tumor effect in patients bearing different metastasizing solid tumors such as BCC and medulloblastoma (Lorusso et al., 2011). The mutable therapeutic outcome of GDC-0449, however, may be ascribed to the lower clinical doses used with respect to in vitro preclinical studies, as well as to the high probability that both BCCs and medulloblastomas may hold mutations in their SHh pathway components (Gibson et al., 2010). Nevertheless, most human cancers, including MM, have been found to accumulate no SHh pathway mutations, thus providing the rationale for testing GDC-0449 in novel clinical studies. On the other hand, distinct agents targeting SMO or other components of the SHh signalling pathways,

such as the GLI transcription factors (Gould et al., 2011; Hyman et al., 2009), may provide alternative strategies to target CSCs in experimental tumor models, including MM (Fig. 5).

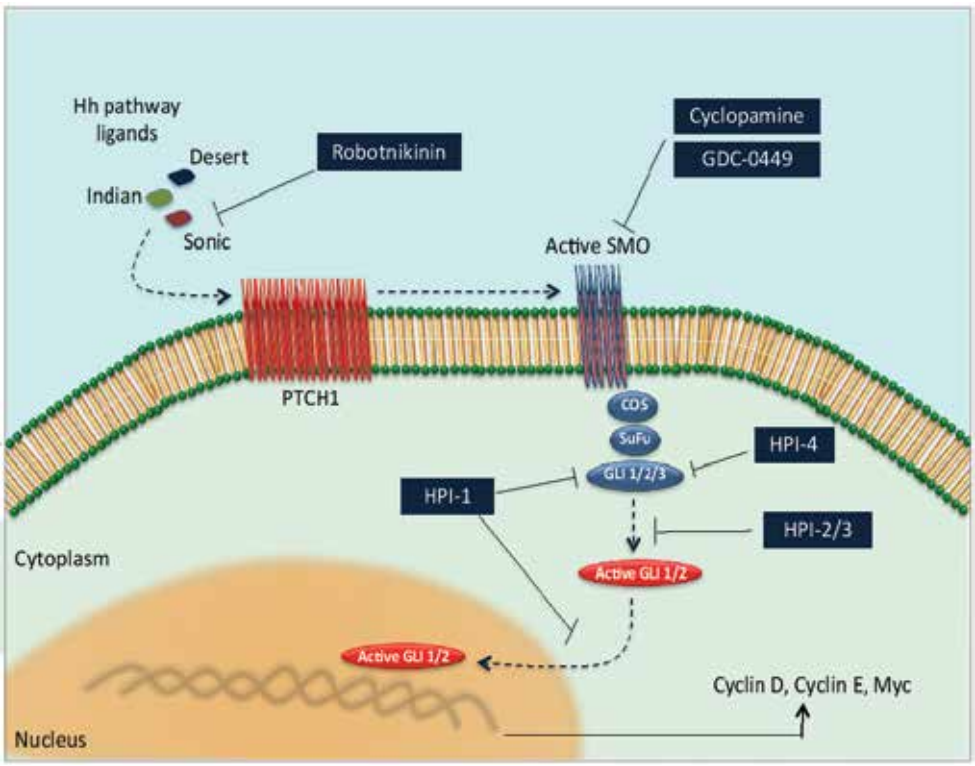

Fig. 5. Preclinical agents targeting Hedgehog signalling pathway

The first step of Hedgehog (Hh) pathway activation, that is the binding of Hh ligands (Sonic, Desert and Indian) to the receptor patched 1 (PTCH1), includes the activation of the transmembrane protein smoothened (SMO), which in turn enables the release from the SMO protein complex, including the Costal (COS) and Suppressor of fused (SuFu) proteins, of GLI 1 and GLI 2 proteins, which translocate to the nucleus where they activate the transcription of major cell cycle regulator genes (Cyclin D, Cyclin E, Myc). Novel agents inhibiting the Hh signalling pathway include natural compounds such as Cyclopamine which targets SMO, and other small synthetic molecules. Among these, Robotnikinin targets the extracellular Sonic ligand, GDC-0449 inhibits SMO activation, whereas the Hh Protein Inhibitors (HPI) 1-4 interfere with the SMO-dependent activation of GLI proteins or with their nuclear translocation.

2.2.2 Inhibition of CSC telomerase activity

Similarly to most human tumor cells (Kim et al., 1994), myeloma cells express high levels of telomerase activity (Wu et al., 2003) that correlate with both disease severity and prognosis (Shiratsuchi et al., 2002). Several in vitro and in vivo studies, in fact, demonstrate that the inhibition of telomerase activity in MM cells considerably affects their survival and growth (Shammas et al., 2004). In fact, the new-generation telomerase inhibitor GRN163L

(Imetelstat), used either alone or in combination with Hsp90 inhibitor 17AAG, exerts a powerful apoptotic effect on MM cells both in vitro and in a mouse model of MM (Shammas et al., 2008). Moreover, identification of high telomerase activity as a MM CSC requirement to self-renewal and clonal expansion may represent a new rationale for evaluating the effect of Imetelstat in this tumor. Interestingly, long-term treatment with Imetelstat induces progressive reduction of telomere length in MM CSCs, resulting in a consistent inhibition of their capacity to form colonies in vitro as well as of their engraftment into immunodeficient mice. On the other hand, shorter incubation of these cells with the drug dramatically impairs their clonogenic growth, although this effect is apparently not mediated by significant shortening of the telomere length, but by a differentiation-dependent mechanism (Brennan et al., 2010). These findings provide the experimental proof that telomerase inhibiting strategies are useful anti-CSC therapeutics in MM. However, further studies are necessary to elucidate the molecular mechanisms of the telomerase inhibitors in MM CSCs.

2.2.3 Induction of CSC differentiation

Emerging evidences support the perspective that induction of CSC differentiation may provide a valid alternative to induce stem cell clone exhaustion within tumors. In a variety of solid and hematologic cancers, in fact, treatment with molecules involved in the stem cell maturation have been shown to exert significant inhibition of tumor growth (Garg, 2009). For instance, retinoic acid is well known to induce a therapeutic "differentiation syndrome" in acute promyelocytic leukemia (Rogers & Yang, 2011), whereas bone morphogenetic proteins have been shown to exert similar effect on brain CSCs, thus restraining their tumorigenic potential (Chirasani et al., 2010). Also, certain chemical compounds have been experienced as anti-cancer therapeutics due to their pro-differentiation potential. The anti-inflammatory acetaminophen, for example, has been found to induce both morphologic and functional differentiation of breast CSCs, and to impair their tumor-initiating ability in vivo (Takehara et al., 2011).

Recent observation strengthens the correlation between the differentiation and anti-tumor activity exerted in vitro by molecules as rosiglitazone and retinoic acid in MM (Huang et al., 2009). These data, moreover, are conceptually in line with previous observations that terminal differentiation of MM cells, triggered by interferon-alpha in association with interleukin-6, results in both arrest at G1 cell cycle phase and impairment of their clonogenic proliferation (Matsui et al., 2003). Therefore, the CSC hypothesis further supports the rationale for clinical differentiation therapy in MM. Inhibition of MM CSC developmental pathways or telomerase activity by specific compounds results in a down-modulation of genes involved in maintaining CSCs in undifferentiated state (Peacock et al., 2007), although further studies are needed to elucidate the specific mechanisms of action of this therapy. On the other hand, the findings that gene expression in myeloma tumor cells is highly regulated by epigenetic modifications driving their proliferation, differentiation and survival (Sharma et al., 2010), have supported the development of new therapeutic strategies. For instance, histone deacetylase and DNA methylation inhibitors are under intensive investigation as potential tools to counterbalance epigenetic, rather than genetic, modifications in both cancer cells and CSCs (Kim et al., 2011; Zhang et al., 2010; Hagemann et al., 2011). Several in vitro and in vivo studies have achieved encouraging data regarding the usefulness of epigenetic therapies in MM (Niesvizky et al., 2011), although no experimental data so far have focused their potential in eradicating MM CSCs.

Novel therapeutic perspectives against CSCs have stemmed from the observation that certain types of cancer possess specific microRNA expression patterns, and that microRNAs (miRNAs) are crucially involved in the regulation of stem cell differentiation even by modulating *OCT4*, *NANOG* and *SOX2* coding sequences (Hatfield & Ruohola-Baker, 2008; Santarpia et al., 2009). In fact, the targeting of specific miRNAs has been shown to effectively antagonize growth of glioma CSCs (Moore & Zhang, 2010), whereas the therapeutic use of miRNA inhibiting specific cellular mRNAs such as Notch-1 and -2, resulted in glioma stem cell differentiation (Guessous et al., 2010). A recent study exploring the miRNA expression patterns in patients with MM, showed that selected miRNAs were specifically altered in patients as compared to healthy subjects and, in particular, miRNA-15a and -16 exerted interesting anti-MM activity both in vitro and in vivo (Roccaro et al., 2009).

2.2.4 Alternative molecular targets of MM CSCs

Additional cellular mechanisms may account for the drug resistance of MM CSCs, thus providing new potential therapeutic targets. Several processes, such as increased DNA-repairing capacity, alteration of cell cycle checkpoints, overexpression of ABC transporters and functional defects of apoptotic pathways, have been suspected to influence the CSC biology, although very little is known on their relative contribution to MM CSC biology and no data concerning their usefulness as therapeutic targets are currently available.

A special interest is presently being reserved for developing strategies to induce death by apoptosis in CSCs. Different natural human proteins involved in immunosurveillance have been shown to trigger apoptosis in a wide variety of tumor cell types (Abe et al., 2000) and, particularly, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was demonstrated to promptly induce apoptosis in CSCs from a few solid tumors (Loebinger et al., 2010; Sussman et al., 2007). New experimental data, however, indicate that CSCs are mostly resistant to TRAIL-induced apoptosis as a consequence of their deregulated apoptotic machinery. TRAIL effectively kills differentiated myeloma cells (Labrinidis et al., 2009), but little is known concerning the sensitivity of MM CSCs to the apoptosis inducer. In this context, only a single report has shown that these cells display both minor levels of TRAIL receptors and TRAIL resistance in vitro. However, the combinatory treatment with Doxorubicin dramatically enhances their sensitivity to the ligand, resulting in complete and lasting eradication of tumor cells in a mouse model of MM (Vitovski et al., 2011).

On the other hand, recent data emphasize the central role of microenvironmental factors, such as stromal cytokines and chemokines, neovascularization and oxygen tensions, in ensuring survival, differentiation and chemoresistance of CSCs within their "niche" (Borovski et al., 2011). Therefore, targeting the major components of the "CSC niche", at both cellular and molecular level, is an attractive perspective to eradicate CSCs, with particular regard to MM whose development and progression are mediated by tight interactions between malignant cells and tumor microenvironment. As a matter of fact, MM CSCs isolated from both cell lines and patient BM samples have been shown to display high expression and functional activity of ABCG2 transporter and, interestingly, increase their viability and tumorigenic potential when co-cultured with bone marrow stromal cells (BMSC). In this single study, moreover, treatment with Lenalidomide exerted a significant reduction of both viability and clonogenicity of MM CSCs, and this effect was particularly evident in CSCs co-cultured with BMSCs. These data emphasize the role of stromal environment in the maintenance of MM CSC compartment and suggest that

immunomodulatory compounds disabling interactions between CSCs and their niche are potential therapeutic tools to eradicate CSCs in MM (Jakubikova et al., 2011).

3. Conclusion

New evidence emphasizes the hypothesis that circulating CBCs with clonogenic potential and properties similar to normal memory B cells are pathogenetically relevant in MM and may be considered tumor stem cells in this malignancy. To investigate this, based on the assumption that putative MM stem cells express markers of normal B cell development, including CD19 and CD20 molecules, two clinical trials have been aimed at evaluating the efficacy of the anti-CD20 monoclonal antibody Rituximab in MM patients, but, beyond the expected reduction of circulating B cells, no curative effect was observed, suggesting that this therapy fails to eradicate the self-renewing tumor cell fraction in MM (Treon et al., 2002; Zojer et al., 2006). Moreover, though the development of drug resistance in MM CSCs is favoured by the BM environment, which may account for the clinical uselessness of Rituximab (Basak et al., 2009), these preclinical results are in line with early studies suggesting that all clonal malignant plasma cells are highly capable of self-renewal in vitro as well as initiating MM growth in vivo (Yaccoby et al., 1998).

In addition, other experimental studies clearly deny the existence of clonotypic MM B cells as an effect of the absence of similar genetic alterations between B cells and tumor plasma cells in MM patients (McSweeney et al., 1996). Therefore, there is no definitive proof that these B cells compose the tumor-propagating compartment in MM and, similarly to other human cancers (Dalerba et al., 2007; O'Brien et al., 2007), several questions concerning their exact phenotype remain unanswered.

The discrepancies in the MM CSC phenotype include the excessive variability of cell isolation methods used by different research groups, whether based on positive or negative selections or on the derivation of tumor cells from BM or peripheral blood. In addition, both the clinical stage of individual patients and any previous treatments may also affect the biology of isolated MM cells and their tumorigenic precursors. Not only the discrepancies in defining the exact phenotype of MM CSCs, but also conflicting results on their functional properties have contributed to generate skepticism in the scientific community regarding the real existence of stem cells in this disease. The clonogenic potential of putative MM stem cells has been examined by a variety of both in vitro and in vivo assays, and different types of MM mouse models have been used to assess the self-renewing capacity of CSCs, thus resulting in potential discrepancies among experimental results from different research groups. Particularly in the in vivo experimental approaches, distinctly different modalities and sites of cell injection, as well as the different microenvironmental factors among the different MM animal models, may profoundly impact on the ability of specific cell types to home, survive, grow and self-renew in vivo, thus reflecting the inconsistency of the results. Therefore, improvements in discriminating both advantages and limitations of the specific assays used to assess the phenotype and the functional properties of MM CSCs are necessary, so that they can provide important knowledge in defining their actual role in MM biology.

At the same time, new clinical strategies aimed at achieving long-term outcomes in MM patients should be based on the current knowledge in the biology of CSCs, and one of the major challenges of clinicians and researchers involved in the field of MM CSCs will hopefully be the translation of the CSC theory into a clinical evaluation of the efficacy of

novel anti-MM therapeutics. In fact, the development of new agents targeting MM CSCs to be used in combination with both conventional and new-generation compounds represents a useful tool to test the CSC hypothesis in this tumor and obtain relevant results for many other types of cancer.

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Part 2

Competing Models of Cancers' Stem Cells

7

Systems and Network Understanding of Cancer Stem Cells

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USA

1. Introduction

The notion that tumors arise from a rare population of cells with stem cell characteristics was first proposed more than a century ago when pathologists like Virchow and Cohnheim formulated the hypothesis that cancer results from the activation of embryonic-tissue remnants (Weiss 2000). Since then, advances in different fields have provided support to this original proposal that has led to the increasingly accepted yet controversial "cancer stem cell (CSC)" hypothesis that explains the development of multiple forms of human cancers (Wicha et al. 2006). The first experiments indicating the existence of these cells were performed in animal models in the 1970s where it was concluded that only a low percentage of transplanted murine lymphoma cells formed colonies in the spleen of recipient animals (Park et al. 1971a; Bruce and VAN DER 1963). Likewise, only a minimum number (1 in 100 to 1 in 100,000) of murine myeloma cells were able to form colonies in *in vitro* experiments. This low *in vivo* and *in vitro* clonogenic potential of tumor cells was subsequently observed for cells isolated from human solid tumors and led to the proposal that only a restricted set of cells "tumor stem cells" that have the propensity to differentiate, give rise to the entire population of cells that are present in certain tumor (Hamburger and Salmon 1977). Over the last few years, the isolation, characterization and functional analysis of CSCs have been facilitated rapid advancements in tissue culture, cell sorting, transgenic animal models and mouse-xenografting techniques (Rasheed et al. 2010). These advances have generated considerable newer insights, and thus contributed in improving our knowledge of CSCs role in cancer and have made their selective targeting a focus of central attention for cancer therapy (Toda 2009; Dodge and Lum 2011). Nevertheless, the precise origin and functional properties of CSCs remains unclear or controversial in several aspects (Hill 2006). Cancers that contain a hierarchy of epigenetically distinct populations of tumorigenic and non-tumorigenic cells might be more effectively studied and treated by focusing on the rare or cancer initiating (causing) cells (Singh et al. 2004). But this field will only achieve its promise if we carefully distinguish between cancers that follow a cancer stem cell model and those that do not (Vermeulen et al. 2008). Therapies designed to eliminate only a small subpopulation of cancer cells will likely not have a clinical impact on cancers in which tumorigenic cells represent most of the cancer cells in the patient (Huff et al. 2006; Massard et al. 2006). Additional testing of the cancer stem cell model will be required in different

cancers to determine what fraction of cases actually follow the model, and how often existing markers are informative. Such testing is likely to yield a complex picture involving differences between cancers that may vary between patients with the same cancer, in terms of the frequency of tumorigenic cells or tumor initiating cells. This is especially true for complex malignancies such as pancreatic ductal adenocarcinomas that are well recognized to be very heterogeneous in nature. Their can also be differences in the degree of hierarchical organization, and the extent to which markers can distinguish tumorigenic from non-tumorigenic cells. In this regard, the use of xenograft tumor models is considered an attractive approach for better understanding of tumorigenesis *in vivo*, the developmental relationship between cancer cells, and even new therapies. However, it is critical that such models be optimized for the engraftment of human cells if we are to draw conclusions regarding the frequency of tumorigenic cells. In this chapter, an attempt is made to revise and extend some current ideas regarding the CSC hypothesis, and how newer technologies such as systems and network biology can aid in this field.

2. Cancer stem cell *versus* clonal selection hypothesis

The fundamental concept of cancer stem cells came from early studies in leukemia and the blood forming hematopoietic stem cells (HSC). Seminal works by Till and McCulloch in the early 1960's established the existence of bone marrow HSC capable of forming myelo-erythroid colonies in the spleen of lethally irradiated hosts. These cells were later isolated by Weissman and his group where they showed that the cells were capable of self-renewal exhibiting multipotent differentiation giving rise to all the blood cell lineages (Spangrude et al. 1988). Studies in human leukemia using *in vitro* and *in vivo* colony-formation assays demonstrated that only a small subset of leukemia cells possess extensive proliferative capability, suggesting that leukemia may actually be derived from a small leukemic stem cell (LSC) population (Park et al. 1971b). This concept was further proved by the successful isolation of myeloid leukemia-initiating cells using cell surface phenotype CD34+CD38- and subsequent *in vivo* transplantation into severe combined immune-deficient (SCID) mice. Even though compelling evidence exists on the existence of stem like cancer cells, yet the hypotheses are considered controversial by purist believing in clonal evolution theory. In the following passages we will discuss the existing concepts and also demonstrate how newer technologies such integrated network and systems biology can help to understand these differences in a more comprehensive way.

2.1 Cancer stem cell hypothesis

It is well established that cancer is in essence a genetic disease that arises from sequential accumulation of mutations in oncogenes and tumor suppressor genes, leading to a malignant clone (Balmain 2001). If the CSC theory is correct, then the result of this accumulation of genetic hits is, at least, one cell with CSC features that can give rise to more CSCs and create more differentiated progeny (Buzzeo et al. 2007). At what stage in the process of malignant transformation this CSC arises, is highly disputed (Potten and Loeffler 1990). An important aspect of the CSC model is the implication that in a malignancy with a defined set of genetic alterations, cells with a different malignant potential are present. In a tumor, both differentiated cells that have lost the capacity to propagate a tumor, and cells that retain a clonogenic capacity, exist. This implies that cells showing the same genotypic alterations can show a completely different potential to initiate a tumor in mice. Here we

first present evidence that this proposed difference in malignant potential is not as striking as it has been initially envisioned. It is believed that CSCs give rise to more differentiated progeny that have lost the ability for self-renewal and the capacity to initiate the formation of a tumor (Figure 1). This would imply that remnant regulatory mechanisms are present in cancer cells that guide the differentiation process in analogy to normal cell differentiation. Indeed, there are examples of malignant cells that are transformed in nonmalignant cells by non-genetical pathways such as epigenetic effects (Kim et al. 2010; Jaenisch and Bird 2003). There is growing body of evidence showing that one of the most studied epigenetic abnormalities in cancer, abnormal gene silencing associated with gene promoter DNA hypermethylation, is linked to key aspects of chromatin regulation of gene expression which maintains the state of embryonic stem (ES)/progenitor cells. This is a timely juxtaposition since there is also a growing body of data, suggesting that cancer "stem/initiating cells", especially when they may dominate in the most aggressive forms of human tumors, have a gene expression signature reminiscent of ES cells.

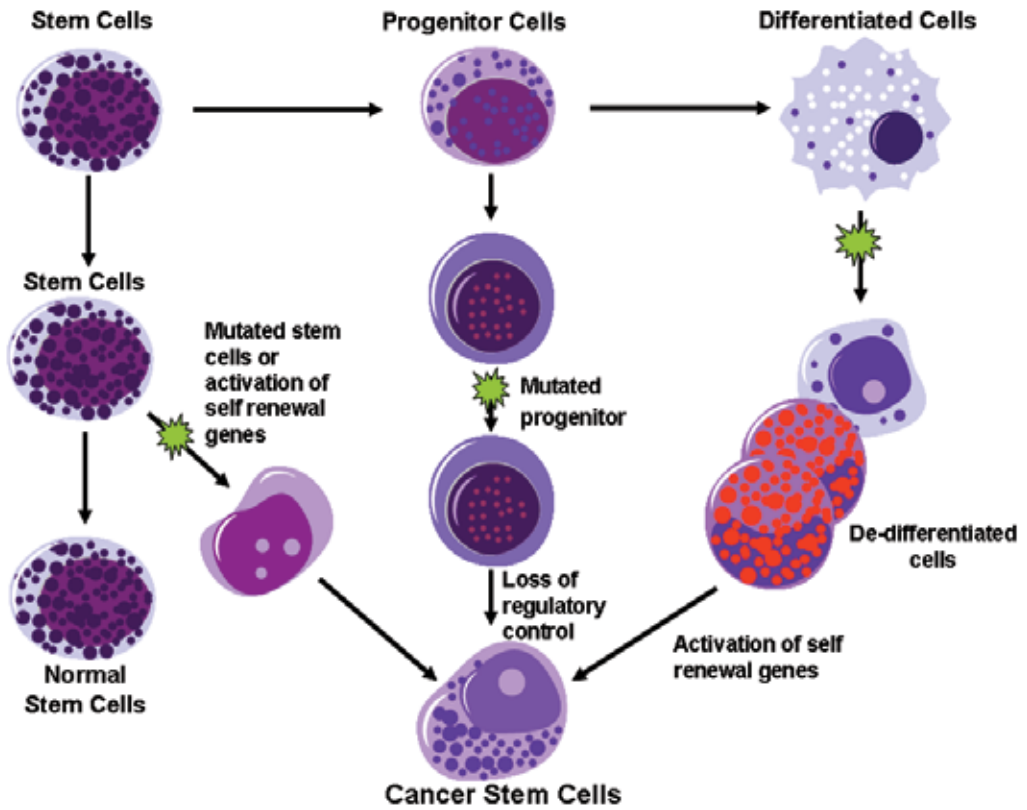

Fig. 1. Cancer stem cell hypothesis: In ideal situation normal hematopoietic stem cells give rise to progenitor cells that form differentiated cells. They can also self renew to give rise to normal cell counterparts that helps in retaining the stem cell number. However, during cancer progression multiple rounds of genetic insult/mutations in normal stem cells or progenitor cells, leads to a progressive loss of regulatory control networks that ultimately causes de-differentiation of these cells. De-differentiated cells can give rise to cancer stem cells that are different from normal progenitor or differentiated cells

Earlier studies have indicated that de-differentiated malignant cells can give rise to both malignant as well as benign cells. In these studies it was shown that mutations are not the only factors that predict the malignant potential of cells (Bissell and LaBarge 2005). Other researchers have recorded that malignant squamous cell carcinoma cells could give rise to more differentiated, non-malignant offspring (Pierce and Wallace 1971). Similarly another study, showed that subcutaneous injection of embryonal carcinoma cells can give rise to teratocarcinomas, while the same cells injected into a blastocyst developed a normal chimeric mouse (Mintz 1965; Mintz and Illmensee 1975). Refining this concept, Hochendlinger et al., demonstrated that transfer of a nucleus from a melanoma cell into an oocyte (to generate embryonic stem cells) generated chimeric mice with a normal phenotype, despite the fact that a clear increase in cancer incidence was observed (Hochedlinger et al. 2004). This work suggests that the epigenetic profile, environmental factors and proteome of the cell cytoplasm of the oocyte influences the events at the time of nuclear transfer and can compensate for mutations to a large extent. This difference in epigenetic profile could also explain the variety in tumorigenic potential of CSCs and differentiated cells in a malignancy. Indeed there is some evidence showing that epigenetic differences between CSCs and more differentiated cells exist, as there is for example, a hypermethylation described for TGFβ-RII in the mammary carcinoma non-CSCs (Shipitsin et al. 2007). Although this suggests that purely genetic models of tumor selection could go hand in hand with the CSC hypothesis, yet several crucial issues remain and can only be answered through a clonal selection perspective that is discussed in the following passages.

2.2 Clonal selection hypothesis

Proponents of clonal selection theory claim that instead of stem cell theory, the hierarchical organization of a malignancy could be easily integrated in the classical clonal selection theory of Nowell (Nowell 1971; Nowell 1976; Nowell 1989). This theory views a malignancy as a clonally-derived cell population, which acquires new potentially advantageous mutations that give rise to new more rapidly proliferating clones. This leads to a process referred to as 'tumor Darwinism', which selects for the cell type most suitable for unlimited proliferation in the given environment (Sottoriva et al. 2010) (Different cell lineages and clonal cells generations depicted in Figure 2). When one integrates the CSC theory in this model, the selection pressure is predicted to act at the level of the CSC compartment, implying that de-differentiation in CSCs results in an increase in expansion of the CSCs due to self-renewal by symmetrical divisions. This does not mean, however, that certain features present only in the more differentiated cells in the tumor could not be the subject of selection, especially if this increases the expansion rate of the CSCs from which they are derived. For example, the more differentiated cells may provide the CSC from which they are derived and which they surround a possible advantage over other clones. In this respect one could think of growth factor production, promoting angiogenesis or the production of immunosuppressive cytokines. Although this suggests that purely genetic models of tumor selection could go hand in hand with the CSC hypothesis although several other crucial issues remains to be fully elucidated.

3. Utilizing systems biology to understand cancer stems cells

Systems approaches including but not restricted to computational modeling have proven of great utility in the study of cancer, with increasing power expected to continue to emerge in the future (Wang et al. 2007). Despite notable and significant challenges that remain, three

areas that have shown significant promise is in the mining of global gene/protein expression data sets to identify molecular signatures that can be used for identification of lineage differences in cells, diagnosis of disease and treatment selection (Araujo and McElwain 2004a; Araujo and McElwain 2004b).

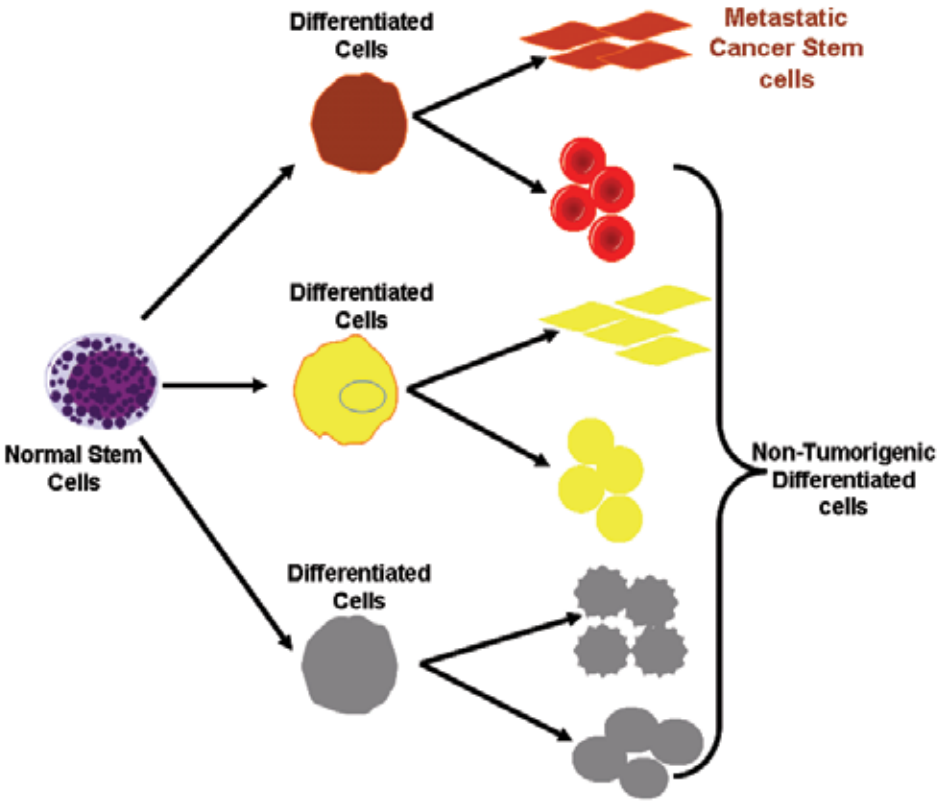

Fig. 2. Tumor clonal selection theory: Stem cells with tumor initiating capacity give rise to more differentiated nontumorigenic offspring. During the process a selection pressure is predicted to act as tumor Darwinism (here depicted by different colors) that can be beneficial for the clone yellow' or metastatic and cancerous (as shown in 'red')

As with any complex biological system, cancer (including CSCs) can be interrogated at the genome/proteome-scale using integrated systems biology approaches. Systems approaches stress three concepts regarding biological information (i) there are two fundamental types of biological information — the digital information of the genome and the environmental information that is outside our DNA. (ii) this digital genome information encodes two types of biological networks — protein interactions and gene regulatory networks. Protein networks transmit and use biological information for development, physiology and metabolism. Gene regulatory networks — transcription factors and RNAs that regulate networks of other transcription factors and other RNAs —receive information from, for example, signal-transduction networks, integrate and modulate it, and convey the processed information to networks of genes and proteins that execute developmental and physiological functions. In biological systems, these two types of networks are closely integrated. The organization of

these networks can be inferred from various different types of measurements including, for example, global measurements of dynamically changing levels of mRNAs and proteins during developmental and physiological responses, as well as large-scale measurements of protein–protein and protein–DNA interactions. (iii) the hierarchical levels of organization and information (for example, DNA, RNA and protein networks, cell signaling and metabolic networks, and organization and responses of organ systems). To understand biological systems, information must be gathered from as many information levels as possible and integrated them into models that generate testable hypotheses about how biological systems may function.

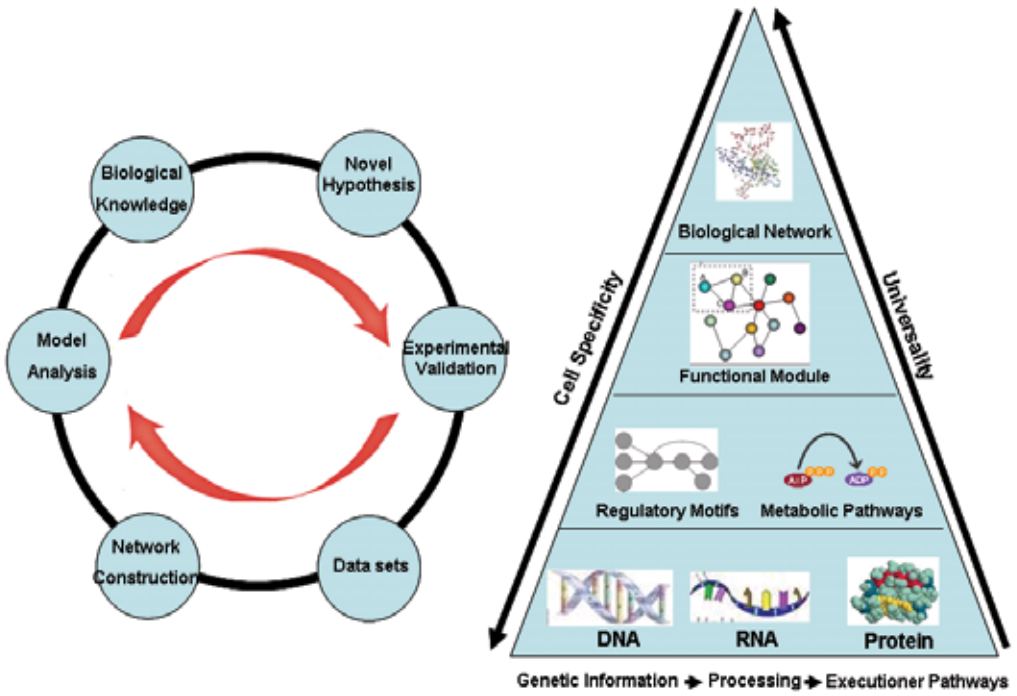

Fig. 3. Systems biology: [Left panel] complex data sets, and complex networks, can rarely be understood using intuition, or traditional biological tools. Instead, an interdisciplinary approach, involving techniques from the mathematical, integrated/computational, physical and engineering sciences is required. To be fully effective such an approach needs to repeatedly traverse an interactive cycle of collaborative interaction between biological knowledge and the proposed hypothesis that has to be validated by robust experimentation. The obtained datasets can be constructed into networks that can be correlated to the available biological knowledge and analyzed in light of the hypothesis. [Right panel] life's complexity pyramid showing hierarchy of structures from basic genomic information at the base to regulatory motifs, functional modules and large scale biological networks. The upward directed information is universal in nature while the information flow downstream of a network is cell/organism specific. Systems biology can help in understanding the inherent differences between CSCs and other cancer cell types through analyzing differences in biological networks in this complex pyramid. (Figure adapted from Oltvai ZN and Barabasi OL, (2002) Science 25, 763-764)

Following on the successes of molecular profiling in identifying prognostic signatures for many cancers, researchers have begun to perform profiling of CSCs as well (Cabanillas and Llorente 2009). Here we discuss such efforts in the context of different tumor models such as leukemia, brain, and breast. In addition to profiling for signatures of specific cancer stem cells, interesting work has also been done to find general signatures for "stemness" in tumors. For example, an 11 gene signature for "stemness" in multiple cancer types has been identified that predicts short interval to disease recurrence, distant metastasis and death from cancer (Glinsky et al. 2005). This signature/analysis reflects a BMI-1 oncogene-driven gene expression pathway, where the BMI-1 gene is essential for the self-renewal of hematopoietic and neural stem cells. Using retrospective survival analysis, this signature for "stem-ness" was found to show predictive ability in 11 different cancers, including epithelial cancers (prostate, breast, lung, ovarian, and bladder) and nonepithelial (lymphoma, mesothelioma, medulloblastoma, glioma, and acute myeloid leukemia). Thus, there is evidence that the property of "stemness" (defined with this signature) is predictive of outcome in a wide variety of tumors. If validated, it is anticipated that the observations could have a major impact on patient care. Additionally, recent studies have indicated that cancer and normal stem cells share the same self-renewal mechanisms, such as the Bmi1 and Wnt canonical pathways (Reya and Clevers 2005), further strengthening the link between stem cells and cancer stem cells. However, it is expected that normal stem cells and cancer stem cells will have certain genotypic differences, which could be further exploited for designing targeted therapy for the elimination of CSCs without affecting normal stem cells.

As mentioned earlier, tumor growth is generally accepted to be the result of several highly complex interacting processes. Fundamental cellular characteristics such as genetic and epigenetic features influence signal transduction activities that, in turn, control cellular functions. Additionally, environmental factors including nutrients and growth factor concentrations interplay with these processes. To study the emergent properties of such systems regarding proliferation speed, infiltrative growth, and phenotypical evolution of cancer, a number of advanced mathematical models have been developed (Anderson and Quaranta 2008). Using these models, some inroads have been made in understanding such hierarchical organized cancer cell populations on solid tumor growth dynamics and progression. In this study, it has been described that implementing the developing concept of CSCs in a mathematical tumor growth model directly results in an invasive morphology. Moreover, it was found that hierarchical organized malignant clones have highly altered evolutionary dynamics. Most strikingly, the CSC organization promotes phenotypical heterogeneity, a feature that could have immediate consequences for therapeutic resistance.

4. Molecular networks of cancer stem cells

Systems approaches to CSC characterization require not only the identification of the key components of a system through global analyses, but also require information about how these components interact in biological networks. Network models of multiple types have been applied to CSCs. The most commonly applied technique to CSC are interaction networks, including protein-protein interaction networks, protein-DNA interaction networks and so forth. Gene expression data can be used to identify differentially expressed genes which could then be visualized on interaction networks, as has been done for different cancers. Various properties of these networks have been studied, with reported findings including, for example, the enrichment of CSC related genes among the "hubs" of the networks. While these interaction networks are very useful tools for visualizing large data

sets, they are not computable whereas predictive network models could hold the most promise for predictive medicine and drug development. Predictive models stemming from mathematical descriptions of biochemical reaction networks and statistical influence models, where CSCs should prove highly useful and are currently being worked upon towards refinement.

Another area of network modeling that should prove very beneficial in research of cancer and CSCs is that of metabolic networks. Key metabolic differences have been shown to exist in normal stem cells vs. CSCs which have been hypothesized to be exploited using Positron Emission Tomography (PET) to do *in vivo* imaging of tumors and even to predict treatment response. If key metabolic differences can be found between CSC and the rest of the tumor, such approaches could potentially even be used to identify the location of CSC populations *in vivo*. One enabling resource for large-scale quantitative modeling of metabolic networks in cancer is the recent stoichiometric reconstruction of known human metabolism at the genome-scale (Radrich et al. 2010). With this global reconstruction, gene expression and other data can be used to create initial models of the genome-scale metabolic networks of a variety of human cell types, including cancer stem cells. These biochemical reaction networks can be useful to make numerous quantitative simulations that have been shown previously to match well with experimental data in model organisms (Wilkinson 2009). These successes with model organisms have also been extended to models of simple systems in yeast to human erythrocyte models (Duarte et al. 2007; Duarte et al. 2004b; Duarte et al. 2004a), with the global metabolic reconstruction poised to allow for larger human metabolic networks that could now be modeled. These studies may well provide insights into the unique metabolic features of cancer cells—allowing one to identify both metabolic features that are shared among cancer cells and features that are unique to individual types of cancer.

More detailed dynamic models of specific biochemical networks in cancer have been made for important signaling networks in cancer, leading to insightful biological observations that have been derived from among many others, the NF-κB signaling network (Lee and Covert 2010; Tay et al. 2010; Covert et al. 2005; Hoffmann et al. 2002; Werner et al. 2005). As isolated CSC populations become better characterized, it will be possible to model these systems to identify differences in their regulation of CSCs and further identify possible therapeutic targets. Dynamic simulations of large-scale signaling networks in cancer cells have also been performed (Christopher et al. 2004). Large amounts of high-throughput data (i.e. transcriptomes) can be used to infer networks that can explain statistical dependencies seen in the data, indicating candidate novel interacting partners, and quantitatively predict the gene expression resulting from knockouts or environmental perturbation. For model systems, such approaches are now being successfully applied at the genome-scale for gene-regulatory networks (Bonneau et al. 2006; Bonneau 2008; Hayete et al. 2007). Such approaches are now also being applied to mammalian systems as was done for normal and cancerous B-cells with the development of an algorithm called Reconstruction of Accurate Cellular Networks (ARACNe) (Basso et al. 2005; Basso and la-Favera 2007). As CSC populations are profiled extensively, these same approaches will be useful to identify predictive networks for CSCs. Comparing these networks to those in normal stem cells and other tumor cells should prove highly informative for identifying drug targets unique to the CSC population of interest. By generating networks of CSCs in particular and comparing them with networks of normal stem/progenitor cells, we should be able to greatly enhance our understanding that could lead to the characterization of cells that becomes cancerous. Computational modeling and systems approaches will be key to catalyzing the future of drug discovery (Hendriks 2010; Kumar et al. 2006; Hood and Perlmutter 2004), and thus

drug discovery focused specifically on CSCs offers tremendous promise for advancing cancer therapies. Therefore, computational modeling of CSC networks to identify potential therapeutic targets and to predict the effect of drug-induced perturbations is critical for this field moving forward.

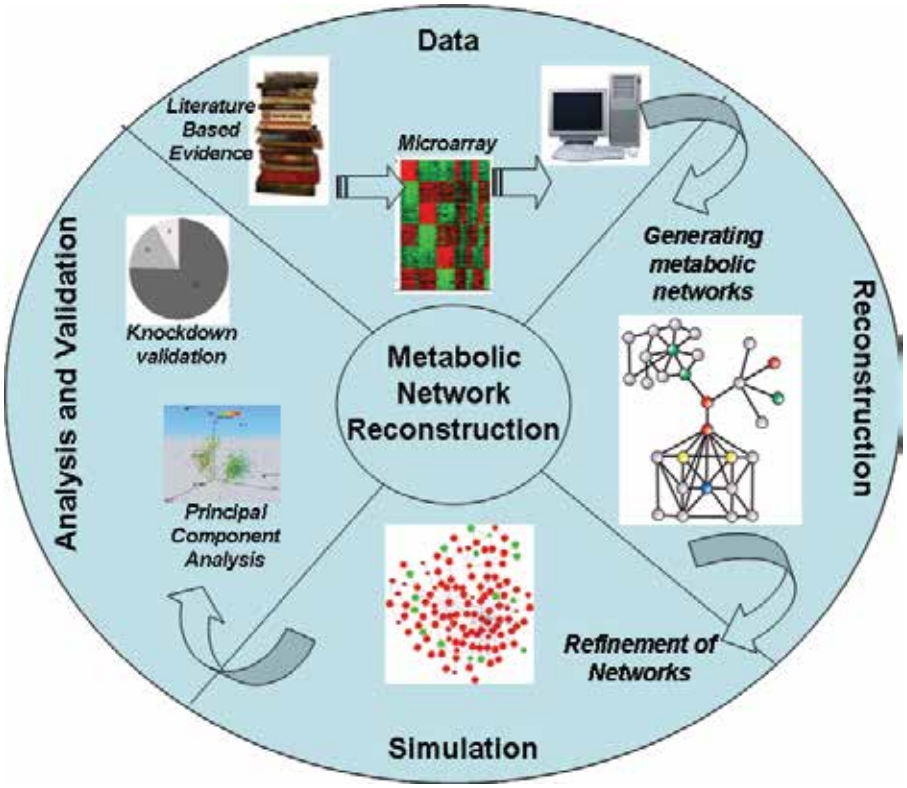

Fig. 4. Designing metabolic networks to understand CSCs: Datasets obtained from microarrays of regular cells and CSCs can be constructed to obtain complete metabolic networks. After refinement of these networks, validation can be done to differentiate key differences between these cells using molecular network silencing technologies. Finally the outcome can be correlated with literature based evidence

5. Systems understanding of CSC response to therapeutic interventions

Once the CSCs are indentified and causal link between CSC and tumor growth is established then the burning question would be how one can investigate the therapeutic intervention, tumor response and tumor relapse. It is well recognized that CSCs are more resistant to therapeutic interventions such as chemotherapy or irradiation compared with their differentiated counterparts (Jordan et al. 2006). More significantly, tumors that relapse after seemingly successful therapy are believed to regrow from the CSCs that survived the therapeutic regimen (Rich 2007; Rich and Bao 2007b). Recently, a number of studies have investigated the dynamics associated with therapeutic interventions that are either selective for CSCs or equally efficient against both cell types. It has been found that the morphology and growth kinetics of relapses for both types of therapeutic interventions are very much

different (Rich and Bao 2007a). Relapse after therapy that specifically targets non CSCs is accompanied by enhanced invasive growth patterns whereas relapsing tumors after stochastic tumor cell killing are similar to the malignancy before treatment. Simultaneously, in case CSCs are resistant to therapy, the pace at which the malignancy relapses is greatly enhanced due to the presence of relatively high fraction of CSCs directly following therapy. Also, the invasiveness of the recurrent tumors is markedly increased following intervention that is not effective against CSCs. These findings are in line with a range of clinical observations describing increased growth speed and enhanced invasion in the relapsing malignancy that are mostly attributed to the selection of more aggressive clones by the drug (Huff et al., 2006). Nevertheless, these observations could be partially explained by the failure of conventional therapies to eradicate the CSC compartment and the subsequent relapse dynamics in CSC-driven tumors. Furthermore, evaluation of evolutionary dynamics during relapse after both types of intervention reveals significant differences as well. Following therapy which is ineffective against CSCs, relapsing tumors display a marked increase in heterogeneity, whereas therapy that does target CSCs results in a dramatic decrease of heterogeneity (analyzed in article by Sottoriva et al 2010). This latter scenario is related to the fact that relapses are very much different compared with the primary malignancy with respect to the clonal lineages that contribute to the relapse of the tumor. In summary, these observations clearly indicate that applying therapy that is ineffectively targeting the CSC population is not only unsuccessful in curing the patient but would also promote malignant features including rapid expansion, increased invasion, and further stimulates heterogeneity directly after therapy. Therefore, overall understanding of the molecular expression differences and network modeling would allow for designing targeted therapy in the future for overcoming therapeutic resistance in order to eliminate tumor recurrence and metastasis.

6. Conclusion

The identification and prospective isolation of CSCs from leukemia, pancreatic and a number of other solid tumors has spawned a new paradigm in cancer research. Incremental progress has been made in understanding the critical differences between CSCs and other counterparts in the tumors, ranging from gene expression, protein expression, metabolic expression and microRNAs that are becoming emerging areas of network research. However, much needs to be learned on the differences between these cells in order to make progress towards the development of novel therapeutics that will specifically target CSCs but not the normal stem cells. Although traditional science has been helpful in understanding few differences but has been restricted to marker identification. In order to make substantial progress in characterization of these elusive cells, newer and integrated technologies are needed that take a holistic view of the cellular system in the context of tumor microenvironment. Systems biology along with molecular network modeling can be utilized with the goal of predictive, preventive, personalized, and participatory medicine for specifically targeting CSCs. This technology utilizes global assessment of cancer stem cells and their microenvironments (niche) at the level of complete transcriptome, proteome, and epigenome, using empowering new high throughput technologies. The resulting gene expression profile signatures of CSCs would serve as more accurate indicatives for cancer diagnosis and prognosis. Emerging proteomic technologies employing mass spectrometry and protein chip platforms would allow for identification of better cell-surface markers and their interaction with the resident stem cell niche, which will provide the potential

diagnostic markers from both body fluids and tumor tissues. Similar exploitation could also be done for microRNAs that are becoming important regulator of gene expression in tumors and body fluids. Although the systems biology methodologies are still developing and error prone, nevertheless, the initial version of the interactomes are of sufficient quality to provide insight into the differences between normal hematopoietic cell and CSCs. It is anticipated that incorporating these data into biological networks will provide fundament insights into the biology of CSCs and their abilities for self-renewal and differentiation. These combined efforts will ultimately lead to newer therapeutic strategy specifically by targeting CSCs for unprecedented design of personalized cancer therapy.

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What do We Know About Cancer Stem Cells? Utilizing Colon Cancer as an Example

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1. Introduction

Cancer is a heterogeneous disease that begins locally before spreading and developing extended metastasis. The initial tumor initiation and development is the results of a cells dysfunction which occurs when accumulated genetic abnormalities transform a normal cell into a tumoral cell with the ability of self-renewal and proliferation. While the exact chronology of this evolution remains unknown, two major models of tumor cell initiation have been proposed (figure 1) (Wang and Dick, 2005):

In the stochastic model, each cell contains a low but similar probability to acquire the necessary genetic mutations resulting in the capacity of proliferation and survival. During the life of the cell, accidentals genetics modifications or mutations may occur and result in the acquisition of the self-renewal potential and thus the ability to sustain neoplasic growth. Then, throughout clonal evolution, cells are subjected to further genetic variations resulting in a heterogeneous tumor. In this model, the relative genetic instability of tumoral cells accounts for the cellular heterogeneity.

In the Stem cell model, each cell has a different probability of acquiring a specific tumoral phenotype. As a result, beyond a simple monoclonal expansion of transformed cells, tumors are more likely considered as a complex tissue where tumor initiation and growth is driven by a minority of tumor cells. This population of initiating cells or cancer stem cells (CSC) is functionally distinct and exhibit specific activated pathways compared to the bulk cells. CSC's display specific properties such as the capability of self-renewal, asymmetric cell division, and capacity to differentiate. The progeny cells - which composed the bulk part of the tumor - undergo specific genetic or epigenetic changes resulting in their limited capacity to divide and survive. In this model, these genetic changes are not rules by accidental genetic events but more likely driven by specific regulations pathways. While the origin of these CSC remains unknown, it is hypothesized that they derive from normal tissue stem cells, or from partially differentiated progenitor cells acquiring unlimited self-renewal potential. Stem cells seem to be the ideal candidates to support this model because of their long life span, which allows for the accumulation of multiple mutations events contrary to the differentiated one.

In hematological malignancies, tumor-initiating cells have been identify and strongly supports the stem cells model. In solid tumors, emergent observations argue for this theory but still require further investigation and validation.

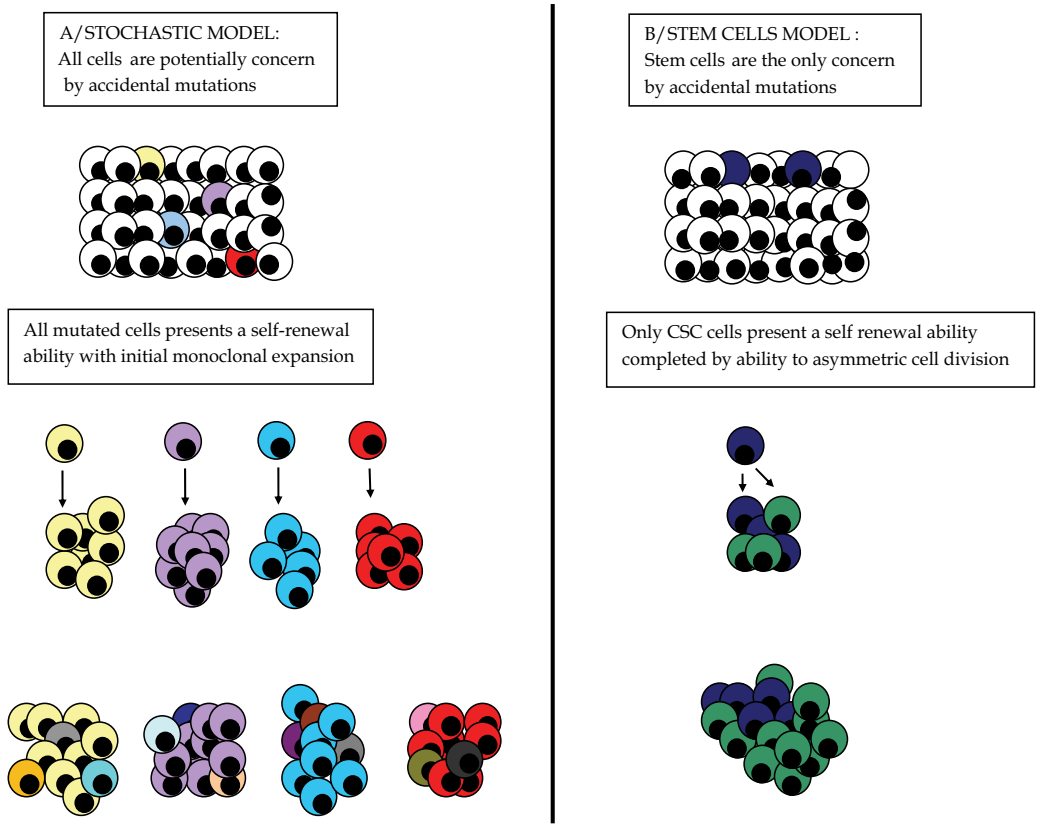

Fig. 1. Two models of tumor initiation

Using colon cancer as an example, this chapter will provide an overview of the complexity of CSC identification and characteristics. Based on the description of different implicated pathways, we'll try to tackle possible clinical applications.

2. Cancer stem cell: identification

As suggested above, CSC's can be differentiated from the bulk part of the tumor either by their specific surface markers or by the specific pathways involved. Considering the example of hematologic malignancy and the supposed origin of the CSC, the first markers used to identify this specific cells subset were common with the normal stem cells of the tissue of origin. The CD 133 antigen is for example commonly expressed both by malignant stem cells and the stem cells of the tissue of origin. On the contrary the CD20 antigen, highly express in colon tissue cells, is not express by the CSC.

2.1 Colon cancer stem cells: CD133

The CD133 antigen (Human prominin 1, PROML1) is a 5-transmembrane glycoprotein of 865 amino acids (120kDa) that is localized to membrane protrusions or microvilli in the colon. This antigen has been used as a marker to enrich for human hematopoietic stem cells and its expression has been correlated with CSC in solid tumors including prostatic cancer

(Richardson et al., 2004), kidney cancer (Florek et al., 2005), non-small cell lung carcinoma (Zhang et al., 2007), and ovarian cancer (Ferrandina et al., 2008).

Using 17 samples of human colonic cancer (6 primary, 10 liver metastases, 1 lung metastases), O'Brien *et al.* conducted serial xenograft implantations through the sub-renal capsule of diabetic (NOD)/severe-combined immunodeficient (SCID) mice (O'Brien et al., 2007). Only the CD133+ cells implanted for xenografts generated tumors. By immunohistochemistry (IHC) the CD133 expression ranged from 1.8 to 24% in the colon cancer samples and from 0.4 to 2.1% in the normal colon cells. The frequency of CSC in the CD133+ cells fraction was estimated to be 1 in 262 cells.

At the same time, Luccia Ricci-Vitani *et al.* reported their observation of both *in vitro* and *in vivo* culture of CD133+ cells sorted by flow cytometry shortly after tissue dissociation of colon cancer samples (Ricci-Vitiani et al., 2007). Again, only the CD133+ cells generated tumors in their xenograft models. The samples analyzed exhibited a very low frequency of CD133+ cells, with only 2.5 ± 1.4 % among a large excess of CD133- cells. In this study, the presence of CD133+ cells was barely detectable from normal colon tissues (Ricci-Vitiani et al., 2007). Several other reports confirmed the implication of CD133+ cells in tumor initiation (Vermeulen et al., 2008, Dalerba et al., 2007). Furthermore, the level of CD133 expression has been shown to be correlated between the primary tumor and corresponding metastasis in 94% of cases (15 patients out of 16) (Horst et al., 2009).

Thus, the definition and identification of colon CSC remains incomplete. As suggested by O'Brien *et al*, among these CD133+ cells only a few selected cells are expected to be "real CSC"(O'Brien et al., 2007). The heterogeneous cell population in colon cancer is partly highlighted by its multiplicity of the genetics combinations disorders found. Hence, it is likely that among CSC, several phenotypic profiles may exist, sharing some common markers and signaling pathways.

Several studies have investigated other potential CSC markers. It is important to note, that all the presented studies focused on colon CSC identification, actually isolated a "CSC-containing" subpopulation with different degree of sensitivity and specificity as there is probably no ideal single marker for CSCs in any tumor system.

2.2 Colon cancer stem cells: EpCAM, CD44 and CD166

The CD44 antigen is a cell surface glycoprotein expressed on lymphocyte, monocyte and granulocyte cells which has also been correlated to undifferentiated cells. Dalerba *et al.* explored therefore that alternative profile to identify CSC (Dalerba et al., 2007). They examined the expression profile of two markers, CD44 and EpCAM previously described as a key to identify CSC in breast cancer (Ponti et al., 2006).

EpCAM is a glycosylated 40 kDa type I transmembrane glycoprotein and functions as an intercellular adhesion molecule modulating cadherin-mediated adhesions and thereby adhesion strength. The physiologic expression of EpCAM in an adult's human tissue is restricted to the basolateral cell membrane of glandular, pseudo-stratified and transitional epithelia cells. Although the biological role of EpCAM is not fully understood, its overexpression has been observed in several cancers' types, including colon cancer (Stoecklein et al., 2006, Todaro et al., 2007, Munz et al., 2009, Gires et al., 2009). The study discriminated between two main expressions' profiles, $EpCAM^{high}$/CD44+ and $EpCAM^{low}$/CD44-, and measured their detectable in both colon cancer cells and normal epithelial colon cells. The frequency of the $EpCAM^{high}$/CD44+cells profile was higher in

some cancer cells population than in normal epithelial colon cells (mean frequency of 1.6% vs. 5.4%, respectively). The study went on to test the ability of these cell populations to form xenografts. While 10^4 EpCAM^{low}/CD44- cells graft failed to form a tumor *in vivo*, as few as 200 to 500 EpCAM^{high}/CD44+ cells consistently generated tumors.

To specify the characterization of this EpCAM^{high}/CD44+ cell population, another set of surface markers was tested and included CD133, CD49f, ALDH and CD166 (Dalerba et al., 2007). Aldehyde dehydrogenase (ALDH) is an enzyme involved in intracellular retinoic acid production and has been linked to cellular differentiation during development, playing a role in stem cell self-protection (Croker et al., 2009). ALDH enzymatic activity was measured in the EpCAM^{high}/CD44+ and EpCAM^{low}/CD44- cells and found to be higher in the majority of the EpCAM^{high}/CD44+ cells. CD49f, also known as integrin alpha 6, functions in cell adhesion and cell-surface mediated signaling. CD49f expression was measured on the tumor cells and correlated with CD44 expression. The study found CD49f expression on tumor cells with higher levels of CD44.

In these experiments using EpCAM^{high}/CD44+ as CSC markers, the expression of CD133 in the selected cells appears heterogeneous. Some tumors displayed a homogeneous negative or positive expression, while others were composed of a mixture of positive and negative CD133 cells. When CD133 was expressed, the CD133+ population includes the CD44+ cells. The CD44+ antigen may be therefore more specific to identify the CSC than the CD133+, while the results do not always correlate between studies.

In Dalerba *et al.* experiments, the CD166 (cluster of differentiation 166), was found to be differentially expressed on colon cancer cells but all colon tumors contained a distinct fraction of EpCAM^{high}/CD44+/CD166+ cells. The study went on to compare the tumorigenic potential of the fraction of CD44+/CD166+ and CD44+/CD166- cells and found that in serial xenografts, only the CD44+/CD166+ cell population was tumorigenic.

Haraguchi *et al.* demonstrated that utilizing both CD133 and CD44 may enhance the selection of tumor initiation cells for colon cancer and by treated colon cancer cells with the differentiation's inducer, sodium butyrate (NaBT), there was a decrease in the expression of CD133 and CD44 (Haraguchi et al., 2008). In this study, the expression of CD44 and CD133 in clinical samples varied from 11.5% to 58.4% (mean 30%) and from 0.3 % to 82% (mean 35.5%) respectively, while the frequency of the CD133+/CD44+ cells population ranged from 0.2 to 15.73% (mean 7%). As expected, when injected in NOD/SCID mice, the CD44+ or CD133+ populations induced tumor formation, whereas the CD44- and CD133- did not. Interestingly, only the CD133+/CD44+ subset population was able to initiate tumors whereas CD133+/CD44- and CD133-/CD44- could not. The subset of CD133-/CD44+ population was too small to evaluate in a xenograft study.

Based on these observations, Du *et al.* proposed to discriminate the respective functional importance of CD44 and CD133 (Du et al., 2008). In contrast with previous reports, this study found that CD44+ cells displayed cluster growth and did not co-localize with CD133+ cells within colorectal cancer (CRC). As few as 100 CD44+ cells were able to initiate tumor formation in a xenograft model utilizing NUDE mice. Knockdown of CD44+ prevented clonal formation and inhibited tumorigenicity in a xenograft model, whereas knockdown of CD133+ did not.

2.3 Colon cancer stem cells: CD24 and other markers

Within spheroid cultures of primary cancer cells, Vermeulen *et al.* identified several heterogeneous subpopulations expressing stem cells markers such as CD133+, CD24+,

CD44+, CD166+, Lgr5 (Vermeulen et al., 2008). Using single-cell sorting of spheroid cells by flow cytometry, they established that only 1 out of 20 cells has the ability to induce monoclonal culture. Among these cells, those presenting a co-expression of CD133 and CD24 were identified as the clonogenic subset, whereas the co-expression of CD133 with CD44, CD166, Lgr5 or CD29 did not increase the selection.

Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) was initially identified as an orphan receptor and a Wnt target gene in colon cancer. On intestinal tissues, Lgr5 expression has been identified to be restricted to the bottom of the crypt and not the villi (Takahashi et al.). However, in premalignant lesions, Lgr5+ cells were not restricted to the crypt base, but were found also at the surface of the crypt (Becker et al., 2008). While its expression has not been precisely related to CSC, it has been recently shown to be overexpressed in ovary, liver cancers and colon cancer (Takahashi et al.).

Marker	Colon cancer	Other solid cancers	Function
CD133	(O'Brien et al., 2007), (Ricci-Vitiani et al., 2007), (Vermeulen et al., 2008, Dalerba et al., 2007), (Haraguchi et al., 2008), (Zhu et al., 2009)	Prostate (Richardson et al., 2004), Kidney (Florek et al., 2005) , Non-small lung (Zhang et al., 2007), Ovarien (Ferrandina et al., 2008), Hepatocarcinoma (Tomuleasa et al.)	transmembrane glycoprotein, self-renewal
CD44	(Dalerba et al., 2007), (Haraguchi et al., 2008), (Du et al., 2008)	Breast (Ponti et al., 2006), Head and neck (Joshua et al.), Glioblastoma (Anido et al.), Non-small cell lung (Leung et al.), Gallbladder (Shi et al.)	cell surface glycoprotein, cell adhesion, hyaluronic acid receptor
EpCAM	(Ponti et al., 2006)	Breast (Ponti et al., 2006), Esophagus (Stoecklein et al., 2006)	transmembrane glycoprotein, intercellular adhesion molecule
CD24	(Vermeulen et al., 2008)	Pancreatic (Rasheed et al.), Breast (Huang et al.), Gastric (Takaishi et al., 2009)	cell surface glycoprotein Cell adhesion
CD166	(Ponti et al., 2006)	Prostate (Rowehl et al., 2008)	cell surface glycoprotein Cell adhesion
ALDH	(Huang et al., 2009)	Lung (Sullivan et al.), Sarcoma (Awad et al.)	Enzyme, cellular differentiation
Lgr5	(Takeda et al., Takahashi et al.)	Colon, Rectum,	G protein-coupled receptor, Wnt targeted gene

Abbreviations: EpCAM: Epithelial cell adhesion molecule; ALDH: Aldehyde dehydrogenase; Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5

Table 1. CSC markers explored according to the cancer type

Using the same process, cells expressing ALDH1 were demonstrated to be highly tumorigenic compared to the ALDH1- ones. Among them further isolation of cancer cells using a second marker like CD133 or CD44 modestly increased the enrichment in tumor-initiating cells (Huang et al., 2009).

3. Cancer stem cell, definition

The CSC are defined by their ability of self-renewal, asymmetric cell division and differentiation. In colon cancer, the evidence of existing CSC was first reported in 2007 by two groups independently (Ricci-Vitiani et al., 2007, O'Brien et al., 2007). Using a xenograft model of colon cancer cells into nude mice, these studies demonstrated that only a small subset of tumor cells was able to generate a tumor. Based on previously known stem cells markers, they demonstrated that only the tumor cell fraction harboring the CD133 or CD44 marker was tumorigenic.

3.1 Tumor initiation (in vivo)

All tumors are comprised of a heterogeneous population of cancer cells. In xenograft experiments, it has been demonstrated that only a minority of the cells from heterogeneous cell population retain the ability to generate a tumor.

Since the early 1900's, xenografts of colon cancer cells into irradiated hamsters were successfully performed (Murphy, 1914). The implantation of human colonic cancer cells into diabetic NOD/ SCID mice to induce tumor formation was later routinely used to investigate cancer metabolism. Yet, limiting dilutions experiments demonstrated that this tumor initiation was dependent on the amount of injected cells (O'Brien et al., 2007, Ricci-Vitiani et al., 2007). These studies demonstrate that if $1x10^5$ cells or more were injected, tumor formation occurred systematically but below this, the efficiency of these grafts decreased. To clarify this decrease in engrafting efficiency, cancer cells were separated in several fractions before being injected into NOD/SCID mice at different dilutions of cells. The cells were fractioned utilizing the CD133 stem cell marker into CD133+ and CD133- cells. After injecting CD133- cells, less than 2% of the mice transplanted with the higher cell dose generated a tumor. In contrast, tumors were generated systematically after injection of 1×10^3 or more CD133+ cells. Moreover, only the CD133+ cells were able to initiate tumor growth in secondary and tertiary mice, providing the first evidence of the existence of a small sub-population of cancer initiating cells (O'Brien et al., 2007, Ricci-Vitiani et al., 2007, Ieta et al., 2008, Varnat et al., 2009). By extension, this suggests that only this small fraction of cancer cells may be responsible for the metastasis development, while it has never been demonstrated.

3.2 Self-renewal (in vitro)

Cancer cells are defined by their ability to divide and renew endlessly. This observation has been confirmed by the *in vitro* experiments, yet this tumoral property is limited to a specific subset of cells among a tumor.

Studies have shown that colonic tumoral cells can be cultivated in serum-free medium containing epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2). In this medium, the culturing of four weeks of CD133+ cells resulted in colon spheres formed by aggregates of exponentially growing undifferentiated cells whereas CD133- cells invariably died. This CD133+ tumor spheres maintained their tumorigenic potential when injected into

SCID mice and generated rapidly growing tumors. Tumor xenografts derived from colon sphere can be their self-maintained in culture for at least twelve months (Ricci-Vitiani et al., 2007, Todaro et al., 2007).
By contrast, in serum-containing medium without EGF and FGF-2, CD133- cells demonstrated growth for 2 weeks before declining in number. In this medium, it was observed that CD133+ cells gradually migrated and began to differentiate.
In these experiments, the CD133- population cells exhibited only limited self-renewal ability, resulting in cell death when cultured *in vitro*. Yet this population of cells is still classified as tumoral due to their displaying features of gastro-intestinal cancer like carcinoembryonic antigen (CEA) expression, and adenomatous polyposis coli (APC) or p53 mutation. These observations clearly demonstrated *in vitro* the differential property of tumoral cells to survive in culture conditions.

3.3 Differentiation ability

3.3.1 *In vitro*

The differentiation's ability of the CSC accounts for the tumor heterogeneity. In serum-containing medium without EGF and FGF, after one day of culture, CD133+ cells gradually migrate and differentiate into large and adherent cells. During their differentiation, these cells acquired CK20 expression and a similar morphology to the original tumor. In the meantime, they lost their ability to transfer tumor in SCID mice (Ricci-Vitiani et al., 2007).
This differentiation is not correlated to accidental mutations as the experiments with CD133 or CD44 cells consistently reproduced the same behavior *in vitro*. This asymmetric division is more likely the result of highly specific cells controls supporting the stem cells model compared to the stochastic model.

3.3.2 *In vivo*

The relative proportion of "CSC-cells" profile varied among xenograft lines but is conserved within each line of successive *in vivo* transplantation in immunodeficient mice. CD133+ tumor cells isolated from xenograft of colon cancer samples demonstrated a similar ability to generate tumors that contained the same range of CD133+ and CD133- cells compared to the original tumor (O'Brien et al., 2007, Todaro et al., 2007, Ricci-Vitiani et al., 2007, Vermeulen et al., 2008). Similarly, tumor generated from EpCAM ^{high}/CD44+ cells (other putative CSC markers) reproduced the same phenotypic heterogeneity as their parents and contained both and in same proportion EpCAM^{high}/CD44+ and EpCAM^{low}/CD44- populations (Vermeulen et al., 2008).
During the differentiation process, the cells gradually lose their stem cell markers. These markers allow us to precisely discriminate the subset of CSC among a large excess of other cells, including bulk tumor part cells and circulating cells. It is critical to note that this differentiation process always reproduces the heterogeneity of the parental tumor. A tumor must be therefore considered as a complex hierarchically organized tissue involving specific pathways and regulatory mechanism.

3.4 Treatment resistance

Because the CSC are the only tumor initiating cells, it is suggested that the cancer treatment will not be successful unless this population of cells can be completely eradicated. Yet, there is growing evidence that CSCs are naturally resistant to both radiation, and the majority of chemotherapies (Pajonk et al.).

The CSC intrinsic radio-resistance, has been demonstrated in different solids tumors such as breast cancer (Phillips et al., 2006), medulloblastoma (Blazek et al., 2007), or glioma (Chang et al., 2009) with an enrichment of the CSC fraction post tumor treatment. In colon cancer, the tumor exposition to chemotherapy and radiotherapy has been shown to increases the CD133+ cells fraction proportionally to the time and intensity of the application (Saigusa et al.).
The relative chemoresistance of the CSC compared to the stemness cells is now becoming a part of the CSC definition (Song et al., 2009). The CSC chemoresistance resulted from the conjunction of two phenomenon. First, several pathways are differentially expressed by the CSC compared to the bulk counterpart. Some of these have been implicated in chemo-resistance and will be further described. Second, in analogy with the adult stem cells, the CSC are postulated to remain quiescent in their niche and therefore responsible for late recurrent disease. As most of the chemotherapeutic agents act through disruption of the mitotic phase of cancer cell-cycle, quiescent CSC are relatively protected against these cytotoxic agents. This hypothesis has been confirmed by several experiments. In xenograft model, the CSC from colon cancer labeled with dye injected to nude mice demonstrated to remain in a state of quiescence. This quiescence was shown to be reversible when mice were exposed to chemotherapy (paclitaxel) (Kusumbe and Bapat, 2009).
Consequently to this chemo-resistance, it has been shown that tumor submitted to chemotherapy demonstrated enrichment in the stem cell fraction. In hepatocarcinoma, CSC were injected into nude mice treated with different doses of chemotherapy (cyclophosphamide). Cells sorted from these generated tumors presented in vitro a self-renewal potential increasing with the increasing dose of chemotherapy. Similarly, in a secondary xenograft, the ability of these enriched cells to produce xenograft in mice was also dependent on the chemotherapy dose (Tan et al., 2009). In breast cancer, doxorubicin-selected cells demonstrated a higher tumorigenic potential in Matrigel when compared to the parental cells (Calcagno et al.).

4. CSC pathway and possible applications

Considering the CSC theory, effective anticancer drugs should target not only the tumor bulk but also specifically the tumor initiating cells. As CSC are considered to be drug-resistant compared to their bulk counterpart due to their elevated expression of the family of ATP-binding cassette (ABC) transporters and their low proliferation rate. This drug resistance is thought to be responsible for tumor recurrences, as these cells persist after treatment and compose the "minimal residual disease". Characterizations of specific signaling pathway exclusively used by CSC, and not by the tumor bulks cells or by the normal colon cells or the normal colon stem cells is indeed a new challenging area for research. First it enriches the CSC definition by physiological understanding, and second it allows the development of new specific CSC targeted therapy. Similarly to normal stem cells, CSC's rely on pathways that govern development, cell-renewal and apoptosis. It is excluded to realize a thorough review of the explored pathway as the variety of explored pathways is important and as most of these publications consisted in initial observations which need to be further examinated. This chapter will thus focus on several of the major implicated pathways in CSC pathology.

4.1 Wnt/β-catenin signaling pathway

The Wnt signaling pathway plays a critical role in cellular processes including proliferation, differentiation, mobility, survival and apoptosis (Takahashi-Yanaga and Kahn). The

relevance of Wnt signaling is underlined by the frequency of its aberrant activation in a large diversity of malignancy. The regulation of this pathway is provided by the cytoplasmic concentration of β-catenin. In the cytoplasm, β-catenin concentration is maintained at a low level by the adenomatous polyposis coli (APC) complex. This complex of axin, APC and glycogen synthase kinase-3β (GSK- 3β) phosphorylates β-catenin which results in its degradation mediated by the proteasome. Wnt binds to a transmembrane receptor complex comprised of Frizzled (Fz)/ low-density lipoprotein receptor-related protein (LRP) and disheveled (Dvl) leads to its phosphorylation thereby inhibiting GSK-3 β (so the APC complex) and unphosphorylated β-catenin then localizes to the nucleus. In the nucleus, β-catenin functions in conjunction with a transcriptional complex composed of TCF (T-cell transcription factor), LEF (lymphoid enhancer-binding factor), and co activators including CBP, cAMP, and p300 to activate the expression of "Wnt-targeted genes". E-cadherin functions as a sequestering protein of β-catenin on the cell membrane (Wang et al., 2004). In spheroidal culture, CSC's (defined as CD133+/CD166+cells) showed heterogeneity in the Wnt signaling network accompanied by heterogeneity in β-catenine localization, although these cells all carried an APC mutation (Vermeulen et al.). On microarray analysis of these CSC CD133+/CD166+, two capital fractions were described. The TOP-GFP^{high} cells fraction (reporter that provides the evidence of Wnt signaling activation) demonstrated up-regulation of the expression of stem-cell-associated genes like LGR5, and revealed a higher clonogenic potential *in vitro*. *In vivo*, this fraction demonstrated the ability to induce tumors in immunodeficient mice. In contrast, the TOP-GFP^{low} cells fraction expressed epithelial differentiation associated gene like mucin 2 (MUC2), cytokeratin 20 (CK20), keratin 20 (KRT20) and fatty acid binding protein 2 (FABP2) (Vermeulen et al., Sikandar et al.).

As in spheroidal culture, each cell lines remains independent and therefore it can be concluded that the regulation of the Wnt pathway is insured at least in part by the cells intrinsic features.

As expected, the TOP-GFP^{high} fraction cells, when cultivated in serum-containing medium progressively acquire differentiation marks and lost CSC markers. Interestingly, when co-cultured with myofibroblast cell lines (MCFM), their morphological and molecular differentiation was prevented and their clonogenicity highly improved (50 fold). A cytokine antibody array revealed that the hepatocyte growth factor (HGF) was one of the most abundant factors present in MFCM. This HGF modulated nuclear β-catenin activity though c-MET. Exposure of TOP-GFP^{low} cells to MCFM induced their re-expression of CSC markers and restored their clonogenic potential. Regarding these results, CSC are not only independent cells clones driving the tumor growth but their activity is highly related to their microenvironment.

This cooperation between the CSC and the corresponding stromal cells is crucial and established a link between the CSC and previous reports concerning the tumor progression. These results has been confirmed in several solid tumors models including the pancreatic cancer (Moriyama et al.).

Another study, focused on the respective correlation between CD133+ cells and Wnt pathway. Mice model were generated with a knock down for one or two of the CD133 alleles were created (Zhu et al., 2009). Surprisingly, mice completely knockout for CD133 were viable and demonstrated normal development. The tissue expression of CD133 in heterozygote embryos (CD133+/CD133-) was initially found to be restricted to the central nervous system, kidney, intestine and skeletal system. As the mouse developed, the CD133 expression expanded to other organs either by differentiated or undifferentiated cells. In the small bowel, the CD133+ expression was relatively restricted in the crypt base and

overlapped with that of LGR5. The activation of the endogenous Wnt pathway in heterozygous CD133+/CD133- mice resulted initially in a complete disruption of the crypt architecture related to a major proliferation of CD133+ cells at the base of the crypt. Lineage-tracing demonstrated that the entire intestine mucosa was replaced by the progeny of these cells resulting in high-grade focal neoplasic formation.

Wnt pathways play an important role in cells maintenance of pluripotency, however it is also involved in differentiation of embryonic cells. Recently it has been demonstrated by Kahn *et al.* that in the Wnt pathway, the co-activateurs CBP and p300 are the mediator of this balance pluripotency/differentiation (Ma et al., 2005). Indeed, CBP/β-catenin-mediated transcription is involved in undifferentiated stem cells maintenance while CBP/β-catenin-mediated transcription is involved in cells differentiation. They demonstrated *in vitro* that combined treatment with imatinib mesylate (IM) and ICG-001, which is specific inhibitor of binding to the N-terminus of CBP, significantly inhibited colony formation of chronic myeloid leukemia progenitors isolated from two patients resistant to IM (Kim et al.). The Wnt pathway is one of the most important pathways being evaluated in stem cell research and is therefore a target for new cancer therapy development.

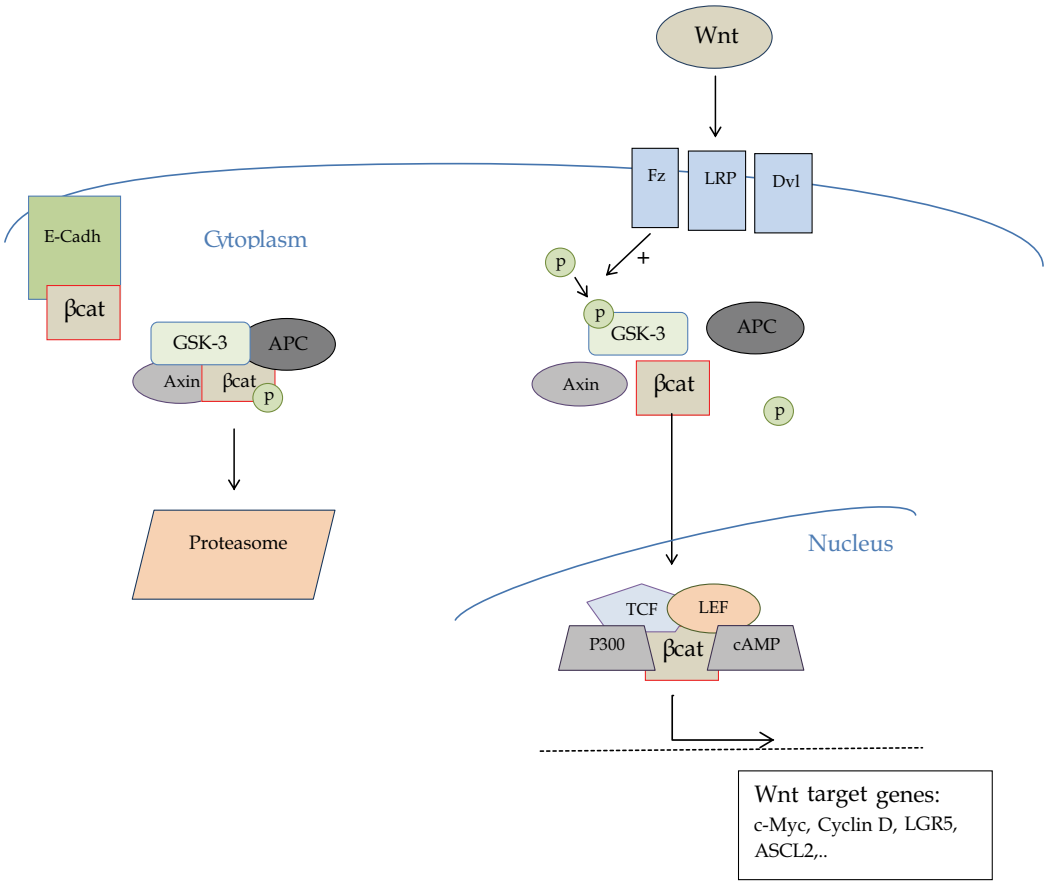

Fig. 2. Wnt/βcatenin signaling pathway

4.2 NOTCH signaling pathway

In mouse models, it has been shown that NOTCH signaling plays an important role in the intestinal tumor initiation. In colon CSC, the NOTCH signaling components are highly expressed compared to the usual colonic cell lines. This expression plays a critical role in CSC self-renewal. Knockdown of the NOTCH pathway in APC mutant cells resulted in cells differentiation into post-mitotic goblet cells. In plate culture, colon CSC treated with NOTCH inhibitors could no longer form adenocarcinoma glands but only disorganized cells cluster without self-renewal capacity (Sikandar et al.).

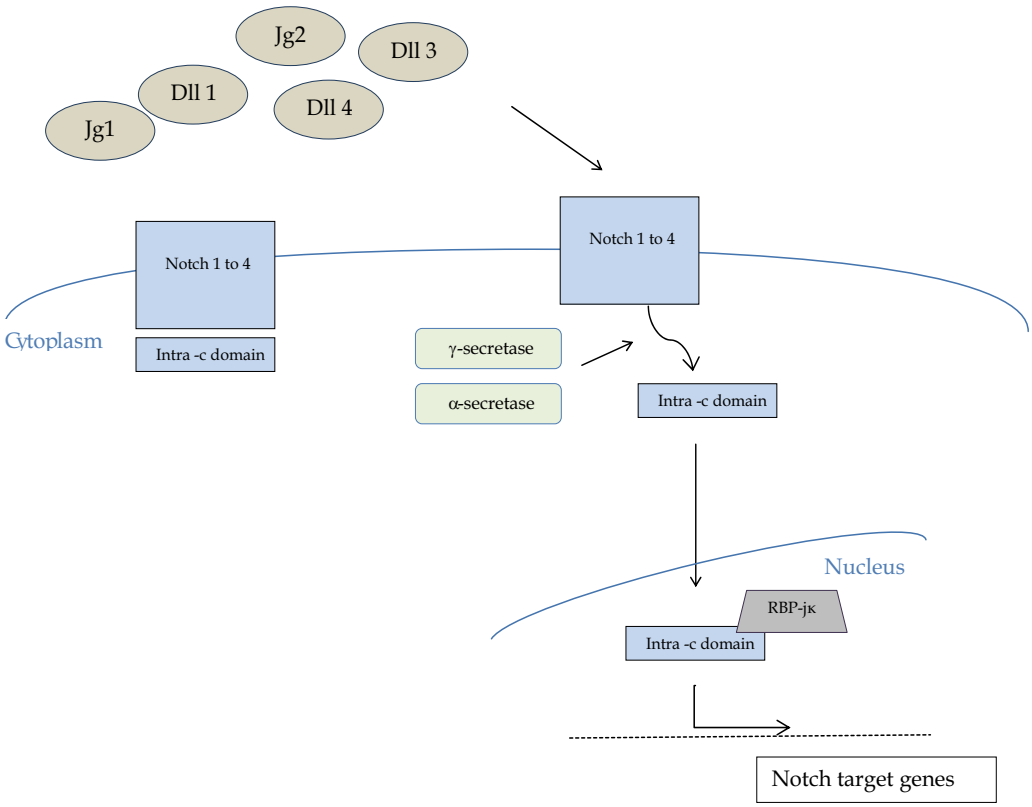

Fig. 3. Notch signaling pathway

4.3 Akt and MAPK signaling pathway

An activation of the epidermal growth factor receptor (EGFR) turns on at least five different signaling pathways: the mitogen-activated protein kinase (MAPK), phospholipase C, phosphatidylinositol 3-kinase (PI3K)/AKT, signal transducer and activator of transcription (STAT), and SRC/FAK pathways. These pathways form an intersecting biochemical network that, when mutated, drives cell growth in a manner unrestricted by environmental cues (Laurent-Puig et al., 2009).

To assess the critical role of these pathways in CSC, a cDNA GeneChip analysis was performed in cells marked CD133+ or CD133- extracted from samples of metastatic colon cancer (Wang et al., 2006). In total, 321 genes were up-regulated and 65 down-regulated in

CD133+ cells compared with the CD133- cells. The gene expression (examined by real-time PCR) confirmed that the changes preferentially concerned PI3K/AKT, NOTCH, Janus kinase/signal transducer and activator of transcription STAT, MAPK and transforming growth factor (TGF)-β pathways. More precisely, AKT was significantly activated and Erk1/2 up-regulated in CD133+cells. When cultured in soft-agarose in the presence of the AKT inhibitor II (SH-5), AKT inhibitor IV or MAPK inhibitor (U0126), there was a decrease in the ability of the CD133+ cells to form colonies formation by 3 to 11 fold. In gene knockdown experiment, cells transduced with AKT and Erk shRNA also demonstrated a reduction in their ability to form colonies.

4.3.1 AKT

Previous studies have suggested the implication of the AKT pathway in colorectal CSC. Thymosin-β-4 (Tβ4) is an ubiquitous G-actin sequestering molecule and has been shown to be involved in a great number of cellular functions such as adhesion, differentiation, migration, angiogenesis, apoptosis and metastasis. Aberrant expression of Tβ4 has been reported in CRC and associated with inducing tumoral progression therefore, Ricci -Vitani *et al.* focused on the Tβ4 expression as a potential critical factor in CSC metabolism (Ricci-Vitiani et al.). Real-time PCR confirmed by Northern and Western blot analysis showed an over-expression of Tβ4 in CSC (CD133+/CD44+) compared to normal epithelial colon cells. Transduced knockdown cells for Tβ4 resulted *in vitro* in their reduced tumor growth ability (50% lower) and their impairment of migration capacity. These knockdown cells showed a decrease of ALDH1 and LGR5 expression (mainly expressed by colon stem cells) whereas differentiation genes like CK20 and trefoil factor 1 were up-regulated. In a xenograft model, tumor growth ability was decreased. Further analysis reveals that Tβ4 over-expression was responsible for increasing integrin-linked kinase and decreasing PTEN expression resulting in AKT pathway activation.

In other experiment, PI3K inhibition of CD133+/CD24+ CSC cells resulted in their "enterocyte-like" differentiation (Vermeulen et al., 2008).

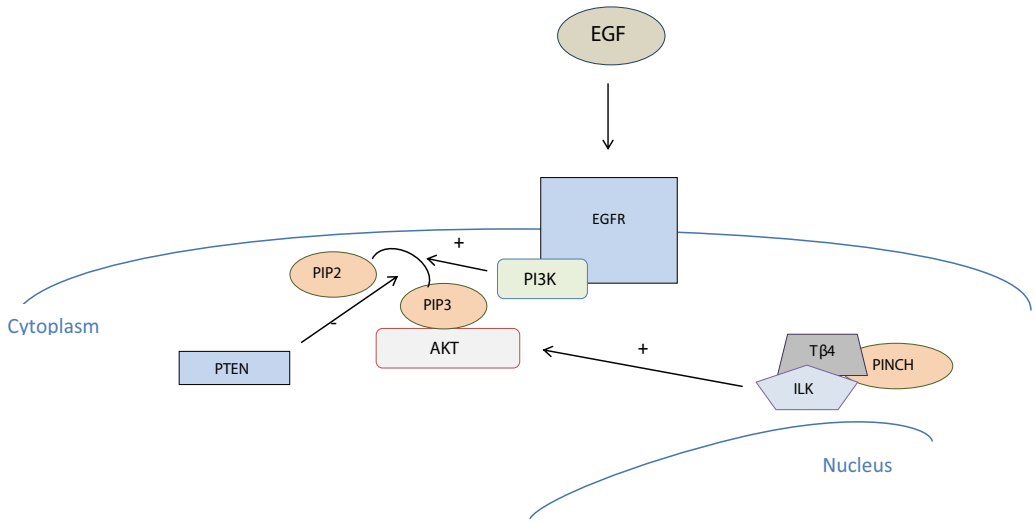

Fig. 4. AKT signaling pathway

4.3.2 TLR and MAPK

Toll-like receptors (TLR) are a family of transmembrane receptors that contribute to activate the MAPK pathway via the adaptator molecule myeloid differentiation primary-response protein 88 (My88). In their study, Grimm *et al.* gave evidence of a correlation between TLR expression and tumor progression (Grimm et al.). Tumoral colon tissues demonstrated a higher expression of TLR 7, 8, 9 or 10 when compared to normal colon tissue. The intensity of this expression correlated with tumor stage and was higher for the late stage tumor (in the UICC classification). The TLR 7 and 8 were shown to be co-expressed with CD133+ in tumor cells.

4.4 CSC and Interleukin-4

Confirming the CSC drug resistance, Todaro *et al* (Todaro et al., 2007) analyzed the cell viability of primary colon cancer cells following exposure to oxaliplatin and/or 5-fluorouracil (5-FU). *In vitro*, the CD133-cells fraction showed a dose-dependent high sensitivity to these drugs whereas CD133+ cells were largely resistant, even at higher dose. Among the different pathways implicated in drug resistance the interleukin-4 (Il 4) mediated signaling has been shown to strongly modulate the death receptors and chemotherapy-induced apoptosis. In this study, colon cancer samples showed a high level expression of IL4 compared to normal colon specimens. More precisely, the CD133+ cells fraction was shown to express both IL4 and IL-4Receptorα. A significantly increase in overall death was observed *in vitro* when CD133+ cells treated with either oxaliplatin and or 5-FU alone or in combination with an IL4-neutrilizing antibody ($p<0.001$). Anti-IL4 treatment resulted in a decrease in the protein expression of anti-apoptotic molecules, cFLIP, Bcl-xl, and PED. *In vivo*, nude mice were engrafted with CD133+ cells and treated by an intraperitonal injection with IL-4DM (IL-4 Rα antagonist) followed (24 hours later) by chemotherapy (oxaliplatin or/and 5-FU). The co-treatment resulted in a marked synergistic effect on the tumor growth compare with single agent chemotherapy.

4.5 CSC and Taxoid-T-1214

To assess the efficiency of a new-generation taxols, Botchkina et al designed an experimental protocol to discriminate the respective effects of Taxoid (SB-T-1214) both on CSC and on the tumor bulk (Botchkina et al.). CSC were isolated form three tumor CRC cells lines (DLD-1, HCT116 and HT29) according to their CD133high/CD44high phenotypic expression. The cytotoxic effect of SB-T-1214 on CSC was studied in two different setting which promoted the stemness phenotype. First, the CSC's were seeded as an adherent monolayer to type I collagen in the serum-free medium for 2 days and then incubated with increasing doses of SB-T-1214 for 48 hours (100nM to 1μM). The vast majority of cells underwent apoptosis (89-96%). The 4-11% of survival cells displayed multiple abnormality including enlarged size, multiple nuclei and severe vacuolization.

Secondly, the experiments were performed in spheroidal cultures. Again, administration of of increasing does SB-T-1214 induced apoptosis in greater than 90% of the CRC cells. The few surviving cells lost their ability to form secondary spheroids colonies.

4.6 Aurora Kinase-A

Aurora-A (STK15/BTAK), is a member of a serine/threonine kinase family, and is involved in mitosis entry, control of centrosome maturation, and segregation during mitosis. Aurora-

A is a key regulator of the p53 pathway, and it's over expression abrogates the wild-type function of p53 such as growth regulation and apoptosis and further confers resistance to chemotherapeutic agents. Cammereri et al. explored this pathway regulation in CSC isolated from colon cancer specimen (defined CD133+CD29+CK20- cells phenotype) (Cammareri et al.). RNA analysis showed that Aurora-A, barely was undetectable in normal colon control cells whereas it was clearly expressed in tumoral cells (CD133+ and CD133-). Immunoblot analysis revealed a higher expression of Aurora-A in tumor specimen compared with normal tissue. Moreover, although in primary tumor cells immunoreactivity was cytoplasmic-located, the Aurora expression in CSC was also nuclear-located. The rare CD133+ cells presents in the normal colon population were mostly negative for Aurora-A expression. Eight of the 15 Aurora-A over expressing CSC cells lines were p53 wild type. Further investigation revealed that knockdown of Aurora-A in CSC resulted in significant growth inhibition, inhibition of migration in vitro, and in a limitation in their tumorigenic capacity on xenograft models with an increase in their susceptibility to chemotherapy induced death.

5. Limits

The experiments discussed above provide evidence that within a tumor, the high cell diversity is not result of a heterogeneous accumulation of diver mutations, but it is more probable that the diversity is the result of a complex regulation program where only a subset of cells are responsible for tumor initiation and development. However, the isolation, characterization and the driving pathways of CSC's are poorly understood and need to be further investigated.

5.1 Stem cells and cancer stem cells

The origin of CSC's cells is thought to be from tissue stem cells. Thus it has been demonstrated that under specific conditions, it is possible to reprogram cells to have a stem-like phenotype. Yu et al. demonstrated that the induction of expression of several gene expression (like Oct4, sox2, nanog, and LIN28 (Yu et al., 2007) or Oct3/4, sox2, Klf4, and c-Myc) in human dermal fibroblasts can convert them into pluripotent cells with a phenotype virtually indistinguishable from embryonic stem cells. In another report, Takahashi et al. (Takahashi et al.) showed that expression of c-Myc can achieve the same result. The evidence that the proto-oncogene c-Myc may be part of the reprogramming of genes supports the hypothesis that under some conditions, it may be possible to reprogram a cell to have a stem-like phenotype.

5.2 Stem cells identification

As an example, while CD133 is supposedly one of the most efficient markers to identify CSC, its biological function on the cell remains unknown. In CRC cell lines (Caco-2 and LoVo) the knock-down for CD133 by siRNA resulted in a significant decrease in both the level of CD133 mRNA and protein expression without any evidence of impairing the cells in vitro rate of proliferation, migration or invasion.

While widely utilized as a CSC marker, CD133 may not be the best marker due its expression as not being restricted to stem cells. Analysis of CD133 knockout mice revealed that this antigen is expressed in epithelial differentiated tissues of several adults' organs including: parietal layer of the Bowman capsule, epithelium of proximal tubules, bile ducts,

and pancreatic duct (Shmelkov et al., 2008). On the colon of mice, CD133 was mostly expressed on the surface and the center of the intestinal crypt, which is typically composed of differentiated columnar absorptive cells.

It has been shown that some tumors do not exhibit CD133+ cells (Dalerba et al., 2007, Ricci-Vitiani et al., 2007, Ieta et al., 2008). In those cases, some of the CD133- cells have been reported as initiating cells with various and contradictory phenotypic profiles such as CD133-/CD44+/CD24- (Shmelkov et al., 2008) or CD133-/CD44-/CDX-2+/CK20+/CK7- (Navarro-Alvarez et al.).

In addition to the characterization of a unique CSC profile, it is of critical importance to be able to discriminate the best sets of markers needed identify the CSC. Consequently to the genetic diversity of colon cancers, the CSC may also be varied among divers tumors but sharing similar phenotypic and function specificity.

Different pathways implicated in CSC functions have been investigated. The hyper-activation of pathways implicated in tumor proliferation and self-renewal has led to the development of several specifically targeted treatments to down regulate these pathways and improve the chemo sensitivity. Until now, these pathways are not specifically used by CSC and the efficiency and side effects when distributed throughout the whole body remains unknown.

6. Conclusion

In conclusion, research has demonstrated that with the heterogeneity of cells within a tumor, only a portion of these cells are tumorigenic. The characterization of these cells needs to be further investigated. The understanding of the implicated pathways will be critical for the development of new targeted therapies that are able to selectively treat the CSC population thereby to reduce a tumor's ability to recur.

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Epithelial-Mesenchymal Transition and Cancer Stem Cells

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1. Introduction

The epithelial to mesenchymal transition (EMT) is a highly coordinated process and a multi-step event during which epithelial cells lose numerous epithelial characteristics and assume properties that are typical of mesenchymal cells, which requires complex changes in cell architecture and behavior. The conversion of epithelial cells to mesenchymal cells is critical for the formation of the body plan and in the differentiation of multiple tissues and organs during embryonic development and involves profound phenotypic changes such as the loss of cell-cell adhesion, the loss of cell polarity, and the acquisition of migratory and invasive properties (Thiery *et al*. 2009). EMT is also involved in the physiological response to injury and in the pathological processes such as organ fibrosis. Accumulating evidence suggests that aberrant activation of the EMT developmental program contributes to tumor initiation invasion, metastatic dissemination and acquisition of therapeutic resistance (Yang, *et al*., 2004; Yang and Weinberg, 2008; Thiery *et al*., 2009; Singh and Settleman, 2010; Acloque *et al*., 2009; Kalluri and Weinberg, 2009). EMT induction can participate in cancer initiation to promote the clonal expansion of premalignant epithelial cells (Tellez *et al*., 2011). Cancer cells undergoing EMT acquire the capacity to migrate, invade the stroma and metastasise. During the process of metastasis, the EMT program enables these cancer cells to disseminate from a primary tumor and also promotes their self-renewal capability to ensure generation of the critical tumor mass required for progression from micro- to macro-metastases (Ruan *et al*., 2009a; Ruan *et al*., 2009c; Ouyang *et al*., 2010). EMT-inducing signalling pathways, such as TGF-β, Wnt, Notch and Hedgehog (Hh), along with other tumor microenvironmental cues, induce well-differentiated epithelial cells to convert into motile mesenchymal cells via the activation of multiple EMT transcription factors, including Twist1, Twist2, Snai1, Slug, ZEB1 and ZEB2. Similarities between developmental and oncogenic EMT have led to the identification of common contributing pathways, suggesting that the reactivation of developmental pathways in cancers contributes to tumor progression. For example, developmental EMT regulators including Twist1, Twist2, Snail, Slug and Six1, and Cripto, along with developmental signaling pathways including TGF-β and Wnt/β-catenin, are misexpressed in breast cancer and correlate with poor clinical outcomes.

Evidence has recently been accumulating to support the hypothesis that tumors contain a subpopulation of tumor cells called cancer stem cells (CSCs), also known as tumor initiating cells or tumorigenic cells, which exhibit stem-like cell properties to self-renew, form tumor spheres, differentiate into heterogeneous populations of cancer cells, and seed new tumors in a xenotransplant system (Dontu *et al*., 2003; Gupta and Weinberg, 2009). In addition to initiating tumors, CSCs are thought to be capable of initiating metastasis. The CSC hypothesis provides an attractive model of tumor development and progression, holding that solid tumors are hierarchically organized and sustained by a small subset of the tumor cell population with stem cell properties. Under this hypothesis, sustained metastatic growth requires the dissemination of a CSC from the primary tumor followed by its re-establishment in a secondary site. The CSC hypothesis has fundamental and important clinical implications, as the current development of cancer therapeutics is largely based on screening agents with the ability to cause bulk tumor regression in animal models or in clinical trials (Bao *et al*., 2006; Rich and Bao, 2007; Bao *et al*., 2008; Gupta and Weinberg, 2009). Strategies aimed at efficiently targeting CSCs are critical for monitoring the progress of cancer treatment and for evaluating new therapeutic agents. The elucidation of signalling pathways which regulate CSC self-renewal and survival provides potential therapeutic targets. In addition, CSC behaviors are constantly regulated both by inside regulators such as transcription factors and external signals from their niches, including neighboring stromal, immune, and non-stem tumor cells. Targeting the neighboring non-stem cancer cells, stromal cells or the paracrine factors secreted by these cells may target CSCs indirectly, and thereby contribute to long-term remissions (Polyak and Hahn, 2006).

Both the EMT and CSCs play a critical role in tumor metastasis, therapeutic resistance and recurrence; however, each alone can not explain the sum of the cellular events in tumor progression and the significance of EMT in regulating the stemness of CSCs remains unknown until very recently. Balancing these two concepts has led researchers to investigate a possible link between EMT and the CSC phenotype. Brabletz *et al*. (2005) proposed an integrated model–the migrating cancer stem cell concept that covers all aspects of human tumor progression. Mobile CSCs are located predominantly at the tumor-host interface and are derived from stationary CSCs through the acquisition of a transient EMT phenotype in addition to stemness. In a recent report, Mani *et al*. (2008) found that Twist1, Snai1 or TGF-β can transform nontumorigenic, immortalized human mammary epithelial cells (HMLEs) into mesenchymal-like cells and dedifferentiate *HER2/neu*-infected HMLE (HMLEN) cells into $CD44^{high}CD24^{low}$ cancer stem-like cells via EMT. The resulting populations that have undergone an EMT and display mesenchymal morphology and stem cell markers can efficiently form mammospheres, soft agar colonies, and tumors. Moreover, other EMT inducers or regulators such as ZEB1, YB-1, LBX1 and Six1 have also been shown to induce well-differentiated cells and cancer cells to form populations with stem cell-like characteristics via promoting EMT, indicating that there is a crosstalk between the EMT program and the pathways involved in regulating stemness in stem cells (Mani *et al*., 2008; Morel *et al*., 2008; Evdokimova *et al*., 2009; Illopoulos *et al*., 2009; McCoy *et al*., 2009; Polyak and Weinberg, 2009; Wellner *et al*., 2009; Yu *et al*., 2009; Ouyang *et al*., 2010; Singh and Settleman, 2010). The critical roles of TGF-β, Wnt, Notch and other signaling pathways in promoting EMT and the stemness maintenance of stem cells adds to a growing body of evidence that cancer cells often reactivate latent developmental programs to regulate the multistep process in tumorigenesis. Furthermore, the expression of stemness and EMT markers in CSCs were associated with resistance to conventional anti-cancer therapies and

treatment failure, highlighting the urgency of improving tools for detecting and eliminating minimal residual disease. In this chapter, we focus on recent findings regarding the role of EMT signaling pathways in regulating the stemness of cancer stem cells.

2. EMT signaling pathways in regulating stemness of CSCs

During the EMT process, epithelial cells undergo specific series of events and dramatic phenotypic changes, lose expression of E-cadherin and other components of epithelial cell junctions, adopt a mesenchymal cell phenotype and acquire motility and invasive properties that allow them to migrate through the extracellular matrix. The functional loss of E-cadherin expression is considered a one of the hallmarks of EMT and a crucial event in the progression of papilloma into invasive carcinoma because the reduction of cell adhesion between cancer cells facilitates their ability to migrate individually and invade (Thiery *et al*., 2009; Yilmaz and Christofori, 2009). *E-cadherin* promoter is repressed directly or indirectly by specific developmental transcription factors such as Twist1, Snai1, Slug, ZEB1, ZEB2, FOXC2, KLF8 and E47, which disrupts the polarity of epithelial cells and maintains a mesenchymal phenotype (Kang and Massague, 2004; Yang and Weinberg, 2008; Thiery *et al*., 2009). Knockdown of E-cadherin by shRNA triggered EMT and resulted in acquisition of a mesenchymal phenotype and increased CSC activity in HMLER breast cancer cells (Gupta *et al*., 2009).

2.1 EMT signaling from the microenvironment in regulating stemness of CSCs

Tumor development is a continuous reciprocal interaction between tumor cells and their surrounding microenvironment, in which stromal cells and the extracellular matrix (ECM) play a decisive role in tumorigenesis (Bissell and Radisky, 2001; Hanahan and Weinberg, 2011). Tumor microenvironment not only provides support for initiation and growth of the primary tumor, but also facilitates tumor metastatic dissemination to distant organ as an active participant. Tumor cells can only thrive in an aberrant microenvironment composed of altered ECM and various non-transformed neighbor cells. Cross-talk between cancer epithelial cells and their neighboring stromal cells is known to be critical to the growth and progression of tumors (Hanahan and Weinberg, 2000; Bissell and Radisky, 2001; Bhowmick *et al*., 2004; Bissell and Labarge, 2005; McAllister *et al*., 2008; Hanahan and Weinberg, 2011). In adult tissues, normal stem cells reside within highly defined anatomical niches that provide both cell-intrinsic and cell-extrinsic factors to maintain stem cells in undifferentiated states to self-renew or give rise to the full repertoire specialized cells in the tissues. Like their normal stem cells, CSCs have the ability both to self-renew and to differentiate to specialized cells with limited proliferation potential. Accumulating evidence has emerged that factors derived from the tumor microenvironment serve to regulate the stemness of CSCs. CSC niche can be considered as the tumor microenvironment surrounding CSCs that contributes to maintain the stemness of CSCs. CSCs may reside in and constantly affected by their aberrant niches, where cell-cell and cell-matrix interactions can provide unregulated external signals to support and maintain the undifferentiated phenotype of CSCs. CSCs may remain dormant in their aberrant niches until they are activated by the altered signals in the microenvironment. Recent work has begun to address the importance of the tumor microenvironment in regulating the EMT during tumorigenesis and also found that the emergence of CSCs occurs in part as a result of EMT, for example, through cues from tumor microenvironment components.

TGF-β signaling. TGF-β is a multifunctional cytokine that plays critical roles in tumor suppression and tumor progression, cell differentiation and tissue morphogenesis, and extracellular matrix production through activation of Smad and non-Smad signaling pathways. Current data show that TGF-β signaling pathway has a dual role in tumorigenesis as a tumor suppressor in early stage tumors or as a promoter of tumor progression and metastasis (Derynck *et al*., 2001; Massague, 2008). In the Smad-dependent pathway, TGF-β ligands bind to heterotetrameric complexes of receptors with serine-threonine kinase activity leading to an increase in their ability to phosphorylate the receptor-related Smad (R-Smad) proteins. The phosphorylated Smad2 and Smad3 then form heteromeric complexes with Smad4 and translocate into the nucleus to regulate the transcription of target genes. The amplitude and duration of Smad2/3-based signaling transpires through their physical interaction with a plethora of transcription factors, and with a variety of transcriptional co-activators and co-repressors in a gene- and cell-specific manner. Currently, TGF-β is recognized as a master regulator of EMT, during embryogenesis and tissue morphogenesis (i.e., type 1 EMT), wound healing and tissue fibrosis (i.e., type 2 EMT), and tumor invasion and metastasis (i.e., type 3 EMT). Multiple transcription factors, including ZEB1, ZEB2, and Snai1, are induced by TGF-β-Smad signaling and play important roles in TGF-β-induced EMT. TGF-β employs HMGA2 (high-mobility group A2) to induce the expression of Twist1, Snai1 and Slug to promote EMT (Thuault *et al*., 2006). Non-Smad signaling activated by TGF-β also plays important roles in induction of EMT. Independent of Smad activity, TGFBR2 can directly phosphorylate the cell polarity protein, Par6, to promote the dissolution of cell junction complexes (Ozdamar *et al*., 2005; Thuault *et al*., 2006). In addition, TGF-β signaling also cross-talks with other signaling pathways to act in concert to trigger EMT programs. Of these, Ras and Wnt signaling pathways synergize with TGF-β signaling, and play a critical role in the induction of EMT (Polyak and Weinberg, 2009; Vincent *et al*., 2009).

TGF-β family members and their signaling pathways also play a key role in the self-renewal and maintenance of stem cells in their undifferentiated state. A recent report about the role of TGF-β-induced EMT in human breast cancer demonstrated that the TGF-β pathway is specifically activated in $CD44^+$ breast cancer cells (Shipitsin *et al*., 2007). The specific activation of TGF-β signaling in $CD44^+$ breast cancer cells is due to the restricted expression of TGFBR2 in these cells and its epigenetic silencing in $CD24^+$ cells. TGFBR inhibitor treatment specifically induces $CD44^+$ cancer cells to undergo a mesenchymal-to-epithelial transition (MET) (Shipitsin *et al*., 2007). $CD44^{high}/CD24^{low}$ cells isolated from HMLEs display a mesenchymal phenotype (Mani *et al*., 2008). After treatment with TGF-β1, HMLEs adopt the $CD44^{high}/CD24^{low}$ expression profile. The $CD44^{high}/CD24^{low}$ subpopulations also display many characteristics of stem cells including self-renewal, tumorigenic and metastasis capability, and the ability to differentiate into myoepithelial or luminal epithelial cells. In addition, treatment of HMLER with TGF-β accelerates the emergence of $CD44^+CD24^{-/low}$ cells from $CD44^{low}CD24^+$ non-tumorigenic mammary epithelial cells via the activation of the Ras/MAPK signaling pathway (Morel *et al*., 2008). In MCF-10A cells, the knockdown of Akt1 promotes TGF-β-induced EMT and a stem cell-like phenotype (Iliopoulos *et al*., 2009). Recently, the activating transcription factor 3 (ATF3) is induced by TGF-β in the MCF10CA1a breast cancer cells and plays an integral role for TGF-β to upregulate its target genes *Snail*, *Slug* and *Twist1*, and to enhance cell motility. Interestingly, ATF3 increases the expression of the TGF-β itself, forming a positive-feedback loop for TGF-β signaling. Moreover, ectopic expression of ATF3 promotes EMT and increases

$CD24^{low}$-$CD44^{high}$ population of cells, mammosphere formation and tumorigenesis (Yin *et al*., 2010).

TGF-β may exert a similar effect on regulating the stem cell-like pool of other tumors. TGF-β is highly expressed in high-grade gliomas and upregulated TGF-β activity confers poor prognosis in glioma patients. TGF-β and LIF have been reported to induce the capacity to self-renew and prevent the differentiation of glioma-initiating cells (GICs) isolated from patient-derived glioma tissues (Penuelas *et al*., 2009). TGF-β increases GIC self-renewal through the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway. The induction of GIC self-renewal by TGF-β and LIF promotes tumorigenesis *in vivo* (Penuelas *et al*., 2009). TGF-β-FOXO signaling is shown to be essential in the maintenance of leukemia-initiating cells in chronic myeloid leukemia (CML) (Naka *et al*., 2010).

Wnt signaling. Among many embryonic signaling pathways, Wnt pathway is one of critical pathways involved in regulating the stemness of CSCs and in the acquisition of EMT characterics during tumorigenesis. Wnt signals are transduced to the canonical pathway for cell fate determination, and to the noncanonical pathway for control of cell movement and tissue polarity. In the absence of active Wnt ligands, β-catenin is complexed with scaffold proteins Axin and APC, and phosphorylated by GSK-3β and CKIα. Phosphorylated β-catenin is then ubiquitinated and undergoes proteasome-mediated degradation. Canonical Wnt signals are transduced through membrane Frizzled (FZD) receptors and LRP5/LRP6 co-receptor to the β-catenin signaling cascade. In the presence of active Wnt signaling, Wnt ligands bind to FZD and LRP, resulting in the phosphorylation of LRP6 by GSK-3β in its cytoplasmic region, leading to the recruitment of Dishevelled (Dvl) and Axin. β-catenin is then released from phosphorylation by GSK-3β and degradation by proteosome. The accumulated β-catenin translocates to the nucleus and regulates the expression of target genes. Noncanonical Wnt signals are transduced through FZD receptors and ROR2/RYK co-receptors to the Dishevelled-dependent or the Ca^{2+}-dependent signaling cascades. The inappropriate expression of the Wnt ligand and Wnt binding proteins and the inappropriate activation of the Wnt signaling have been found in a variety of human cancers. In epithelial cells, β-catenin-E-cadherin complexes locate at adhesion junctions. Translocation of β-catenin from adhesion junctions to the nucleus might result in the loss of E-cadherin and, subsequently, the EMT. Consistent with its role in embryonic development, many β-catenin target genes are involved in promoting stemness (Brabletz *et al*., 2005). Aberrant nuclear expression of β-catenin might confer cancer cells with these two capabilities, EMT and stemness, which promote malignant tumor progression. GSK-3β is an endogenous inhibitor of Snail and can phosphorylate Snai1. GSK-3β down-regulation by the FGF-dependent PI3-K/Akt pathway directly results in the activation of the Snai1-EMT signaling cascade. Therefore, inhibition of GSK-3β function by Wnt and other pathways can promote Snai1 stability and nuclear import to induce EMT (Zhou *et al*., 2004; Bachelder *et al*., 2005). In patients with a CML blast crisis, a β-catenin mutation may confer self-renewal properties on granulocyte-macrophage progenitors (Jamieson *et al*., 2004). In skin cancer, β-catenin signaling is essential to maintain the stemness properties of CSCs. Ablation of the β-catenin gene results in the loss of CSCs and a complete tumor regression (Reya and Clevers, 2005; Malanchi *et al*., 2008). Inhibiting of Wnt pathway through LRP6 decreases the ability of cancer cells to self-renew and seed tumors *in vivo* (DiMeo *et al*, 2009). Moreover, inhibition of Wnt signaling blocks tumor formation by promoting epithelial differentiation and repressing the EMT transcription factors, Twist1 and Slug. These data indicate that Wnt

pathway is involved in CSC self-renewal, EMT and metastasis in basal-like breast cancer (DiMeo *et al*, 2009).

Notch signaling. Notch signaling is important for development and tissue homeostasis and regulates cell fate specification through local cell interactions in invertebrate and vertebrate organisms. For example, Notch activity promotes EMT during cardiac development via transcriptional induction of Snai1 and induces EMT in immortalized endothelial cells *in vitro* (Luika *et al*., 2004). Notch pathway is also activated in many human cancers and contributes to EMT and to cancer stem-like cell characteristics in tumorigenesis. Notch signaling pathway is essential for both nonneoplastic neural stem cells and embryonal brain tumors. The activation of Notch signaling is a hallmark of $CD133^+$ CSCs in embryonal brain tumors, and blocking the Notch pathway by pharmacologic inhibitors of γ-secretase results in a depletion of $CD133^+$ stem-like cells in these tumors (Fan *et al*., 2006). Notch signaling is associated with chemo-resistance and EMT phenotypes in gemcitabine-resistant pancreatic cancer cells (Wang *et al*., 2009). Recently, miR-200 members has been shown to target Notch pathway components, such as Jagged1 (Jag1) and the mastermind-like co-activators Maml2 and Maml3, thereby mediating enhanced Notch activation by ZEB1 (Brabletz *et al*., 2011).

Hedgehog signaling. As an ancient cell signaling system, the Hedgehog (Hh) signaling is an important developmental pathway. In the absence of Hh ligands Shh, Ihh or Dhh, Hh receptor Ptch inhibits a second transmembrane protein Smo. This repression is relived when Hh ligands bind to Ptch. Subsequently Smo causes activation of Hh pathway targets via the Gli family of transcription factors (Gli1, Gli2, and Gli3). Hh signaling is essential for embryonic pattern formation, hematopoiesis, and also plays an important role in tumorigenesis and stem cell maintenance (Trowbridge *et al*., 2006; Dierks *et al*., 2008; Zhao *et al*., 2009). Hh signaling components such as *Ptch*, *Gli1*, and *Gli2* are highly expressed in normal and malignant human breast stem/progenitor cells. Activation of Hh signaling increases mammosphere-initiating cell number and mammosphere size, these effects are mediated by the polycomb gene, *Bmi-1* (Liu *et al*., 2006). Hh signaling is also activated in Bcr-Abl-positive leukemic stem cells (LSCs) by the upregulation of Smo. Loss of Smo in Bcr-Abl-positive hematopoiesis effectively inhibits the development of Bcr-Abl-positive leukemias in mice and abrogates the ability of the disease to re-transplant, indicating that the expansion of the Bcr-Abl-positive LSC pool is dependent on Hh signaling activation (Dierks *et al*., 2008). Another paper also revealed that the loss of Smo impairs hematopoietic stem cell renewal, lowers the propagation of Bcr-Abl-positive chronic myelogenous leukemia (CML), and decreases the growth of imatinib-resistant mouse and human CML (Zhao *et al*., 2009). However, a conditional Smo deletion or over-activation has no significant effects on adult HSC self-renewal and function, and the Hh signaling pathway is dispensable for adult HSC function (Gao *et al*., 2009). These results confirm recent findings that pharmacological Smo inhibition may only affect short-term repopulating HSCs in regular hematopoiesis; however, long-term repopulating HSCs and the long-term regeneration of hematopoiesis are not affected (Dierks *et al*., 2008). In addition, medulloblastomas arising from Patched-1-deficient or Patched-mutant mice contain $CD15^+$ CSCs (Read *et al*., 2009; Ward *et al*., 2009). Hh/Wnt feedback is involved in regenerative proliferation of epithelial stem cells in bladder (Shin *et al*., 2011). A recent report directly demonstrated a key and essential role of Hh signaling in regulating the stemness of CSCs via EMT. Stem cells of human colon carcinomas at all stages acquire a high Hh-Gli signature coincident with the development of metastases. The growth of colon cancer xenografts, their

recurrence and metastases require active Hh-Gli. Moreover, the self-renewal of colon CSCs *in vivo* relies on Hh-Gli activity, which induces a robust EMT (Varnat *et al*., 2009).

Extracellular matrix proteins. The extracellular matrix is a complex and dynamic structural network that is composed of structural proteins, proteoglycans, latent or active growth factors, and matricellular proteins. Cancer cell attachment and invasion of the ECM are crucial events leading to the initial disengagement from neighbor cells. Cancer cells can modify the composition of the adjacent stroma by secreting their own ECM proteins and by using the ECM proteins secreted by their neighbor stromal cells to create a permissive and supportive microenvironment for their survival, growth and invasion (Erkan *et al*., 2007; Ruan *et al*., 2009a). Type I collagen is highly expressed at the invasive front of human colorectal cancer. Type I collagen can decrease E-cadherin and β-catenin at cell-cell junctions and promote EMT on human colorectal carcinoma cells. Moreover, Type I collagen promotes a stem cell-like phenotype with an increased clonogenicity and expression of stem cell markers CD133 and Bmi-1 (Kirkland, 2009), indicating that Type I collagen may be involved in generating and maintaining human colorectal CSCs via EMT.

Other microenvironment cues. In addition to TGF-β, Wnt, Notch, and Hh which play a critical role in inducing EMT and regulating the stemness of CSCs, several other autocrine and paracrine growth factors such as FGFs, IGF, HGF, EGF family members and PDGF, together with their receptors, are also involved in regulating the EMT program in development and tumorigenesis (Huber *et al*., 2005; Yang and Weinberg, 2008). These data suggest that these autocrine- or paracrine-mediated EMT may be associated with the maintenance of self-renewal in cancer stem-like cells. However, whether these secreted growth factors from tumor microenvironment and their receptors regulate the stemness of CSCs via EMT remains to be established. Interleukin-6 (IL-6) is a tumor microenvironment-derived extracellular signaling factor capable of inducing EMT (Sullivan *et al*., 2009). IL-6 is overexpressed in human breast tumors as well as breast cancer patient sera and is associated with a poor prognosis in breast cancer. IL-6 is secreted by cancer cells and/or stromal cells and induces MCF-7 breast cancer cells to undergo EMT characterized by impaired E-cadherin expression and induction of Vimentin, N-cadherin, Twist1 and Snai1 via the activation of STAT3 (Sullivan *et al*., 2009). Moreover, IL-6 can induce malignant properties in mammospheres from human ductal breast carcinoma and normal mammary gland (Sansone *et al*., 2009). Furthermore, oncogenic Ras induces the secretion of IL-6 in different cell types. Knockdown of IL-6, genetic ablation of IL-6, or treatment with a neutralizing IL-6 antibody can thwart Ras-mediated tumorigenesis (Ancrile *et al*., 2007). Recently, IL-6 signaling has also been shown to contribute to glioma malignancy by promoting glioma stem cell (GSC) growth and survival (Wang *et al*., 2009). GSCs preferentially express IL-6 receptors IL-6Rα and gp130. Knockdown IL-6Rα or IL-6 ligand expression in GSCs significantly decreases growth and neurosphere formation but promotes apoptosis. Furthermore, STAT3 is a downstream mediator of pro-survival IL-6 signals in GSCs. The levels of IL6 ligand and receptor are enhanced in gliomas and are associated with poor survival of glioma patients. Inhibiting IL-6Rα or IL-6 expression in GSCs promotes the survival of mice bearing intracranial human glioma xenografts (Wang *et al*., 2009). A recent report revealed that carcinoma-derived IL-6 is involved in activation of cancer-associated fibroblasts. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness (Giannoni *et al*., 2010).

2.2 EMT transcription factors in regulating stemness of CSCs

E-cadherin is a central adhesion molecule located at cell-cell adhesion junctions and is essential for the formation and maintenance of the epithelial cell phenotype. Loss of E-cadherin is consistently observed at sites of EMT in embryonic development and tumorigenesis. Transcription factors such as Twist1, Snai1, Slug, ZEB1, ZEB2, FOXC2, KLF8 and E47, can repress the *E-cadherin* promoter directly or indirectly (Kang and Massague, 2004; Yang and Weinberg, 2008; Thiery *et al*., 2009). These transcription factors play critical roles in mediating type 1 EMT during embryogenesis and tissue morphogenesis; however, their aberrant activation of EMT developmental programs during tumorigenesis is considered a hallmark of disease progression and metastasis initiation. Among these developmental transcription factors, the Twist, Snai1 and ZEB family members are well-investigated in EMT and CSCs.

Twist1 and Twist2. Twist proteins are highly conserved basic helix-loop-helix (bHLH) transcription factors that play an important role in embryogenesis and tumorigenesis. Twist1 and Twist2 are significantly over-expressed in various human solid tumors and are involved in tumor invasion and metastasis through their ability to promote EMT (Ansieau *et al*., 2008). Twist1 and Twist2 mediate the growth and commitment of human mesenchymal stromal/stem cells (MSC) (Isenmann *et al*., 2009). The levels of Twist1 and Twist2 are very high in freshly purified human bone marrow-derived MSCs but decrease following ex vivo expansion. Over-expression of Twist1 and Twist2 in human MSC cultures up-regulates the level of the MSC marker, STRO-1, and the early osteogenic transcription factors, Runx2 and Msx2. Therefore, Twist1 and Twist2 are potential mediators of MSC self-renewal and lineage commitment. Also these proteins may act to regulate critical transcription factors and osteo/chondrogenic inductive factors that are important in early events to determine cell fate decisions in human MSC populations (Isenmann *et al*., 2009).

In a recent report, Mani *et al*. (2008) found that Twist1 can transform nontumorigenic, immortalized human mammary epithelial cells (HMLEs) into mesenchymal-like cells and dedifferentiate *HER2/neu*-infected HMLE (HMLEN) cells into $CD44^{high}CD24^{low}$ cancer stem cells via EMT. Induction of EMT in nontumorigenic, immortalized mammary epithelial cells by ectopic expression of either Twist1 results in a population of stem-like cells. Moreover, the stem-like cells isolated from mouse and human normal and neoplastic mammary glands express markers associated with an EMT. Compare to the level in $CD44^{low}/CD24^{high}$ cells, the expression of *E-cadherin* mRNA in stem-like $CD44^{high}/CD24^{low}$ cells is strongly decreased (~150-fold), while the levels of mRNAs encoding mesenchymal markers and EMT-inducing transcription factors are significantly upregulated, specifically *N-cadherin* (~200-fold), *Fibronectin* (~60-fold), *Twist1* (~198-fold), *Snai1* (~9-fold), *ZEB2* (~30-fold), and *FOXC2* (~16-fold). Furthermore, Twist1 can considerably increase the number of tumor-initiating cells in HER2/neu- or Ras-activated human mammary epithelial cells. The resulting populations that have undergone an EMT and display mesenchymal morphology and stem cell markers can efficiently form mammospheres, soft agar colonies, and tumors. This study provided direct support for a potential association between EMT and cancer stem-like cell phenotype. Vesuna *et al*. (2009) further demonstrated that Twist1 is directly involved in generating a breast CSC phenotype through down-regulation the expression of CD24. Twist2, like Twist1, overrides oncogene-induced premature senescence by promoting EMT in human epithelial cells (Ansieau *et al*., 2008).

Twist2 has been shown to be overexpressed in several types of human cancers, but the expression pattern of Twist2 is different from that of Twist1 in these cancers, suggesting that

Twist1 and Twist2 may have overlapping but distinct roles in different set of tumors. Twist2 is involved in $p12^{CDK2\text{-}Ap1}$-induced EMT of hamster cheek pouch carcinoma-I cells (Tsuji *et al*., 2008). Our recent studies have suggested a role of Twist2 in regulating EMT and CSC stemness in human breast cancer progression (Fang *et al*., 2011). Twist2 is a potent inducer of EMT in human mammary epithelial cells and breast cancer cells. Ectopic expression of Twist2 in mammary epithelial cells and breast cancer cells increases the size and number of their $CD44^{high}/CD24^{low}$ stem-like cell subpopulations, promotes the expression of stem cell markers and increases the self-renewal capabilities of stem-like cells. Moreover, exogenous expression of Twist2 leads to constitutive activation of STAT3 and down-regulation of *E-cadherin* (Fang *et al*., 2011). In addition, we also showed that the Twist2-driven EMT plays critical roles in ovarian cancer progression by promoting a cancer stem cell phenotype to augment tumor metastasis and therapeutic resistance (Mao *et al*., our unpublished data). Therefore, Twist2 may contribute to breast and ovarian cancer progression by activating the EMT program and enhancing the self-renewal of cancer stem-like cells.

Snai1 and Slug. The Snail family is highly conserved zinc-finger transcription repressor and plays a pivotal role in embryonic development and tumorigenesis. Both Snai1 and Slug can be activated by the TGF-β, Wnt, FGF, HGF and ER signaling pathways and Snai1 is specifically activated at the tumor-stroma interface. Snai1 has a critical role in EMT both during embryonic development and in tumor progression by inhibiting junction components such as E-cadherin, claudins, occludin and desmoplakin (Vincent, *et al*., 2009). Snai1-induced EMT accelerates tumor metastasis through enhanced invasion and the induction of multiple immunosuppression. Inhibition of Snai1-induced EMT can simultaneously suppress both tumor metastasis and immunosuppression in cancer patients (Kudo-Saito *et al*., 2009). Casas *et al*. (2010) reported that direct induction of Slug is essential for Twist1 to induce EMT and that Twist1 and Slug act together to promote EMT and tumor metastasis. In addition, Snai1 is a cofactor for Smad3/4 and these transcription factors form a transcriptional repressor complex to inhibit *CAR*, *occluding* and *E-cadherin* transcription during TGF-β-induced EMT in mammary epithelial cells (Vincent, *et al*., 2009).

The well-established roles of Snai1 and Slug in EMT during embryogenesis and tumor progression indicate that they may also be involved in generating and maintaining the stemness of CSCs. Slug can protect hematopoietic progenitor cells from radiation-induced apoptosis *in vivo* (Inoue *et al*., 2002). A recent report demonstrated that Snai1 and Slug are critical for ovarian cancer cells to acquire stem cell characteristics, and upregulation of Snai1 and Slug in ovarian cancer cells is associated with increased cell survival and acquisition of radioresistance and chemoresistance (Kurrey *et al*., 2009). Furthermore, Mani and colleagues (2008) found that Snai1 can generate cells with properties of stem cells via EMT induction like Twist1. When EMT is transiently induced in HMLEN cells through the ectopic expression of Snai1, the cells undergo an EMT and form more colonies in soft agar suspension culture but fail to form tumors more efficiently than untreated cells *in vivo*. However, constitutively expressing Snai1 in $H\text{-}Ras^{V12}$-infected HMLE (HMLER) cells augments the stem-like cell pool, mammosphere formation and tumorigenic property *in vivo*. This study also demonstrated that the long-term maintenance of the EMT/stem cell state may depend on continuous EMT-inducing signals (Mani *et al*., 2008).

ZEB1 and ZEB2. The ZEB family proteins, ZEB1 and ZEB2, are implicated in the malignancy of various human tumors, and are important regulators in EMT and contribute to the drug resistance and stemness of CSCs (Peinado *et al*., 2007). Interestingly, ZEB1 can promote tumorigenesis and link the activation of EMT with the maintenance of CSC stemness by

repressing stemness-inhibiting microRNAs (miRNAs), which reinforces the direct relationship between EMT and the stemness of CSCs (Wellner *et al*., 2009).

Bmi-1. Bmi-1, a member of the polycomb-repressive complex 1 (PRC1), is commonly deregulated in various tumors and plays an important role in maintaining self-renewal in normal and malignant human mammary stem cells (Dimri *et al*., 2002; Liu *et al*., 2005; Liu *et al*., 2006). Bmi-1 inhibits PTEN and induces EMT in human nasopharyngeal epithelial cells and is also involved in the regulation of self-renewal and differentiation of stem cells (Song *et al*., 2009b). A recent report showed that *Bmi-1* can be regulated by Twist1 directly. Bmi-1-containing PRC directly represses *E-cadherin* expression. Bmi-1 and Twist1 are mutually essential to promote EMT and tumor-initiating capability of human head and neck squamous cell carcinoma cells (Yang *et al*., 2010). We also showed that Bmi-1 is involved in Twist2-induced EMT of mammary epithelial cells and breast cancer cells and cancer stem cell self-renewal (Fang *et al*., 2011). The current findings highlight the critical role of the polycomb group protens in regulating EMT and the stemness of CSCs.

LBX1. Ladybird homeobox 1 (LBX1) is a well established homeobox regulator implicated in normal myogenesis and neurogenesis. Recent work has shown that LBX1 is over-expressed in the unfavorable ER/PR/HER2 triple-negative basal-like subtype of human breast cancer (Yu *et a*l., 2009). Moreover, LBX1 is a potent activator of EMT and can regulate the expression of the known EMT inducers TGF-β2, Snai1, ZEB1 and ZEB2. LBX1 induces EMT, enhances cell migration, enlarges the $CD44^{high}/CD24^{low}$ progenitor cell population in mammary epithelial cells, and cooperates with activated H-Ras to cause tumorigenesis and correlates with the basal subtype of human breast cancer (Yu *et a*l., 2009). These results suggest that LBX1 is an important developmental regulator of oncogenic EMT and stemness of breast cancer stem cells and contributes to breast cancer aggressiveness.

Six1. Six1, one of member of Six family of homeodomain proteins, is involved in the expansion of the precursor cell population during embryogenesis. In addition to the role of the Six family members in epithelial plasticity during muscle and kidney development, Six1 is frequently overexpressed in various cancers and has been shown to play an important role in inducing features of EMT in both a mammary carcinoma cell line and in mammary tumors derived from mammary specific Six1 overexpressing transgenic mice (McCoy *et al*., 2009; Micalizzi *et al*., 2009). Overexpression of Six1 in immortalized mammary epithelial cells induces malignant transformation and facilitates mammary carcinoma cells to undergo EMT and metastasis by increasing TGF-β signaling (Coletta *et al*., 2008; Micalizzi *et al*., 2009). Six1 also promotes the expansion of the stem/progenitor cell population in the mouse mammary gland and subsequent mammary tumor development via EMT (McCoy *et al*., 2009. Therefore, over-expression of Six1 in breast cancer induce highly aggressive and invasive mammary tumors with EMT and cancer stem cell features.

YB-1. Mammalian Y-box binding protein-1 (YB-1) is a member of the cold-shock domain (CSD) protein superfamily. Targeted disruption of YB-1 in mice causes severe developmental defects and embryonic lethality. YB-1 is involved in tumorigenesis and exhibits both pro-oncogenic role and tumor-suppressive functions by regulating gene expression through transcriptional and translational ways. YB-1 is over-expressed in ~75% of human breast cancers and high YB-1 levels provoke remarkably diverse breast carcinomas through the induction of genetic instability (Bargou *et al*., 1997; Bergmann *et al*., 2005). Increased expression of YB-1 in premalignant mammary epithelial cells with elevated Ras-ERK signaling inhibits proliferation, disrupts mammary morphogenesis, and induces EMT and promotes invasive properties and cell dissemination (Evdokimova *et al*., 2009). YB-1

regulates EMT by directly promoting the cap-independent translation of mRNAs encoding Snai1, LEF-1, ZEB2 and other transcription factors involved in EMT and by suppressing cap-dependent translation of growth-related genes. Furthermore, premalignant MCF-10AT human mammary epithelial cells ectopically expressing YB-1 appear to obtain various stem cell properties such as low proliferation rates, upregulation of the stem cell markers p63, CD44, and downregulation of CD24 (Evdokimova *et al*., 2009). Therefore, MCF-10AT cells with ectopic upregulated YB-1 may acquire cancer stem cell phenotypes by inducing EMT.

Hypoxia-inducible factors (HIFs). Intratumoral hypoxia occurs when tumor cells are located greater than the distance from functional blood vessels for adequate diffusion of oxygen as a result of rapid tumor cell growth and abnormal blood vessels. As one of the most pervasive microenvironmental stresses, hypoxia is now considered a common feature of solid tumors and promotes tumor angiogenesis, invasion and metastasis (Ruan *et al*., 2009c). Hypoxia is also involved in regulating the stemness of stem cells. Low oxygen tensions promote the maintenance of pluripotency in hESCs and prevent differentiation. Interestingly, the subpopulation of brain tumor cells expressing a stem cell marker is enlarged by hypoxia *in vitro*. HIF-2α can regulate stem cell function and differentiation through the activation of *Oct-4*, which in turn contributes to the tumor promoting activity of HIF-2α (Covello *et al*., 2006). In glioblastomas, CSCs differentially respond to hypoxia with a distinct induction of HIF-2α (Li *et al*., 2009). HIF-2α-specific target genes such as *Oct4*, *Glut1* and *SerpinB9* are expressed at significantly higher levels in GSCs compared to matched non-stem cancer cells under hypoxic treatment. HIF-2α is also required for VEGF expression in GSCs, but not in non-stem cancer cells. Thus, HIF-2α-mediated upregulation of these genes may provide CSCs with advantages in proliferation, survival, angiogenesis, metabolism, and escape from immune surveillance. Furthermore, targeting HIFs in GSCs inhibits self-renewal, proliferation and survival *in vitro*, and suppresses tumor initiation potential of GSCs *in vivo* (Li *et al*., 2009).

Hypoxia can also induce EMTs in tumors through the upregulation of HIF-1α, Snai1, Twist1, ZEB1, ZEB2, lysyl oxidase (LOX) and by activating Wnt and Notch pathways (Erler *et al*., 2006; Pouyssegur *et al*., 2006; Yang *et al*., 2008). Twist1 has a critical role in EMT and metastatic phenotypes induced by hypoxia or over-expression of HIF-1α. In primary tumors of head and neck cancer patients, co-expression of HIF-1α, Twist1 and Snai1 correlates with metastasis and a poor prognosis (Yang *et al*., 2008). Hypoxia can inhibit the expression of E-cadherin via the activation of the LOX-Snai1 pathway to promote tumor invasion and metastasis, indicating that LOX may cooperate with Snai1 and Twist1 in hypoxia-mediated EMT and invasion (Pouyssegur *et al*., 2006; Yang *et al*., 2008). Jagged2 is upregulated in bone marrow stroma under hypoxia and promotes the growth of cancer stem-like cells by activating their Notch signaling. Hypoxia-induced Jagged2 activation in both tumor invasive front and normal bone stroma has a critical role in breast cancer metastasis and self-renewal of cancer stem-like cells (Xing *et al*., 2011). Therefore, high levels of HIFs in hypoxic tumor cells may promote cancer cells to acquire the properties of CSCs including self renewal and multi-potency by activating Oct4, c-Myc, Notch, Snai1 and other critical signaling pathways (Keith and Simon, 2007). Hypoxic microenvironment may be not only a critical niche favorable for expansion and stemness maintenance of CSCs in solid tumors, and also a breeding ground for generating CSCs from differentiated tumor cells by promoting EMT, and a critical microenvironmental condition that is associated with radioresistance, chemotherapy resistance and a poor clinical prognosis of solid tumors (Keith and Simon, 2007; Li *et al*., 2009).

In addition to Twist, Snail and ZEB family members and the transcription factors mentioned above, developmental transcription factors such as Goosecoid and FOXC2 have also emerged as key factors that regulate E-cadherin and promote EMT during embryonic development and tumorigenesis. Furthermore, these transcription factors may play a critical role in the stemness maintenance of CSCs via EMT. Goosecoid, a conserved transcription factor, is overexpressed in human breast tumors and can elicit an EMT to promote cell motility and significantly enhance the ability of breast cancer cells to form pulmonary metastases in mice (Hartwell *et al*. 2006). FOXC2 is associated with aggressive basal-like breast cancer and also confers stem cell properties on epithelial cells. FOXC2 specifically promotes mesenchymal differentiation via EMT and may serve as a critical mediator to orchestrate the mesenchymal component of the EMT program (Mani *et al*., 2007; Mani *et al*., 2008).

2.3 Other players of EMT in regulating stemness of CSCs

microRNAs. microRNAs (miRNAs) are a newly discovered endogenous class of small non-coding RNAs of 18-25 nucleotide in length that modulate gene expression as negative regulators at the post-transcriptional level by specifically binding and cleaving target mRNAs or inhibiting their translation. Current reports demonstrated that the deregulation of miRNAs correlates with various human cancers and is involved in the initiation and progression of human cancers (Ruan *et al*., 2009b). Recently, miRNAs have also been identified as a new class of EMT regulators due to their regulation of EMT-inducing transcription factors, such as Twist1, Snai1, ZEB1 and ZEB2 (Ma and Weinberg, 2008).

The miR-200 family of miRNAs (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) is both an important marker for epithelial cells and a powerful master regulator of EMT in embryonic development and tumorigenesis (Park *et al*., 2008). miR-200 suppresses the expression of ZEB1 and ZEB2 to favor an epithelial phenotype and inhibit EMT (Gregory *et al*., 2008; Korpal *et al*., 2008; Park *et al*., 2008). Moreover, let-7, miR-335, miR-205, miR-206, miR-126, miR-146a and miR-101 have also been reported as metastasis suppressors (Yu *et al*., 2007; Gregory *et al*., 2008; Tavazoie *et al*., 2008; Varambally *et al*., 2008). Conversely, miRNAs such as miR-155, miR-10b, miR-21, miR-373 and miR-520c are involved in promoting tumor invasion and metastasis via regulating EMT (Ma *et al*., 2007; Huang *et al*., 2008; Kong *et al*., 2008; Yan *et al*., 2008). For example, TGF-β stimulation of normal mammary epithelial cells elicits their increased expression of miR-155 via a Smad4-dependent pathway. Once expressed, miR-155 participates in EMT by inhibiting RhoA expression, leading to the dissolution of tight junctions (Kong *et al*., 2008).

Recently, various miRNAs are also involved in regulating the stemness of embryonic stem cells, adult stem cells or CSCs. miRNAs are crucial for normal embryonic stem cell self-renewal and cellular differentiation (Marson *et al*., 2008). Recent reports demonstrated that a subset of the miR-290 cluster in the mouse and the miR-371 cluster in humans are direct regulators of the cell cycle in ES cells (Judson *et al*., 2009). A subset of the miR-290 cluster, including miR-291-3p, miR-294 and miR-295, increased the efficiency of reprogramming by *Oct4*, *Sox2* and *Klf4*, but not by these factors plus *c-Myc* (Judson *et al*., 2009). A recent report demonstrated that the level of miR-145 is low in self-renewing hESCs but is much higher during differentiation. Furthermore, the pluripotency factors *OCT4*, *SOX2*, and *KLF4* are direct targets of miR-145. miR-145 upregulation is sufficient to inhibit hESC self-renewal and induce lineage-restricted differentiation of hESCs (Xu *et al*., 2009).

Multiple members of the let-7 family of miRNAs are often inhibited in human cancers. A recent paper showed that let-7 is reduced in breast CSCs and can negatively regulate the stemness of breast CSCs and tumorigenesis by silencing H-Ras and HMGA2, regulators of self-renewal or differentiation of breast CSCs, respectively. Ectopic over-expression let-7 in breast CSCs reduces proliferation, mammosphere formation, and the proportion of undifferentiated cells *in vitro*. Also, in NOD/SCID mice, tumor formation and metastasis is reduced when let-7 is over-expressed (Yu *et al*., 2007). These findings indicate that a low level of let-7 is required to maintain CSCs, and let-7 may link EMT with CSCs. Interestingly, a recent paper demonstrated that miR-200c is differentially expressed between human breast CSCs and nontumorigenic cancer cells. miR-200c can target *Bmi*, a known regulator of stem cell self-renewal, and strongly inhibits the ability of normal breast stem cells to form mammary ducts and tumor formation driven by human breast CSCs (Shimono *et al*., 2009). Iliopoulos *et al*. (2010) demonstrated that downregulation of miR-200 lead to increased expression of Suz12, a subunit of the polycomb repressor complex 2, increased binding of Suz12 to the *E-cadherin* promoter, and upregulated H3-K27 trimethylation and polycomb-mediated inhibition of *E-cadhrein* expression. The interactions between the miR-200 family are required for CSC formation. Xia *et al.* (2010) reported that miR-200a not only regulates EMT by targeting ZEB2 but also stem-like transition via differentially and specifically by β-catenin signaling in nasopharyngeal carcinoma cells. This finding demonstrates for the first time the function of miR-200a in shifting nasopharyngeal carcinoma cell states via a reversible process coined as epithelial-mesenchymal to stem-like transition through differential and specific mechanisms. In addition, the stem cell factors, Sox2 and KLF4, are also targets of miR-200c. ZEB1 links EMT-activation with the maintenance of stemness of CSCs by suppressing stemness-inhibiting miRNAs such as miR-200c and miR-203 (Wellner *et al*., 2009). Induction of EMT can be controlled by miR-200 family whose abundance depends on the balance between Akt1 and Akt2 rather than on the overall activity of Akt (Iliopoulos *et al*., 2009). A recent report showed that EMT induction is epigenetically driven, initially by chromatin remodeling through H3K27me3 enrichment and later by ensuing DNA methylation to sustain silencing of miR-200b, miR-200c, and miR-205 (Tellez *et al*., 2011). These current data highlight the central role of miRNAs in regulating EMT and self-renewal and/or proliferation of normal and neoplastic stem cells. The miRNA signatures of CSCs likely represent a new layer of regulatory control over cell fate decisions of CSCs via EMT.

p53. The tumor suppressor p53 is known to function as transcription factor. Recently, p53 has been shown to exhibit a role in regulating both EMT and EMT-associated stem cell properties through transcriptional activation of miR-200c (Chang *et al*., 2011). Loss of p53 in human mammary epithelial cells decreases the expression of miR-200c and activates the EMT program, accompanied by an increased mammary stem cell population. Moreover, loss of p53 correlates with a down-regulated level of miR-200c, but an increased expression of EMT and stemness markers, and development of a high tumor grade in a cohort of breast tumors. Therefore, the p53-miR-200c pathway most likely accounts for regulating the EMT-associated cancer stem cell population (Chang *et al*., 2011).

3. Concluding remarks

EMT is regarded as a critical step in tumor invasion and metastasis. During tumor metastasis, disseminated cancer cells from primary tumors are associated with a loss of epithelial

differentiation and the acquisition of a mesenchymal phenotype. Furthermore, these cancer cells appear to require the capability to self-renewal in order to spawn macroscopic metastases. The majority of disseminated cells are destroyed in the process of tumor metastasis; however, only a small number of cancer cells are able to survive and initiate the formation of micrometastases at the secondary sites, and even a smaller subpopulation of these micrometastases can develop into macrometastases (Bao *et al*., 2004). Current evidence supports that metastasis is a relatively inefficient process and the overwhelming majority of cells that shed from a primary tumor and disseminate to distant secondary sites lack the capability to self-renew and their ability to form macroscopic metastasis in the new microenvironment is compromised from the outset. The discovery that EMT generates cells with properties of self-renewing stem cells has linked EMT with both tumor metastasis and acquisition of stem-like cell properties, indicating that cancer cells undergo an EMT are capable of metastasizing through their acquired invasiveness and, following dissemination, through their acquired self-renewal potential, which enables them to spawn the large cell populations that constitute macroscopic metastases (Taube *et al*., 2010).

EMT occurs in a variety of distinct physiological and pathological settings, including normal embryogenesis, tissue morphogenesis, tissue remodeling and repair and fibrosis, and cancer progression. A number of developmental signaling pathways have been shown to play a role in EMT such as TGF-β, Wnt, Notch, Hh and other microenvironmental cues. These EMT-inducing signaling pathways promote the well-differentiated epithelial cells to convert into motile mesenchymal cells via the activation of multiple EMT transcription factors such as Twist1, Twist2, Snai1, Slug, ZEB1 and ZEB2. Each of these factors is capable, on its own, of inducing an EMT in various normal and cancer cell lines. However, the overlapping and unique contributions of each inducer to the EMT program have not been adequately explored. The critical roles of TGF-β, Wnt, Notch, Hh and other signaling pathways in promoting EMT and the stemness maintenance of stem cells adds to a growing body of evidence that cancer cells often reactivate latent developmental programs to regulate the multistep process in tumorigenesis. Therefore, the knowledge gained from the multifaceted players of EMT during development and from the acquisition of CSC traits with the EMT transdifferentiation program may provide useful information to uncover the roles of these EMT players in tumorigenesis and metastasis, and offer new avenues of therapeutic intervention with the potential to go beyond traditional anti-cancer approaches.

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10

Cancer Stem Cell Niche: The Role of Mesenchymal Stem Cells in Tumor Microenvironment

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1. Introduction

Niche, a specialized physiological microenvironment in which stem cells reside, plays a crucial role in the maintenance of stem cell characteristics such as pluripotency and self-renewal. The stem cell niche is composed of a group of cells providing a physical anchoring site for stem cells with the interaction of adhesion molecules between stem cells and niche cells or extracellular matrices (Li & Xie, 2005). The niche controls stem cell number, proliferation and fate of determination. Thus, a better understanding of the nature of stem cells and their niche will lead to alternative treatment strategies for various diseases, including malignant tumors.

More than a decade ago, the existence of a rare population with both stem cell-like properties and tumor initiating capability was first identified in acute myeloid leukemia and, subsequently, in several solid tumors. These populations with stem cell-like properties were termed 'cancer stem cells (CSCs)', indicating that only a subset of cancer cells were tumorigenic and able to initiate and produce the bulk of tumors, thus also termed 'tumor initiating cells' (Reya, et al., 2001). Although CSCs may not harbor all of the properties of normal stem cells, they are characterized by their ability both to self-renew and to differentiate into certain cell types with limited potential for differentiation and proliferation.

Some functional environments, namely 'cancer stem cell niche', a counterpart of normal stem cell niche, may support CSCs like normal stem cells (Sneddon & Werb, 2007). There are similarities in the maintenance system for the stem cell niches between normal and cancer stem cells such as the presence of molecules involved in adhesion and chemokine-chemokine receptor interaction. On the other hand, it might be possible that the behaviors of CSCs and normal stem cells are regulated by the niche to different degrees (Li & Neaves, 2006). The niche is supposed to control the balance between quiescence and proliferation/differentiation of the stem cells. In cancers, the characteristics of the niche that function to support proliferation and differentiation could be more dominant than is necessary to maintain quiescence. However, the similarities and differences between the normal stem cell niche and the tumor microenvironment are still unclear.

Mesenchymal stem cells (MSCs) are non-hematopoietic stromal cells, mainly existing in the bone marrow and possibly in various tissues, that are capable of differentiating into and contributing to the regeneration of mesenchymal tissues such as bone, cartilage, muscles, tendons and adipose tissue (Chamberlain, et al. 2007).

MSCs secrete several paracrine factors including chemoattractants for endothelial lineage cells, monocytes and macrophages as well as inflammatory factors such as various chemokines and interleukins (Kinnaird, et al. 2004). MSCs could be involved in cell survival, invasion, motility, and interactions with the extracellular matrix through the chemokine signaling that results in the transcription of target genes in cancer cells as well as macrophages and lymphocytes (Roorda, et al. 2010). MSCs are also considered to be the source of tumor-associated fibroblasts (TAFs) that are important components of tumor stroma. Therefore, MSCs play an important role in orchestrating the tumor microenvironment through angiogenesis, modulation of both immune system and tumor stromal architecture.

MSCs may also be involved in the metastatic process (Karnoub, et al. 2007). Tumor metastasis requires numerous cell types and molecules to undertake sequential, multi-step processes such as intravasation and circulation, extravasation and migration, and angiogenesis to form the metastatic foci (Honoki, et al. 2007). The metastatic process is considered to be very complex and inefficient as only ~2% of disseminated cells form micrometastasis and only ~0.02% of cells develop into macrometastasis. One of the possible explanations for this inefficiency is the need for the metastatic cells to find an appropriate environment for themselves. MSCs are thought to play some roles in providing the homing sites for those cells, leading to the establishment of the metastatic foci, either directly or indirectly through the cooperation with extracellular matrices and other cells like macrophages and vascular endothelial cells.

The composition of the niche is very complex and varied in specific tissues even in normal conditions. Thus, the interaction of CSCs with their niche could be even more complex. The current review describes the potential roles of MSCs in tumor progression as the constituents of tumor stroma and niche that affect the cell fate of CSCs. Although the environments constituting a true cancer stem cell niche are still not fully elucidated, the exploration of the precise role of MSCs in cancer could potentially uncover the pathogenesis and mechanisms of progression such as metastasis. This is likely to lead to novel targeted therapies against cancers.

2. Role of mesenchymal stem cells in tumor microenvironment

The tumor stroma consists of a compilation of cells and matrices including fibroblasts/myofibroblasts, immune/inflamatory cells, blood vessels, connective tissues and extracellular matrix (ECM). Tumor-associated stromal cells like fibroblasts/myofibroblasts, immune/inflamatory cells and vascular endothelial cells are considered to be recruited from surrounding normal tissue or from circulation, and contribute to diverse aspects of tumor development and progression. Mesenchymal stem cells (MSCs) have also been demonstrated thier homing ability to the primary tumor site and metastatic sites in several studies (Studeny, et al. 2002, Dwyer, et al. 2007, Hall, et al. 2007), and possibly play a role as a co-conspirator of tumor development. MSCs may interact with tumor cells to promote tumor growth directly or indirectly through autocrine/paracrine manners with soluble

factors as well as anigiogenesis. In addition, MSCs could be the origin of tumor-associated fibroblasts or myofibroblasts contributing to the formation of tumor microenvironment that eventually lead to expansion and progression of tumors (Spaeth, et al. 2009).
It is possible that MSCs have a potencial to provide a specific microenvironment or a niche for cancer stem cells by themselves or as a source of its components. The interaction between stem cells and their specific microenvironment/niche cells will enhance the understanding of cancer development, including metastasis. We will firstly discuss here the general concept of stem cell and niche, then expand it to cancer stem cells and finally focus on the role of mesenchymal stem cells in the tumor microenvironment.

2.1 Stem cell and niche

Stem cells are the subset of cells that have the capacity to self-renew and generate functionally differentiated mature cells. Adult stem cells, including mesenchymal stem cells, are an essential component of tissue homeostasis, which support tissue regeneration, and are considered to reside in a special microenvironment. The fate of stem cells is regulated with a delicate balance between self-renewal and differentiation by both intrinsic programs and extrinsic signals from the environment. There are various intrinsic programs that control stem cell self-renewal and potency, such as Hox genes family for hematopoietic stem cells and Bmi, a polycomb family, for hematopoietic and neural systems (Kyba, et al. 2002, Lessard, et al. 2003, Park, et al, 2003). These intrinsic genetic programs have been shown to be subject to environmental regulation. Therefore, environmental regulatory signals are required to stem cell properties as well as intrinsic genetic programs.
Schofield first proposed the 'niche' hypothesis to define some specific microenvironment that supports stem cells (Schofield, 1978). The niche is a physical anchoring site for stem cells, composed of a group of cells in a special location that functions to maintain stem cells. In recent years, niches have been identified for stem cells in the intestinal, neural, epidermal, and hematopoietic system. The niche maintains stem cells primarily in a quiescent state and the stem cells become activated to divide and proliferate when a stimulating signal reaches to them (He, et al. 2005). The niche generates extrinsic factors that control stem cell fate through regulatory signal molecules, including sonic hedgehog, Wnts, bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), and Notch (Ivanova, et al. 2002). Stem cells exhibit an asymmetric division, indicating that one daughter cell is maintained in the niche as a stem cell, in terms of self- renew, while other daughter cell leaves the niche to proliferate and differentiate into a matured cell. Above extrinsic factors orchestrate the appropriate regulation of stem cell self-renewal and lineage commitment.
The component of niche or niche cells and involved environmental signals differ in tissue by tissue. For instance, hematopoietic stem cell niche is considered to be composed of osteoblastic and perivascular niche (Fig.1), and Wnt, Notch and BMP signals play a role in self-renewal, differentiation and proliferation (Reya, et al. 2003, Duncan, et al. 2005, Zhang, et al. 2003). While, for intestinal stem cells, they are located at the 4^{th} or 5^{th} position above the Paneth cells, and mesenchymal cells adjunct to the intestinal stem cells function as the niche. There, Wnt signalling plays a positive role in promoting stem cell activation/self-renewal and crypt cell fate, in contrast, BMP signalling restricts stem cell activation/self-renewal and crypt cell fate (van de Wetering, et al. 2002, He, et al. 2004).
When the balance between these controls is disrupted, stem cell may proliferate without restraint that might lead to tumorignesis.

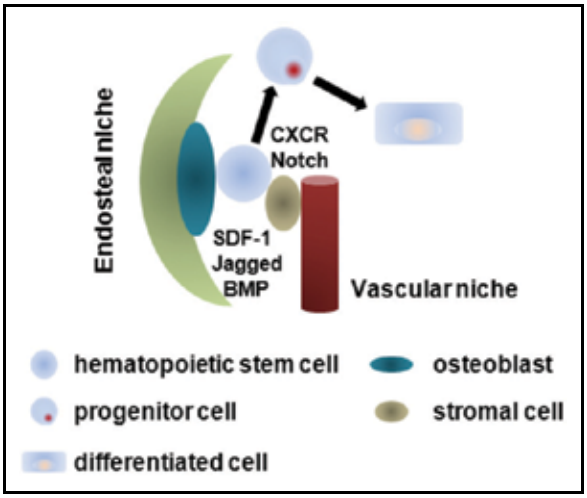

Fig. 1. Model of hemtopoietic stem cell (HSC) niche. The HSC niche is located on the surface of trabecular bone, and spindle-shaped N-cadherin-positive osteoblastic cell plays an important role to regulate HSC behavior, with an interaction of different types of stromal cells by secreting different microenvironmental signals

2.2 Normal stem cell and cancer stem cell, and their niches

Recent numerous researches have generated a new concept of tumor progression modeling for the cancer growth based on the cancer stem cell hypothesis (Fig. 2, Dick, 2008). The properties of cancer tissues have been defined the following characteristics; (i) limitless capacity to replicate, (ii) self-sufficiency for growth signals, (iii) insensitivity to antigrowth signals, (iv) evasion of apoptosis, (v) sustained angiogenesis, (vi) tissue invasion and metastasis (Hanahan et al., 2000). Since majority of those characteristic are considerably sharing with normal stem cell properties, the hypothesis has emerged indicating that tumor tissue, like normal tissue, is continuously repopulated from pools of self-renewing stem–like cells, termed as 'cancer stem cells' (Table 1). Cancer stem cells exist as a side population on the top of hierarchy within tumors, possessing stem-like properties like self-renewing and expression of stem cell-related genes/markers, and they are truly involved in tumor initiation.

Somatic Stem Cell	Cancer Stem Cell
Origin of cellular pedigree	Origin of cellular pedigree
Organ generation Tissue regeneration	Cancer initiation Relapse and Metastasis
Self - renew Pluripotency	Self - renew (Pluripotency)
Tissue reconstruction	Tumor formation

Table 1. Comparison between normal somatic and cancer stem cell

As described above, in normal adult tissues, stem cells depend on the integration of both cell-intrinsic and cell-extrinsic factors for proper, homeostatic tissue maintenance. It is likely that there is a functional microenvironment to support cancer stem cells, a counterpart of normal stem cells and their niche.

Involvements of several factors have been indicated in the interaction between cancer stem cells and their microenvironment. Dick and colleague and Van Etten and colleague respectively showed that CD44 is essential for the homing and engraftment of the cancer stem cells to the niche for acute myeloid leukemia and chronic myeloid leukemia (Jin, et al. 2006, Krause, et al. 2006). Interestingly, the molecular mechanisms of leukemia cell homing to the niche resemble those of the interaction between normal hematopoietic stem cells and the vascular niche.

Another example is that CD133-positive brain tumor cells, that contain cancer stem cells, selectively adhere to the endothelial cells that possibly form a vascular niche (Calabrese, et al. 2007). CD133-positive cells have been extensively studied in colon, prostate and pancreatic cancers, and further study will reveal the molecular mechanism underlying the relationship between CD133-positve cancer stem cells and the niche.

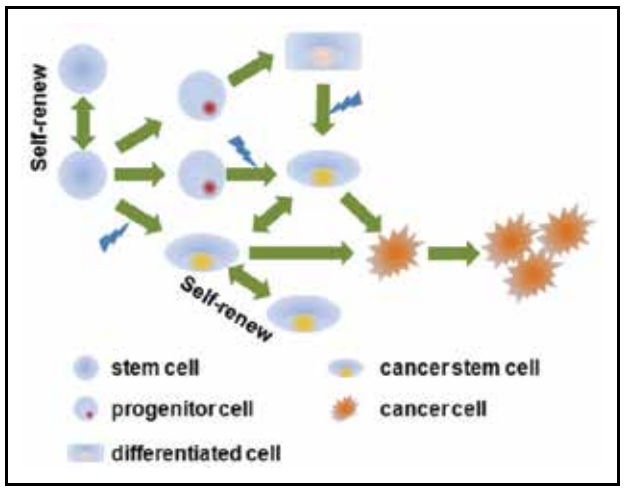

Fig. 2. Hypothesis for the development of cancer stem cells. Stem cell and/or progenitor cells with accumulated genetic alteration show sustained or reacquired self-renewal capacity, and at certain point, these cells, so called 'cancer stem cells' give rise to more differentiated, but not fully matured cancer cells with aggressive proliferation

Several cellular components have also been indicated as the cancer stem cell niche with specific signal transduction pathways. For instance, endothelial cells in the vasculature of the brain maintain neural stem cell properties, in part through Notch signalling (Shen, et al. 2004), and this is the same as the brain cancer stem cells and their vascular niche (Hovinga, et al. 2010). Another extensively studied example is the mesenchymal stromal cells in cancer stem cell niche in the intestine. In normal intestine, stem cells reside in a stem cell niche composed of epithelial cells and mesenchymal cells of the myofibroblast lineage lining the crypt. Currently, the Wnt signalling cascade is considered to be a prominent force controlling cell proliferation, differentiation, and apoptosis to maintain stem cell fate at this region (Clevers, 2006). As the counterpart of the normal intestinal stem cell niche, tumor -

associated myofibroblasts and mesenchymal stem cells are shown to be major components of the colon cancer stroma (Mishra, et al. 2008). Colon cancer stem cells have been shown high Wnt activity and orchestrated by myofibroblasts residing in the tumor stroma through the hepatocellular growth factor pathways (Vermeulen, et al. 2010).
These data suggest that there is a strong link between cancer stem cells and the microenvironment as well as that between normal stem cells and their niche, courageously indicating the presence of cancer stem cell niche.
However, unlike normal stem cell niche, the behaviours of cancer stem cells might be regulated by the niche to different degrees (Li & Neaves, 2006). The stem cell niche under normal conditions predominantly inhibits stem cells from both proliferation and differentiation, and a transient proliferating signal is required to activate tissue regeneration. Contrary, in tumors, cancer stem cells could be self-sufficient to undergo uncontrolled proliferation due to their internal mutations and/or changes in the niche signals. This leads the hypothesis of cancer stem cell niche as an environment with dominant signals to tumor cell proliferation and growth more favourably compared to the normal stem cell niche.

2.3 Homing of mesenchymal stem cells in tumor stroma

The concept of adult mesenchymal stem cells (MSCs) was first proposed almost four decades ago (Friedenstein, et al. 1970), then have been extensively investigated on molecular and functional characterization of these cells. Although the definition of MSCs is commonly accepted as bone marrow-derived, adherent to plastic surface, with the ability of self-renew and differentiation into bone, cartilage and adipose tissues, the specific marker or phenotype to definitively identify or describe these cells are still lacking. This is because MSCs reside not only in bone marrow but also in many other tissues through the entire body, and presumably MSC-like cells or MSC subsets exist with retaining MSC features.
It has been shown that MSCs can be recruited to the site of wound healing to repair damaged tissues, and so as to tumors like breast cancer, colon cancer, ovarian cancer, glioma, melanoma and osteosarcoma (Karnoub, et al. 2007, Studeny, et al. 2004, Nakamizo, et al. 2005, Komorava, et al. 2006, Djouad, et al. 2003, Brune, et al. 2010). These MSCs exhibit specialized features tailored for each particular tissue. As described above, tumor-associated fibroblast (TAFs), including myofibroblast, are part of tumor stroma that functionally and structurally supports tumor progression and development in conjunction with other components like vasculature, inflammatory cells and extracellular matrix (Sullivan, et al. 2008). There are also several evidences indicating that MSCs invade tumors and function as TAFs (Direkze, et al. 2004). TAFs have been shown to promote tumor progression through the production of growth factors like TGF-β, chemotactic factors like SDF-1 and monocyte chemotactic protein-1, angiogenic factors like VEGF and matrix metalloproteinases (MMPs) leading invasion and spread of tumor cells. Although the origin of tumor stromal fibroblasts is not fully elucidated, it is possible to consider some of these stromal cells including TAFs as a 'specialized' subset of MSCs. (Haniffa, et al. 2007, Stagg, 2008). Therefore, either MSCs themselves or specialized MSCs like TAFs in the stroma are key players directly or indirectly involved in tumor progression (Fig. 3).
As cancers are sometimes referred to as 'wounds that never heal' (Dvorac, 1986), the microenviroment of a solid tumor is very similar to the environment of an injured tissue. Thus, tumor growth is often associated with a variety of cells and factors in a similar manner with the wound healing and tissue repair sites. Several studies have shown the involvment of MSCs for injured tissue repair. In that process, chemokines and chemokine

receptors such as CXCR4, CXCR12 and CCL5 have been indicated in the tissue-homing ability of MSCs (Dwyer, et al. 2007), and TGF-β family and Wnt signals play important roles in MSCs-mediated tissue repair (Mishra, et al. 2005). Like these factors, many players involved in MSCs for tumor progression are very similar to the wound repair response (Dvorac, 1986).

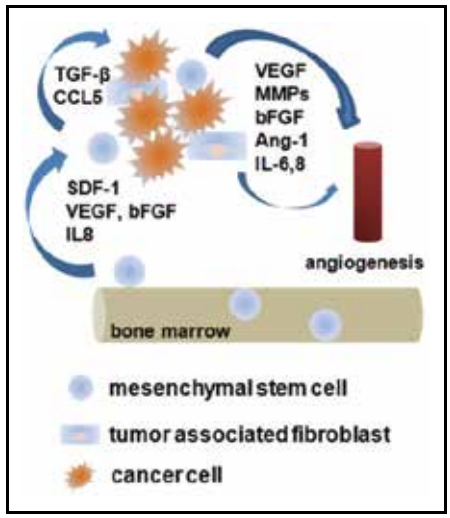

Fig. 3. Hypothesis for the involvement of mesnechymal stem cells in tumor progression. Mesnechymal stem cells migrate in tumor tissue and incorporate into TAFs. TAFs stimulate cancer cell proliferation through the growth factor signals like TGF-β. MSC itself or TAFs could participate in angiogenesis by secreting factors like VEGF and MMPS

Another contribution of MSCs in tumor development would be pro-invasive potential homing to the metastatic site for cancer cells, possibly the cancer stem cells, to create a so called 'premetastatic niche' (Kanoub, et al. 2007). The ability of a tumor to metastasize may depend on the ability to establish distant niches suitable for them as well as the characteristics of tumor cells themselves. MSCs colonize the premetastatic niche before metastatic tumor cells have arrived in response to signals from the tumor cells. Factors like CCL5, SDF-1, VEGF and MMPs are also involved in this phenomenon (Kaplan, et al. 2007). Although numerous studies indicated the homing ability of MSCs to primary and metastatic site, one might argue that the homing of MSCs might not be completely tissue specific and could distribute to a wide range of organs. So far, no data are available where MSCs detected in tumor stroma originate from whether the local mesenchyme or bone marrow. Therefore, detailed mechanisms of participating molecules involved in homing of MSCs to tumor site should be further investigated for the development of therapies targeting MSC-tumor cell interaction.

2.4 Role of mesenchymal stem cells in tumor progression

Intrinsic capabilities of tumor cell itself have been focused on the research of tumor progression including invasion and metastasis. On the other hand, recent advances of the research on tumor microenvironment indicate that components of heterogeneous population of stromal cells such as fibroblasts, immune cells can enhance tumor progression

with their dynamic interactions. MSCs are considered to be involved in many aspects as the origin of tumor stromal fibroblasts, source of soluble factors to attract immune cells and angiogenesis.
In fact, the relationship between MSCs and tumor cells could be dual. Thus, both primary and metastatic tumor cells actively attract MSCs from either bone marrow or surrounding tissues where they contribute to the tumor microenvironment as described above; vice versa, MSCs stimulate tumor cells to produce a variety of cytokines and other growth factors.

2.4.1 An immunomodulator in the process of tumor development

Immunosuppressive properties of MSCs contribute in part to the tumor developments. MSCs have been shown to suppress the lymphocyte proliferative response in a broad way regarding the types of stimulation and of lymphocyte population (Di Nicola, et al. 2002, Djouad, et al. 2003). A number of mechanisms have been reported for these effects, such as secretion of anti-inflammatory factors (Rasmusson, 2006), modulation of the function of the major immune cell population (Glennie, et al. 2005), cell cycle arrest in the G0/G1 phase on B lymphocyte (Corcione, et al. 2006), modulation of development and function of cytotoxic T cells and dendritic cells (Rasmusson, et al. 2003, Jiang, et al. 2005), inhibition of interferon-γ production by activated natural killer cells (Aggarwal & Pittenger, 2005).
These mechanisms could be clue to explain how tumor cells evade the immune system. One might hypothesize that tumor cells can harness MSCs that manipulate the immune system through several ways described above, and eventually evade the immune attack (Yen & Yen, 2008).
Beside immunosuppressive effects, recruitment of tumor infiltrating macrophages or tumor associated macrophages (TAMs) could be another role of MSCs. The role of TAMs is multifaceted, as not only providing supportive microenvironment for malignant cells but also inducing oncogenic mutations in surrounding cells in earliest carcinogenesis (Pelham, et al. 2006). TAMs could be a heterogeneous population and whose possible common origin would be monocytes actively recruited into tumor site. Monocytes can migrate in response to chemokines such as CCL2, CCL5, CXCL8/IL-8 and SDF-1, and also to growth factors like vascular endothelial growth factor (VEGF) produced by MSCs as well as tumor cell itself (Murdoch, et al. 2004, Barleon, et al. 1996). Migrated monocytes differentiate into TAMs (Mantovani, et al. 2002), and eventually contribute to tumor progression *via* production of growth factors and regulation of pro-angiogenic switch (Lin, et al. 2006).

2.4.2 A source of soluble factors involved in angiogenesis

The formation of tumor vessels requires the proliferation and directional migration of vascular endothelial cells through basement membrane and stroma toward pro-angiogenic stimuli, such as soluble chemoattractants like VEGF, basic fibroblast growth factor (bFGF) and angiopoietins (Ang-1).
Specialized MSCs in tumor stroma, like TAFs as described above, play an important role for the formation of tumor vessels. MSCs and/or TAFs produce several pro-angiogenic factors including VEGF, FGFs, Ang-1, platelet derived growth factor (PDGF) as well as cytokines such as IL-6, 8 and TNF-α (Kinnaird, et al. 2004). These molecules are involved in the recruitment and activation of endothelial cells through paracrine manner as well as direct cell – cell contacts (Zacharek, et al. 2007).

Tumor angiogenesis requires active remodeling and integration of new cells into existing structures and is the result of a complex tumor – stroma crosstalk, involving multiple ligands and cellular signaling pathways. SDF-1/CXCL12 axis and MMPs are critical players to recruit and engraftment of marrow derived endothelial progenitors for growth of new vessels from pre-existing vasculature (Genis, et al. 2006).
MMPs secreted from stromal cells including MSCs and TAFs play the diverse roles in not only degradation of the extracellular matrices, but also cleavage of proteinases, chemokines and chemotactic factors, growth factors and cell surface receptors, and cell matrix adhesion molecules (Coussens, et al. 2002) to participate in restructuring tumor microenvironments including angiogenesis. Proteolytic cleavage of angiogenic factor, secreted protein acidic rich in cysteine (SPARC)/osteonectin is also indirect effect of MMPs to regulate the tumor angiogenesis (Sangaletti, et al. 2003).
Then, VEGF from MSCs and TAFs in tumor stroma stimulate budding of existing vessels and proliferation of new vascular channels. Although VEGF is a potent vascular growth factor and the main stimulus for the formation of tumor vessels, it is implicated in many aspects of cancer growth such as extracellular matrix remodeling and generation of inflammatory cytokines and other molecules involved in the pro-angiogenic and pre-metastatic niche (Kaplan, et al. 2005). The main stimulus for secretion of VEGF is hypoxia, which results in the recruitment of endothelial progenitor and mesenchymal stem cells to sites of ischemia, that in turn additional VEGF production leading to tumor angiogenesis and generation of tumor stroma (Okuyama, et al. 2006).

2.4.3 A source of niche cells for cancer cells / cancer stem cells

For normal stem cells, the microenvironment or niche where they reside is important to maintain the stemness, and to regulate proliferation and differentiation. The similarities between normal stem cells and cancer stem cells as shown in Table I could extend the attractive concept of stem cell niche to the presence of cancer stem cell niche. There have been several studies indicating the specialized cancer stem cell niches may participate in the tumor development (Calabrese, et al. 2007, Gilbertson & Rich, 2007), and is considered to play an important role in virtually every aspect of the tumorigenic cascade including the metastatic process as well as drug resistance.
Usually, metastasis occurs in an organ-selective manner described as the 'seed and soil' hypothesis which indicate that local microenvironment of specific organs seems to be more receptive to particular tumor cells than other organs. Thus, disseminated tumor cells need to meet a suitable microenvironment in order to survive and initiate a secondary tumor. The metastatic process is very complicated and inefficient with multi-steps such as intravasation, circulation, arrest, extravasation migration and angiogenesis (Honoki, et al. 2007). Tumor stromal cells including MSCs might be involved in several phases of the metastatic process with the interaction of cancer stem cells (Fig. 4).
Mesenchyaml stem cells have the ability to enhance growth and metastasis of certain cancers like colon cancer (Shinagawa, et al. 2010), and have been proposed to give rise to tumor-associated fibroblasts that further promote tumor progression. The metastatic potential of breast cancer cells and osteosarcoma cells has also been shown to be strongly enhanced when coinjected with MSCs through the paracrine signaling events (Karnoub, et al. 2007, Xu, et al. 2009). In this context, MSC-derived CCL5 appears to be an essential factor to enhance the growth and invasiveness of tumor cells. IL-6 from MSCs is also involved in

growth promotion effect for osteosarcoma cells (Bian, et al. 2010). MSCs also produce chemoattractant proteins like SDF-1 and MCP-1 that attract circulating tumor cells like B leukemia cells, breast cancer cells (Burger & Kipps, 2002, Molloy, et al. 2009).
Transition to TAFs from MSCs has been shown to contribute to the tumor growth through fibrovascular network expansion and production of tumor-stimulating paracrine factors (Spaeth, E.L. 2009). Activated fibroblasts or TAFs have been found also at liver metastasis, where they promote tumor outgrowth (Olaso, et al. 1997). Fibroblast activation is probably involved in priming the premetastatic niche with fibronectin deposits (Kaplan, et al 2005) to attract tumor cells to their preferable sites for metastasis. Moreover, the infiltration of activated fibroblasts or myofibroblasts precedes the recruitment of vascular endothelial cells in the hypoxic avascular metastatic environment, and they produce VEGF to promote angiogenesis and transition to a vascular stage (Olaso, et al. 2003). In addition to paracrine signaling, MSCs protect tumor cells against apoptosis and promote initial tumor cell proliferation mainly by direct cell – cell contact interactions (Roorda, B.D. 2010). All of these data suggest that MSCs are directly or indirectly involved in the metastatic process as the source of niche for metastatic tumor cells.

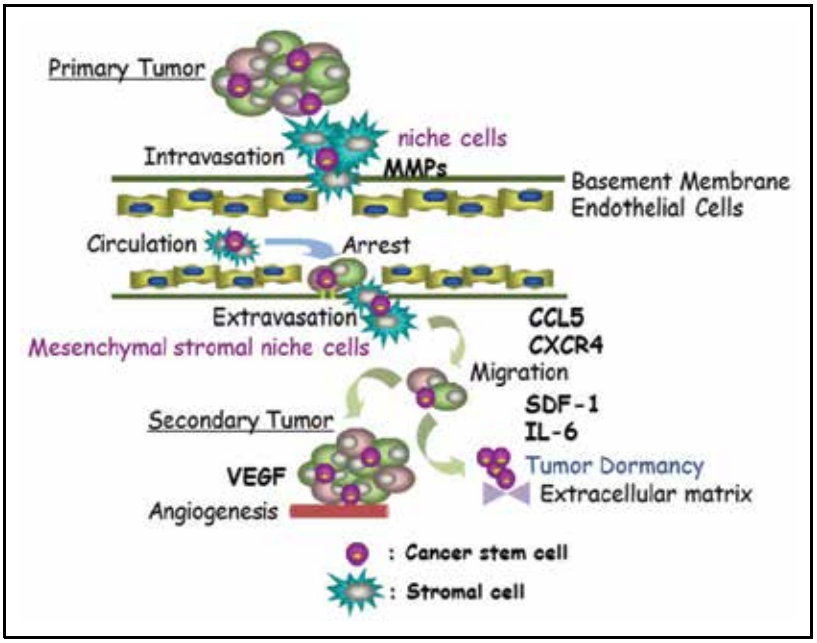

Fig. 4. Possible involvement of tumor stromal cells including MSCs/TAFs in clustering metastatic model. Metastatic tumor cells, possibly including cancer stem cells, complete a very complicated metastatic process cooperating with tumor stromal cells. Soluble factors such as MMPs, CCL5, CXCR4, SDF-1, IL-6 and VEGF secreted from MSCs/TAFs participate in the metastatic process

Besides of the effect on tumor progression, tumor stromal cells may contribute to the drug resistance through complex mechanisms such as the direct cell contact, the interaction of extracellular matrices (ECM) and soluble factors (Nefedova, et al. 2003). Soluble factors that mediate drug resistance are produced by a dynamic interaction between tumor cells and

stromal cells. IL-6 and SDF-1 are known to mediate resistance to various chemotoxics in hematological and epithelial cancers. IL-6 activates c-FLIP and STAT3 signaling that result in protecting tumor cells from TNF-related apoptosis-inducing ligand (TRAIL) and FAS-mediated apoptosis (Duan, Z, et al. 2006, Perez, et al. 2008). SDF-1 increases β1 integrin-mediated adhesion of myeloma and small lung cell carcinoma, leading to drug resistance in ECM-adhered tumor cells (Sanz-Rodriguez, et al. 2001, Hartmann, et al. 2005). Integrin-mediated adhesion increases the activation of cytokine signaling pathways and also leads to a state of tumor dormancy. For instance, β1 integrin amplifies IL-6-induced STAT3 signaling (Shain, et al. 2009) as well as leading tumor cells to cell cycle arrest (White, et al. 2006) that result in drug resistance eventually. β1 integrin-mediated adhesion to ECM components also leads to cell cycle arrest through p21 and p27 up-regulation (Fischer, et al. 2005), which contribute to drug resistance (St. Croix, et al. 1996).

Taken together, although there are no direct evidences indicating MSCs or TAFs provide the niche for cancer stem cells nor they are not the only player for cancer stem cell niche, these tumor stromal cells could have bystander effects to participate in tumor progression as the niche cells, providing dominant signals to tumor cell proliferation and growth, and recessive signals to lead cancer cells into the dormant state with the interaction of ECM. Both signals eventually contribute to tumor progression such as metastasis and drug resistance.

2.5 Therapeutic implications of mesenchymal stem cells in cancer

There are several possible different ways to imply targeting MSCs into therapeutic purpose. Firstly, targeting paracrine loop of soluble factors could disrupt the interaction between tumor cells and MSCs, which contributed many aspects of tumor progression and drug resistance. Inhibition of VEGF receptor tyrosine kinase activity and receptor tyrosine kinase for FGF blocks a paracrine IL-6 production by stromal cells, and leads to apoptosis of tumor cells (Lin, et al. 2002, Bisping, et al. 2009). Small molecule inhibitor of SDF-1 receptor CXCR4 increases the chemo-sensitivity of leukemic cells and prolongs survival of leukemic patients (Zeng, et al. 2008). Since both IL-6 and SDF-1 also play an important role in the microenvironment of solid tumors as described above, the therapeutic approaches that disrupt communication between tumor cells and stromal cells such as MSCs/TAFs could be feasible against solid malignancies.

Secondly, MSCs that migrate to tumor site would be useful as carriers of oncolytic or immune-modulatory adenoviruses or as direct target of 'activated' or genetically altered stromal cells. MSCs have been engineered in a number of different ways as the vehicles delivering cytotoxic drugs, stimulating the immune response or blocking angiogenesis. MSCs modified to express IFN-β and γ were able to inhibit tumor growth (Studeny, et al. 2004, Li, et al. 2006). MSCs infected with adeno- or retrovirus encoding IL-12 showed inhibitory effects on tumor growth through the activation of NK cells and CD8+ T cells (Elzaouk, et al. 2006). Bone marrow-derived progenitors with suicide gene integration used as delivery vehicles have also been shown to reduce tumor size and vascularity (Komarova, et al. 2006). Tumor-associated angiogenesis was inhibited by MSCs infected with adenovirus to express NK4 *via* antagonizing HGF-c-met signalling pathway (Kanehira, et al. 2007).

Several studies suggested the direct inhibitory effects of MSC itself against tumor growth of, for instance, mouse Lewis lung carcinoma, B16 melanoma (Maestroni, et al. 1999), and rat colon cancer model (Ohlsson, et al. 2003). Involvements of non-inflammatory cytokines and Akt activity have been suggested on these effects, however, the exact factors mediating the anti-tumor activities of MSCs have not been clearly identified.

Although MSCs have a potential in cell-based anti-cancer therapy, the safety of using MSCs is still questionable, because homing of MSCs is not selective and genetically engineered MSCs could undergo malignant transformation. Thus, the precise action of MSCs will need to be studied to develop the safe and specific treatments.
Another possible target would be the homing mechanisms by which cancer stem cells migrate to metastatic sites or the factors that govern the cellular dynamics within their 'pathological niche' or 'cancer stem cell niche', where MSCs possibly play a role in several aspects of tumor progression.
Further study will be required to understand the details of components, regulation and interaction between cancer stem cell and their niche to develop a novel treatment strategy.

3. Conclusions

Pro-tumorigenic contribution of MSCs is still not fully uncovered. This is in part explained by the presence of heterogeneous population due to the lack of exact definition of MSC population, and the complexity of their interaction with tumor cells and the large range of cytokines and growth factors. However, a number of evidences suggest that MSCs are actively recruited into tumor site, and contribute to tumor microenvironment as either themselves or as the tumor-associated fibroblasts. They directly or indirectly regulate tumor cell proliferation, differentiation, immune tolerance, angiogenesis, metastasis and drug resistance through the interaction with numerous cytokines and growth factors as well as providing niche to the cancer cells or cancer stem cells in cooperated with ECM. Although MSCs can be friends or foes of tumor cells, depending on their constituents of population including the origin and stage of differentiation and also the type of tumor cells to be interact with, further investigation will define the role of MSCs in tumor progression that leads to novel strategy for cancer therapy.

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11

Transformation of Mesenchymal Stem Cells

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1. Introduction

As part of normal organismal homeostasis, the human body loses various types of cells like hepatocytes, keratinocytes and certain types of blood cells and needs to replace them. These cells are replaced using stem cells as the source. Many different names are used for cells with stem cell-like properties, such as precursor cells, progenitor cells, somatic stem cells and adult stem cells. Stem cells are unique cells that have the ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types. In addition, stem cells, with few notable exceptions, are cell types that show telomerase activity and therefore, actively maintain telomere length to some degree. The ability to maintain telomere length allows them to have an extended proliferative capacity compared to somatic cells.

There are three kinds of stem cells: Embryonic, germinal and somatic or in other terms adult stem cells. The differentiation potential of stem cells varies according to type from totipotency to unipotency. Cells such as the fertilized oocytes, up to the 8-cell blastocyst, are considered to be totipotent since they can differentiate and generate a complete organism. Embryonic stem (ES) cells are derived from the inner cell mass of a blastocyst. ES cells posses all the characteristics of true stem cells. In addition to the self-renewal capacity, they are pluripotent, being able to produce derivates of all three germ layers (endoderm, mesoderm and ectoderm) but not the complete organism (Burdon *et al*., 2002). A high telomerase activity prevents the ES cells from undergoing crisis and reaching senescence, which is an advantage for long-term culturing. ES cells have furthermore been shown to retain their developmental identity even after reintroduction into the blastocyst (Beddington & Robertson, 1989). Somatic stem cells are differentiated forms of embryonic stem cells that are known as multipotent stem cells. They are capable of self-renewal and are responsible for the regenerative property of the e.g. hematopoietic system and the gastrointestinal system. These cells can be isolated from the developing organism (the fetus and the postnatal organism) as well as from the adult organism. Depending on their origin, the offspring of the somatic stem cells is also specific to the original tissue. A stem cell is said to be unipotent like the epidermal stem cells if they can persistently give rise to only a specific cell type. It has for many years been known that stem cells – contrary to other cell types –

can perform asymmetric cell divisions, whereby one daughter cell remains undifferentiated, while the other is committed to differentiation. It is important to also note that through transdifferentiation, somatic stem cells are also able to produce progeny different from their tissue of origin. These cells can be isolated from the developing organism in fetal and postnatal stages as well as the fully developed organism. Examples of somatic stem cells will include; stem cells which are harvested from the brain which are capable of differentiating into the three lineages of the central nervous system (CNS) (neurons, astrocytes and oligodendrocytes). Another example of somatic stem cells is the bone marrow stem cells that include hematopoietic and mesenchymal stem cells which are able to repopulate the blood and the bone cell systems.
In this chapter, we will focus on Mesenchymal Stem Cells (MSCs), their therapeutic potential, risks of neoplastic transformation of Mesenchymal Stem Cells (MSCs) during expansion, issues related to the use of MSC during therapeutic use and possible ways of addressing these issues.

2. Mesenchymal stem cells

2.1 Properties and isolation of mesenchymal stem cells

Bone marrow contains a supporting tissue called the stroma which was thought of as a simple structural framework for the hematopoietic system. It is now known to have very diverse functions. One of its most important aspects is that it contains mesenchymal stem cells (MSCs). These cells are strongly adherent and therefore, they can be isolated by culturing marrow on a special substrate, which allows the other cells to be washed off (Dennis *et al*., 2004; Pittenger *et al*., 1999; Terskikh *et al*., 2006). Their properties were described as early as 1968 by Friedenstein (Friedenstein, 1968). The human mesenchymal stem cells (MSCs) are multipotent precursor cells and can differentiate into various types of mesenchymal tissue cells. Mesenchymal stem cells (MSCs) undergo self-renewing divisions but also give rise to more committed progenitor cells, which can differentiate into cells of the mesodermal lineage, such as adipocytes, osteocytes and chondrocytes, as well as cells of other embryonic lineages (Jiang *et al*., 2002).
In addition to bone marrow, MSC-like cells have been shown to be present in a number of other adult and fetal tissues, including circulating blood (Zvaifler *et al*., 2000), cord blood (Weiss & Troyer, 2006), placenta (Miao *et al*., 2006), amniotic fluid (Tsai *et al*., 2004) heart (Chen *et al*., 2008), skeletal muscle (Peault *et al*., 2007), adipose tissue (Zuk *et al*., 2001) synovial tissue (De Bari *et al*., 2001) and pancreas (Di Rocco *et al*., 2008). In addition to healthy tissue, MSC-like cells have even been isolated from pathological tissue like rheumatoid arthritic joints (Marinova-Mutafchieva *et al*., 2000). Cells that carry MSC characteristics might be present in nearly all postnatal organs and tissues (Chamberlain *et al*., 2007). Historically, the broad variety of tissue sources from which MSCs are isolated, in conjunction with disparate culture conditions like media formulations and plating density, has led to a lack of consensus regarding the phenotype of the MSC. However, recent reports are pointing to a strong consensus regarding the morphology of fresh MSCs, irrespective of the method by which they were isolated. This morphology of MSCs has been described as large cells with a prominent nucleoli and bleb-like projections. These projections extend further as MSCs adhere. The morphology is different from the spindle-shape of typically shaped MSCs (Jones & McGonagle, 2008). Despite the historic variation in reported phenotypes of MSCs, it is widely accepted that cultured cells, regardless of the methods

employed in their isolation and culture, lack expression of prototypic hematopoietic antigens including CD45, CD34, CD11b and CD14 and express SH2 (CD105), SH3/SH4 (CD73), CD29, CD44, CD90, CD71, CD106, CD166, STRO-1, GD2, and CD146 (Martinez *et al*., 2007; Pittenger *et al*., 1999; Shi & Gronthos, 2003; Simmons & Torok-Storb, 1991; Sordi *et al*., 2005).
The methodology used in the isolation and enrichment of human mesenchymal cells is essentially based on the ability of these cells to adhere to and subsequently proliferate on tissue culture plastic with 10% fetal calf serum. The cells might experience a lag phase but then divide rapidly. The doubling time of the MSCs *in vitro* depend on the donor and the original plating density (Chamberlain *et al*., 2007). Indeed, culture selection is still widely employed as a means of MSC isolation. Pre-enrichment through cell separation strategies using cocktails of antibodies that deplete the bone marrow of specific cell populations (negative selection) (Louis *et al*., 2001; Reyes *et al*., 2001) or FicollTM separation are most widely used as an initial step in MSC isolation. To date no single and unique marker allowing for MSC isolation has been reported thus a range of composite cell surface phenotypes are being used. Enriched populations of MSCs have been isolated from human bone marrow aspirates using a STRO-1 monoclonal antibody in conjunction with antibodies against VCAM-1/CD106 (Simmons & Torok-Storb, 1991), CD271 (Quirici *et al*., 2002), D7-Fib30 and CD49a.31. In order to harvest and isolate MSCs, a needle is used to aspirate the bone marrow from the trabecular of the bone. The bone marrow can then be manipulated within the laboratory to remove the red blood cells, macrophages, and other extraneous material (Boiret *et al*., 2005). MSCs can then be enriched by their adherence to a plastic culture dish. They can also be sorted via flow cytometry based on MSC surface proteins and viewed under a microscope to determine that the cells look like MSCs (Campagnoli *et al*., 2001; Li *et al*., 2006; Quirici *et al*., 2002). MSCs have also been isolated from other species such as mice, rat, cats, dogs, rabbits and baboons with varying success. However, MSCs from different species do not express the same molecules as the human cells (Javazon *et al*., 2004).

2.2 Therapeutic potential of mesenchymal stem cells

MSCs have many desirable characteristics that make them great therapeutic tools for many diverse illnesses. MSCs can be isolated from adult donors and can easily be expanded in culture without greatly compromising genetic stability; an in depth discussion on genetic stability and neoplastic transformation will be presented in the following section. Their lack of immunogenicity allows for allogenic transplantation and their homing capacity creates room for treatment with minimal invasion (Teng, 2010). Isolation of MSCs has been discussed in greater detail in the previous section. They can be isolated not only from blood tissue but also from many diverse tissues such as adipose tissue and trabecular bone and placenta.
Many studies have been performed using MSCs. These studies were mainly based on site-directed and/or systemic administration of MSCs and both delivery methods of hMSC have shown their ability of engraftment in a number of tissues after injury (Barbash *et al*., 2003; Horwitz *et al*., 2002; Orlic *et al*., 2001; Ortiz *et al*., 2003). In order for a stem cell to work as an effective therapeutic tool it must be able to access the target organ to deliver their therapeutic effect. Access to the target organ might not be a problem in some cases, however, if the illness is systemic in nature or if the target organ is not anatomically accessible effective delivery might become a problem. MSCs provide us with a very valuable tool in the latter two scenarios as they have been shown to spread to various tissues after their intravenous

administration (Devine *et al*., 2003). These systemically administered MSCs home to the site of injury and aid in functional recovery. In earlier studies, cultured MSCs have been infused into patients to support bone marrow transplant for osteogenesis imperfecta and glycogen storage disease where the therapeutic options are limited (Pittenger & Martin, 2004). They have also been used in graft versus host disease and their role in the treatment of Crohn's disease is being explored (Mittal *et al*., 2009). MSCs are also used in diverse variety of clinical trials for ischemic stroke, multiple sclerosis, acute leukaemia, critical limb ischemia, articular cartilage and bone defects.

Although the exact mechanism is not well understood, introduction of MSCs into the infarcted heart directly or through intravenous administration, have resulted in improved recovery and prevented deleterious remodeling (Pittenger & Martin, 2004). The ability of MSCs to repair damaged tissue is thought to be primarily from their capacity to secrete paracrine mediators such as interleukin-10, interleukin-1ra, keratinocyte growth factor and prostaglandin (Matthay *et al*., 2010). However, the mechanisms underlying migration/homing of hMSC have yet to be clarified, even though there are some evidence suggesting the involvement of chemokines and their receptors (Kortesidis *et al*., 2005; Von Luttichau *et al*., 2005; Wynn *et al*., 2004). In addition to cellular adhesion molecules, the following chemokine/receptor pairs have been implicated in MSC migration; SDF-1/CXCR4, SCF/c-Kit, HGF/c-Met, VEGF/VEGFR and PDGF/PDGFr. However, the migration to the tumor site is believed to be non-specific since the administered MSCs have been shown to localize to lung, bone marrow and lymphoid organs. Additionally, it was observed that whole body irradiation increases the distribution of MSCs to multiple organs (Momin *et al*., 2010).

Because of their stem cell properties, facilitation of engraftment of the transplanted hematopoietic stem cells and promotion of the structural and functional repair of damaged tissues are some of the first studies the scientists have focused in the MSC field. However, because of the immunomodulatory properties of MSCs, they might have potential uses in immune-related diseases as well (Uccelli *et al*., 2008). One of the advantages of using MSCs as therapeutic agents is their poor immunogenicity *in vitro*, in preclinical trials and in human studies. This would allow the use of MSCs from allogenic donors. There still remains the possibility of using autologous MSCs even in autoimmune conditions. (Bartholomew *et al*., 2002; Le Blanc *et al*., 2004; Murphy *et al*., 2002; Tse *et al*., 2003). Recently reported findings present the observation that MSCs derived from the bone-marrow suppress T-cell proliferation (Uccelli *et al*., 2008). MSCs have been shown to have a role in both innate and adaptive immunity. In innate immunity, MSCs can decrease the pro-inflammatory response potential of dendritic cells by inhibiting their production of tumor-necrosis factor (TNF). They have also been shown to have an inhibitory effect on the maturation of monocytes and cord-blood and CD34+ hematopoietic progenitor cells (Jiang *et al*., 2005; Li *et al*., 2008; Nauta *et al*., 2006; Ramasamy *et al*., 2007). Neutrophils, an important part of innate immunity, can also be regulated by MSCs. The MSCs, through an IL-6 regulated mechanism, can dampen and delay the spontaneous apoptosis of resting and activated neutrophils (Raffaghello *et al*., 2008). In adaptive immunity, T cells are maintained in a state of quiescence through an MSC-mediated anti-proliferative effect, which can be partially reversed through IL-2 stimulation (Zappia *et al*., 2005). It has also been suggested that MSCs are able to modulate the intensity of immune system response through an inhibition of antigen-specific T-cell proliferation and cytotoxicity and through promotion of regulatory T cell generation (Hwa Cho *et al*., 2006; Pevsner-Fischer *et al*., 2007; Tomchuck *et al*., 2008).

2.3 Limited proliferation capacity of hMSCs

Most of the human somatic cells can undergo 60-70 population doublings on average, and then enter senescence (Meyerson, 2000). Cellular senescence is the process by which normal cells lose the ability to divide. This limited number of cell divisions is called the "Hayflick limit" (Hayflick, 1976) (Figure 1, top panel).

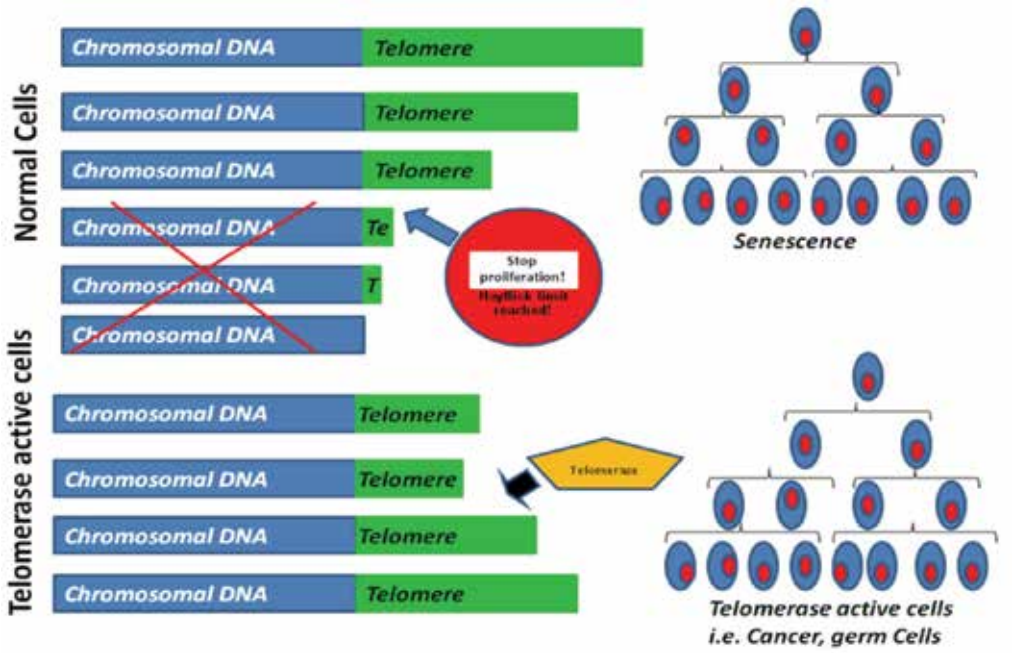

Fig. 1. Hayflick limit, senescence and telomerase reactivation

In normal cells, cell divisions are preceded by replicating the DNA to form two daughter molecules each having an original strand from parent cell and one newly synthesized strand. Replication of the leading DNA strand is simple and complete, but on the lagging strand replication uses small Okazaki fragments, to ensure 5′ to 3′ addition of bases. This results in the incomplete replication of the extreme ends of the lagging strand of chromosomes, and loss of genes. This is called the "end replication problem". The normal senescence involves the p53 and pRb pathways and leads initially to the arrest of cell proliferation (Campisi, 2005). The telomere signal that activates the senescence program operates through the Rb and p53 pathway. Rb and p53 deficient primary cells that continue cellular growth beyond the Hayflick limit exhibit severe telomere shortening, marked genetic instability and massive cell death—this period is referred to as crisis (Counter *et al*., 1992; Shay *et al*., 1991). It is thought that senescence plays an important role in the suppression of cancer emergence. Once the telomeres reach a critical short length, by means of the end replication problem, the cell enters the M1 stage (mortality 1) and goes into senescence (Kim *et al*., 1994). If the cell escapes senescence and continues to proliferate with further shortening of telomeres, it will undergo crisis or mortality 2 (M2). Cells that overcome M1 and M2 by acquiring mutational changes become immortal. Most cancers are the result of "immortal" cells that have evaded programmed cell death. At this point the cells will have acquired telomerase reactivation to maintain a constant length of telomeres (Fig 1).

Despite their stem cell characteristics when pressed to proliferate strongly, mesenchymal stem cells can also suffer from replicative senescence with critically short telomeres (Kim *et al*., 1994). This brings about certain limitations in therapeutic use of stem cells. One of these limitations is as mentioned above, the limitation in proliferative capacity, which undoubtedly can be overcome by introduction of certain genes that will enable them to continue to proliferate. It has been shown that there are numerous genes like the TERT gene that are capable of extending the proliferative capacity (Fig 1) and immortalizing the stem cells or progenitor cells, however, with the risk that malignant transformation may occur. Therefore, telomere dynamics becomes an important issue in stem cell function especially when expanding a population of stem cells is needed.

Considering the fact that stem cells in general tend to give rise to a high number of daughter cells, one would expect, that stem cells would express telomerase to maintain telomere length. But the real picture is inconsistent. In primary stem cells the replicative capacity is, however, limited. The telomere length pattern (also termed the telomere profile) can be monitored until the cells reach replicative arrest after approximately 10 population doublings (PD) and it has been shown that the telomere profile is conserved for this number of doublings. In addition, the possible conservation of profile has been studied in telomerase-immortalized mesenchymal stem cells where these cells were grown for 205 PD's and still they maintained the profile. This surprisingly long term conservation of the telomere profile clearly suggests that also in mesenchymal stem cells there is a very low degree of random fluctuation in the telomere dynamics, as previously suggested in lymphocyte progenitor cells. Overall, there is a general agreement, that adult stem cells have very low levels of telomerase, thus telomeres are slowly shortened during life. The primary mesenchymal stem cells have also been shown to obtain a diminished mean telomere length during long term culturing periods (Serakinci *et al.*, 2007). (Fig2). Serakinci *et al.* have shown that the mean telomere length of the primary cells is 8 kb and the length is continuously decreasing with the population doubling level.

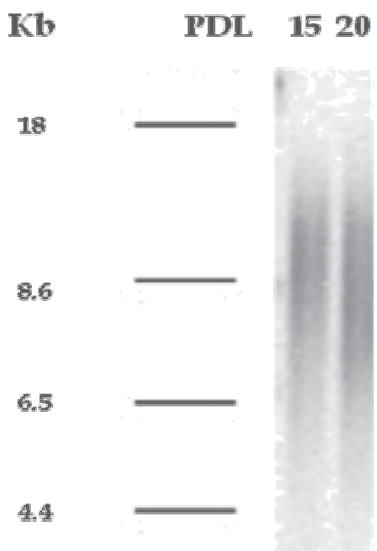

Fig. 2. Deccreased telomere length in primary hMSC with population doubling levels (PDL)

3. MSC expansion for therapeutic purposes: Neoplastic transformation

Within the last decade, human mesenchymal stem cells have been validated as potential tools in different therapeutic approaches. Many of these therapeutic potentials for MSCs have been discussed in the previous section in detail. Initial results were promising but there are some challenges for the use of MSCs in clinical applications. During the isolation process, only 1 in every 10^5 cells is MSCs and there is also the issue of low grafting efficiency and potency of MSCs. Although hMSCs have great potential in therapeutic use the main rate limiting factor is that hMSCs exhibit limited mitotic potential, especially considering that, in a human system very many cells are needed for injection. Therefore, a need for large scale MSC expansion is obvious (Momin *et al*., 2010; Teng, 2010). To overcome this proliferative limitation introduction of the TERT gene has been highlighted. Hence, telomere dynamics is an important issue in stem cell function especially when expanding a population of stem cells. Serakinci *et al.* by introducing a retrovirus carrying the hTERT gene has established an immortalized hMSC-telo1 cell-line, which maintain their stem cell characteristics and have an expanded life span (Serakinci *et al*., 2007). But extending the proliferative capacity of stem cell populations through manipulation of the telomere-telomerase system brings certain risks that are associated with the possibility that stem cells may show increased susceptibility to carcinogenesis. This manipulation bypasses the naturally built-in controls of the cell that govern the delicate balance between cell proliferation and senescence and carcinogenesis.

Given the critical role of telomere dynamics and telomerase in tumor progression and the fact that the cancer cells rely on telomerase for its survival, it is not surprising that telomeres are rather unique structures in a given cell. On one hand they have a role in protecting the chromosome ends from being recognized as DNA double strand breaks by the DNA repair machinery. On the other hand telomeres, when critically shortened, can lead to cellular senescence, which can be regarded as a barrier against cancer formation via the so-called telomere-mediated checkpoints. However, in the cells that manage to avoid being destroyed, dysfunctional telomeres can affect the genomic stability through initiating the so called Break-Fusion-Break (BFB) cycles leading to severe genomic aberrations and ultimately to cancer development (Furlani *et al*., 2009; Serakinci *et al*., 2008) (Fig 3). These events have been reported in literature in adipose-derived human MSCs (Rubio *et al*., 2005) and bone marrow-derived mouse MSCs (Miura *et al*., 2006; Tolar *et al*., 2007; Zhou *et al*., 2006). These MSCs showed phenotypic and genotypic alterations such as chromosome instability, rapid cell proliferation, loss of contact inhibition, gradual increase in telomerase activity and increased c-myc activity (Furlani *et al*., 2009).

Besides the immortalized cell line examples, there are also studies in both rodent models and human mesenchymal stem cells that have suggested that during long-term culturing mesenchymal stem cells acquired chromosomal aberrations and subsequently exhibited a malignant transformation (Rubio *et al*., 2005; Zhou *et al*., 2006). Such studies raised the concerns that hMSCs that are forced into extensive expansion can undergo spontaneous transformation. Several groups have shown results showing spontaneous transformation of human mesenchymal stem cells that are expanded long term (Serakinci *et al*., 2004; Rubio *et al*., 2005; Momin *et al*., 2010; Wang *et al*., 2005). Most studies have been done in mouse but Rubio *et al*., have shown that adipose tissue–derived human MSC populations, after a long-term *in vitro* expansion, can transform spontaneously. Rubio *et al*. further characterized the molecular mechanisms implicated in the spontaneous transformation. They have shown that

the transformation process occurred after the hMSC had bypassed senescence by up regulating c-myc and repressing p16 levels. Then, through acquisition of telomerase activity, deletion at the Ink4a/Arf locus and hyperphosphorylation of Rb followed (Rubio *et al*., 2008). It was also reported by Wang *et al*. that human MSCs derived from the bone marrow would produce a sub-population of cells when cultured. These cells would have high levels of telomerase activity, chromosomal aneuploidy and translocations and were able to form tumors in multiple organs in NOD/SCID mice (Momin *et al*., 2010; Wang *et al*., 2005). Contrary to these studies, several groups have reported no transformation of hMSC after long-term culture (Bernardo *et al*., 2007; Meza-Zepeda *et al*., 2008). Using comparative genomic hybridization, karyotyping and subtelomeric fluorescent *in situ* hybridization analysis Bernardo *et al.* performed extensive studies on the genetic changes in the hMSCs at different stages of the long-term culture but they did not find any evidence for spontaneous transformation of hMSCs during long term culture (Bernardo *et al*., 2007). In addition, there are studies demonstrating that telomerase-immortalized hMSC accumulate various genetic and epigenetic changes in spite of maintaining a normal karyotype, and ultimately showed spontaneous transformation (Burns *et al*., 2005; Serakinci *et al*., 2004). These studies supported the existence of neoplastic transformation of hMSCs during *in vitro* expansion. However, the spontaneous transformation potential of hMSCs is still a controversial issue and more evidence is needed. In addition to *in vitro* expansion studies, there are studies suggesting a role for hMSC in the carcinogenesis progression. Recent evidence thus suggests that exogenously administrated hMSCs can be recruited to the stroma of developing tumors when systemically infused in animal models for glioma, colon carcinoma, ovarian carcinoma, Kaposi's sarcoma and melanoma (Lazennec & Jorgensen, 2008).

Christensen *et al* have investigated and shown that the telomerase expression in long-term cultured telomerase-immortalized hMSCs (hMSC-telo1 cells) did not give rise to a tumor formation (Christensen *et al*., 2008). Telomerase activation most often occurs at the transition to cancer which is highly specific to cancer cells. Neoplastic transformation occurred when the cells were subjected to 2.5 Gy of gamma irradiation and subsequently cultured long-term. We, therefore, could conclude that transducing hMSC with hTERT did not unmask the neoplastic potential on its own but subsequent DNA damage such as irradiation-induced damage contributes to mesenchymal tumor development (Fig 3). These findings are in agreement with observations made by a number of other groups. Thus, it appears that using telomerase to help production of large numbers of cells is effective but it has impact on neoplastic transformation. Therefore, more information about adult stem cells and how their growth is regulated is required. Thus, close monitoring is crucial for clinical applications.

4. Two-faces of mesenchymal stem cells in cancer

It has been shown that exogenously administered hMSC may contribute to tumor stroma formation in animal tumor models by promoting angiogenesis or by creating a niche to support cancer stem cell survival (Sullivan & Hall, 2009). These tumor models include melanomas, colon adenocarcinomas, lung cancer, multiple myelomas and glioblastomas (Momin *et al*., 2010). The reason for this is thought to be the immunosuppressive ability of MSCs. As the MSCs interact with many of the cells of the innate and acquired immune system and suppress them, the tumor cells may get the chance to better evade the immune surveillance (Lazennec & Jorgensen, 2008; Momin *et al*., 2010).

Recently, Correa *et al.* has induced the development of a gastric cancer by *Helicobacter felis* infection in a C57BL/6 mouse model where normal bone marrow was replaced with bone marrow cells tagged with either beta galactosidase or GFP. With this study, they could demonstrate that in this model cancer cells are directly derived from bone marrow stem cells. During the helicobacter inflammation, these cells have been recruited to the site, where the gastric cancer later occurred (Correa & Houghton, 2007). Similar results have recently been achieved in a rat model of Barrett's metaplasia (Sarosi et al., 2008). In addition to these examples, there is substantial literature in rodent models, demonstrating the ability of MSCs' homing and transdifferentiation to inflammatory lesions at extra-medullar sites (Hayashi et al., 2008; Kahler et al., 2007; Serikov et al., 2007; Suzuki et al., 2008).

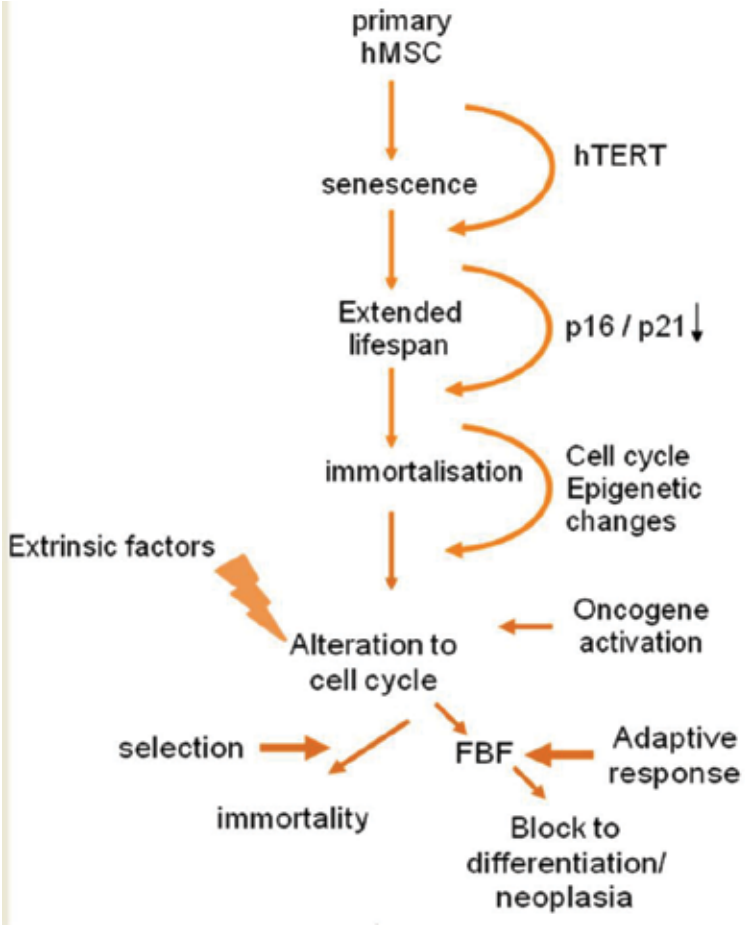

Fig. 3. Illustration of the sequence of events during the neoplastic transformation of hMSCs

Another face of the cancer and MSC interplay is that, the stromal cells of the tumor might exert transforming effects on the mesenchymal stem cells. It is thought that after a prolonged exposure to the tumor-conditioned media the MSCs can assume a carcinoma associated fibroblast (CAF)-like phenotype. CAF are known to support the growth of cells and angiogenesis in breast cancer. These observations have lead us to consider that upon

exposure to the tumor environment, MSCs could transform into CAFs and may help in tumor growth and even spread (Momin *et al*., 2010). However, the current animal models are not enough to help us understand this complex relationship that exists between MSCs and tumors. The animal models used now rely on harvesting the human tumor cells and culturing them *in vitro* before grafting them into the animal. The problem with this approach is that during the culturing process the cells are away from the tumor stroma and the epithelial cells. And even when the cells are inside the animals the stroma will be formed from the animal's own cells leading to a chimeric tumor that would not reproduce the interaction between the MSCs and the tumor stroma. Perhaps the new generation of immunocompromised mice such as the NOG mouse will be able to help in the investigation of this relationship as it is better able to accept heterologous cell populations (Hahn & Weinberg, 2002; Ito *et al*., 2002; Kim *et al*., 2004; Momin *et al*., 2010; Rangarajan & Weinberg, 2003; Rosen & Jordan, 2009).

Today, cancer still remains as one of the most challenging diseases with regards to treatment. One of the biggest problems is that there is really no selective killing towards tumor cells. Thus, therapies that are more specifically directed towards cancer stem cells might result in much more durable responses and even might also cure the metastatic tumors. While hMSCs play a role in supporting tumor formation, based on their homing abilities hMSC have been used as cellular vehicles for local delivery of biological agents to brain tumors. Human MSCs were transduced with a lentivirus expressing secretable TRAIL (S-TRAIL) and mCherry (red fluorescent protein) and injected into established intracranial glioma tumors in mice. The genetically modified hMSCs were able to inhibit tumor growth, resulting in significantly longer animal survival. Thus, the study demonstrated the therapeutic efficacy of hMSC S-TRAIL cells and confirmed that hMSCs can serve as a powerful cell-based delivery vehicle for the site-specific release of therapeutic proteins (Menon *et al*., 2009). In earlier experiments looking at targeted delivery, MSCs genetically engineered to secrete IFN-β were able to successfully home and engraft at melanomas growing in mice lungs and locally deliver IFN-β. When delivered locally, IFN-β was able to inhibit the growth of malignant cells both *in vivo* and *in vitro*. The same effect could not be achieved with systemically delivered IFN-β or IFN-β produced away from the tumor site (Studeny *et al*., 2002; Studeny *et al*., 2004). In a recent experiment, MSCs were shown to be recruited to and incorporated into the prostate epithelium during prostate regrowth (after testosterone reintroduction). The incorporated MSCs were used to deliver frizzled related protein-2 (SFRP2) to antagonize the Wnt-mediated cancer progression by reducing tumor growth, increasing apoptosis and causing potential tumor necrosis (Placencio *et al*., 2010).

hMSC having the potential to home to the tumor stroma allows them to be a promising tool for the delivery of anticancer drugs to the tumor microenvironment. This strategy has been shown to work by the observation of specific homing of intravenously administered hMSCs, engineered to produce interferon-β (IFN-β), to tumors with subsequent tumor regression in a xenogenic mouse model (Studeny *et al*., 2004). This study showed that mesenchymal stem cells expressing IFN-β could inhibit the growth of tumor cells *in vivo*. The approach required integration of the hMSC at the tumor site, because non-tumor site integrated or systemic delivery of IFN-β did not have enough tumor regressing effect. These findings have recently been supported by Serakinci *et al.* where they have shown that hMSC can home to tumor site and furthermore could deliver a therapeutic agent to the site (Serakinci *et al*, in press).

Besides the abovementioned studies, Seo *et al*. have demonstrated that IL-12M-expressing MSC injection directly into the tumor had the strongest antitumor effect compared with other injection routes such as intravenous or subcutaneous. In addition to the inhibition of solid tumor growth, the same study also demonstrated anti-metastatic effects for MSCs/IL-12M when embedded in the Matrigel (Seo *et al*., 2011). Furthermore, Correa *et al*. have demonstrated that gastric cancers may originate from hMSCs (Correa & Houghton, 2007). Based on these and similar studies, we can suggest that hMSCs can modulate the tumor growth, although this issue still remains controversial and not fully understood.

It seems that to incorporate cancer-fighting genes inside stem cells grown from a patient's bone marrow mesenchymal stem cells are promising therapeutic and newly emerging approaches. Success of such approaches is dependent on well designed vectors or viruses carrying therapeutic agents. Then those vectors or viruses can be inserted into stem cells e.g. mesenchymal stem cells, which are grown from a sample of a patient's bone marrow. Such stem cells would not be rejected as foreign objects by the immune system when they are injected back into the patient. Once inside the patient's bloodstream, the stem cells would migrate to tumor site and release the anti-carcinogenic agent. The basic approach would be to harvest MSCs, modify them such that they secrete an anti-neoplastic compound and as the last step, administer them into an animal that has a tumor. To date, various stem cells have been modified to secrete several different compounds that would either reduce the tumor size or prolong the survival of the organism. These compounds had varying degrees of success (Aboody *et al*., 2008; Momin *et al*., 2010).

Gene and viral therapies for cancer have shown some therapeutic effects, but there has been a lack of real breakthrough. Based on others and our studies it seems that the identification of reservoirs of multipotential stem cells within adult tissue provides exciting prospects for developing novel vehicle for stem cell-mediated gene therapy. This new strategy seems to produce stronger and more specific anti-tumor effects. But considering that one of the hallmarks of cancer development is continued cell growth, which is most often correlated with activation of telomerase, the question must be raised if there is a potential cancer risk of genetically engineered cells. Critical to such approaches will be an ability to remove or inactivate the genetically engineered stem cells that homed to tumor site at the time of delivery of the targeted treatment.

One approach might be to use telomerase inhibitors that may be a way to stop proliferation of these particular cells. Since the telomerase is essential to the life of a cell, treatments with modified structures called telomerase inhibitors will eventually lead to cell death. But this approach might face another problem namely that some of these cells will escape the cell death and this will lead to genomic instability, ultimately causing development of a new cancer. Another and perhaps safer approach might be that after delivery of the targeted treatment, the vehicle cells should be engineered to commit suicide. Such a strategy has been used in connection with the tumor-selective viruses that mediate oncolytic effects on tumors due to genetically modified viruses, which is engineered to replicate in and kill targeted cancer cells. Such viruses have been engineered with tumor-specific transcriptional response elements based on the telomerase promoter sequence thereby attacking telomerase-positive cells (Abdul-Ghani *et al*., 2000; Bilsland *et al*., 2005; Komata *et al*., 2001b; Plumb *et al*., 2001). These studies have suggested that combinational therapy approaches (genetically engineered vehicle stem cell therapy and the suicide gene therapy) might improve targeted therapies and at the same time reduce the risk of secondary tumors.

Numerous clinical trials have been published involving MSCs. In many of these studies the MSCs were evaluated only in a non-cancerous context like myocardial infarctions or the role of MSCs in graft versus host disease (Momin *et al*., 2010). No acute or long-term effects have been reported so far including any reports of carcinogenesis. The longest follow up reported in these studies is 3 years. However, more information and studies are needed regarding these studies as carcinogenesis might be a long process. Another point to consider is that most of these studies were carried out on patients with poor prognosis which might be masking any adverse effects from MSCs. Furthermore, there has been no appropriate imaging methods used in clinical trials to look for carcinogenesis in the body. The last point we will need to keep in mind regarding the use of MSCs in therapy is that so far not many genetically-modified MSCs have been used in clinical trials published to date (Momin *et al*., 2010). Carcinogenesis issues remain a hot topic with regards to MSCs and will always need to be carefully evaluated. The risk from the illness, the risk of not getting a treatment will have to be weighed against the risk of transformation of MSCs, which may lead to carcinogenesis.

5. Future directions

Considering the neoplastic transformation risk of genetically engineered hMSC and their role in tumor formation different systems should be devised to address this risk during the conduction of therapeutic approaches. It is critical to place a fail-safe system that will remove or inactivate the genetically engineered stem cells once they have homed to the tumor site and delivered the therapeutic agent. To overcome the expansion related risks Momin *et al*. have suggested using MSCs from different sources to reach to a larger number of cells with fewer number of passages. Another approach might be to use telomerase inhibitors in an effort to stop the proliferation of the genetically modified MSCs once they were done with delivery. Since the telomerase is essential to the life of these telomerased cells, treatment with telomerase inhibitors will eventually lead to cell death. However, this approach might raise yet another problem; namely, some of these cells might escape cell death which will lead to genomic instability and ultimately cause the development of a new cancer. Alternatively, another and perhaps safer approach might be to cause the vehicle cells to commit suicide after delivery of the targeted treatment. This strategy has been used in connection with tumor-selective genetically modified viruses that mediate oncolytic effects on tumors by replicating in and killing cancer cells in a targeted manner. These viruses contain transcriptional response elements based on the telomerase promoter sequence thereby attacking telomerase-positive cells, which includes the genetically modified MSCs in this case (Abdul-Ghani *et al*., 2000; Bilsland *et al*., 2005; Komata *et al*., 2001a; Plumb *et al*., 2001).

Taken together, studies on the impact of neoplastic transformation of the hMSC and the studies on impact of using telomerase to help production of large numbers of cells, one could conclude that caution is warranted for stem cell-based therapies. However, this does not mean that these studies need to be put off. Thus, considering their susceptibility to neoplastic transformation and potential to home to tumor site one of the key questions that still needs to be addressed is; how safe is to use hMSC for targeted cancer therapy? And the answer to this question is that there is to date good indications that human bone marrow MSC, which will be isolated from individuals and *in vitro* expanded, can be used for cancer therapy especially as delivery vehicles targeting tumor stroma after a careful safety

monitoring and preclinical tests. Still, for successful clinical applications more information about human mesenchymal stem cells and their growth regulation is still required.

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Part 3

Glioma Stem Cells

12

Brain Tumor Stem Cells and Anti-Angiogenic Therapy

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1. Introduction

The hypothesis that tumor growth may be sustained by a rare subpopulation of cells, termed cancer stem cells, is currently demonstrated in different types of cancer (Al-Hajj *et al.*, 2004; Jordan *et al.*, 2006; Reya *et al.*, 2001). Brain tumor stem cells were isolated from primary brain tumors, such as malignant glioma (Galli *et al.*, 2004; Hide *et al.*, 2008). Glioma stem cells share some characteristics with normal neural stem cells, including the expression of neural stem cell markers, such as CD133 and Nestin (Hadjipanayis and Van Meir, 2009; Singh *et al.*, 2004). Brain tumor stem cells possess the capacity for self-renewal and multipotency (ability to differentiate into neurons, astrocytes, and oligodendrocytes) and the proliferative ability for generation of many progeny. Furthermore, they are able to initiate new tumors in vivo when transplanted into immunocompromised mice even at low cell numbers (Galli *et al.*, 2004).

Glioma stem cells play an important role in tumor invasion and therapy resistance (Bao *et al.*, 2006a; Calabrese *et al.*, 2007; Dean *et al.*, 2005; Hirschmann-Jax *et al.*, 2004). Complete surgical resection is almost impossible because of the deep invasion to the normal brain parenchyma. Glioma stem cells have the ability to divide slowly and infinitely, which leads to the resistance to chemotherapy and radiotherapy. These stem cell-like properties allow glioma stem cells to survive selectively and initiate recurrence (Hide *et al.*, 2008). Therapeutic strategy that target glioma stem cells may improve the prognosis of malignant glioma. Recent evidence has revealed that glioma stem cells are located in the highly vascular region, and glioma stem cells' properties are tightly regulated by the microenvironment, so called vascular niche, similar to normal neural stem cells (Gilbertson and Rich, 2007; Yang and Wechsler-Reya, 2007).

The development of new therapeutic strategies that target the glioma stem cells and vascular niche may result in more effective treatment of malignant glioma. In this article, we review the recent evidence on the biology of glioma stem cells associated with vascular endothelial growth factor (VEGF)- vascular endothelial growth factor receptor (VEGFR) signalling pathways for anti-angiogenic therapy.

2. Brain stem cells and vascular niche

Recent studies reveal a close relationship between the stem cells and the vascular niche. In the adult brain, neural stem cells were demonstrated to be concentrated around blood

vessels where they had access to signalling molecules, nutrition, and nascent vasculature for migration (Shen *et al.*, 2004). Similarly, glioma stem cells were shown to be located around vascular niches (Calabrese *et al.*, 2007). Vascular niche supplies oxygen and nutrition, and at the same time regulates the glioma stem cells' characteristics, such as self-renewal and differentiation. In addition, glioma stem cells not only receive the signals from the surrounding niche but also modulate the signals through the secretion of VEGF (Bao *et al.*, 2006b; Folkins *et al.*, 2009; Oka *et al.*, 2007). Glioma stem cells and vascular niche represent integral factors for invasion and expansion. Therefore, comprehension of the interactive structural units may lead to development of therapeutic innovations.

2.1 Neural stem cells

Reynolds and Weiss were the first to isolate neural stem cells from the adult striatum that could proliferate and generate multipotent cells *in vitro,* termed neurospheres (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992). Neurosphere culture relies on a serum-free, selective growth factor (epidermal growth factor and fibroblast growth factor 2) system. Neural stem cells are responsive to the growth factor, and they can be passaged and expanded indefinitely with little change in characteristics. Removal of growth factors induces the differentiation of the progeny of neural stem cells into neurons, astrocytes, and oligodendrocytes. Neural stem cells have been isolated also from the subventricular zone lining of the lateral ventricles, dentate gyrus within the hippocampus, and subcortical white matter (Ayuso-Sacido *et al.*, 2008; Eriksson *et al.*, 1998; Nunes *et al.*, 2003; Sanai *et al.*, 2004).
The subventricular zone is the largest of the germinal regions in humans. It is located between the ependymal layer of the lateral ventricles and parenchyma of the striatum. This zone is also thought to be the likely source of glioma stem cells (Sanai *et al.*, 2005).

2.2 Glioma stem cells

Glioma stem cells are thought to originate from transformed neural stem cells and progenitor cell populations (Hadjipanayis and Van Meir, 2009). Clonogenic, neurosphere-forming precursor cells were isolated from glioblastoma specimens by applying the same conditions used for the isolation of human neural stem cells. However, only 0.01%-1.0% of cells even in this selected population from malignant glioma can reinitiate tumors in immunodeficient mice (Hide *et al.*, 2008). There are two major techniques for enrichment of glioma stem cells.
One technique is cell sorting using a specific cell-surface antigen or a combination of the cell surface antigens. Purification of CD133-positive cells from human gliomas by flow cytometry can allow the isolation of glioma stem cells. CD133 was first reported as a marker for hematopoietic stem/progenitor cells (Yin *et al.*, 1997). This cell-surface antigen was later reported as a marker for neural stem/progenitor cells (Uchida *et al.*, 2000). Singh et al. demonstrated that as few as 100 CD133-positive glioma cells could initiate tumors in vivo when transplanted into immunocompromised mice; whereas, the injection of 5×10^4 to 1×10^5 CD133-negative cells is not capable of tumor initiation (Singh *et al.*, 2004). Recently, several reports have suggested that there is no difference in the ability of CD133-positive and CD133-negative cells to form orthotopic tumors (Zheng *et al.*, 2007). CD133-negative cells isolated from glioblastoma were reported to form orthotopic tumors similar to CD133-positive subpopulation (Chen *et al.*, 2010; Wang *et al.*, 2008). This may reflect the presence of other types of glioma stem cells. Further researches are needed to identify more specific cell-surface antigen.

The other technique is side population technique using the ability to efflux Hoechst 33342 dye (Hirschmann-Jax *et al.*, 2004). The side population technique is a method to identify cancer stem cells in various cancers, including the brain (Hide *et al.*, 2008). Cancer stem cells are thought to maintain their drug efflux ability, which makes it possible to separate cancer stem cells in unstained cell fractions. These cells appear on the lower left side of dot graphs analyzed by a cell sorter; hence, they are called side population cells.
The current lack of a single marker to identify all cancer stem cells in malignant glioma may suggest the molecular heterogeneity among these cells.

2.3 Glioma vascular niche

Excessive and grossly disorganized blood vessel formation is a hallmark of glioblastoma. This aberrant vascularity has been presumed to be important for satisfying the demand for nutrition of the rapid tumor growth. Normal neural stem cells within the subventricular zone and hippocampus are concentrated in regions of the brain that are rich in blood vessels, called the vascular niche (Palmer *et al.*, 2000). This organization places the stem cells in a close relationship with endothelial and other vascular cells, which facilitate communication among these cell types. The vascular niche protects neural stem cells from apoptotic stimuli to maintain a good balance between self-renewal and differentiation. Similarly, glioma stem cells were intimately associated with the vascular niche in the tumor. CD133-positive/nestin-positive glioma stem cells were frequently discovered close to capillaries within glioblastoma (Calabrese *et al.*, 2007). Endothelial cells have been shown to be one of the most important components in the vascular niche. Endothelial cells secrete paracrine factors that promote normal stem cell survival and self-renewal (Shen *et al.*, 2004). In the same manner, CD133-positive glioma stem cells when transplanted with endothelial cells grew more rapidly than when transplanted alone (Calabrese *et al.*, 2007). In addition, tumors established in the presence of endothelial cells contained up to 25 times more CD133-positive cancer stem cells. Thus, endothelial cells are demonstrated to develop the self-renewal capacity of CD133-positive glioma stem cells.
Recent evidence has suggested that a functional relationship between the glioma stem cells and vascular niche may be bidirectional; such that, the glioma stem cells may maintain the vascular niche just as the vascular niche helps in the proliferation and self-renewal of glioma stem cells. Bao et al. showed that high-level production of VEGF by CD133-positive glioma stem cells could develop their tumor-initiating capacity (Bao *et al.*, 2006b). The authors demonstrated that freshly resected CD133-positive glioma cells increased endothelial migration and tube formation by producing VEGF, leading to vascular-rich and hemorrhagic tumors in the brains of immunocompromised mice, but not for the CD133-negative glioma cells.

3. Angiogenesis and VEGF family

Angiogenesis is a tightly regulated process in which the development of new blood vessels arises from a pre-existing vascular network. It is regulated by endogenous activators and inhibitors during development and by normal physiological processes, such as wound healing. However, angiogenesis is also involved in tumor growth, progression, and metastasis (Hoeben *et al.*, 2004). As a key step in tumor development, the angiogenic switch occurs when endogenous activators of angiogenesis exceed endogenous inhibitors (Hanahan and Folkman, 1996). This phenomenon results in increasing blood vessel

formation and supplying tumors with oxygen and nutrition for growth. However, tumor vasculature is disorganized and poorly structured; but is nonetheless essential for continuous tumor growth. Folkman et al. firstly proposed that tumor growth was restricted in size because there was limitation in the diffusion of oxygen without the blood supply, and so tumor angiogenesis could be a potential therapeutic target (Folkman, 1971; Folkman, 1972; Folkman *et al.*, 1971). Of the angiogenic factors, VEGF has been demonstrated to play a crucial role in angiogenesis and progression of malignant glioma (Plate *et al.*, 1992).

3.1 New blood vessel formation in glioma

Formation of new blood vessels is classified into three models: angiogenesis, vasculogenesis, and arteriogenesis (Tate and Aghi, 2009). Angiogenesis is the formation of new blood vessels by rerouting or remodeling pre-existing vessels, and it is thought to be the primary method of vessel formation in gliomas. Vasculogenesis is classically considered an embryonic process, but recently it has been identified in adults, too. Vasculogenesis in tumors has been demonstrated to be the formation of blood vessels from circulating marrow-derived endothelial progenitor cells that are recruited to the tumor. Finally, arteriogenesis is a process in which arteriolar networks hypertrophy in order to sustain increased oxygen demands. A current model of tumor vessel formation suggests that this process involves recruitment of sprouting vessels from existing blood vessels and incorporation of endothelial progenitors into the growing vascular bed. Thus, only angiogenesis and vasculogenesis were thought to play important roles in tumor biology. The necrotic and hypoxic nature of glioblastoma is thought to cause angiogenesis and vasculogenesis through the induction of angiogenic factors including VEGF (Onishi *et al.*, 2011).

3.2.1 VEGF family

The vascular endothelial growth factor (VEGF) family consists of five members: VEGF-A (thereafter called VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Kowanetz and Ferrara, 2006). In addition, multiple isoforms of VEGF, VEGF-B, and PIGF are generated through alternative splicing of pre-mRNA (Sullivan and Brekken, 2010). VEGF family ligands show different affinities for the three VEGF tyrosine kinase receptors: VEGF receptor (VEGFR)-1, VEGFR-2, and VEGFR-3. Moreover, several co-receptors, such as neuropilins (Nrp)-1 and Nrp-2, also regulate VEGF family interaction with VEGFRs.

3.2.2 VEGF(-A)

The *VEGF* gene contains eight exons and seven introns. VEGF binds to VEGFR-1, VEGFR-2, Nrp-1, and Nrp-2. VEGF induces vascular permeability and also functions as an endothelial cell mitogen and survival factor, and an inducer of endothelial cell and monocyte migration (Kowanetz and Ferrara, 2006). Knock out studies in mice showed that homozygous or heterozygous deletion of the *VEGF* gene was embryonically lethal, resulting in defects in vasculogenesis and cardiovascular abnormalities. These studies have demonstrated that VEGF is essential for development. VEGF is important to postnatal angiogenic processes such as wound healing, ovulation, and pregnancy. VEGF is also involved in tumor angiogenesis, arthritis, macular degeneration, and diabetic retinopathy. VEGF is considered to be a strong angiogenic effector under most physiological and pathological conditions (Hicklin and Ellis, 2005; Sullivan and Brekken, 2010).

3.2.3 VEGF-B

VEGF-B binds to both VEGFR-1 and Nrp1. The function of VEGF-B is still controversial. *VEGF-B* null mice were viable and largely healthy, except for some abnormalities in cardiac conduction (Sullivan and Brekken, 2010). VEGF-B plays some roles in heart function in adults, but not in developmental angiogenesis or cardiovascular development (Sullivan and Brekken, 2010). Therefore, VEGF-B has been thought to have a negligible role as an angiogenic factor even under pathological conditions. However, recent studies revealed that VEGF-B was a potent survival factor for blood vessels, and the inhibition of VEGF-B lead to pathological angiogenesis by abolishing blood vessel survival in animal models (Hicklin and Ellis, 2005).

3.2.4 VEGF-C

VEGF-C binds to VEGFR-2 and VEGFR-3. It is involved in developmental lymphangiogenesis and in the maintenance of adult lymphatic vasculature (Sullivan and Brekken, 2010). *VEGF-C* null mice were embryonic lethal and heterozygous *VEGF-C* loss was characterized by lymphedema because of defective development of the lymphatic vasculature. VEGF-C is not necessary for blood vessel development because vessels appear normal in *VEGF-C* null animals (Sullivan and Brekken, 2010). Although VEGF-C is not expressed in a normal brain, recent reports that show the high expression of VEGF-C in malignant glioma suggest the ligands' role in glioma angiogenesis (Grau *et al.*, 2007; Jenny *et al.*, 2006; Witmer *et al.*, 2001).

3.2.5 VEGF-D

VEGF-D binds to both VEGFR-2 and VEGFR-3. VEGF-D is also involved in developmental lymphangiogenesis and adult lymphatic vasculature (Sullivan and Brekken, 2010). *VEGF-D* null mice were viable and had a normal lymphatic vasculature during development and in adults, which suggests that VEGF-C and other growth factors may substitute for VEGF-D function. VEGF-C and VEGF-D bind to VEGFR-2 and they might also play a role in angiogenesis as well, especially during pathological states such as tumor growth. However, the role of these ligands in tumor angiogenesis is unclear. Similar to VEGF-C, VEGF-D is shown to be expressed highly in malignant glioma but not in the normal brain, which suggests the ligands' contribution to glioma angiogenesis (Grau *et al.*, 2007; Witmer *et al.*, 2001).

3.2.6 VEGF-E

VEGF-E is not a mammalian VEGF homolog, but rather a viral protein encoded by the parapoxvirus Orf virus (Shibuya, 2003). VEGF-E binds to only VEGFR-2. It is also involved in angiogenesis like VEGF-A but its role still remains unclear.

3.2.7 Placental growth factor

Placental growth factor (PlGF) binds to only VEGFR-1 and is also involved in angiogenesis (Hicklin and Ellis, 2005). PlGF is also suggested to play the role in recruitment of monocyte and vascular progenitor cells from bone marrow to tumors. This ligand is primarily expressed in the placenta, and also in other organs such as the heart, retina, and muscle (Sullivan and Brekken, 2010). Although *PlGF* null mice were viable and displayed no defect in embryonic angiogenesis or developmental abnormalities, the loss of PlGF impaired angiogenesis, plasma extravasation, and collateral growth during ischemic conditions,

inflammation, wound healing, and tumor growth (Hicklin and Ellis, 2005). Lastly, PlGF may play important roles on pathologic states in adult.

3.2.8 VEGF family expressions in glioma cells

The over-expression of VEGF in glioma was demonstrated (Plate *et al.*, 1992), and recent studies also revealed the over-expression of VEGF in glioma stem cells by evaluating expression levels of VEGF in conditioned media from matched CD133+ and CD133- glioma cultures (Bao *et al.*, 2006b). High expression of VEGF-B mRNA was shown in low and high grade gliomas (Gollmer *et al.*, 2000). Grau SJ et al. analyzed expressions of VEGF-C, VEGF-D, and VEGFR-3 in glioblastomas, and showed strong protein expression of VEGFR3 on tumor endothelium, while VEGF-C and VEGF-D were expressed on numerous cells in areas of high vascularization (Grau *et al.*, 2007). Although VEGF-C, VEGF-D, and VEGFR-3 are not expressed in normal brain tissue, expressions of VEGFR3, VEGF-C and VEGF-D were found on the protein and RNA levels. Nomura et al. investigated the relationship between PlGF and primary brain tumor angiogenesis. PlGF mRNA was expressed in all the hypervascular primary brain tumors (Nomura *et al.*, 1998). In addition, they conducted hypoxic experiments with cultured U-251MG human glioma cells to determine the mechanism of PlGF gene regulation. As the atmospheric oxygen concentration was decreased, the PlGF mRNA level in the U-251MG cells was markedly increased. These results suggested that PlGF may contribute to the pathogenesis of brain tumor angiogenesis.
Until now, it has been demonstrated that only VEGF is expressed highly in glioma stem cells.

3.3.1 VEGF receptors

There are three receptor tyrosine kinases that mediate the angiogenic functions of VEGF family members (Kowanetz and Ferrara, 2006). They are structurally very similar. The VEGF receptors contain a seven immunoglobulin-like domain extracellular region, a single transmembrane domain segment, a juxtamembrane segment, a split intracellular protein-tyrosine kinase domain, and a carboxyterminal tail. Unlike other VEGFR genes, alternative splicing of *VEGFR-1* produces a soluble form of the receptor (sVEGFR-1) that contains only extracellular domain (Sullivan and Brekken, 2010). This receptor does not possess the activity of tyrosine kinase in intracellular signaling, and inhibits the function of VEGF. VEGFR-1 and VEGFR-2 were originally identified on endothelial cells, and VEGFR-3 was identified on lymphatic endothelial cells (Kowanetz and Ferrara, 2006).

3.3.2 VEGFR-1

VEGFR-1 is a receptor for VEGF, VEGF-B, and PlGF. It binds VEGF with at least 10-fold higher affinity than VEGFR-2, but the kinase activity is weaker than that of VEGFR-2 (Sullivan and Brekken, 2010). Although *VEGFR-1* null mice were embryonic lethal, the mice that did not express the tyrosine kinase domain of VEGFR-1 but retained the ligand-binding extracellular domains and transmembrane segment (*VEGFR1-TK-/-*) were viable. Thus, VEGFR-1 was initially considered to be a negative regulator of VEGF activity by acting as a decoy receptor for VEGF. VEGFR-1 was also expressed by monocytes, macrophages, and other bone marrow-derived progenitor cells (myeloid cells) with cell-surface marker of CD11b (Kaplan *et al.*, 2005). VEGFR-1 was thought to mediate the migration of bone marrow-derived cells into cancerous tissues and recruitment of endothelial progenitor cells

as another function resulted in tumor growth and angiogenesis (Sullivan and Brekken, 2010). *VEGFR-1* null mice were embryonic lethal because of excessive hemangioblast proliferation and poor organization of vascular structures, which seemed to be due to the inhibition of the function as a negative regulator for VEGF signaling and the inhibition of angiogenesis via recruitment of endothelial progenitor cells. In addition, VEGFR-1 was also shown to be expressed by the subsets of liquid and solid tumor stem cells, which resulted in tumor cell survival and growth (Kowanetz and Ferrara, 2006). Although VEGFR-1 must be critical for physiologic and developmental angiogenesis, the precise function of VEGFR-1 remains unclear. Recent studies have shown that during pathologic conditions such as tumorigenesis, VEGFR-1 is a potent, positive regulator of angiogenesis (Hicklin and Ellis, 2005). Hence, current evidence has suggested that the function of VEGFR-1 differs with each stage of development, various states of physiologic and pathologic conditions, and cell types in which it is expressed.

3.3.3 VEGFR-2

VEGFR-2 is the major mediator of VEGF-induced angiogenic signalling. The functions of VEGFR-2 include endothelial cell survival, migration, proliferation, and vascular permeability. This receptor has the most important role in vessel formation during both physiologic and pathologic conditions (Hicklin and Ellis, 2005). Recent studies have shown that VEGFR-2 is also expressed in subsets of liquid and solid tumor cells in addition to endothelial cells, which imply the additional role of VEGF in cancer through stimulation of VEGFRs on tumors cells (Hicklin and Ellis, 2005; Kowanetz and Ferrara, 2006). VEGFR-2 is a receptor for VEGF, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGFR-2 has a lower affinity for VEGF than VEGFR-1, but it has a stronger kinase activity. *VEGFR-2* null mice were embryonic lethal (Sullivan and Brekken, 2010). These animals had severe defects in endothelial and hematopoietic cell development with no organized blood vessel found at any point within the developing embryo or the yolk sac.

3.3.4 VEGFR-3

VEGFR-3 binds both VEGF-C and VEGF-D and is a key regulator of normal and tumor lymphangiogenesis (Hicklin and Ellis, 2005; Kowanetz and Ferrara, 2006; Sullivan and Brekken, 2010). During development and in adulthood, its expression is limited to lymphatic endothelial cells. VEGFR-3 is also expressed in the embryonic vasculature. *VEGFR-3* null mice were embryonic lethal and displayed cardiovascular failure as a result of the abnormal structure and organization of large vessels. VEGFR-3 is not expressed in the brain, but recent studies have shown that VEGFR-3 is expressed highly with VEGF-C and VEGF-D in malignant glioma endothelium, which may suggest that VEGFR-3 and these ligands contribute to glioma angiogenesis (Grau *et al*., 2007; Jenny *et al*., 2006).

3.3.5 VEGFRs expressions in glioma cells

The expression of VEGFRs on liquid and solid tumor cells has been reported already (Dias *et al*., 2001; Dias *et al*., 2000; Ferrer *et al*., 1999). VEGF may also have another role in cancer through the stimulation of VEGFRs on tumor cells. Although the significance of this expression is still under investigation, it has been hypothesized that VEGF ligands promote tumor growth not only in a paracrine manner, but also in an autocrine manner. Rafii et al. showed that functional VEGF/VEGFR-2 autocrine loop was present in subsets of human

leukemias and supported *in vivo* leukemic cell survival and migration (Dias *et al.*, 2000). Fan F et al. demonstrated that VEGFR-1 activation by VEGF or VEGF-B led to activation of the MAPK pathway in tumor cells and phenotypic changes including an increase in cell migration and invasion (Fan *et al.*, 2005).
Knizetova et al. demonstrated that the expression of VEGFR-1 and VEGFR-2 genes in glioma cells was shown by PCR (Knizetova *et al.*, 2008). They reported autocrine regulation of glioblastoma cell proliferation. Lucio-Eterovic et al. showed the mRNA and protein levels of VEGFRs in glioma stem cell line, suggesting that autocrine VEGF signalling blockade played an important role in glioma invasion (Lucio-Eterovic *et al.*, 2009).
The expression of VEGFR-3 in glioma cells, including glioma stem cells, is still unreported.

3.4 VEGFR signaling molecular pathways

Receptor tyrosine kinases (RTKs) are transmembrane proteins that mediate the transmission of extracellular signals to the intracellular environment. RTKs are activated through the binding of a growth factor ligand to the extracellular domain, leading to receptor dimerization and subsequent autophosphorylation of the receptor complex by the intracellular kinase domain, using ATP. The phosphorylated receptor then interacts with a variety of cytoplasmic signaling molecules, leading to signal transduction.
In VEGFR-2 intracellular signaling pathways, main signalling cascades include the phospholipase Cγ(PLCγ)-protein kinase C-Raf kinase-mitogen-activated protein kinase kinase (MEK)-MAPK pathway and Phosphatidylinositol-3-Kinase(PI-3K)/AKT pathway. The PLC-γ pathway regulates cell proliferation and cell migration. The PI-3K pathway regulates cell migration and cell survival via anti-apoptosis effect. The PI-3K pathway also regulates vascular permeability via heat shock protein 90 (Hsp90) and endothelial nitric oxide synthase (eNOS), but the signal is still not well understood (Kowanetz and Ferrara, 2006). Unlike other RTKs, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), the Ras pathway is not involved so strongly in the VEGFR-2 signalling pathway.
In VEGFR-1 and VEGFR-3 intracellular signaling pathways, a limited number of signalling effectors have been shown to act downstream of these receptors. Further investigation for these receptors in cancer cells is required.

4. VEGF/VEGFR-related molecular biology of malignant glioma

4.1 Glioma invasion and angiogenesis

Marked proliferation, angiogenesis, and invasion are hallmarks of malignant gliomas. Magnetic resonance imaging (MRI) showed two characteristic regions of malignant glioma; the central region with gadolinium-enhanced mass and the marginal region with high-intensity signals on T2-weighted images. Histopathological analysis of malignant glioma invasion showed that the clusters of glioma cells and necrotic tissue were seen in the central region with gadolinium contrast enhancement. In the marginal region with high-intensity signals on T2-weighted images, diffuse infiltrating glioma cells were seen around and inside the normal brain parenchyma. Vascular proliferation was seen in both regions. (Onishi *et al.*, 2011) Glioma cells, including glioma stem cells, are beside newly developed vessels and had a close interaction with vascular niche especially in the paracrine manner. High-level expression of VEGF family and VEGFRs has been shown in glioma vasculature and glioma

cells, including glioma stem cells (Hicklin and Ellis, 2005). Glioma stem cells expressed higher levels of VEGF than the matched non-stem glioma cells and displayed greater angiogenic potential *in vitro and in vivo (Bao et al., 2006b).* Furthermore, recent studies have demonstrated that the relationship between glioma stem cells and vasculature is complex and bi-directional. Therefore, anti-angiogenic therapy has the potential to function as an anti-glioma stem cell therapy (Folkins *et al.*, 2009; Oka *et al.*, 2007). Calabrese et al. have demonstrated that the treatment of mice with VEGF inhibitor after glioblastoma implantation resulted in a large reduction in the number of glioma stem cells and blood vessels of the tumors. In contrast, VEGF inhibitor had minimal effect on the proliferation or survival of most cells in the tumors, suggesting that the glioma stem cells were targeted (Calabrese *et al.*, 2007).

Necrotic tissue in the central region of malignant glioma was surrounded by pseudopalisading cells. Recently, Rong et al. demonstrated that pseudopalisading cells were present in severely hypoxic regions, over-expressed hypoxia-inducible factor, and secreted VEGF (Rong *et al.*, 2006). Pseudopalisading cells were cell populations composed of a series of actively migrating glioma cells, moving away from a central hypoxic region. Hypoxia activated the VEGF promoter and transcription in glioma cells, which could have led to the cell activation of a migrating phenotype toward the viable vessels (Brat *et al.*, 2004). Both *in vitro* and *in vivo* models have demonstrated that tumor hypoxia results in increased glioma cell migration (Elstner *et al.*, 2007).

The autocrine function of VEGF in cancer invasion was first shown in invasive breast cancer cell lines (Bachelder *et al.*, 2002; Price *et al.*, 2001). In malignant glioma, tumor hypoxia may also increase tumor invasion in a VEGF/VEGFR autocrine manner independent of angiogenesis. Autocrine stimulation of VEGFRs on glioma cells including glioma stem cells, has been shown to be important for cell survival, proliferation and invasion (Knizetova *et al.*, 2008). In addition, hypoxia can promote the expansion of glioma stem fraction and regulate the expression of stem cell markers (Heddleston *et al.*, 2009; McCord *et al.*, 2009; Soeda *et al.*, 2009). Hypoxic condition induced VEGF expression in both glioma stem cells and non-stem glioma cells, but the VEGF levels were consistently higher in glioma stem cells (Li *et al.*, 2009). Under normoxic condition, glioma stem cells also expressed a higher level of VEGF than non-glioma stem cells.

In addition, other histopathological findings as glioma invasion models were reported; such that glioma cell infiltrations into normal brain parenchyma independent of vasculature (Onishi *et al.*, 2011). Cancer stem cells have been shown to promote metastasis (Hermann *et al.*, 2007; Li *et al.*, 2007). Although malignant gliomas rarely metastasize beyond the central nervous system, MRI sometimes shows glioma cells infiltration to contralateral hemisphere. This infiltrative feature away from tumor vascular niche may be equivalent to metastasis. Recent evidences in lung cancer have demonstrated that immature myeloid cells can be recruited to the metastatic sites and immature myeloid cells prepare to make the pre-metastatic state for cancer cells (Kaplan *et al.*, 2005). Immature myeloid cells express VEGFR-1, and a neutralization antibody for VEGFR-1 has been shown to significantly suppress the metastasis in lung cancer (Hiratsuka *et al.*, 2002). This evidence may suggest that bone marrow-derived cells, such as immature myeloid cells, are other components of the niche, and these cells are associated with glioma cells invasion via VEGF signalling pathways. Folkins et al. investigated that bone marrow-derived cells recruitments in mice with glioma stem cell-rich xenograft tumor (Folkins *et al.*, 2009). Glioma stem cells were shown to

increase the mobilization of endothelial progenitors, but not myeloid cells. Further researches are required to show the possibility that the myeloid cells may be associated with glioma cells invasion.
In summary, the VEGF paracrine signals between glioma cells and vascular niche enhance glioma cells invasion in the vascular-rich regions. In addition, the VEGF autocrine signals on glioma cells also enhance self-invasion in the hypoxic regions. As another component of the niche, myeloid cells may be involved in glioma cells invasion via VEGF signalling pathways. In particular, glioma stem cells have stronger signals of VEGF under various situations.

4.2 Therapeutic resistance of glioma stem cells

As well as regulating stem cell proliferation and survival, niche may also play a protective role of shielding stem cells from environmental insults (Moore and Lemischka, 2006). It was demonstrated that vascular niche could protect glioma stem cells from chemotherapy and radiotherapy (Huang *et al.*, 2010). In fact, it was shown that the postoperative first-line chemotherapy for malignant glioma, temozolomide, was not so effective for glioma stem cells (Liu *et al.*, 2006).
Anti-angiogenic therapy is thought to have the potential to improve such therapy resistance. VEGF was one of the most characteristic permeability factors and it was demonstrated to contribute to BBB breakdown in gliomas directly (Tate and Aghi, 2009). Increased permeability of tumor blood vessels induced by VEGF resulted in elevated interstitial pressure and significant intracerebral edema. The elevated interstitial pressure decreases the transport of medication to tumor cells. In addition, chemotherapy and radiotherapy are less effective in the hypoxic area. Jain et al. proposed that the normalization of tumor vasculature by anti-angiogenic therapy could decrease brain edema, enhance drug delivery and increase radiation sensitivity (Jain, 2005). Vredenburgh et al. has reported that bevacizumab (VEGF inihibitor) in the combination with irinotecan (cytotoxic drug) was an effective treatment for recurrent malignant glioma (Vredenburgh *et al.*, 2007). The normalization of tumor vasculature resulted in decresed interstitial pressure, less hypoxia, and increased delivery of irinotecan to tumor. The auther concluded that the efficacy seen with the combination of bevacizumab and irinotecan could be explained by the anti-tumoral stem cell effect of bevacizumab and by the anti-differentiated glioma tumor cell effect of irinotecan.
Radiation therapy was demonstrated to induce VEGF expression in glioma cells (Hovinga *et al.*, 2005). It was also demonstrated that VEGF blocked the killing of endothelial cell by radiation (Gorski *et al.*, 1999). The radiation-enhanced VEGF secretion was associated with an increased angiogenic potential of the tumor, which thought to be a factor in radioresistance. Anti-VEGF therapy has the potential to prevent VEGF secretion after radiation and enhance radiation sensitivity. Recent studies have shown that glioma stem cells were more resistant to radiation than the matched non-stem glioma cells (Bao *et al.*, 2006a; Rich, 2007). In response to radiation-induced DNA damage, glioma stem cells preferentially activated several critical DNA damage checkpoint proteins. As a result of the preferential DNA damage checkpoint activation, glioma stem cells were more efficient in repairing the damaged DNA and more rapidly recover from the DNA damage than the matched non-stem tumor cells (Bao *et al.*, 2006a). Thus, anti-angiogenic therapy enhances glioma cells' and glioma stem cells' sensitivity for cytotoxic chemotherapy and radiotherapy.

5. VEGF/VEGFR-related therapeutic target of malignant glioma

An upregulation of VEGF family and the VEGF receptors has been shown in malignant gliomas and that can be a target for cancer therapy. In the anti-angiogenic therapy, drugs targeting anti-VEGF/VEGFR pathways have recently attaracted considerable attention.

5.1 VEGF inhibitor

Bevacizumab is a humanized monoclonal antibody that binds to VEGF-A, preventing it from binding to receptors and activating signaling cascades that lead to angiogenesis. The proof of the concept that targeting VEGF-A could inhibit the growth of tumors was demonstrated in a mouse model in 1993 (Kim *et al.*, 1993). The first clinical trial was performed for the treatment of colorectal cancer. The first line chemotherapy (irinotecan, fluorouracil (5-FU), and leucovorin) with bevacizumab for colorectal cancer significantly increased the progression-free survival (PFS), as well as the median overall survival (OS), leading to FDA approval of bevacizumab as the first drug developed for anti-angiogenesis and anti-cancer use in humans (Hurwitz *et al.*, 2004).

Vredenburgh et al. performed a phase II trial to evaluate the efficacy of bevacizumab in combination with chemotherapy for malignant gliomas (Vredenburgh *et al.*, 2007). Bevacizumab and irinotecan were administrated to 32 patients with recurrent high-grade glioma. The radiographic response was observed in 14 of 23 patients (61%). The median PFS for treated patients was 24 weeks, the 6-month PFS was 30%, and the overall median survival time was 42 weeks. This study suggested that bevacizumab in combination with irinotecan is an effective treatment for recurrent glioblastoma. Recently, Albert Lai et al. reported an open-label, prospective, multicenter single-arm phase II study that combined bevacizumab with radiation therapy (RT) and temozolomide (TMZ) for the treatment of newly diagnosed glioblastoma (Lai *et al.*, 2011). In this study, 70 patients with newly diagnosed glioblastoma were enrolled between 2006 and 2008. Patients received standard RT starting within 3 to 6 weeks after surgery with concurrent administration of daily TMZ and biweekly bevacizumab. After completion of RT, patients resumed TMZ for 5 days every 4 weeks and continued biweekly bevacizumab. OS and PFS were 19.6 and 13.6 months, respectively. Patients treated with bevacizumab and TMZ during and after RT showed an improvement of PFS but not OS compared to the control group.

5.2 VEGF-Trap

Aflibercept (VEGF-Trap, AVE0005) is a soluble fusion protein of the human extracellular domains of VEGFR-1 and VEGFR-2 and the Fc portion of human immunoglobulin (Ig) G. Aflibercept binds to both VEGF-A and PlGF with higher affinity than monoclonal antibodies and prevents theVEGF-A and PlGF ligands from binding and activating cell receptors. In vitro, aflibercept was shown to have an antiproliferative activity and completely blocked the VEGF-induced VEGFR-2 phosphorylation (Sullivan and Brekken, 2010). In xenograft models, tumor growth and tumor-associated angiogenesis were inhibited by aflibercept (Holash et al., 2002). Candelaria Gomez-Manzano et al. reported that treatment of animals bearing human gliomas with VEGF Trap resulted in a significant increase in the mean survival (Gomez-Manzano et al., 2008).

5.3 VEGFR inhibitor

In addition to VEGF inhibitors, small molecule inhibitors of VEGFR have been tested in recurrent malignant gliomas. They often target more than one type of receptor and affect

both endothelial cells and cancer cells because the receptors are expressed on both types of cells. Because the target kinase specificity between inhibitors can vary, different compounds have shown various levels of efficacy and activity between cancers (Sullivan and Brekken, 2010; Kowanetz and Ferrara, 2006). Some of these agents include compounds that bind to the ATP binding site of the RTK, which block receptor activation, or with antibodies that bind to the growth factors or their receptors, which prevent the binding and subsequent activation of receptors.

Cediranib (AZD2171) inhibits all known subtypes of VEGFR and has been evaluated in a phase 2 trial of patients with recurrent glioblastoma (Batchelor *et al.*, 2010; Batchelor *et al.*, 2007). Results were comparable to those reported for bevacizumab, with a response rate of 56% and a 6-month PFS rate of 26%. A striking steroid-sparing effect was observed. The drug was largely well tolerated, with hypertension, diarrhea, and fatigue as the most common adverse effects. Using dynamic contrast-enhanced MRI, it was demonstrated that cediranib therapy reduced blood vessel size and permeability. These are the first clinical data to support the hypothesis that antiangiogenic therapy may transiently "normalize" the dilated, abnormally permeable tumor vasculature. Other VEGFR inhibitors in which clinical studies were conducted for malignant gliomas are listed in Table 1.

Inhibitor	Primary targets (other targets)	Mechanism of action
Bevacizumab	VEGF-A	Monoclonal antibody
Aflibercept	VEGF-A (VEGF-B, PlGF)	Soluble decoy receptor
Cediranib	VEGFR-2 (other VEGFR, PDGFR-β, c-kit)	Tyrosine kinase inhibitor
Sorafenib	VEGFR-2 (other VEGFR, Raf, PDGFR-β, c-kit, Ras, RET)	Tyrosine kinase inhibitor
Sunitinib	VEGFR-2 (other VEGFR, PDGFR-β, Flt3, c-kit)	Tyrosine kinase inhibitor
Pazopanib	VEGFR-2 (other VEGFR, PDGFR-α,β,c-kit)	Tyrosine kinase inhibitor
Vandetanib	VEGFR-2 (other VEGFR, EGFR)	Tyrosine kinase inhibitor
Vatalanib	VEGFR-1,2 (PDGFR-β, c-kit)	Tyrosine kinase inhibitor
Brivanib	FGFR (VEGFR)	Tyrosine kinase inhibitor
CT-322	VEGFR-2 (other VEGFR)	Adnectin
XL-184	VEGFR-2 (c-Met, RET, c-Kit, Flt3, Tie-2)	Tyrosine kinase inhibitor

Table 1. VEGF/VEGFR related anti-angiogenic drugs in clinical development for high-grade glioma

6. Summary and future outlook

The existence of glioma stem cells prompts us to review the cancer biology. Gliomas appear to have a cellular hierarchy rather than a bulk of equally potent tumor cells. Within this hierarchy, glioma stem cells have an extraordinary capacity for tumor-initiation, and glioma stem cells are thought to be attractive targets for anti-glioma therapies. The development of novel treatments against glioma stem cells is expected as an urgent task. Glioma stem cells'

properties are maintained with vascular niche, and vascular niche is maintained with VEGF secreted by glioma stem cells. Glioma stem cells and vascular niche make the bi-directional functional units. Of anti-angiogenic factors, VEGF family/VEGFRs are one of the most important therapeutic targets for these units.

As the use of anti-angiogenic drugs such as bevacizumab becomes widespread, some problems surfaced. Firstly, the potential side effects that occur in the short-term or long-term use of angiogenesis inhibitors are becoming apparent (Norden *et al.*, 2008). These side effects include gastrointestinal perforations, impaired wound healing, bleeding, hypertension, proteinuria, and thrombosis. These occurrences are unpredictable and further studies are needed to measure the risk for patients, understand the cause of complications, and find prophylactic measures to minimize risk. Secondly, it has been found that most tumors become to be resistant to anti-angiogenic drugs as a consequence of the long-term administration of anti-angiogenic drugs (Norden *et al.*, 2008). Because multiple signaling pathways are involved in angiogenesis, blocking a single pathway may not be highly effective. The administration of single anti-angiogenic agent can lead tumors to acquire the resistance when the tumor cells develop other angiogenesis pathways. The combination drug therapies targeting multiple pathways may be able to overcome this problem. Therefore recently, clinical trials of combination drug therapies are performed such as other anti-angiogenic drugs, cytotoxic drugs or anti-invasion drugs (Van Meir *et al.*, 2010). Thirdly, the further studies are needed to evaluate the administration protocol of anti-angiogenic agents. For example, metronic dosing, which is administration of small doses of drugs in a rapid cycle, has the potential to improve in outcomes over standard dosing (Kesari *et al.*, 2007). Kesari S et al. performed the phase 2 study of metronomic four drugs chemotherapy (etoposide, cyclophosphamide, thalidomide and celecoxib) for reccurent malignant gliomas (Kesari *et al.*, 2008). Although this study did not show a significant improvement of OS in heavily pretreated patients who were generally not eligible for conventional protocols, there were some responders. It was suggested that further studies using metronomic chemotherapy combined with more potent anti-angiogenic agents in patients with less advanced disease may be warranted.

Brain tumor stem cells have a bi-directional relationship with vascular niche. Anti-angiogenic therapy is the strategy targeting for vascular niche, which may result in the strategy targetting for brain tumor stem cells. Accumulated evidence suggests that VEGF family/VEGFRs are strongly related with the biology of brain tumor stem cells. Thus VEGF family/VEGFRs signaling pathway is expected to be one of the most important targets of anti-angiogenic therapies for malignant gliomas.

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Targeting Glioma Stem Cells: Path Leading to the Cure

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1. Introduction

In the United States, 22020 new cases of cancer in central nervous system (CNS) are expected to occur in 2010 (CBTRUS 2010). Although the incidence of cancers in CNS is much lower than that of malignancies in other organs such as lung, breast and colorectal cancers, CNS cancers are the second lethal cancer for males younger than 40 years (Jemal et al. 2010). In addition, with the unconstraint growth, brain cancers can often involve eloquent area. As a result, the neurological and psychological deficits may severely damage the health-related quality of life (QOL) in patients with brain cancers. Improvement of QOL and the prognosis of brain cancers is the goal of both physicians and basic investigators.

Glioblastoma multiforme (GBM) is the most frequent primary brain cancer, accounting for 17% of all primary tumours in CNS. In the past five decades, despite the advances in the fields of neurosurgery, radiotherapy and pharmaceutics, the prognosis of patients with GBM remains dismal, with a 5-year survival of only 9.8%(Stupp et al. 2009). The nature of extensive proliferation, diffuse infiltration and resistance to conventional treatments makes the chance to cure GBM slim. Exploration of mechanisms underlying therapeutic resistance of GBM and developing novel strategies against GBM are of urgent necessity.

The emergence of brain tumour stem cell (BTSC) theory is a great breakthrough in the field of neuro-oncology. BTSC theory assumes that brain tumour is a hierarchy of cancer cells maintained by a small population of cells sharing characteristics of normal embryonic and somatic stem cells. BTSC theory is confirmed by the isolation of BTSCs from established brain tumour cell lines and freshly surgical samples. Accumulated evidence suggests that BTSCs are responsible for the initiation, progression, recurrence and treatment resistance. Therefore, BTSCs are promising therapeutic targets. In this chapter, we aim to summarize advances in BTSC biology with the focus on the treatment strategies against BTSCs.

2. Identification of BTSCs

The hypothesis that tumours arise from cancer stem cells (CSCs) is not new. The histological similarity between cancers and embryonic tissues was observed by Rudolph Virchow more than one century ago (Huntly and Gilliland 2005). The heterogeneity of cancer and only a small cancer population capable of forming clones *in vitro* and reconstituting tumour *in vivo* have also been wildly recognized for decades. However, because of the limitation of

knowledge and technology in molecular biology, CSCs have not been confirmed in leukemia and solid tumours including brain tumours until late 1990's (Clevers 2011).

2.1 Isolation and characterization of BTSCs

In 2002, based on the finding that the expression of markers specific for undifferentiated neural cells was detected in malignant glial tumours, Ignatova and coworkers assumed the existence of cells sharing features of normal neural stem cells (NSCs) in gliomas (Ignatova et al. 2002). By using *in vitro* serum-free NSC culture system supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF), the authors successfully isolated NSC-like cells from human glioma tissues. These cells are capable of forming clones morphologically resembling those generated by NSCs. And populations from the tumour-derived clones demonstrate positive for NSC marker nestin, differentiated neuronal marker β-III tubulin or glial lineage-specific marker glial fibrillary acidic protein (GFAP). Singh and coworkers confirmed the existence of BTSCs and did further feature analysis (Singh et al. 2004). Their investigation demonstrated that CD133-positive tumour cells in gliomas possess obvious stem cell characterization of extensive proliferation, self-renewal and lineage-restricted differentiation that recapitulates the original tumour phenotype. In addition, the CD133-positive tumour cells show a strong ability to form tumours in xenograft models. One hundred CD133-positive glioma cells efficiently initiate tumours in immunocompromised mice while 100,000 CD133-negative glioma cells fail to establish tumours. The BTSC model appears to be more robust in preclinical research than traditional established cell lines kept in serum-containing medium, because the former has been shown to mirror both the phenotype and genotype of their parental tumours (Lee et al. 2006). With transcriptome analysis, Fine's group found that GBM cells maintained in serum-free NSC media have gene expression profiles similar to NSCs and harbour all the genetic aberrations detected in primary tumours. Genetic stability maintains even after repeated passage. Moreover, these NSC-like glioma cells demonstrate a high tumourigenic potential and establish tumours with extensive infiltration into normal brain *in vivo*, which is frequently observed in human GBMs. By contrast, GBM cells under standard *in vitro* conditions with serum-containing media, undergo dramatic *de novo* genomic rearrangement over time, with a divergence from genotype of parental GBMs but displaying resemblance to genomic features with commonly used glioma cell lines. A majority of these cells lose the ability to initiate tumours in murine models. Even for a small subset of glioma cells that retain tumourigenic potential, the xenografts they generate phenotypically differ from their parental human GBMs. Collectively, BTSCs are defined as a small population in brain tumours, with the ability to maintain the BTSC pool by self-renewal and with the ability to extensively proliferate into differentiated non-tumourigenic brain tumour cell phenotypes that reconstitute the cellular heterogeneity of the parental brain tumours.

2.2 Identification of BTSCs

2.2.1 CD133

Human CD133, also known as prominin-1, is a pentaspan transmembrane glycoprotein localized in membrane protrusions, which contains 865 amino acids with a nonglycosylated molecular weight of 97 kDa. The predicted structure of CD133 composes an 85-amino acid N-terminal extracellular domain, five transmembrane domains with two large extracellular loops containing eight potential N-linked glycosylation sites, and a 50-amino acid cytoplasmic tail. It was first identified as a hematopoietic stem cell antigen (Miraglia et al. 1997).

Although CD133 mRNA is found to be strongly expressed in multiple organs including adult kidney, trachea and digestive tract, glycosylation immunoreactivity of CD133 appears to be restricted to undifferentiated cell types and reduce during differentiation (Florek et al. 2005; Corbeil et al. 2000). Subsequently, CD133 has been demonstrated to be successfully used as a marker to define and purify stem and progenitor cell populations in various organs such as fetal liver, kidney, prostate and brain (Rountree et al. 2007; Angelotti et al. 2010 ; Vander Griend et al. 2008 ; Barraud et al. 2007).

Because the resemblance of cancer stem cells to normal stem cells, CD133 is also widely employed as a marker to identify and isolate cancer stem cells in multiple solid tumours such as prostate cancer, colon cancer and melanoma (Collins et al. 2005, O'Brien et al. 2007; Monzani et al. 2007). In addition, the clinical relevance of CD133 expression with the prognosis of brain tumour patients and the association of CD133 immunoreactivity with BTSCs are well documented. The proportion of CD133-postive cells in WHO grade 2 and 3 gliomas is found to be an independent risk factor for tumour recurrence and time to malignant progression (Zeppernick et al. 2008). Consistently, the presence of CD133 has been demonstrated to be correlated with a decreased survival in patients with high-grade oligodendroglial tumours (Beier et al. 2008). Preclinical studies demonstrated that the CD133-positive BTSCs are capable of extensive self-renewal and recapitulation of original tumours. Moreover, ectopic overexpression of CD133 in rat C6 glioma cells significantly enhances the chemoresistance of tumour cells to camptothecin and doxorubicin. Tumour cells with increased expression of CD133 appear to be more reluctant to undergo apoptosis after the treatment of cytotoxic agents, which is demonstrated as a result of elevated efflux of drugs by up-regulating ATP-binding cassette transporters ABCB1 (Angelastro and Lame 2010). The studies aforementioned suggest CD133 as a *bona fide* marker for BTSCs. However, the emergence of conflicting evidences indicates that CD133 is not necessarily required for BTSC phenotype. The existence of CD133-negative BTSCs is confirmed as well. It has been shown that a hierarchy of self-renewing BTSC types exists in GBMs. Both CD133-positive and CD133-negative glioma cells in individual GBMs exhibit self-renewal *in vitro* and initiate highly aggressive tumours *in vivo*. Notably, CD133-negative glioma cells can even give rise to CD133-postive cells (Chen et al. 2010). Moreover, CD133 expression has been proposed as an indicator of bioenergetic stress rather than to be obligatorily related with BTSC phenotype in human gliomas. Reduced oxygen levels have been shown to increase the CD133 expression in gliomas (Bar et al. 2010). Mitochondrial dysfunction can also up-regulate CD133 expression that is inversely correlated with changes in mitochondrial membrane potential. Genetic depletion of mitochondrial DNA results in a remarkable increase of CD133 expression, which can be reversed by re-introducing parental mitochondria (Griguer et al. 2008).

To reconcile the discrepancies in the CD133 studies, several hypotheses are proposed. First, the limitations of current antibodies against epitopes of CD133 can lead to inconsistent results. AC133 and AC141 mAb, reported to bind the extracellular glycosylation epitope of CD133, are the most commonly used antibodies to identify and purify BTSCs. However, little is known about the molecular nature of the epitopes recognized by these two antibodies. Although AC133 and AC141 mAbs are sometimes used interchangeably, discordant results about the expression of the AC133 and AC141 epitopes were observed in other cancer stem cell types (Green et al. 1999). Additionally, the presence of alternatively spliced CD133 isoforms is another complicating factor. Transcription of CD133 can be started at five tissue-restricted promoters, producing several alternatively spliced transcripts,

among which there are possible spliced isoforms without AC133 and AC141 epitopes. In this case, some BTSCs are actually CD133 false negative. Secondly, BTSC phenotypes that are independent of CD133 status may exist. Several lines of evidence support this hypothesis. With genetic approach, BTSC population depleted of CD133 was generated. BTSCs lacking CD133 expression can proliferate as tumour-spheres *in vitro* and produce gliomas when transplanted into mouse brain, indicating the dispensable role of CD133 in BTSC maintenance and gliomagenesis (Nishide et al. 2009). In addition, transcriptional profiles demonstrated a distinct gene expression in CD133-positive BTSCs compared to CD133-negative BTSCs. CD133-positive BTSCs, growing as neurospheres, display "proneural" transcriptional profiles with a resemblance to fetal neural stem cells, while CD133-negative BTSCs with a semiadherent/adherent growth pattern show "mesenchymal" signature genes similar to adult neural stem cells. The molecular heterogeneity of CD133-positive and –negative BTSCs implied the different origins of these two BTSC populations (Lottaz et al. 2010).

2.2.2 Side population technique

Side population (SP) cells are referred to a small population of cells which are capable of excluding vital dye Hoechst 33342 and featured in flow cytometry plots as the "side" of the bulk of positively labeled cells. The exclusion of Hoechst 33342 by SP cells has been suggested as a result of the cells' ability to pump out drugs mediated by ABCG2, an ATP-binding cassette (ABC) transporter (Scharenberg, Harkey, and Torok-Storb 2002). SP cells were first identified in mouse bone marrow, with the enrichment of stem cell properties. The observations that SP cells share stem cell features have been extended in other normal and tumour tissues. In glioma, SP cells demonstrate characteristics of pluripotency *in vitro* and high tumourigenicity *in vivo*. Kondo and coworkers successfully isolated SP cells from the rat glioma cell line C6, which only account for 0.4% of the cells maintained in serum-containing medium. C6 SP cells can form neurospheres in culture free of serum and produce SP as well as non-SP cells *in vitro*. Moreover, C6 SP cells are capable to initiate tumours containing multiple lineages such as neurons and glia when transplanted into various tissues in nude mice (Kondo, Setoguchi, and Taga 2004). In addition, SP cells have also been associated with the resistance to anti-cancer treatment. When exposed to temozolomide (TMZ) at DNA-damaging dose, SP cells purified from astrocytoma cell lines demonstrate a profound resistance to the cell arresting effects of the drug and maintain a robust proliferative capacity, compared with non-SP cells. The percentage of SP cells increases as high as 8-fold (Chua et al. 2008). Consistently in a platelet-derived growth factor (PDGF)-induced glioma mouse model, SP cells are shown to be less sensitive to TMZ relative to non-SP cells. Moreover, the *in vivo* aggressiveness of SP cells increases greatly after treatment with TMZ. Mechanistically, an increased level of O6-methylguanine-DNA methyltransferase (MGMT) in SP cells contributes to the chemoresistance to TMZ (Bleau et al. 2009).

Although SP technique is effective in enriching BTSCs, there is disagreement regarding the connection between SP and BTSC phenotypes. As mentioned above, only a small fraction of C6 glioma cells (0.4%), namely, SP cells, fulfill the criteria of BTSCs. However in a study using clonal and population analyses, Zheng and coworkers revealed that most single C6 cells have similar clone formation ability *in vitro* and tumourigenic potential *in vivo*, suggesting that most of C6 glioma cells are BTSCs. Furthermore, Hoechst 33342 is shown cytotoxic and can impair the clonogenicity and proliferation of individual C6 cells. The authors therefore postulated that Hoechst 33342 labeling and sorting can deprive non-SP cells of their stemness (Zheng et al. 2007). In addition, Srivastava and coworkers

demonstrated that both the SP and non-SP fraction in medulloblastoma can produce neurospheres and regenerate both fractions. The toxicity of Hoechst 33342 to medulloblastoma cells, especially non-SP cells, is also confirmed in this study and suggested as the reason for the biological differences between SP and non-SP fractions (Srivastava and Nalbantoglu 2008).

Collectively, CD133 and SP technique are successful in the identification and purification of BTSCs. However, these two approaches are unlikely to be enough to define all BTSC phenotypes because of their intrinsic limitations and the complexity of BTSCs. As a result, cautions should be exerted in interpreting results generated with these two methods. The development of novel approaches to enrich BTSCs is also critical.

2.3 Maintenance and expansion of BTSCs *in vitro*

The establishment of models in which BTSCs can be properly propagated is critical for the studies of BTSCs. To date, neurosphere culture is the most commonly used protocol to expand BTSCs. According to this protocol which was first developed by Reynolds and Weiss for neural stem cell studies, BTSCs are cultured in serum-free medium supplemented with growth factors, for example, EGF and FGF. Under this condition, BTSCs grow as floating aggregates with extensive self-renewal (Reynolds and Weiss 1992; Galli et al. 2004). When transferred to medium with serum, these cells differentiate and express neuron and glia markers. Notably, BTSCs maintained as neurospheres are highly tumourigenic and initiate tumours with phenotypical and genetical resemblance to their origins. Neurosphere culture successfully maintains the BTSC phenotype and is therefore widely used to explore key issues in BTSC biology. However, several studies call into question neurosphere culture as a reliable method to expand and analyze BTSCs. First, neurosphere culture protocol has a low efficiency to establish BTSC lines (Laks et al. 2009). Secondly, the majority of cells in neurospheres are more differentiated and/or dying progeny (Martens, Tropepe, and van Der Kooy 2000). Thirdly, the aggregating growth manner of BTSCs and the fuse between neurospheres make the evaluation of the efficacy of treatment difficult (Woolard and Fine 2009). In order to overcome the limitations of neurosphere culture, novel methodological approaches have been investigated, among which the adherent culture developed by Pollard and coworkers is of great promise (Pollard et al. 2009). With this adherent culture, tumour cells from surgical glioma tissues are maintained and passaged in laminin-coated flask with neural stem cell medium. BTSC phenotypes are retained under this condition, harbouring genetic aberrations consistent with parental tumours. The efficiency of successful establishment of BTSC lines is 100% for adherent culture but is only around 30% for neurosphere culture. In addition, the adherent population composes more true BTSCs compared with neurosphere culture, because of less differentiation and apoptosis. Furthermore, the adherent monolayer growth pattern of BTSCs is more suitable for chemical screens. Therefore, adherent culture appears to be superior in the maintenance of BTSCs.

2.4 Animal models for BTSCs

Although the *in vitro* culture protocol offers a convenient and less expensive method to maintain BTSCs, it has apparent limitations. Most of all, the *in vitro* culture is unable to establish a specific and interactive microenvironment as BTSCs have in brain tumour tissues (Sanai, Alvarez-Buylla, and Berger 2005). As a result, BTSCs may undergo genotypic and/or

phenotypic changes so as to adapt to the *in vitro* environment, which potentially weakens the clinical relevance of BTSCs, especially when to test the efficacy of novel chemotherapeutic drugs and small molecule inhibitors.

In order to overcome the drawbacks of the *in vitro* culture systems, different categories of *in vivo* animal models were developed to maintain BTSCs and were employed to dissect the molecular events in BTSCs. Transgenic murine model is one of them. In this model, mouse lines genetically engineered with specific genetic alterations frequently observed in human brain tumours were created. Notably, with the advances in genetic techniques, the gain and loss of one or multiple genes can be conditionally manipulated in a tissue- and/or time-specific manner. For instance, tet-regulated or CRE-inducible alleles of genes can allow for the control of the timing, duration, and tissue compartment of gene expression or inactivation (Glaser, Anastassiadis, and Stewart 2005). The spontaneous brain tumours generated from these transgenic murine models are appropriate resources of BTSCs. In a *S100β-verbB;Trp53* transgenic murine model that develops spontaneous gliomas, the existence of BTSCs was confirmed, which were enriched in the side-population cells and characterized with self-renewal, multipotentiality and enhanced tumour-initiating capacity (Harris et al. 2008). Similarly, Ward and coworkers harvested BTSCs from medulloblastomas arising from genetically engineered *Patched-1*-deficient mice. These BTSCs displayed a neural precursor phenotype and were capable to generate medulloblastomas following allogeneic orthotopic transplantation (Ward et al. 2009).

Besides, transgenic murine models provide valuable information on fundamental and mechanistic facets of BTSC biology. Until now, amounting evidences supports the role of BTSCs in the tumourigenesis of brain tumours. But questions are coming with the BTSC theory. Among them, where BTSCs originate is the most intriguing one. Three hypotheses on the origin of BTSCs are proposed: (1) mature astrocytes dedifferentiate to have stem cell-like properties, (2) committed progenitors acquire mutations that endow them with unstrained "stemness", (3) neural stem cells become tumourigenic as a result of the chaotic regulation in mitosis and differentiation. Physiologically relevant transgenic murine models that spontaneously generate gliomas provide an ideal and powerful approach to tackle this question. Studies with somatic cell mouse models demonstrated that the loss function of pivotal tumour suppressor genes render early cortical astrocytes susceptible to oncogenic transformation and dedifferentiate to initiate gliomas, suggesting the possibility of the reprogrammed astrocytes as the precursors of BTSCs (Uhrbom et al. 2005, ; Xiao et al. 2005). Committed progenitors have also been shown to be able to derive BTSCs. Overexpression of oncogenic $HRas^{L61}$ in *p53*-deficient oligodendrocyte precursor cells (OPCs) can result in the generation of BTSCs in mice (Hide et al. 2011). By using RAS/tv-a mouse model, Lindberg and coworkers specifically transferred PDGF-B into OPCs and successfully induced gliomas *in vivo*. The introduction of PDGF-B resulted in the expression of SOX2, OLIG2 and NG2 in tumour cells, implying a slight dedifferentiation of the targeted OPCs (Lindberg et al. 2009).

Because of the similarities that BTSCs share with neural stem cells, it is logical to assume that BTSCs may derive from neural stem cells. Clinical and genetic studies provide clues that some GBMs may arise from the subventricular zone where neural stem cells originate. Wang and coworkers employed a transgenic murine model with an in-frame *p53* deletion mutation specially targeted into the nervous system to investigate the role of neural stem cells in *p53*-mediated gliomagenesis (Wang et al. 2009). This study revealed that *p53* deficiency provides no significant growth advantage to adult brain cells, but can induce accumulation of cooperative oncogenic alterations in neural stem cells in subventricular

zone and subsequently result in glioma formation in experimental animals, which links BTSCs with neural stem cells. In addition, neural stem cells derived from mice with concomitant specific deletion of *p53* and *Pten* in the central nervous system have an enhanced self-renewal and impaired differentiation and initiated acute-onset infiltrative high-grade gliomas (Zheng et al. 2008). The oncogenic transformation of neural stem cells is demonstrated to be driven by the up-regulation of Myc protein.

As mentioned above, transgenic murine models shed light on the initiation of BTSCs and play an important role in the studies to analyze the functionality of specific genes and cooperative gene networks in BTSCs. But there are ongoing concerns about transgenic murine models: (1) targeting selected genes in transgenic these models are not likely to fully replicate the clinical and biological heterogeneities of brain tumours, (2) the mouse-derived brain tumours may biologically divert from human counterparts because of species difference.

Xenograft model is another option to investigate BTSCs. In this model, human glioma tissue or cells that enrich BTSCs are implanted heterotypically (in nonautochthonous site) or orthotopically (in the original site) in immunodeficient animals. Although heterotypical subcutaneous xenograft model is widely used to investigate brain tumours, subcutaneous implantation of GBM cells has been shown to generate tumours with less clinical relevance because organ-specific environment plays a vital role in glioma behaviour (Antunes et al. 2000). Therefore, orthotopic intracranial xenograft models appear to be superior in the preclinical studies of brain tumours and BTSCs. Orthotopic xenograft models of multiple brain tumour types such as GBM, medulloblastoma and ependymoma have been successfully established. The tumours generated from these models bear histopathological resemblance to their origins and BTSCs can be isolated from the orthotopic models (Fei et al. 2010 ; Shu et al. 2008,; Yu et al. 2010). Because of the advantage to faithfully recapitulate the biological phenotypes of original patient tumours and stably preserve BTSC pool, the orthotopic model is an optional approach to facilitate biological studies of BTSCs. In addition, novel therapeutic strategies such as telomerase angtagonist, oncolytic picornavirus and Akt inhibitors have been pre-clinically tested in orthotopic xenograft models and yield promising outcomes by preferentially targeting BTSCs (Marian et al. 2010; Yu et al. 2011; Eyler et al. 2008).

3. BTSC, the prime culprit for treatment resistance

The identification of BTSCs provides insight into the therapeutic resistance of brain tumours and lead to a reassessment of current treatment against brain tumours. Most of current therapies have the power of mass destruction against the non-BTSC population but fail to precisely strike BTSCs.

3.1 Radioresistance

Radiotherapy is a mainstay in the management of GBMs and eliminates glioma cells mainly through inducing a DNA double-strand break. Although effective, radiotherapy can seldom eradicate all the glioma cells and the recurrence of GBM after radiotherapy seems inevitable. A growing body of evidence supports that BTSCs play a pivotal role in the resistance of radiotherapy. With magnetic resonance (MR) imaging and L-[methyl-11C] methionine positron emission tomography (MET-PET) scanning, Taumura and coworkers demonstrated that most of malignant gliomas treated with Gamma Knife surgery (GKS) plus external

beam radiation (EBRT) recur even with initially well responding. Malignant glioma cells are found within the areas exposed to irradiation. In histological sections after GKS plus EBRT, CD133-positive tumour cells markedly accumulate while are infrequent in primary sections obtained before irradiation. These CD133-positive glioma cells are postulated as BTSCs and are capable of surviving high dose irradiation (Tamura et al. 2010).

The radioresistance of BTSCs may stem from an increase in DNA repair. After irradiation, CD133-positive BTSCs are enriched because of their capability to preferentially activate the DNA damage checkpoint and repair radiation-induced DNA damage more efficiently than those CD133-negative non-BTSCs. The radioresistance of CD133-positive BTSCs can be reversed with a specific inhibitor of Chk1 and Chk2 cell cycle checkpoint kinases (Bao et al. 2006). In addition, autophagy has been shown to contribute to the radioresistance of BTSCs. Autophagy is a process of self-cannibalization whereby cells maintain homeostasis and survive under stress via lysosomal degradation of cytoplasmic proteins and organelles. In a study carried out by Lomonaco and coworkers, the expression of autophagy-related proteins, such as LC3, ATG5 and ATG12, are found higher in CD133-positive BTSCs compared with CD133-negative cells. Gamma-radiation induces a larger degree of autophagy in CD133-positive BTSCs. Autophagy protects cancer cells from radiation damage by decreasing cytoplasmic acidification, by providing catabolites required for DNA repair and by removing toxic substances. Inhibition of autophagy can significantly decrease the viability of BTSCs in response to γ-radiation (Lomonaco et al. 2009). The mechanism underlying the induction of autophagy in BTSCs by γ-radiation is not elucidated. But recent studies suggested that the activation of ataxia-telangiectasia-mutated (ATM), a checkpoint protein, following radiation, can lead to an inhibition of mTOR pathway through the phosphorylation of LKB-1, which may initiate the process of autophagy. The role and the mechanism of autophagy in the radioresistance of BTSCs still need further investigation.

More recently, the microenvironment that BTSCs reside in has been found to contribute to the radioresistance of GBM (Jamal et al. 2010). When intracranially transplanted, BTSCs demonstrate a less susceptible to irradiation and have a faster repair of radiation-induced DNA damage reflected by the dispersal of γH2AX foci, compared with their counterpart grown *in vitro*. Microarray analysis revealed that genes involved in reactive oxygen species (ROS) scavenging and antioxidant response are significantly influenced in BTSCs in xenografts, which appears to subsequently render BTSCs to be more resistant to radiotherapy *in vivo*.

3.2 Chemoresistance

GBMs are notorious for their insensitivity to chemotherapeutic agents. Recently, BTSCs have been demonstrated to be involved in the chemoresistance of GBMs. *In vitro*, BTSCs isolated and cultured from surgical GBM specimens display marked resistance to cytotoxic drugs including TMZ, cisplatin, epotoside and vincristine. BTSCs can recover and proliferate quickly following treatment with antineoplastic agents (Eramo et al. 2006). Moreover, glioma cells isolated from multiple tumour areas have different sensitivity to TMZ. More committed glioma cells are found to distribute along the peripheral area and to undergo apoptosis in response to TMZ, while more immature cells with CD133 positivity localized in the inner core of GBMs and are more insensitive to TMZ (Pistollato et al. 2010). Gene expression analysis of GBM patients treated with concomitant chemotherapy with TMZ revealed that an expression gene set comprising CD133 is associated with treatment-resistance and predicts poor survival in this group of patients. Intriguingly, the gene set

includes a cluster that is reminiscent of a self-renewal gene signature identified in murine MLL-AF9-induced leukemic stem cells derived from committed progenitors, which suggests the relevance of a stem-like cell phenotype in the treatment resistance of GBMs (Murat et al. 2008). Pallini and coworkers conducted a prospective investigation to explore the prognostic potential of *in vitro* BTSC analysis and the presence of CD133-positive cells in 44 consecutive GBM patients treated with concurrent chemoradiation followed by TMZ. Fourteen GBMs which can generate BTSC *in vitro* have a less favourable prognosis, with a median overall survival of 8 months compared with 14 months among GBMs without generation of BTSCs. In addition, the presence of more than 2% CD133-positive cells in GBM lesions is associated with an early progression. The median progression-free survival is 10 months for GBM patients with less than 2% CD133-positive cells while it was only 5 months for those with more than 2% CD133-positive cells (Pallini et al. 2008). Similarly, the prognostic value of the presence of BTSCs and CD133 expression is also shown in high-grade oligodendroglial tumours treated with chemotherapy. Patients with tumours neither containing BTSCs nor showing CD133 expression have a favourable clinical outcome. In this study, the possibility of presence of CD133 vascular progenitor cells is excluded with immunohistochemical double staining of CD133 and the panvascular marker CD31. Weakly double-positive cells for CD133 and CD31 are only detected in one of 20 tumours investigated, which indicates that the CD133-positive cells are derived from tumours instead of vasculatures (Beier et al. 2008).

BTSCS have been found able to modulate expression of multidrug resistance related genes to reverse the cytotoxic effect of chemotherapeutic agents. Liu and coworkers demonstrated that CD133-posive BTSCs are significantly resistant to conventional chemotherapeutic agents including TMZ, carboplatin, paclitaxel and etoposide when compared to autologous CD133-negative GBM cells (Liu et al. 2006). Further real-time PCR analysis revealed that CD133-positive BTSCs have an enhanced gene expression of multi-drug resistance and DNA mismatch repair, as well as genes inhibiting apoptotic cascade. For instance, ATP-binding cassette sub-family G member 2 (ABCG2), a gene accounting for chemoresistance of multiple cancer types, is increased 6.5 times in BTSCs than in CD133-negative cells. Of note, the expression of MGMT, a pivotal DNA repair enzyme that confers resistance to alkylating agents, in CD133-positive cells, is found 32.4 times as high as that in non-BTSCs. In addition, anti-apoptotic genes, including FLIP, BCL-2 and BCL-XL, are also found markedly up-regulated while the expression of pro-apoptotic gene BAX is repressed in CD133-positive BTSCs. Hussein and coworkers established a panel of pediatric BTSC cell lines and analyzed their sensitivity to etoposide (Hussein et al. 2011). BTSCs are found to be enriched when they are maintained as neurospheres in serum-free medium supplemented with EGF and FGF compared to as monolayers in serum-containing FBS. The clonogenic survival analysis demonstrated that neurosphere-derived cells are significantly more resistant to etoposide. Two ATP-binding cassette multidrug transporters, ABCB1 and ABCC1, are enriched in CD133-positive BTSCs and are further increased by etoposide treatment.

3.3 Resistance of BTSCs to immunotherapy

Immunotherapy represents a promising strategy for the treatment of cancers in addition to conventional therapeutic approaches. The virtue of immunotherapy is that it mobilizes the patient's immune system to specifically recognize and eradicate cancer cells while spares normal cells at the same time. The researches of immunotherapy against gliomas were once hindered by the perception that the brain is an immune privileged organ, basing on several

evidences. For example, the existence of blood-brain barrier (BBB) separates brain parenchyma from systemic circulation, which was assumed to hamper the entrance of immune effector cells into the brain (Pachter, de Vries, and Fabry 2003). In addition, the paucity of major histocompatibility complex (MHC) expression on brain cells, and the lack of organized lymphoid tissue and lymphatic drainage also suggest the difficulty to initiate effective immune responses (Read et al. 2003; Walker, Calzascia, and Dietrich 2002). However, several studies threw doubt on the assumption of the complete immunological silence in CNS. The findings in CNS autoimmune diseases including multiple sclerosis indicate the capability of activated T lymphocytes to traverse the BBB (De Simone et al. 1995). In addition, BBB in patients with infection and tumour appears to be compromised (Avison et al. 2004; Davies 2002). Moreover, MHC antigens are found to be up-regulated at sites of brain injury, degenerative disease and tumour (Yang et al. 2006). All the observations mentioned above lead to intense interest in the application of immuotherapy against gliomas. However, until now, only limited clinical efficacy has been observed. Recent studies indicated that BTSCs employ various mechanisms of immune evasion. The generation of a successful classic specific immune response requires antigen uptake by antigen-presenting cells (APC) and subsequent activation of immune effector cells by APCs. Simply, APCs ingest antigenic peptides of target cells and then prime $CD8^+$ (cytotoxic T cells, CTLs) for MHC class I and $CD4^+$ (T-helper cells) for MHC class II, which will take effect to kill target cells (Sikorski and Lesniak 2005). BTSCs are found to express lower level of MHC than their differentiated counterpart in FBS medium. Since MHC I plays a critical role in the immune response, the altered MHC I expression shelters BTSCs from the recognition and subsequent lysis by CTLs (Di Tomaso et al. 2010). In addition to escaping immune response, BTSCs can profoundly influence the function of immune system components. BTSCs but not differentiated glioma cells are able to inhibit allogeneic T-cell proliferation. With enzyme-linked immunosorbent assay, Wei and coworkers found that BTSCs produce multiple immunosuppressive cytokines including transforming growth factor -β1 (TGF-β1) and prostaglandin E2 (PGE2) (Wei et al. 2010). TGF-β is a family comprising a large number of structurally related polypeptide growth factors and has been reported to be involved in immune response (Kirkbride and Blobe 2003). TGF-β inhibits the maturation and antigen presentation of APCs. As well, TGF-β has been found to inhibit T cell proliferation and activation. Moreover, TGF-β can impair CTL function through inhibiting the synthesis of cytotoxic molecules including FasL, IFN-γ and perforin. As for PGE2, it is a product of arachidonic acid metabolism, which is produced at sites of inflammation or tissue injure (Wang and Dubois 2006). PGE2 can suppress T cell activation and inhibit the anti-tumour activity of NK cells. Besides, PGE2 are shown to inhibit immune response through down-regulating the production of Th1 cytokines (IFN-γ and TNFα) and up-regulating Th2 cytokines (IL-4 and IL-10). PGE2 is also reported to enhance the suppressive activity of regulatory T cells. In addition, BTSCs are found to trigger T cell apoptosis mediated by co-stimulatory molecule B7-H1 and soluble Galectin-3. These findings suggested that BTSCs are able to escape immunotherapy through diminishing signals for immune recognition and inducing profound immune suppression.

4. BTSCs, promising therapeutic target

Although much effort has been made to aggressively treat malignant brain tumours, the prognosis is far from satisfactory. An accumulation of recent studies suggested the pivotal

role of BTSCs in the treatment resistance to conventional therapies. The current treatment strategies appear to effectively reduce the bulk of brain tumour cells but spare BTSCs. The improvement in the understanding of mechanisms underlying BTSC regulation provides insight into the development of novel treatment specifically targeting key signalling pathway and microenvironment of BTSCs, which appears to be a promising path leading to the cure of malignant brain tumours.

4.1 PI3K/Akt signaling

PI3K/Akt signaling is one of the most important and widely investigated pathways in brain tumours. As a major intracellular effector, PI3K transduces membrane-based activation from various sources, including G proteins such as Ras and receptor tyrosine kinases such as EGFR. Activation of PI3K/Akt pathway is implicated to regulate cellular proliferation, metabolism, differentiation and survival (Engelman, Luo, and Cantley 2006). Dysfunction of PI3K/Akt pathway is frequently observed in gliomas and suggested to mediate the gliomagenesis, progression and the resistance to therapy, which can be resulted from persistent activating signals from cell membrane (i. e. EGFR mutations), gain-of-function mutations in PI3K components (i. e. PIK3CA) and loss of tumour suppressor (i. e. PTEN deletion). In fact, PI3K-activating mutations are detected in almost all patients with GBMs (Comprehensive genomic characterization defines human glioblastoma genes and core pathways 2008, ; Parsons et al. 2008). Recently, PI3K/Akt pathway has been reported to be essential for the survival and treatment resistance of BTSCs. An animal model study demonstrated that the activation of PI3K/Akt pathway due to the loss of PTEN in gliomas greatly increases the SP cells and renders them to be more resistant to multiple chemotherapeutic agents including TMZ (Bleau et al. 2009). In addition, activated PI3K/Akt pathway protects BTSCs in their niche from radiation-induced apoptosis through the induction of p53-dependent cell cycle arrest (Hambardzumyan et al. 2008). Based on the critical role in BTSC biology, activated PI3K/Akt pathway can be a promising target against BTSCs. Eyler and coworkers revealed that BTSCs isolated from primary GBMs are more sensitive to Akt inhibition. Treatment with an Akt inhibitor resulted in a more remarkable reduction of BTSCs compared with non-BTSCs, which is associated with a preferential induction of apoptosis and a suppression of neurosphere formation. Furthermore, inhibition of Akt activity impairs the tumourigenicity of BTSCs and significantly prolongs the survival of mice bearing glioma xenografts initiated by BTSCs (Eyler et al. 2008). In another study performed by Sunayama and coworkers, dual blockade of PI3K and mTOR has a profound effect on BTSCs. Exposure to LY294002 combined with NVP-BEZ235 disrupts the self-renewal and triggers differentiation in BTSCs, with an inhibition of neurosphere formation and tumour initiation, as well as an up-regulation of neuronal differentiation marker, βIII-tubulin (Sunayama et al. 2010).

4.2 STAT3

Signal Transducer and Activator of Transcription 3 (STAT3) is a potentially attractive therapeutic target for BTSCs based on its dual role in regulation of stem cell functions and oncogenesis. STAT3 belongs to a family of latent transcription factors that are activated by multiple extracellular and intracellular signals including the Janus kinase (JAK) family and receptor tyrosine kinases (RTKs) (Darnell 1997). After phosphorylation of a tyrosine residue in the transactivation domain, activated STAT proteins dimerize, translocate into the nucleus and modulate gene expression by binding to specific DNA-response elements in the

promoter of target genes, including genes associated with cell survival, cell cycle regulation, immune response and differentiation, such as c-myc, cyclin D1, Bcl-2, VEGF and HIF1α. STAT3 is a crucial component in the regulation of embryonic development and the maintenance of multiple normal stem cell types, with mouse models demonstrating that STAT3-deficiency leads to lethality at E6.5 (Takeda et al. 1997). In neural development, STAT3 is necessary for the maintenance and self-renewal of neural stem cells and precursors (Yoshimatsu et al. 2006). In addition, STAT3 is an oncogene that induces transformation through increased expression of target genes that promote cell growth and inhibit apoptosis in multiple cell types. Constitutive activation of STAT3 is observed in a variety of tumours including glioma (Rahaman et al. 2002). STAT3 is found to be a master regulator of mesenchymal transformation in gliomas and correlate with poor prognosis of glioma patients. Elimination of STAT3 and another transcriptional factor C/EBPbeta leads to the collapse of the mesenchymal signature and reduces tumour aggressiveness (Carro et al. 2010). STAT3 has been identified as an important maintenance factor for BTSCs. Under undifferentiated condition, BTSCs have a constitutively high level of phosphorylated STAT3. Genetic knockdown of STAT3 leads to a significant inhibition of proliferation and neurosphere formation of BTSCs. Of note, even a transient inhibition of STAT3 is sufficient to result in irreversible growth arrest and diminishment of neurosphere formation, implying the critical role of STAT3 in the maintenance of BTSC self-renewal (Sherry et al. 2009). In addition, an inhibition of STAT3 synergizes the anti-cancer effect of TMZ against BTSCs and impairs the tumourigenic capacity of BTSCs *in vivo* (Villalva et al. 2011; Li et al. 2010).

4.3 Differentiation induction in BTSCs

Normal neural stem cells are characterized with their multipotency to differentiate into neurons, astrocytes and oligodendrocytes. BTSCs, although aberrantly, can also differentiate into tumour progenies with multi-lineage morphologies and markers. These differentiated cells constitute the bulk of brain tumours but lose the capacity of unlimited proliferation *in vitro* and the tumourigenicity *in vivo*. Therefore, exhaustion of BTSC pool by the induction of differentiation appears to be a practical therapeutic strategy. In fact, differentiation-inducing agents have already been used in the management of brain tumours before the surge of BTSC theory. The clinical activity of retinoids, known as powerful modulators of cellular differentiation and proliferation, was evaluated in patients with recurrent malignant brain tumours. In a Phase II study performed by Yung and coworkers, high-dose 13-cis-retinoic acid (CRA) is found to be effective against malignant gliomas with acceptable toxicity. CRA appears to work as a cytostatic rather than a cytolytic agent. The mechanism underlying the antitumour activity of CRA was not clearly known at that time (Yung et al. 1996). But now, under the BTSC scenario, this effect of CRA is attributed to its ability to induce differentiation in BTSCs. Exposure to all-trans-retinoic acid (ATRA) decreases CD133 level and results in an augmented expression of lineage markers in BTSCs. The disruption of angiogenesis and impairment of motility are also observed in BTSCs after ATRA treatment. In addition, the differentiation induced by ATRA can sensitize BTSCs to chemotherapy and irradiation by increasing cell death and apoptotic susceptibility (Campos et al. 2010).

Key components in several signalling pathways have been identified to be involved in the differentiation induction of BTSCs. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β superfamily and play an important role in promoting differentiation in embryonic stem cells and neural stem cells. BMPs are also assessed for the

differentiation induction in BTSCs. Piccirillo and coworkers demonstrated that non-cytotoxic BMP4 starts the differentiation program in BTSCs, resulting in a depletion of BTSC pool by 50% and an induction of morphological transition into more committed tumour cells. BTSCs transiently pre-treated with BMP4 fail to initiate large invasive and highly vascular tumour masses *in vivo* but only generate small lesion with low capacity of proliferation and infiltration. In addition, intracranial delivery of BMP4 simultaneously or 10 days after the injection of BTSCs into mouse brain retards tumour growth and prolongs the survival of tumour-bearing mice (Piccirillo et al., 2006). This study presents differentiation induced by BMPs as a promising strategy to target BTSCs. However, subsequent investigations demonstrated that not all BTSCs are readily responsive to BMPs at all time. Induction of differentiation in BTSCs by BMPs can be blocked by intrinsic and extrinsic factors. The response of BTSCs to BMPs depends on the expression and normal function of BMP receptors. In a subset of BTSCs, the expression of BMP receptor 1B (BMPR1B) is lost due to epigenetic silencing by an EZH2-dependent mechanism (Lee et al. 2008). In addition, the differentiative effects of BMPs on BTSCs can be regulated by oxygen tension. A hypoxic condition renders BTSCs to be more resistant to BMP-induced differentiation, which is found to be mediated by HIF1α (Pistollato et al. 2009).

Notch, a modulator of neural stem cell fate, has also been demonstrated to play a key role in the differentiation of BTSCs. Notch proteins are cell surface receptors that mediate cell-cell communication. After binding with transmembrane ligands from adjacent cells, Notch releases its intracellular domain cleaved by γ-secretase. The intracellular domain then translocates into the nucleus and functions as a transcription factor to regulate target gene expression. In the embryonic central nervous system, Notch signalling maintains a pool of undifferentiated neural stem cells by promoting the proliferation while inhibiting their differentiation into neurons. The dysregulation of Notch signalling pathway has been reported in a variety of neoplasms, including breast cancer, leukemia, and melanoma. In brain tumours, increased expression of Notch-related genes and elevated Notch activities are also found. Gene expression array demonstrated that Notch ligand Jagged-1, Notch 3 and the downstream target of Notch (HES1 and HES2) are overexpressed in a majority of primary GBMs relative to non-neoplastic brain tissues (Kanamori et al. 2007). Purow and coworkers performed a detailed functional study on Notch signalling pathway in gliomas. In this study, the presence of Notch-1 was found in glioma tissues and six glioma cell lines. Down-regulation of Notch-1 with siRNA leads to decreased cell proliferation, cell cycle arrest and apoptosis in glioma cells. Knock-down of Notch-1 significantly prolongs the survival of mice orthotopically transplanted with glioma cells (Purow et al. 2005). Moreover, Notch signalling is also linked to the maintenance and differentiation of BTSCs. When investigating GBM BTSCs, Fan and coworkers found that BTSCs derived from GBMs are sensitive to Notch signalling blockade by γ-secretase inhibitors (GSIs). GSIs selectively reduce the proliferation of Nestin- or CD133-positive BTSCs, which results in an inhibition of neurosphere clonogenicity *in vitro* and tumourigenicity *in vivo* (Fan et al. 2010). In another study carried by the same group, Notch blockade is found to preferentially target BTSCs in medulloblastomas. GSI exposure reduces the CD133-positive cell fraction by almost 5-fold and totally depletes the side population cells. The viable cells after the GSI treatment have an increased expression of two neuronal markers, Tuj1 and GABRA6. These better-differentiated tumour cells can continue to proliferate but fail to establish soft-agar colonies or tumour xenografts (Fan et al. 2006). These studies indicate that Notch blockade can induce differentiation in BTSC and can be potentially useful in the treatment of malignant brain tumours.

4.4 BTSC niche

It has been recognized that stem cells are not randomly distributed throughout the organ where they remain. In fact, stem cells reside in niche, a specific microenvironment consisted of both cellular and acellular components, which plays an important role in regulating the function of stem cells (Burness and Sipkins 2010). In the brain, neural stem cells (NSC) are concentrated in the subgranular zone of the hippocampus and the subventricular zone of the lateral ventricle, with close proximity to blood vessels (Quinones-Hinojosa et al. 2007). The contact and communication of niche and NSCs are critical in maintaining the quiescence and driving the proliferation and differentiation of NSCs. BTSCs are proposed to origin from the oncogenic transformation of NSCs and appear to occupy similar niches. The subventricular zone has long been proposed as the source of glioma, based on the observation that many gliomas either grow around the ventricle or are contiguous with the subventricular zone (Glantz et al. 2009). In clinical studies, bordering lateral ventricle by gliomas has been associated with decreased survival. Chaichana and coworkers analyzed the relationship of the tumour location and the survival of GBM patients (Chaichana et al. 2008). The authors found that GBM patients with a radiological contrast-enhancing lesion bordering the lateral ventricles (LV CEL) have a more unfavourable prognosis compared to patients with non-LV CEL and therefore postulated that the region of lateral ventricles may have a microenvironment more conducive for potent tumour cells, perhaps BTSCs, to proliferate and/or infiltrate. This area also has been shown to have an increased propensity to form glioma in animal studies. Marumoto and coworkers successfully induced intracranial high-grade gliomas in adult immunocompetent mice by injecting Cre-loxP-controlled lentiviral vetors expressing oncogenes (Marumoto et al. 2009). Of note, more than 75% of mice show tumour formation when viral vectors are injected in neurogenic areas such as the subventricular zone and hippocampus, while there is little tumour formation in the cortex. Moreover, transplantation of these brain tumour cells into naïve recipient mouse brain results in the formation of GBM-like tumours containing BTSCs. This study suggested the susceptibility of cells in neurogenic areas to oncogenic stimuli and the possible origin of BTSCs in these areas.

Malignant gliomas are characterized as highly vascular tumours. The presence of microvascular proliferating structures is recognized as a pathological criterion in diagnosis of high-grade gliomas. The density of microvascular proliferation has been demonstrated to correlate with the prognosis of glioma patients (Wong et al. 2009). Accumulating evidence suggested that vasculatures in glioma are more than pipelines to supply nutrients and oxygen, they can function as niches, interacting with brain tumours and regulating BTSC behaviour (Gilbertson and Rich 2007). A dynamic analysis revealed that gliomas cells extensively infiltrate the brain parenchyma through migrating along blood vessels. Remarkably, the majority of glioma cells divide around vascular branch points, indicating that the microenvironment provides cues to trigger mitosis (Farin et al. 2006). Calabrese and coworkers did the first thorough investigation on the perivascular niche for BTSCs. With 3-dimensional (3D) imaging, the authors clearly demonstrated that Nestin-positive BTSCs directly associate with tumour capillaries in glioma specimens. Coculture with primary human endothelial cells (PHECs) maintains the self-renewing and undifferentiated status of BTSCs. Moreover, PHECs are found to robustly expand the proliferation of BTSCs and promote tumour growth *in vivo*. It is suggested that vascular endothelial cells may support BTSCs by secreting soluble factors similarly as they do for neural stem cells (Calabrese et al. 2007).

Because BTSCs have intimate relationship with their microenvironment, targeting the niche seems to be a desirable therapeutic strategy to eradicate BTSCs. Increasing the dosage of irradiation to BTSC niche has been shown to yield significant benefits for patients with high-grade gliomas. Evers and coworkers performed a retrospective analysis to investigate the effect of the radiation to the periventricular niche on the prognosis of 55 adult patients with malignant glioma (Evers et al. 2010). The progression-free survival of patients whose bilateral subventricular zone (SVZ) received greater than the median SVZ dose (43 Gy) is 15.0 months, which is much longer than that (7.2 months) of patients who received less than the median dose. Besides, disruption of the vascular structures that BTSCs depend on is also a promising option. Preclinically, anti-angiogenic agents are shown to specifically act on BTSCs (Calabrese et al. 2007). Erlotinib and Bevacizumab are successful in inhibiting the self-renewasl of BTSCs and suppressing the tumour growth in glioma-bearing mice, implying the efficacy of the disruption of BTSC niche. Similarly, Williams and coworkers revealed that creation of a barrier between BTSCs and endothelial cells can decrease the number of BTSCs in glioma xenografts (Williams et al. 2010). The authors treated the orthotopic glioma models with IFN-β. Although IFN-β is not directly toxic to BTSCs *in vitro*, treatment with IFN-β leads to tumour growth arrest *in vivo* by affecting the BTSC niche. IFN-β increases perivascular cells investing vasculature, resulting in the disruption of the communication between BTSCs and endothelial cells. Clinically, bevacizumab, a monoclonal antibody targeted against VEGF, and cediranib, a small molecule inhibitor of VEGF show some success. After a systematic review and survival-gain analysis, Xu and coworkers found that combination of bevacizumab and irinotecan largely improves response rates and has a possible moderate effect on overall survival in patients with recurrent adult high-grade glioma (Xu et al. 2010). A Phase II clinical trial performed by Batchelor and coworkers demonstrated that treatment for recurrent GBM with Cediranib monotherapy results in encouraging proportions of radiographic response and 6-month progression-free survival (Batchelor et al. 2010). In addition to depletion of the tumour blood supply, the clinical efficacy of anti-angiogenic drugs may be partly attributed to the disruption of the perivascular niche of BTSCs (Gilbertson and Rich 2007).

5. Future directions and challenges

The emergence and evolution of BTSC concept broaden our horizon in the understanding of brain tumour biology. The roles of BTSCs in the progression and treatment resistance of brain tumours lead us to have a reassessment of the current treatments. Key signalling pathways identified in BTSCs provide clues to develop novel therapeutics, which may pave the road to the cure of malignant brain tumours. However, this road can be winding and bumpy. There are still challenges we should realize. First, BTSCs are heterogeneous and the markers as well as approaches to purify BTSCs currently employed are unlikely to identify all BTSC phenotypes. New markers need to be explored and a combined use of a set of various markers and/or purification techniques appears to be more effective in the identification of different BTSC population. Secondly, many signalling pathways pivotal to the maintenance and self-renewal of BTSCs are also necessary for normal stem cells. Approaches aiming at these signalling pathways may do harm to normal stem cells. Therefore, critical signalling pathways specific in BTSCs are superior targets. Thirdly, the stemness of BTSCs is not unchangeable, which can be tuned in order to adapt tumour cells to the changes in microenvironment. Therefore, BTSCs appear to be running targets instead

of stationary ones. The therapeutic strategies against BTSCs should be continuously modulated. Finally, the microenvironment contributes significantly to the phenotype and plasticity of BTSCs. The effective treatment in future should focus on not only BTSCs *per se* but also the niches BTSCs reside in.

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14

Glioma Stem Cells

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1. Introduction

Malignant gliomas are the most common primary brain tumor, with median survival of less than 15 months from the time of diagnosis. (Stupp R, et al., 2005) Most malignant tumors contain a small subpopulation of cells that are highly tumorigenic and share features with normal stem cells, including the ability for extensive self-renewal and to differentiate into multiple lineages. Many tumors, including glioblastomas, are activated by these cancer stem cells, making them resistant to conventional therapies such as radiation and chemotherapies. In addition, these cancer stem cells adopt the signaling pathways of normal neural stem and progenitor cells, thereby playing a critical and complex role in tumorigenesis, allowing the tumor to rapidly progress, proliferate, and metastasize. Thus, the pathologic pathways directed by these cancer stem cells make gliomas hard to treat and regulate. Therefore, in order to understand gliomas as well as the cancer treatment-related neurotoxicity on a cellular level, it is crucial to be familiar with the concept of glioma stem cells and their lineage relationships with the central nervous system. Furthermore, to effectively target these cancer stem cells, an understanding of the molecular profiling of well-characterized cancer cell populations is necessary to identify novel biomarkers that will provide the foundation to track their targeted pathways. This will help evaluate and personalize treatment options to help advance our knowledge in the biology of glioma and translate these concepts into the clinical arena. Thus, this chapter will focus on the current understanding on progenitor cells and neural stem cells and highlight important findings regarding the identification and characterization of glioma stem cells, and the development of novel-stem-cell-based treatment strategies for brain tumors.

2. Glioma and stem cells

Malignant gliomas, aside from being the most common brain tumor, are very challenging to treat with median survival times of less than 15 months from time of diagnosis (Stupp R, et al., 2005). Many of the malignant gliomas, especially glioblastomas, display aberrant genetic abnormalities that contribute to their pathologic cellular and morphological heterogeneity (Tabatabai & Weller, 2011). This tumor heterogeneity thus poses a critical obstacle in treating malignant gliomas because different cell populations within the tumor tissues respond differently to treatments such as conventional chemotherapy and radiation (Tabatabai & Weller, 2011; Bao, et al., 2006; Dietrich et al., 2008). In addition, the tumor's tendency to aggressively infiltrate into the surrounding brain parenchyma, therefore

preventing a complete surgical tumor resection, hinders the treatment of gliomas and results in fatal tumor recurrences. (Bao, et al., 2006; Cheng L, et al., 2011; Dietrich et al., 2008).

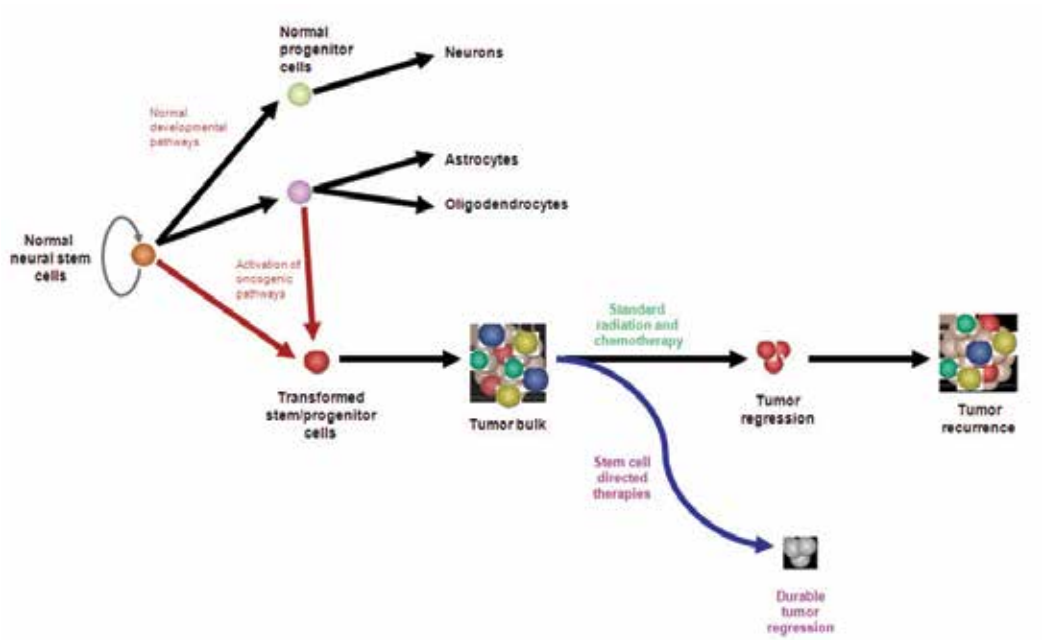

Fig. 1. Resistance mechanisms in glioma cells

Normal neural stem cells self-renew and give rise to multipotential progenitor cells that form neurons, oligodendroglia, and astrocytes. Glioma stem cells arise from the transformation of either neural stem cells or progenitor cells (red) or, less likely, from differentiation of a oligodendrocytes or astrocytes (red arrows) and lead to malignant gliomas. Glioma stem cells are relatively resistant to standard treatments such as radiation and chemotherapy and lead to regrowth of the tumor after treatment. Therapies directed at stem cells can deplete these cells and potentially lead to more durable tumor regression (blue).

To complicate the matters further, early and effective detection of these cancers are extremely difficult, resulting in a poor prognosis for the patients diagnosed with malignant gliomas (Huang Z et al., 2010). Then, these difficulties collectively warrant for reevaluation of current treatments in order to achieve optimal efficacy, prolong the survival, and improve and maximize the quality of life for the patients.

Recent studies suggest that in order to understand the pathologic nature of malignant gliomas, it is crucial to acknowledge the role of cancer stem cells that are involved in the processes of tumor initiation, tumor progression, angiogenesis and resistance to therapy (Huang Z et al., 2010). In a study done by Park et al., even though the correlational relationship between gliomas stem cells and massive tumor is not clear, it is possible that the stem cells contribute to the tumor recurrence after the initial, conventional therapies (Park & Rich, 2009). These cancer stem cells are also known as the tumor-initiating or propagating cells because they display and share some important characteristics with normal stem cells, including self-renewal, multi-lineage differentiation, and maintained proliferation (Huang Z

et al., 2010; Rosen and Jordan 2009; Park & Rich, 2009; Heddleston et al., 2010). Also similar to neural stem cells, cancer stem cells appear to be organized and depend on vascular and nonvascular elements (Dietrich, et al., 2008, Calabrese,et al.,2007). Although the exact origin of the gliomas stem cells is still controversial, there seems to be a consensus that these cancer stem cells arise from genetic and epigenetic changes in neural stem and progenitor cells after many mutations or epigenetic transcription (Huang Z et al., 2010).
Therefore, exploring the signaling pathways and molecular mechanisms that drive these tumor-initiating cells in malignant gliomas is necessary to identify and provide promising novel treatment strategies. In order to understand the exact mechanisms that govern cancer stem cells' role in tumor angiogenesis and extensive proliferation, a careful insight into the cancer stem cell signaling mechanisms warrants further analysis and investigation to optimize brain tumor treatment.

3. Glioma stem cell signaling

The main factor that contributes to the cancer cells' extensive proliferation within their perivascular niche is the cell-extrinsic and cell-intrinsic signals. Therapeutic targeting of these signals within their niche, along with the tumor-associated vasculature may significantly interfere with glioma cancer stem cell growth (Park & Rich, 2009; Dietrich et al., 2010). In clinical studies done by Kreisl et al., and Vredenburgh et al., the administration of bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF) demonstrated a clinical effectiveness in achieving therapeutic response (Kreisl et al., 2009; Vredenburgh et al., 2007). As evident by this and other ongoing studies, providing therapies that target these glioma stem cell signaling pathways not only provide effective treatment by directly targeting these stem cells but also gives us valuable insights into the pharmacodynamics of cancer stem cells' dependence on the perivascular niche for survival, growth, and proliferation (Park & Rich, 2009). Therefore, the following major signal transduction pathway cascades will be extensively reviewed to provide highly discriminating targets and tumor biomarkers of therapeutic response: epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), RAS/Raf/MAPK & AKT/PI3K pathways, Sonic hedgehog (Shh), and PTEN.

3.1 Epidermal growth factor signaling

Epidermal growth factor related signaling has probably been the most extensively studied pathway in malignant gliomas. Epidermal growth factor receptor (EGFR) is a tyrosine kinase that is activated upon ligand binding which induces receptor dimerization. EGFR signaling is involved in many aspects of cellular events including corticogenesis, neural cell survival, proliferation, differentiation, and migration (Ayuso-Sacido et al., 2009; Zhu G et al., 2000). The overexpression of EGFR plays a significant role in GBM and other malignant gliomas' pathologic progression (Ayuso-Sacido et al., 2009). In addition, EGFR is important in neural cell fate and astrocyte differentiation. EGFR expression is often mutated in more than 50% of glioblastomas, with EGFRvIII, a truncated extracellular domain within EGFR that transform into an active ligand-independent kinase, being the most common mutant encountered in gliomas and is responsible for enhanced tumorigenic behavior and genetic instability (Ayuso-Sacido et al., 2009; Frederick et al., 2000, Liu L et al., 2005; Rasheed BK et al., 1999; Ekstrand, 1991; Wong, 1992; Collins, 1993; Mellinghoff, 2005; Aldape, 2004; Pelloski, 2007; Huang, 2007; Li, 2009).

In experimental studies looking at the role of EGFR and the expression of EGFR vIII, the expression of EGFRvIII in the glial lineage in mouse models were significantly similar to human gliomas and in addition, these lesions were mainly occurring within the progenitor cells (Ayuso-Sacido et al., 2009; Dai C et al., 2001; Halatsch ME et al., 2000; Holland EC et al., 2004; Laywell ED et al., 2000). The critical importance of EGF-mediated signaling in gliomas is that these signaling have been shown to contribute to both normal stem cell behavior and to pathologic gliomagenesis. Activated EGFR signaling pathway induce normal stem cells to display characteristics that are seen in glioma stem cells including increased proliferation, migration and survival (Ayuso-Sacido et al., 2000). Consequently, it has been suggested that interference with EGFR and EGFRvIII signaling may offer an attractive strategy for selective glioma therapy (Huang, 2007; Shir, 2006; Zhu, 2009).

Selective small-molecules designed to inhibit EGF/EGFR signaling were among the first targeted molecular therapies used in glioblastoma patients. One of the drugs that is being investigated to treat gliomas is gefitinib (Iressa®), a selective EGFR inhibitor. However, in preclinical studies using gefitinib to observe its activity revealed that the drug showed selective efficacy in tumors that have mutations in exons 19 and 21 of the amplified HER1/EGFR-TK domain (Halatsch ME et al., 2006; Lynch T et al., 2004; Paez J et al., 2004). This result might be the reason why gefitinib is ineffective in treating malignant gliomas, including GBM, because these mutations have not been found in gliomas (Marie Y et al., 2005; Halatsch ME et al., 2006). In addition, preclinical data showed that gefitinib has very low or no activity against tumors that express EGFRvIII (Heimberger AB et al., 2002). Clinical data shows that gefitinib for glioma patients are generally well tolerated with few, minor side effects. However, when compared with historical data, event-free/progression-free survival and median overall survival were same and gefitinib failed to produce effective objective response (Wong et al., 1999; Rich,2004).

Erlotinib (Tarceva®, OSI-774) is another inhibitor of EGFR and the constitutively active mutant EGFRvIII. In pre-clinical studies, erlotinib suppressed GBM cell lines' anchorage-independent growth, suggesting that erlotinib may prevent or even delay GBM recurrence following surgical resection (Halatsch ME et al., 2006). For erlotinib, phase I/II clinical study data indicate that it was well tolerated and findings from phase I trials supported the data from phase II trials in terms of response rates and its antitumor effects (Brewer et al., 2005). The results of a large multicenter Phase II trial, in which patients with progressive glioblastoma were randomly assigned to erlotinib, or conventional chemotherapy with either temozolomide or carmustine (BCNU), revealed that erlotinib had insufficient activity when used as monotherapy (van den Bent, 2009).

Taken together, monotherapy with anti-EGFR agents such as gefitinib and erlotinib, thus far, have not demonstrated significant activity in patients with malignant glioma. However, recent studies suggest that combining the anti-EGFR agents with inhibitors of other molecular target may provide more effective approach in treating malignant gliomas. For example, dual-kinase inhibition of EGFR and ErbB-2 (e.g., lapatinib) (Spector, 2005; Giannopoulou, 2009), or EGFR and VEGF recepetor (VEGFR; e.g., AEE788; Zactima®/vandetanib/ZD6474) (Traxler, 2004; Goudar, 2005; Sandstrom, 2008). Since the stem cell biology suggests that glioma stem cells display a close resemblance to the normal neural cells, our current knowledge of normal stem cell can help analyze and deregulate the pathway of the cancer stem cells to provide promising, therapeutic results.

3.2 Platelet-derived growth factor signaling

Platelet-derived growth factor (PDGF) and its associated receptors play fundamental roles in the developing and adult brain. (Yeh,1993) Studies from *in vivo* animal models show that there is a correlational relationship between the abnormal PDGF signaling and glioma formation (Calzolari & Malatesta, 2009). Often times in gliomas, PDGF ligands are overexpressed along with their PDGFRα receptors. Hence these amplification-dependent receptors or the ligands give rise to aberrant, overactive PDGF signaling pathway (Calzolari & Malatesta, 2009). In the adult brain, PDGFR-a expression is found in the lateral subventricular zone, whereas PDGF is abundantly expressed by neurons and astrocytes (Oumesmar, 1997).

Also in animal model studies increased PDGF signaling blocked neuroblast generation and enhanced neural stem cell proliferation in the subventricular zone with formation of glioma-like hyperplasias (Jackson, 2006). Moreover, the inhibition of PDGF-mediated signaling decreases glioma cell proliferation both in vitro and in vivo (Lokker, 2002), supporting the role of both autocrine and paracrine mechanisms in glioma biology. The fact that many of the malignant gliomas display altered and overactive PDGF pathway suggest that this signaling axis plays an important role in gliomagenesis. Also it seems likely that PDGF signaling is involved and is crucial in tumor proliferation and survival by stimulating growth and supplying nutrients to underlying tumor cells (di Tomaso E et al., 2009).

Imatinibmesylate (Gleevec®) is a small-molecule, oral inhibitor of multiple tyrosine kinases including PDGFR α and β, c-KIT and the BCR-ABL onco-protein (Morris & Abrey 2010). In a phase I/II study done by Wen et al., Imatinib in a monotherapy setting showed disappointing results. Of the 105 patients who were enrolled, 68% of the patients had very low imatinib in their plasma when taking the drug with enzyme inducing anti-epileptic drugs (EIAEDs). This is a serious problem because most glioma patients are on anti-seizure medications, regardless of the treatments that they are receiving. In addition, to the low pharmacokinetic data, imatinib also induced intratumoral hemorrhages and showed no therapeutic responses to patients with anaplastic gliomas (Morris & Abrey 2010; Wen PY et al., 2006). Another phase II showed disappointing results with having 6-moth PFS rate of 16% in GBM, 9% in astrocytomas, and 4.0% in oligodendrogliomas (Raymond et al., 2008). In a combination therapy regimen, administering imatinib with temozolomide found to be tolerable in many patients. However in several clinical studies observing a combined treatment of imatinib with hydroxyurea, a ribonucleoside diphosphate reductase inhibitor, showed disappointing results (Reardon DA et al., 2005 & 2008).While other PDGFR pathway inhibitors are currently under clinical investigation (Roberts, 2005), dual kinase inhibitors, or combinational therapies with conventional cytotoxic agents are also developing including PDGFR and VEGFR dual inhibitors such as PTK787 (vatalanib), sorafenib (Nexavar), sunitinib, AEE788, AZD2171 (cediranib), TKI258, OSI-930 and pazopanib. Also, there are studies observing the efficacy of nilotinib (Tasigna®), which is an oral drug that has greater potency and selectivity for BCR-ABL than imatinib (Saglio G et al., 2010).

3.3 Vascular endothelial growth factor signaling

Antiangiogenic treatments examining VEGF and its associated signaling cascade have been an integral part in modern cancer therapy by targeting extensive tumor vasculatures of the malignant gliomas (Wick et al., 2011). Both endothelial cells and glioma cells may express

and upregulate VEGF and its receptors, resulting in both paracrine and autocrine loops that drive endothelial cell proliferation, tumor invasion, migration and permeability. (Ferrara, 2003; Millauer, 1994) Also, there are circumstantial evidence that elevated VEGF expression in gliomas is associated with the degree of malignancy of the tumor and overall tumor prognosis (Schmidt, 1999; Leon, 1996).

Glioma stem cells appear to be directly involved in this process by stimulating tumor angiogenesis through production of pro- angiogenic factors, such as VEGF (Bao, 2006). In many gliomas, especially in glioblastomas, the neural stem cells and their endothelial compartment closely interact with the vascular niche and by releasing VEGF and promoting angiogenesis (Ricci-Vitiani L et al., 2011). Thus, inhibition of tumor angiogenesis may especially target the cancer stem cell population with the hope of achieving more durable clinical responses. (Folkins, 2007) Antiangiogenic therapy that targets VEGF signaling has evolved into an important therapeutic treatment strategy. Bevacizumab (Avastin®) is a humanized anti-VEGF antibody that demonstrates promising results in treating patients with glioblastomas and have been approved by the United States FDA for the treatment of recurrent of progressive GBMs (Vredenburgh et al., 2007; Huang Z et al., 2010). In a retrospective study of 55 patients with GBM done by Norden et al., the result showed a promising data where the median 6-month progression survival rates were 42% (Brastianos & Batchelor, 2010; Norden AD et al., 2008). Similar promising results have also been reported in other subsequent studies in randomized phase II trials where the patients in the bevacizumab arm showed 42% 6-month progression survival (Therasse P et al., 2000).

Aflibercept (Regeneron®) is a potent, VEGF-trap that is fused to an immunoglobulin constant region. In a study done by De Groot et al., aflibercept brought therapeutic response to 30% of GBM patients. Future trials examining aflibercept in treating malignant gliomas is still underway (De Groot JF et al., 2008).

There are range of novel RTK inhibitors, such as cediranib, vandetanib, vatalanib, sorafenib, sunitinib, pazopanib, AE-788, and CT-322 that have been shown to influence angiogenesis and tumor growth through multiple targets and are currently in various stages of preclinical and clinical investigation. (Brastianos & Batchelor, 2010). Cediranib (Recentin®) is a potent pan-VEGFR, PDGFR and c-KIT inhibitor that showed objective response in 57% of the patients in a phase II trial (Batchelor TT et al., 2007). Vandetanib (Zactima®) is a selective inhibitor of VEGFR2 and EGFR and showed promising antiglioma effects in preclinical studies (Rich JN et al., 2005). Vatalanib is a an oral pan-VEGFR, PDGFR, c-Kit inhibitor that reduced the activity of VEGF-mediated glioma growth (Goldbrunner et al., 2004). In a phase I/II trial, vatalanib produced radiographic responses in 4% of the patients and resulted in stable disease response rate of 56% (Conrad C et al., 2004). Sorafenib (Nexavar®) is a multi-target inhibitor that targets VEGFR, PDGFR, c-KIT, and Raf. It is approved to be used in a single-agent, monotherapy setting and data from phase I trial indicates that patients well tolerated the drug (Jane EP et al., 2006; Nabors L et al., 2007). Sunitinib (Sutent®) is also a multi-target kinase inhibitor that displayed promising and efficacious antiangiogenic activity and antitumor activity in GBM models *in vitro* and *in vivo*. Pazopanib (Voltrient®) is another multi-target kinase inhibitor of VEGFR-1, -2, and -3, PDGFR and c-KIT and in a recent phase II study, pazopanib was well tolerated with median progression-free survival being 12 weeks (Iwamoto FM et al., 2010). Studies exploring the therapeutic efficaciousness of AE-788 and CT-322 are underway, which are oral inhibitor of EGFR, HER2 and VEGFR2 and pegylated protein inhibiting VEGFR-2 signaling pathway cascade, respectively (Brastianos & Batchelor, 2010).

Exploring VEGF signaling pathway is crucial in treating malignant glioma because these anti-VEGF agents can normalize the blood vessels, which will allow to improve the delivery of chemotherapy agents that will produce favorable radiation response. Even though there are many promising data published regarding the therapeutic efficacy of VEGF inhibitors in pre-clinical and clinical settings, more studies are in development to confirm these results and to understand the complexity of molecular signaling pathways.

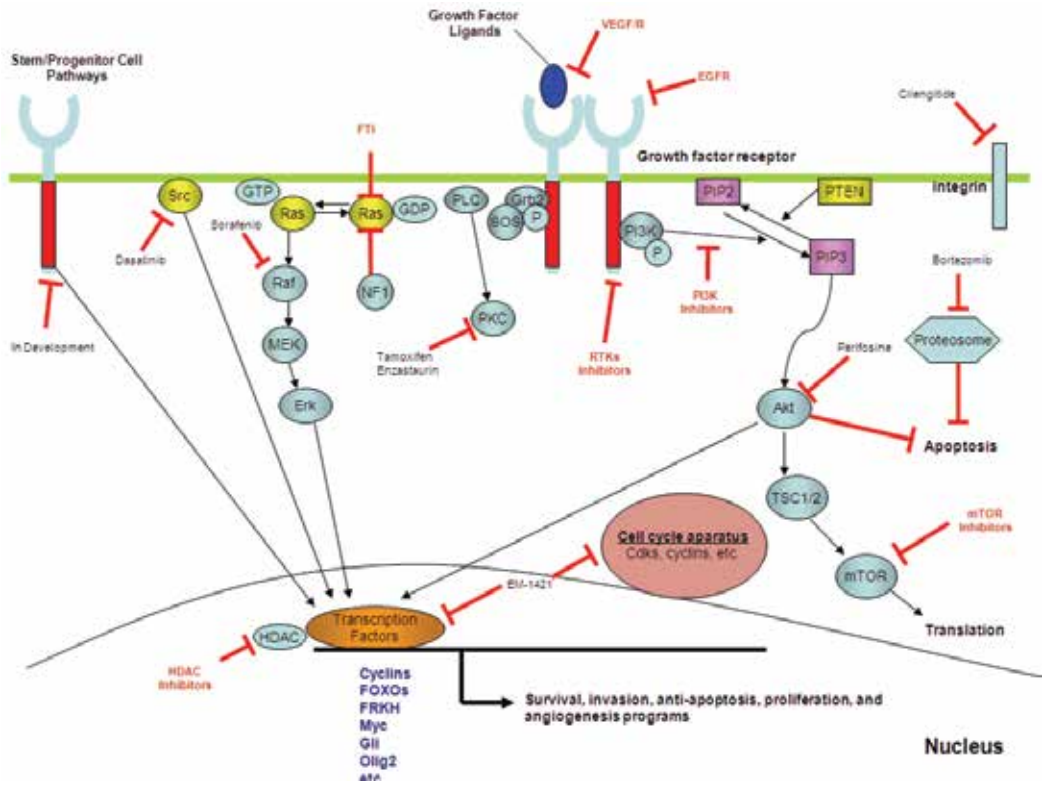

Fig. 2. Signaling pathways important for stem cell signaling

Pharmacological interference with these pathways promises to have therapeutic potential in targeting cancer stem cells. Receptor tyrosine kinase-mediated signaling (FGF, PDGF, EGF, VEGF) promotes progenitor cell proliferation and angiogenesis. PTEN acts as a tumor-suppressor gene and is frequently inactivated in gliomas. The
Wnt/b-catenin pathway leads to accumulation of intranuclear b-catenin and transcription of target genes critical for stem cell and progenitor cell function. Shh binds to its associated transmembrane receptor PTC1, releasing the membrane protein Smo, which results in downstream activation of the Gli proteins and transcription factors. Bmi1 controls maintenance of stem cells by repressing genes that promote differentiation or cell death, such as the tumor suppressors p16Ink4a and p19Arf. Binding of the cell membrane-associated Notch receptor proteins and their associated ligands (not shown) leads to cleaving of the intracellular domain of Notch (NICD) and transcription of target genes essential for maintenance and self-renewal of stem cells. PTC1: Patched 1; Shh: Sonic hedgehog; Smo: Smoothened.

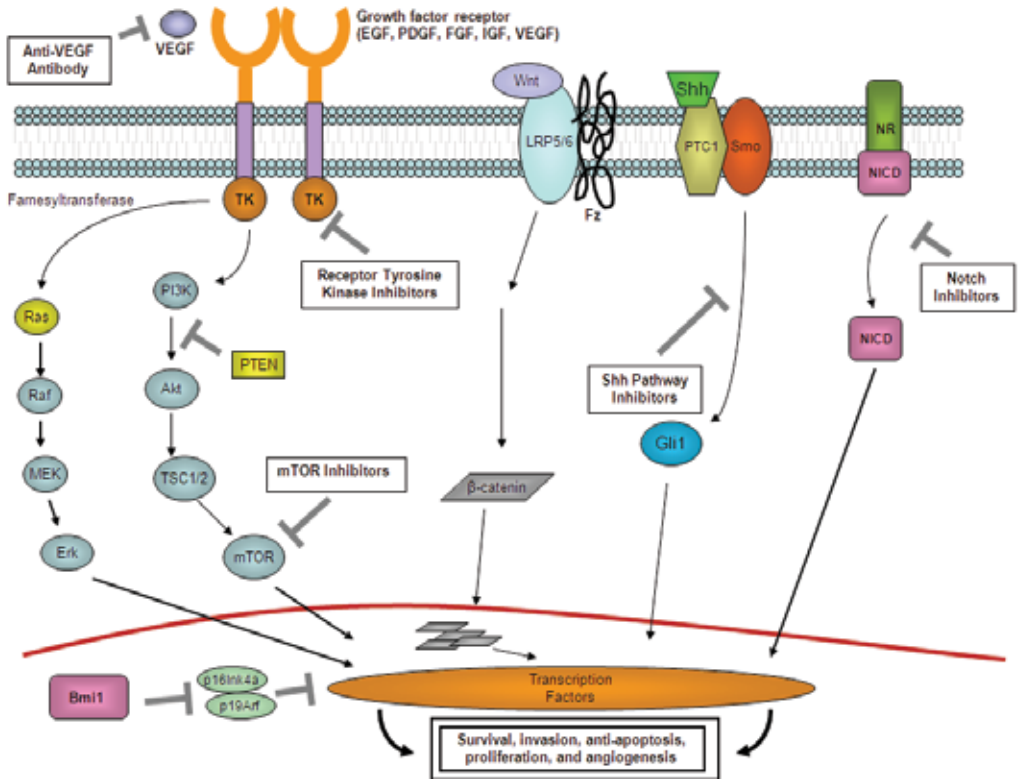

Fig. 3. Major signaling pathways in malignant gliomas and the corresponding targeted agents in development for glioblastoma

RTK inhibitors that target epidermal growth factor (EGF) receptor include gefitinib, erlotinib, lapatinib, BIBW2992, and vandetanib; those that target plateletderived growth factor (PDGF) receptor include imatinib, dasatinib, and tandutinib; those that target vascular endothelial growth factor (VEGF) receptor include cediranib, pazopanib, sorafenib, unitinib, vatalanib, vandetanib, and XL184. EGF receptor antibodies include cetuximab and anitumumab. Farnesyl transferase inhibitors include lonafarnib and tipifarnib; HDAC inhibitors include depsipeptide, vorinostat, and LBH589; PI3K inhibitors include BEZ235 and XL765; mTOR inhibitors include sirolimus, temsirolimus, everolimus, and deforolimus; and VEGF receptor inhibitors include bevacizumab, aflibercept (VEGF-trap), and CT-322. Growth factor ligands include EGF, PDGF, IGF, TGF, HGF/SF, VEGF, and FGF. Stem-cell pathways include SHH, wingless family, and Notch. Akt denotes murine thymoma viral oncogene homologue (also known as protein kinase B), CDK cyclin-dependent kinase, ERK extracellular signal-regulated kinase, FGF fibroblast growth factor, FTI farnesyl transferase inhibitors, GDP guanine diphosphate, Grb 2 growth factor receptor-bound protein 2, GTP guanine triphosphate, HDAC histone deacetylase, HGF/SF hepatocyte growth factor/scatter factor, IGF insulin-like growth factor, MEK mitogen-activated protein kinase kinase, mTOR mammalian target of rapamycin, NF1 neurofibromin 1, PIP2 phosphatidylinositol (4,5) biphosphate, PIP3 phosphatidylinositol 3,4,5-triphosphate, PI3K phosphatidylinositol 3-kinase, PKC protein kinase C, PLC phospholipase C, PTEN phosphatase and tensin homologue, RAF v-raf 1 murine leukemia viral oncogene

homologue 1, RAS rat sarcoma viral oncogene homologue, RTK receptor tyrosine kinase inhibitor, SHH sonic hedgehog, SOS son of sevenless, Src sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue, TGF transforming growth factor family, and TSC1 and 2 tuberous sclerosis gene 1 and 2. Red text denotes inhibitors.

3.4 RAS/Raf/MAPK & AKT/PI3K pathways

Downstream effects of RTK signaling include activation of Ras/ Raf/MAPK and AKT/PI3K, promoting cell survival, cell proliferation, cell migration and angiogenesis. Malignant gliomas and glioblastomas have hyperactivated phosphatidylinositol-3-kinase (PI3K)-Akt , also known as protein kinase B) pathway that serve as the genesis of human cancers (Eyler C et al., 2008; Castellino RC et al., 2007; Wlodarski P et al., 2006; Knobbe CB et al., 2005). Once Akt signaling pathway is hyperactivated it results in the proliferation, invasion, and angiogenesis of tumor cells. AKT/PI3K activation through loss of PTEN in combination with constitutively active EGFR signaling has been shown to induce glial tumor formation along with genetic instability in experimental models (Eyler C et al., 2008). Interestingly, glioma stem cells appear to be more dependent on AKT signaling than non-stem glioma cells, suggesting that AKT inhibition is crucial in managing the tumor stem cells from expanding and treating brain tumors more effectively (Eyler C et al., 2008). Currently, there are various clinical trials observing tem- sirolimus (CCI-779), its active metabolite sirolimus (rapamycin), everolimus (certican), and AP23573, which are mTOR inhibitors that target PI3K signaling pathway (Galanis,2005; Chang,2005; Kuhn,2007).

3.5 Sonic hedgehog signaling

Sonic hedgehog (Shh) is a key regulator and a determinant that is crucial for the generation and maintenance of adult stem cells (Dave R et al., 2011). Hedgehog (Hh) signaling is also involved in patterning, growth, and cell fate determination in many developing organ systems. Upon secretion, the hedgehog molecules bind to Patched 1 protein (PTCH1) and inhibit the receptor. Once inactivated, PTCH1 accumulates Smoothened (SMOH) in its cytoplasm instead of releasing them (Dave R et al., 2011). These results in downstream activation of the Gli proteins – zinc-finger transcription factors that translocate to the nucleus and may either activate or repress downstream targets, such as Wnt, IGF and PDGFR-a, myc and cyclin D1. Shh is important in regulating neural stem cells, neural tube patterning, and neurogenesis (Machold, 2003; Park, 2003; Lai, 2003; Palma, 2005; Cai, 2008; Han, 2008; Komada, 2008). Recent studies show that SHH-neutralizing antibodies help in inhibiting tumor cell growth and reducing its proliferation rates (Chen YJ et al., 2007; Thayer SP et al., 2003; Berman DM et al., 2003; Karhadkar SS et al., 2004). Early clinical trials (e.g., GDC-0449) are ongoing to evaluate inhibitors of Shh signaling (Rudin,2009). Collectively, Shh-mediated signaling is critically important in stem cell and tumor biology and may constitute another attractive target for therapy of malignant brain tumors.

3.6 PTEN

Phosphatase and tensin (PTEN) homologue gene has important functions in both normal neural stem cell physiology and oncogenic processes. It acts mainly as a tumor suppressor gene and is involved in many cellular functions including cell cyle progression, angiogenesis, migration, invasions, and stem cell regulation (Cheng RB et al., 2010; Alexiou GA et al., 2010). However, in many malignant gliomas, PTEN genes are often deleted or

mutated, which contributes to the pathologic progression of the tumor cells. This loss of PTEN leads to constitutive activation of AKT and resistance to apoptosis (Maehama T et al., 1998; Radu A et al., 2003; Stiles B et al., 2002). In turn, this down-regulation of PTEN results in aggressive tumor expansion and poor prognosis. PTEN inactivation in combination with EGFR amplification is sufficient to cause invasive gliomas in experimental mouse models, supporting the critical role of PTEN inactivation in gliomagenesis. Furthermore, it has been suggested that loss of PTEN enhances resistance to EGF RTK inhibitors in glioblastoma patients (Mellinghoff, 2007).

4. Targeted molecular therapy

In recent years, there has been tremendously increased understanding of the molecular abnormalities occurring in malignant gliomas (Maher,et al.,2001; Kitange,et al.,2003; Konopka&Bonni,2003). Molecular analysis of gliomas shows a step-wise progression of genetic changes involving overexpression of proto-oncogenes and loss of tumor suppressor genes. Low-grade astrocytomas (World Health Organization [WHO] grade II) tend to have inactivating mutations of TP53 and overexpression of platelet-derived growth factor (PDGF) and their receptors (PDGFR). Progression to anaplastic astrocytomas (WHO grade III) is associated with inactivation of the p16-cdk4-Rb pathway and allelic loss of 19q, whereas progression further to a secondary glioblastoma (WHO grade IV) is associated with loss of chromosome 10 and other changes. Primary glioblastomas, which originate de novo, often have loss of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), together with amplification, mutation, and overexpression of the epidermal growth factor receptors (EGFR). There is increasing work on molecular profiling of malignant gliomas using a variety of different techniques.

These approaches are beginning to enable genes that are important in tumor progression to be identified (van den Boom et al., 2003). In addition, morphologically indistinguishable malignant gliomas can be differentiated into molecular subtypes that may eventually be used for identifying potential targets for treatment (Mischel et al., 2003; Rao et al., 2003), for patient stratification in clinical studies (Shai et al., 2003), and for determining prognosis (Nutt et al., 2003).

Recently, inhibitors of oncogenes and signaling pathways have shown promising therapeutic potential in the treatment of several systemic cancers (Drucker, 2002). The prototypic targeted molecular agent is imatinib mesylate (Gleevec; Novartis, Basel, Switzerland), a small molecule inhibitor of the abl, c-kit, and PDFGR tyrosine kinases. It has demonstrated significant antitumor activity in chronic myelogenous leukemia (CML) by inhibiting the abl tyrosine kinase and in gastrointestinal stromal tumors (GIST) by inhibiting c-kit (Drucker, 2002). The success of imatinib in these tumors demonstrates the potential of these agents in tumors with well defined molecular targets. Although the complexity of the molecular abnormalities in malignant gliomas and the redundancy of the signaling pathways make it unlikely that single agents will achieve the same success as imatinib in CML, there has been significant interest in this approach (Karpati et al., 2003; Newton, 2004; Mischel, 2003). Over the past years, several of the first generation trials evaluating targeted molecular agents in malignant gliomas have reached maturity, and it is possible to draw some preliminary conclusions. In general, these agents have been well tolerated, but unfortunately only small subsets of patients have benefited.

Molecular therapeutic target	Name of agent
EGFR	Gefitinib (ZD1839; Iressa®)
EGFR	Erlotinib (OSI-774; Tarceva®)
EGFR+Erb-B2	Lapatinib (GW-572016)
EGFR+VEGFR	AEE788
EGFR + VEGFR + RET	Vandetanib (ZD6474; Zactima®)
EGFR	Nimotuzumab
EGFR	Cetuximab (C225; Erbitux®)
PDGFR	Nilotinib (Tasigna)
PDGFR	Imatinib mesylate (ST1571; Gleevec®)
PDGFR + VEGFR	Vatalanib (PTK787; ZK222584)
PDGFR + Src + c-kit + Bcr-Abl	Dasatinib (Sprycel®)
PDGFR + c-Kit + FLT-3	Tandutinib (MLN-518
PDGFR + VEGFR + c-Kit + FLT-3	Sunitinib (Sutent®)
PDGFR	CP-673, 451
PDGFR + VEGFR + c-kit + Raf	AMG706
PDGFR + VEGFR + c-kit	Pazopanib (GW-786034)
PDGFR + VEGFR + c-kit	SUO11248
PDGFR + VEGFR + c-Kit + Raf	Sorafenib (Bay43-9006; Nexavar®)
PDGFR + VEGFR	OSI-930
PDGFR + VEGFR	TKI258
VEGF-A/B Ab	Aflibercept (VEGF-Trap)
VEGFR + c-Met	XL184
VEGFR, PDGFR, c-Kit	Pazopanib (GW786034)
VEGFR + PDGFR + c-Kit	Cediranib (AZD2171; Recentin®)
VEGFR + PDGFR + c-Kit + Raf	Sorafenib (Bay43-9006; Nexavar®)
VEGFR + EGFR + Ret	Vandetanib (ZD6474; Zactima®)
VEGFR + PDGFR + c-kit	Vatalanib (PTK787; ZK222584)
VEGFR + PDGFR + c-Kit	Sunitinib (Sutent®)
VEGFR + EGFR	AEE788
VEGFR + PDGFR + c-kit	SUO11248
VEGFR + FGFR + PDGFR + c-kit	SU6668
VEGFR + PDGFR	OSI-930
VEGFR + PDGFR	TKI258
Raf + VEGFR + PDGFR	Sorafenib (Bay43-9006; Nexavar®)
Raf	Bay549805
Raf + PDGFR + VEGFR + c-kit	AMG706
Raf, VEGFR	AAL881
PKC-b2 + Akt	Enzastaurin
Akt	Perifosine
mTOR	Temsirolimus (CCI-779)
mTOR	Everolimus (RAD001; Certican®)
mTOR	Sirolimus (rapamycin; Rapimmune®)
mTOR	AP23573

Table 1. Selected small-molecule inhibitors with activity against signaling pathways relevant to cancer stem cells in malignant gliomas

5. Conclusion

The discovery that malignant tumors contain small subpopulations of cells that are highly tumorigenic and share features with normal stem cells has stimulated the field of cancer

research and established a novel concept in tumor biology – that most cancers, including glioblastomas, are driven by 'cancer stem cells' responsible for tumorigenesis and resistance to conventional therapies. Understanding the mechanisms and signaling pathways that govern cancer stem cells will be a key to identifying effective therapies to eventually improve tumor control and clinical outcome. A number of problems will have to be overcome in the development of effective therapies targeting cancer stem cells. Cancer stem cells are typically slowly cycling cells, express high-levels of drug-resistance genes and may not necessarily depend on oncogenes and their gene products targeted by small-molecule inhibitors. Further progress in glioma research will come from the molecular profiling of well-characterized cancer cell populations (e.g., after FACS analysis) and the identification of novel cellular markers that will provide the foundation to track cancer stem cells *in vitro* and *in vivo.* There is significant need to improve our ability to monitor treatment response with novel biomarkers so that patients who are resistant to therapy may be identified early in the treatment course. Moreover, novel biomarkers or surrogate markers of activity, and advances in molecular imaging in combination with tumor tissue analysis from patients enrolled into clinical trials will be important to evaluate treatment response and to understand treatment failure.

Several signaling pathways that orchestrate normal neural stem and progenitor cells are adopted by cancer stem cells and drive tumor cell proliferation, migration and treatment resistance.

Further elucidation of the molecular circuitry driving tumorigenesis and treatment resistance will be essential to advance our knowledge in glioma biology and to translate these concepts into the clinical arena. Both targeting the cancer stem cell compartment and individualizing patient treatment based on the unique signaling features in a given tumor have the greatest potential to translate into a successful treatment strategy.

While the first generation of molecular targeted therapies have shown promising results in preclinical studies, most agents have failed to translate into significant clinical benefit in early clinical trials. Preliminary clinical studies suggest that inhibition of a single pathway may not be sufficient to inhibit glioma growth in order to prolong patient survival (Pillay, et al., 2009). Therefore, targeting multiple pathways and signaling components in combined treatment approaches promises to be more successful.

However, given the increasing number of putative targets and agents, and the exponentially increasing number of potential combinations used in patients, it will be important to identify the most promising combinations and to carefully design and plan clinical trials. Recent genomic studies highlight the fact that gliomas are heterogeneous tumors. (Parsons,et al.,2008) Consequently, it will be important to integrate information derived from large genomic studies and combine it with our increasing understanding of mechanisms relevant to cancer stem cells in order to effectively treat brain tumor patients.

With recent efforts to individualize cancer treatment in patients, molecular targeted therapies directed to cancer stem cells and their signaling pathways will be increasingly used in the near future by clinicians and oncologists. An important issue of concern has come from recent studies on the cell-biological analysis of cancer therapy-associated neurotoxicity. Both radiation and cytotoxic therapies have been shown to be highly toxic to neural progenitor cell populations important for the maintenance of normal brain function, and may disrupt neurogenesis and white matter integrity (Dietrich, et al., 2006; Han, et al., 2008; Monje, 2002; Dietrich, et al., 2008). As multiple new molecular agents have been developed to specifically target signaling pathways employed by normal neural stem and

progenitor cells, serious neurotoxic adverse effects may be encountered in long-term survivors. Another concern has come from recent experimental studies demonstrating that molecular targeted therapies with antiangiogenic compounds may promote tumor cell migration and metastasis (Holash et al., 1999; Rubenstein et al., 2000; Loges et al., 2009; Ebos et al., 2009; Paez-Ribes et al., 2009).

Regardless, targeted molecular therapies hold great promise and will become an important component of combined treatment approaches in our effort to fight cancer.

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15

Regulation of Glioma Stem Cells by the Notch Signaling Pathway: Mechanisms and Therapeutic Implications

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1. Introduction

Glioma is the most common malignant tumor of the central nervous system (CNS) following with high fatality. A large body of evidence has been accumulated showing that a group of neural stem cell (NSC)-like tumor cells in glioma, or glioma stem cells (GSCs), play a critical role in the initiation and the progression of glioma. The Notch signaling pathway is a highly conserved pathway maintaining stem cell state and governing the differentiation of NSCs. Several research groups, including ours, have recently shown that Notch signaling is also critically involved in the maintenance of GSCs. Most impressively, blocking Notch signaling appears to result in the exhaustion of GSCs in animal models, leading to the proposition that the Notch signaling pathway might be a potential therapeutic target of glioma. In this review, we will briefly introduce the concepts of cancer stem cells (CSCs) and GSCs, followed by a summary of the cellular and molecular properties so far uncovered about GCSs. We will then discuss the major progresses about the roles and mechanisms of Notch signaling in the proliferation, differentiation, and apoptosis of GSCs, in addition to the function of Notch signaling in tumor microenvironment especially stem cell niches. Finally, we will prospect the application of Notch signaling as a therapeutic target of glioma, emphasizing its potential advantages and disadvantages.

2. Glioma stem cells (GSCs)

2.1 An overview of cancer stem cells

It is believed that the earliest hint of CSCs was proposed about 150 years ago by pathologists Rudolph Virchow and Julius Cohnheim, who discovered certain similarities between developing fetus and terotoma, such as that they both possess proliferation and differentiation abilities (Dick, 2009). However, solid evidence of CSCs has not been available until Dick and co-workers found that only rare cells in progressively diluted leukemia cells, which were harvested from patients, had the ability to reproduce leukemia in immunodeficiency mouse models. They named these cells as leukemia initiating cells (LICs)

that had greater differentiation potential and looked like stem cells in normal organs (Lapidot et al., 1994). Subsequently, CSCs have been isolated from many solid tumors, such as breast cancers (Al-Hajj et al., 2003), myelomas (Niederwieser et al., 2004), brain tumors (Singh et al., 2003, 2004), pancreatic (Meszoely et al., 2001) and prostate cancers (DL Hudson, 2004), among others. All these observations indicate that these stem-like cell populations may represent CSCs in cancer; they have strong potentials for self-renewal and multi-lineage differentiation and may responsible for tumor initiation and growth, invasion and metastasis, chemo- and radio-resistance. As a result, CSC theory was expected to bring promising innovations on finding new tumor therapeutic targets and improving therapeutic strategies.

2.2 Identification of GSCs

Glioma is believed to be derived from the glial tissue and is classified into astrocytoma (70%), oligodendroglioma (10% to 30%), mixed oligoastrocytoma and ependymoma (less than 10%). Low grade giloma, mostly astrocytomas (WHO grade II), are progressively transforming into malignant glioma, that is, anaplastic tumors (WHO grade III) and ultimately into glioblastoma multiforme (GBM; WHO grade IV). Because of their high heterogeneity and infiltrating nature, traditional surgery, unspecific and cytotoxic chemotherapy and radiotherapy can only impede glioma progression for a limited time. In addition, during treatment chemo- and radio-therapy resistance were often concomitant, followed by tumor relapse (Lino et al., 2010).

In 1920s, the famous neuropathologist Bailey postulated that tumor cells in the brain were mostly derived from the transformation of normal astrocytes, oligodendrocytes or neuronal precursors. However, this hypothesis was challenged following the isolation of NSCs by Reynolds and Weiss in 1992. Since then researchers started to speculate if malignant glioma results from the transformation of NSCs (Oliver& Wechsler-Reya, 2004).

The first evidence of cells with stem-like characteristics in malignant was reported by Ignatova et al., who isolated clonogenic, neurosphere-forming precursors from post-surgery specimens of human GBM. These stem-like cells expressed both neuronal and astroglial markers on differentiation, together with several key determinants of NSC fate (Ignatova et al., 2002). Later in 2003, Singh et al. also found a part of cancer cells from medulloblastoma can form neurospheres in vitro culture conditions, and some surface markers for NSCs, such as Nestin, Musashi-1, Bmi-1 and CD133, were also identified on these neurosphere cells. These neurosphere cells even possessed stronger capacities of proliferation, self-renewal and differentiation than neurosphere cells derived from normal NSCs. Corresponding to NSCs, these cancer cells were named as GSCs. GSCs could differentiate in culture into tumor cells that phenotypically resembled tumor cells from the patient, and also these cells can further differentiate into neuron or astrocytes under differentiation condition. These studies suggested that GCSs might originate from the transformation of NSCs (Singh et al., 2003). The further study by Singh et al. showed that injection of as few as 100 $CD133^+$ GSCs into immuno-compromised hosts produced tumors that could be serially transplanted and phenotypically resembled the patients' original tumors. In contrast, the injection of as many as 10^5 $CD133^-$ cells did not grow a tumor (Singh et al., 2004). At the same time, Kondo et al. successfully isolated side population (SP) from glioma cell line C6 by Hoechst 33342 staining, which shared similar prosperities as GSCs (Kondo et al., 2004). All these studies indicate that a minority of stem-like cells can represent GSCs in glioma.

2.3 The roles of GSCs in glioma

GSCs share similarities to normal NSCs in the brain, therefore possess the potential for self-renewal and multi-lineage differentiation. These population cells may thus play important roles in glioma initiation and growth, metastasis, drug resistance and disease relapse.

In contrast to Singh et al. finding that $CD133^-$ neurosphere cells did not recapitulate tumors in SCID mouse, Zheng et al. found that both $CD133^-$ and $CD133^+$ cells isolated from C6 glioma cell line could form neurospheres and show GSC features by neurosphere assay. This result suggested that $CD133^-$ cells could de-differentiate into stem cells or progenitor cells to generate tumorigenic clones again (Zheng et al., 2007). The observation was supported by two other independent groups: Beier et al. and Zhang et al. showed that $CD133^-$ tumor cells possessed apparent stem cell-like properties, but had distinct molecular profiles and growth characteristics from $CD133^+$ tumors in vitro and in vivo (Beier et al., 2007; Zhang et al, 2006). However, no matter with these controversial findings, these stem-like cells indeed exist and are involved in glioma initiation and recurrence.

Although the relationship between CSCs and metastasis has not been elucidated clearly, it has been demonstrated that the number of metastasis cancer colonies is correlated with the frequency of CSCs in primary tumors, and cancer cells undergoing epithelium-mesenchymal transitions (EMT) displayed with some stem-like cells properties (Mani et al., 2008). It is known that on one hand EMT is a differentiation switch between polarized epithelial cells and motile mesenchymal cells, and facilitates cell movements and generation of new tissues during embryogenensis. On another hand, EMT has been found to contribute to tumor invasion and vascular intravasation during cancer metastasis (Yang & Weinberg, 2008). The recent recognition of mesenchymal change in glioblastoma and its association with more aggressive clinical phenotypes suggest that mechanisms promote EMT in carcinoma may be of great clinical relevance in GBM. Recently Mikheeva et al. demonstrated that the transcription factor TWIST1 plays an important role in glioma invasion through activation of mesenchymal change, and suggesting its potential as a therapeutic target (Mikheeva et al., 2010). However, whether TWIST1 is involved in GBM metastasis by EMT process through regulating GSCs still needs to be further investigated.

Except the hallmark of "stemness" discussed above, it has been postulated that tumor stem-like cells may also possess a number of other properties associated with normal stem cells, including a slow proliferation rate, active DNA damage repair and antiapoptotic pathways, and the expression of multidrug transporters on the plasma membrane (Dean et al., 2005). Indeed, it has been shown that glioma cells exhibiting the side-population phenotype, which is characterized by cellular exclusion of the dye Hoechst 33342 primarily attributed to the ABCG2 multidrug transporter, are enriched in GSCs (Kondo et al., 2004). Furthermore, $CD133^+$ GSCs have been found to resist doses of radiation lethal to surrounding non-stem glioma cells in the tumor by preferential activation of the DNA damage response (Bao et al., 2006). The retention of such properties by GSCs suggests that, like normal stem cells, GSCs may be inherently resistant to many traditional anticancer therapies that target rapidly dividing cells. This represents a daunting therapeutic challenge because the characterization of the GSCs as the proliferative driving force in the tumor suggests that GSCs must be eradicated to permanently or significantly arrest tumor growth. As such, there is growing interest in developing therapeutic strategies specifically aimed at eliminating or affecting the GSCs population.

3. The Notch signaling pathway

3.1 Notch signaling

The *Notch* gene was first discovered by Thomas Morgan in 1914 in the fruit fly *Drosophila melanogaster*, with an adult phenotype consisting of "notches" at the wing margin. Genetic analysis of Notch loss-of-function mutations also revealed an embryonic phenotype with an expanded population of neuroblasts at the expense of epidermal cells (Artavanis-Tsakonas et al., 1995). In 1985, Artavanis-Tsakonas et al. successfully cloned the *Notch* gene, which encodes a large type I transmembrane receptor consisting of the extracellular domain (ECD) that containing 36 epidermal growth factor-like tandem repeats and 3 cysteine-rich LIN-12 repeats, a transmembrane domain (TMD), and the intracellular domain (NICD). Therein NICD is composed of a RAM (RBP-J association molecule) domain, nuclear localization signals (NLS), an ankyrin repeats (ANK), transactivation domain (TAD), and a praline/glutamate/serine/threonie-rich (PEST) region that is involved in protein degradation (Bray, 2006).

The Notch signaling pathway is evolutionarily highly conserved. The canonical Notch signaling pathway mainly comprises receptors, ligands, transcriptional complex components in the nucleus, and downstream genes, with a growing roster of regulatory molecules. In mammals, four Notch receptors (Notch1-4) are activated by five type I transmembrane ligands, three Delta-like (Dll1, Dll3 and Dll4) and two Serrate/Jagged (Jag1 and Jag2) receptors. When Notch is triggered by direct interaction with its ligands, the Dll/Jagged family proteins expressed on neighboring cells, NICD is released from the membrane after receptor cleavage executed by a γ-secretase-like protease. NICD translocates into nucleus and associates with RBP-J through its N-terminal RAM domain, and transactivates promoters harboring RBP-J-binding sites, which leads to expression of genes associated cell differentiation, such as the hairy/enhancer of Split (HES) family of basic helix-loop-helix (bHLH) transcription factors. Inhibitors of γ-secretase (GSIs) are widely used to block Notch signaling in vitro and in vivo. Moreover, in the absence of transactivators such as NICD, RBP-J suppresses transcription of promoters recognized by RBP-J (Bray, 2006). Although RBP-J has been generally accepted as the major effector of Notch pathway, a non-canonical RBP-J-independent and Deltex-dependent alternative pathway has also been reported in human and in *Drosophila* (Martinez Arias et al., 2002).

3.2 Modulators of Notch signaling

During Notch signal activation, NICD translocates into nucleus and transactivates downstream gene promoters harboring RBP-J-binding sites through recruiting co-activators such as GCN5, p300/CBP and the Mastermind-like (MAML) proteins (Moellering et al., 2009). In addition to NICD, RBP-J also mediates transactivation of Epstein Barr (EB) virus nuclear antigen (EBNA) 2 and therefore may play a role in the immortalization of cells infected by EB virus (Waltzer, 1994; Henkel, 1994). On another hand, in the absence of transactivators such as NICD or EBNA2, RBP-J suppresses transcription of promoters recognized by RBP-J (Dou et al., 1994). This is mostly attributable to multiple co-suppressors and/or adaptor molecules recruited by RBP-J. Kao et al. and Zhou & Hayward reported that SMRT/N-CoR interacts with RBP-J and suppresses the RBP-J-mediated transcription by competing with NICD and by recruiting histone deacetylases (HDACs), which renders the chromatin into an architecture that is inaccessible to the general transcriptional machinery (Kao, 1998; Zhou & Hayward, 2001). In addition, CIR (CBF1 interacting co-repressor),

another RBP-J-interacting protein, also suppresses the RBP-J-mediated transactivation by associating with HDAC2 and SAP30 (Hsieh et al., 1999). Recently, Oswald et al. and Kuroda et al. demonstrated that MINT (MSX2-interacting nuclear target protein), a nuclear matrix protein interacting with SMRT/N-CoR, also interacts with RBP-J and suppresses the RBP-J-mediated transactivation by competing for binding sites and by recruiting co-repressors (Oswald, 2002; Kuroda, 2003). Taniguchi et al. and Qin et al. subsequently demonstrated that a LIM domain protein KyoT2 can inhibit RBP-J-mediated Notch signal transactivation by competing with NICD, and by recruiting Polycomb suppression complex containing HPC2 and RING1 protein through the LIM domains (Taniguchi, 1998; Qin, 2004, 2005) (Fig.1).

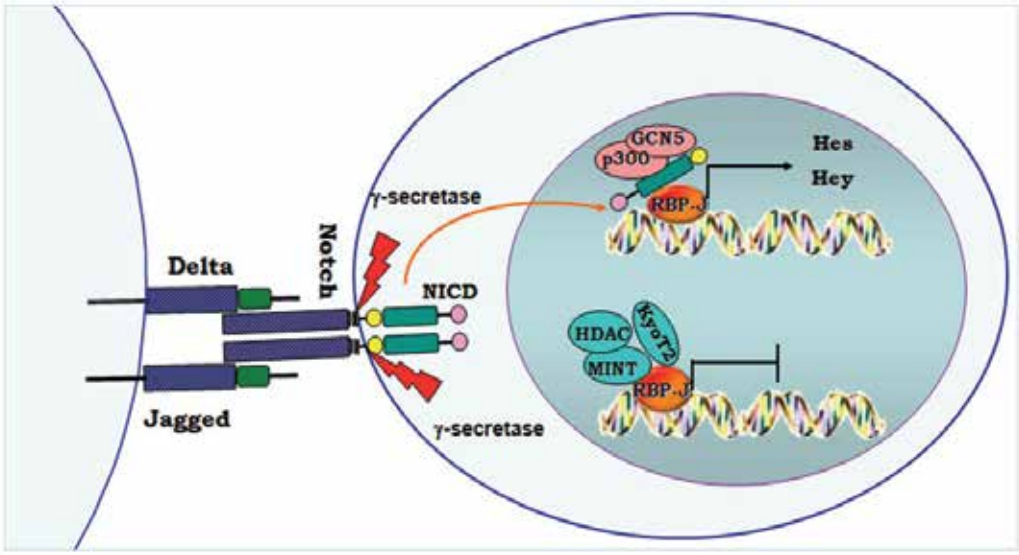

Fig. 1. The canonical Notch signaling pathway and its regulatory mechanisms

Productive Notch ligand-receptor binding depends on posttranslational modifications, such as glycosylation of receptors mediated by OFUT-1 and Fringe (Heines & Radtke, 2003). The half-life of Notch and DSL proteins on membrane is determined by the endocytosis of receptors and ligands, executed mainly by ubiquitin E3 ligase such as Deltex and Mindbomb, respectively (Kopan and Ilagan, 2009). In addition, the local distribution of Notch receptors on the cell membrane are controlled by some polarity proteins, for example, Numb and Crumbs, which result in region-specific Notch activity (Ilagan and Kopan, 2007). Taking all these observations together, Notch signal pathway should be regulated within a subtle and strict molecular network.

4. The roles of Notch signaling in the proliferation and differentiation of GSCs

4.1 Notch signaling in normal NSCs

Proliferation and differentiation of progenitor cells and stem cells during development are controlled by signals from other cells. The Notch signaling pathway plays a pivotal role in the regulation of the progenitor as well as stem cell differentiation by mediating cell-cell interaction, and is involved in multiple human diseases. During the development of

vertebrate CNS, NSCs in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus undergo self-renew and give rise to neurons and glia in a spatially and temporally defined manner (Temple,2001; Doetsch, 2003). NSCs, which possess the characteristics of radial glial cells (RGCs), can proliferate and self-renew through symmetric cell division (Alvarez-Buylla et al., 2001; Merkle and Alvarez-Buylla, 2006). As the development proceeds, some NSCs generate certain types of early-born neurons (e.g., Cajal-Retzius cells), while other NSCs differentiate into intermediate neural progenitors (INPs) through asymmetric cell division (Lillien, 1998). It has been demonstrated that the RBP-J-mediated Notch signaling plays an important role in the maintenance and the differentiation of NSCs into INPs and neuron/glial cells.

During early neurogenesis, activated Notch signaling has been shown to promote RGC identity (Gaiano et al., 2000). This was supported by Mizutani et al., who recently reported that Notch signaling determines the differentiation of NSCs into INPs through differential activation of RBP-J (Mizutani et al., 2007). Notch1 mutant mice have fewer NSCs and INPs, as shown by that the frequency of neurosphere formation is reduced when NSCs and INPs are cultured under the colony-forming condition (Hitoshi et al., 2002; Yoon et al., 2004). This is accompanied by precocious neuronal differentiation indicated by the precocious expression of proneural bHLH genes such as Mash1, Math1, and NeuroD (de la Pompa et al., 1997; Lutolf et al., 2002), and the neuron specific marker β-tubulin-III (Yang et al., 2004), and by the decreased expression of the NSC marker, nestin (Lutolf et al., 2002) as well. Mice deficient for other Notch pathway molecules also displayed similar phenotypes (Ishibashi et al., 1995; de la Pompa et al., 1997; Ohtsuka et al., 1999; Handler et al., 2000; Ohtsuka et al., 2001; Hatakeyama et al., 2004). With conditional RBP-J knockout mice, we have reported that RBP-J-mediated signaling might inhibit the differentiation of NSCs into INPs, while support the generation of certain early born neurons at early neurogenic stages, and the differentiation of neurons and glial cells at later neurogenic stages. This study suggested the RBP-J-mediated Notch signaling may regulate neuronal differentiation by the developmental stage-dependent way (Gao et al., 2009).

In addition, the Notch pathway is also crucial for many other binary cell fate decisions during CNS development (Louvi and Artavanis-Tsakonas, 2006). As Notch signaling inhibits NSCs from differentiating into neurons, Notch was also reported to inhibit neuronal while promote glial fate, and to promote the differentiation of glial progenitors into astrocytes versus oligodendrocytes (Tanigaki et al., 2001; Grandbarbe et al., 2003; Taylor et al., 2007). The regulation of binary cell fate decisions by Notch signaling pathway can be related to the pioneer observation on neuroblast and epidermal cell fate decision during *Drosophila* development (Heitzler and Simpson, 1991).

4.2 Notch signaling in GSCs

The many similarities in the growth characteristics and gene expression profiles between NSCs and GSCs suggest that similar signaling pathways should be required for their survival and growth. Notch signaling is known to promote the survival and proliferation of NSCs and to inhibit differentiation (Gaiano and Fishell, 2002). It is reasonable to assume that Notch signaling plays an important role in GSCs.

Aberrantly activated Notch signaling is involved in the generation and progression of many tumors (Radtke and Raj, 2003). Studies from Purow group showed that Notch1, Dll1 and Jagged1 were overexpressed in many glioma cell lines and primary human giloma. When

glioma cell lines were transfected with si-RNA against Notch1 and its ligands, proliferation inhibition and apoptosis induction were observed. This phenomenon was also observed in vivo with mice injected with si-Dll1 and si-Jag1-transfected glioma cells (Purow et al., 2005). However, it is worth to note that the expression pattern of Notch receptors and ligands are variable in different grade glioma, which may indicate that Notch signaling plays divergent roles in different type of glioma (Purow, 2005; Somasundaram, 2005; Phillips, 2006; Kanamori, 2007; Gao, 2007; Zhang, 2008; Hu, 2011).

Recently, further studies about Notch function in GSC maintenance have been published. By measuring HES1 and NICD2, Fan et al. found that Notch activation was elevated in the $CD133^+$ cell fraction in medulloblastoma cell lines. Inhibiting Notch activation by GSI resulted in diminished proliferation, increased neuronal differentiation, reduced $CD133^+$ cell fraction in vitro, and decreased tumorigenicity in vivo. Conversely, $CD133^+$ cell fraction was expanded by activating Notch2 signaling with constitutively activated NICD2 plasmid. In addition, they also found Notch blockade induced more apoptosis in nestin-positive medulloblastoma cells than that in better-differentiated cells lacking nestin. Their data indicate that Notch signaling is important in maintaining GSCs, and Notch signaling blockade can deplete GSCs that is required for in vivo glioma formation by suppressing proliferation and inducing apoptosis or differentiation (Fan et al., 2006).

We found that human glioma cell line SHG-44 stably transfected with NIC plasmid grew significantly faster and had higher colony- and sphere-forming abilities than the parental and control cell lines. These colonies expressed nestin, and could differentiate into three neural lineages, namely neurons, astrocytes or oligodendrocytes. This study indicated Notch signaling play a role in generation and/or maintenance of GSCs in human glioma (Zhang et al., 2008). In a latest study, Hu et al. further demonstrated Notch signaling maintained patient-derived GSCs by promoting their self renewal and inhibiting their differentiation into INP-like cells. Blocking Notch signaling by GSI in cultured tumor sphere can exhaust GSCs from glioma, although some tumor spheres display resistance to GSI treatment (Hu et al., 2011). Taken together, these studies strongly indicate that Notch signaling maintains GSC stemness in glioma as in normal NSCs.

4.3 Notch in stem cell niches

Stem cells reside within specific microenvironment with defined anatomical location termed "niches". SVZ and SGZ of the hippocampus region are the primary regions in which NSCs reside and support neruogenesis in the brain (Temple et al., 2001; Doetsch et al., 2003). Given the demonstrated similarities between NSCs and GSCs, it is reasonable to presume that GSCs may also, like NSCs, exist within a supportive niche. Through contact-mediated and paracrine signaling interactions between stem cells and the niche microenvironment, the niche maintains and controls critical stem cell properties and functions (Scadden et al., 2006).

The continued tumor growth is often associated with neovascularization (Gimbrone et al., 1972). Soluble factors secreted by tumor cells (such as vascular endothelial growth factor [VEGF]) induce angiogenesis, offering the necessary route for cell dissemination, changing vascular integrity and permeability, and even promoting intravasation and extravasation (Hashizume et al., 2000) . Many studies have shown that proangiogenic growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which would ostensibly be in higher concentration near blood

vessels, permit maintenance and expansion of GSCs in culture (Singh et al., 2003, 2004; Yuan et al., 2004; Kondo et al., 2004). Soluble factors (such as VEGF) secreted by vascular endothelial cells have been found to promote self renewal and to inhibit differentiation of NSCs, suggesting that NSCs have a vascular niche (Shen et al., 2004). This finding raises the possibility that GSCs may also rely on interaction with a vascular niche to maintain their stem-like properties, and consequently, their ability to drive tumor growth. Indeed, a very recent study provides compelling evidence that brain tumor stem-like cells are supported by a vascular niche (Calabrese et al, 2007). According to these studies, Folkins et al. combined both antiangiogenic and cytotoxic drugs to treat glioma, and found a significant reduction in sphere forming units (SFU) in tumor. Their work highlights the possibility that selective eradication of GSCs may be achieved by targeting the tumor microenvironment rather than the GSCs directly (Folkins et al, 2007).

Notch signaling pathway has been found to regulate the vascular formation. For example, haplo-insufficiency of Dll4 in mice results in embryonic lethality due to defective vascular development (Gale et al., 2004). Dll4 Expression had been shown to be up-regulated in tumor vessels of several human tumors and its expression correlates with VEGF level in clear cell-renal carcinoma (Malihos et al., 2001; Patel et al., 2005; Li et al., 2007). Pro-angiogenic factors VEGF has been shown to induce Notch1 expression in arterial endothelial cells, which in turn can promote angiogenesis (Liu et al., 2003). Further studies show that mutant mice with disrupted Notch signaling display various defects in blood vessel formation, and deletion of RBP-J can disturb the vascular homeostasis in mouse neural retina by affecting VEGFR1 and VEGFR2 (Dou et al., 2008; Phng & Gerhardt, 2009). In stem cells niches, cell adhesion seems another key factor for the exact location of stem cells. With Notch1, RBP-J and Hes1 deleted mouse models, some studies showed the adherence junctions between retinal progenitor cells (RPCs) in mouse retina are severely disturbed, indicating Notch function on the maintenance of adherence junctions (Jadhav et al., 2006; Zheng et al., 2009). Hypoxic tumor microenvironment plays a key role in the regulation of the GSC phenotype through hypoxia inducible factor 2α (HIF2α) and subsequent induction of specific GSC characteristic genes, including MAML-3, nuclear factor of activated T cells 2 (NFAT2) and aspartate beta-hydroxylase domain-containing protein 2 (ASPHD2) (Seidel et al., 2010). HIF2α has been demonstrated to activate Oct4 that can accelerate cell proliferation as an oncogene (Keith and Simon, 2007). In response to hypoxia, several components of the Notch signaling cascade, such as Notch1, Hes1, Hey1, Dll1 and Dll4, are induced (Poellinger and Lendahl, 2008). Based on those studies, we can speculate that HIF2α may transactivate Oct4 expression through regulating Notch signaling, and then stimulate GSCs proliferation and maintain GSCs niches. However, the interplay mechanism between Notch and hypoxia in glioma still needs to be explored.

5. Mechanisms of the regulation of GSCs by Notch signaling

5.1 Downstream effectors of Notch signaling

Hes1, 5, 7 and Hey1, 2, L have been confirmed as direct targets of Notch signaling that are involved in transcriptional repression, and regulate the expression and function of pro-neuronal bHLH proteins, such as Mash-1 and Hash-1 in mammalian and Ac-Sc in *Drosophila* (Kageyama et al, 2005). Moreover, accumulating evidence have defined other Notch targets such as GFAP, cyclin D1, p21, p53, which are regulated by RBP-J dependent Notch signal pathway (Stockhausen et al, 2010).

These downstream molecules of Notch signaling are abnormally regulated in different grades of glioma. Somasundaram et al. showed that Hash1 mRNA was elevated in grade II, III, and secondary GBM, whereas its expression was unchanged in primary GBM compared with normal brain tissue. However, Hes1 expression was elevated in primary GBM (Somasundaram et al., 2005). This study may indicate different activation status of Notch signaling existing in different types of glioma. Recently, a study from Hu et al. showed that Hes1, Hes5 and Glast mRNA level was significantly decreased in cultured patient-derived primary glioma spheres when Notch signaling was blocked by GSI, meanwhile Mash1 expression, a pronerual gene antagonized by Hes genes, was up-regulated. This suggests that Notch-RBP-J-Hes signaling axis may play a critical role in the proliferation and differentiation of GSCs, as its function in NSCs (Hu et al., 2011).

5.2 Crosstalk between Notch signaling and other signaling pathways

In the development of brain, EGFR is expressed in neurogenic regions such as SVZ (Weickert et al., 2000). Given the established Notch/EGFR interplay in several other tumor types (Fitzgerald et al., 2000; Weijzen et al., 2002; Miyamoto et al., 2003; Stockhausen et al., 2005), the high frequency of dysregulated EGFR activity in primary GBM (Tohma et al., 1998; Wong et al., 1987), and the important role of Notch signaling in neurogenesis (Shih & Holland, 2006), it is not surprising that the two pathways have cross-talk in glioma too.

A direct correlation between Notch and EGFR in glioma was provided by Purow et al. who showed that EGFR is under the transcriptional control of Notch signaling, and that EGFR and Notch1 mRNA co-existed and correlated significantly in high-grade astrocytomas (Purow et al., 2008). However, their results did not show the activation status of the Notch signaling, so whether Notch is able to drive the expression of EGFR needs further verify.

EGFR itself has been shown to induce TGFα expression, thus providing the autocrine loop for EGFR activity (Tang et al., 1997). TGFα was detected highly expressed in high grade glioma (Schlegel et al., 1990), and recently one study showed TGFα can up-regulate Hes1 expression and its nuclear translocation in glioma. The nuclear translocation of Hes1 was blocked by MEK1/2 inhibition, indicating that ERK1/2 activity is crucial for this process also (Zheng et al., 2008). However, in another brain tumor neuroblastoma, Hes1 up-regulation by TGFα induction was not dependent ERK1/2 activity (Stockhausen et al, 2005). Therefore, the exact cross-talk mechanism of EGFR-TGFα-Hes1 network seems to be more intriguing and needs further investigation.

In addition, the cross-talk between Notch signaling and EGFR signaling has been revealed in tumor angiogenesis (Zeng et al., 2005). VEGF promotes angiogenesis by stimulating Notch signaling. In glioblastoma cells EGFR transcriptionally up-regulates VEGF expression by PI3K signaling. However, inhibition of PI3K or EGFR did not completely abolish induction of VEGF mRNA by hypoxia, indicating that transcriptional regulation of the VEGF promoter by EGFR appears to involve other signals and to be distinct from signals induced by hypoxia (Maity et al., 2000). In fact, in solid tumor such as glioblastoma, although hypoxia elicits an angiogenic response, tumor vessels are often malformed and occlusions are frequent, as such intratumoral hypoxic areas remain. In this hypoxic environment, HIF1α and HIF2α are stabilized, and as a consequence, VEGF is up-regulated and participates in new blood vessels forming, and TGFα is subsequently up-regulated (Birlik et al., 2006). There is growing evidence that the cellular response to hypoxia and Notch signaling are intimately connected both in normal cells and cancer cells (Poellinger &

Lendahl, 2008). For example, Notch ligand Dll4 has been shown to be induced by hypoxia, and more specifically by HIF1α, VEGF and bFGF (Mailhos et al., 2002; Patel et al., 2005; Liu et al., 2003). Consistently, Dll4 expression in a glioma xenograft model significantly enhanced tumor growth accompanying with Hes1 mRNA elevation in host endothelial cells. Here the tumor displayed decreased vessel density and number, although the vessels were larger and better perfused. Recently, our lab also reported that general blockade of Notch signaling in tumor-bearing mice could lead to defective angiogenesis in tumors through inhibition of HIF activation (Hu et al., 2009). These observations indicate a potential cooperation network in glioma angiogenesis among EGFR, hypoxia, VEGF, and Dll4-Notch signaling pathway (Stockhausen et al., 2010).

EMT represents a molecular change on decreasing cell adhesion and acquiring tumor invasion. Together with transforming growth factor (TGFβ), Jagged1-Notch pathway activates Hey1 to trigger EMT of epithelial cells of human, murine and canine origins (Zavadil et al., 2004). The Jagged1-Notch-Snail2 cascade has also been shown to induce EMT in human breast tumor cells (Leong et al., 2007). One extracellular matrix glycoprotein tenascin-c (TNC), that induces proliferation and migration of neuronal precursors in neurogenic zones of embryonic and postnatal mouse brain, has been shown to be highly expressed in invasive GBM (Garcion et al., 2001; Nishio et al., 2005). The molecular mechanism through which Notch signaling induces TNC-dependent glioma cell motility is based on the trans-activation of the TNC promoter by RBP-J (Leins et al., 2003). Recently, a parallel study in childhood ependymomas has shown the association between tumor recurrence and frequent amplification of 9qter, precisely at the location of both Notch1 and TNC genes (Puget et al., 2009). However, the cross-talk between TGFβ signaling and Notch signaling during glioma EMT still needs to be explored.

6. Notch signaling as a therapeutic target

6.1 Inhibitors of Notch signaling

The observations described above suggest that blocking Notch pathway may be a promising strategy for glioma therapy. In fact, GSI MK0752 (Merck) as an inhibitor of Notch activation has recently been used in a phase I clinical trial of relapsed or refractory T-acute leukemia and lymphoma (T-ALL) (NCT00100152), and phase I breast cancer or others solid tumors (NCT00106145). A new clinical trial has just started for treating patients with recurrence or progressive GBM with GSI RO4929097 (NCT01122901).

However, as GSI affects all Notch receptors, it is difficult to distinguish the specific outcome on a particular cell type. In addition, GSI are not specific for Notch receptors and effects on other targets are to be expected. Moreover, unwanted side effects using GSI have been observed, for example gastrointestinal toxicity (Van Es et al., 2005). The shortcoming of GSI administration in patients may suggest that different targeting strategies or drug combinations are needed. When considering the cross-talk between Notch signaling and the RAS/MEK/ERK and PI3K/AKT pathways downstream of EGFR and their roles in experimental glioma, it is tempting to speculate that simultaneous inhibition of several pathways could lead to improved treatment of glioma patients. In addition, based on the specific role of Dll4-Notch1 in neovascularization, anti-Dll4 has been proposed as sharper therapeutic agents that can reduce tumor burden and prolong survival of the Dll4 expressing tumors, especially devoid of side effects (Ridgway et al., 2006).

6.2 Exhausting GSCs by inhibiting Notch signaling

Based on the observations that Notch signaling participates in regulating multiple aspects of GSCs, especially on their "stemness" maintenance, to exhaust GSCs by inhibiting Notch signaling seems a more exciting option. In 2006, Fan et al. reported to block Notch pathway by GSIs to reduce neurosphere growth and clonogenicity in vitro. The putative GSCs markers CD133, Nestin, Bmi, and Olig2 were reduced following Notch blockade. Using in vitro and in vivo assays, these authors demonstrated that Notch pathway blockade depletes stem-like cells in GBMs, suggesting that GSIs may be useful as chemotherapeutic reagents to target GSCs in malignant gliomas. Notch pathway inhibition appears to deplete stem-like cancer cells through reduced proliferation and increased apoptosis associated with decreased AKT and STAT3 phosphorylation (Fan et al., 2006, 2010). Further, Hu et al reported Notch signaling contributes to the maintenance of both normal NSCs and patient-derived GSCs. Inhibition of Notch signaling by GSI can dramatically decrease the number of secondary tumor spheres, and GSCs can be induced to differentiate into mature neural cell types in differentiation medium. However, GSI might not effect in the same way on different gliomas, especially at the early stage of therapy. Thus, this study suggested drug combination may be more useful for targeting GSCs and consequently exhausting GSCs (Hu et al., 2011).

Hoey et al. reported that the application of anti-human and anti-mouse Dll4 can impede tumor proliferation and neovasculogenesis, and that anti-human Dll4 reduces cancer stem cell frequency (Hoey et al., 2009). Zhen et al. investigated the ability of As(2)O(3) to inhibit the formation of tumor in three different glioma cell lines. Their results revealed the negative regulation of GSCs by As(2)O(3). In addition, Western blot analysis revealed decreased levels of Notch1 and Hes1 proteins due to As(2)O(3) treatment. The authors concluded that As(2)O(3) has a remarkable inhibitory effect on GSCs in glioma cell lines in vivo and in vitro; in addition, they determined that the mechanism of GSC inhibition involves the down-regulation of Notch activation (Zhen et al., 2010).

Radiotherapy represents the most effective non-surgical treatments for gliomas. However, gliomas are highly radioresistant and recurrence is nearly universal. Inhibition of Notch pathway with GSI rendered GSCs more sensitive to radiation at clinically relevant doses. In a study by Wang et al., GSI enhanced radiation-induced cell death and impaired clonogenic survival of GSCs, but not non-stem glioma cells. Moreover, knockdown of Notch1 or Notch2 sensitized GSCs to radiation and impaired xenograft tumor formation (Wang et al., 2010). This indicated that the Notch signaling plays a critical role in the regulation of radioresistance of GSCs, and demonstrated that inhibition of Notch signaling holds promise to improve the efficiency of current radiotherapy in glioma treatment.

It has been commonly recognized that CSCs contributes to the tumor angiogenesis. It is found that GSCs produce much higher levels of VEGF in both normoxic and hypoxic conditions than the non-GSC population, and this GSC-mediated VEGF production leads to enhanced endothelial cell migration and tube formation in vitro (Bao et al., 2006). A VEGF-overexpression glioma model has recently provided supportive evidence for this as well by showing that glioblastoma GSCs overexpressing VEGF produce larger, more vascular, and highly hemorrhagic tumors (Oka et al., 2007). When applying the humanized monoclonal anti-VEGF antibody bevacizumab (Avastin) to cultured GSCs, the in vitro endothelial migration and vascular formation were blocked. Moreover, in vivo administration of bevacizumab potently inhibited the growth, vascularity, and hemorrhage of xenografts derived from GSCs, whereas no effects were seen on xenografts from non-GSCs (Bao et al.,

2006). However, drug resistance and recurrence of tumors has been observed, even treatment was initially effective (Bergers & Hnanhan, 2008). This is most likely due to the activation of pro-angiogenic pathways other than VEGF, especially Dll4-Notch signaling involved in tumor angiogenesis (Li et al., 2007). This suggested that targeting Notch by soluble Dll4 plus anti-VEGF antibody in GSCs would result in improved glioma treatment outcome. Moreover, the latest report from Ying et al showed that glioblastoma stem-like cells were depleted with all-trans retinoic-acid (RA) treatment, accompanied with down-regulation Notch signaling pathway targets, such as Hes5, Hey1 and Hey2. These data indicate that Notch pathway downregulation mediates RA effects on stem cell pool loss. However, this paper does not observe the combinatory inhibition effect from GSI blockade of Notch signaling and RA downregulation of Notch signaling, and the detailed mechanism of targeting GBM-stem cells by drug combination still need to be further elucidated (Ying et al., 2011).

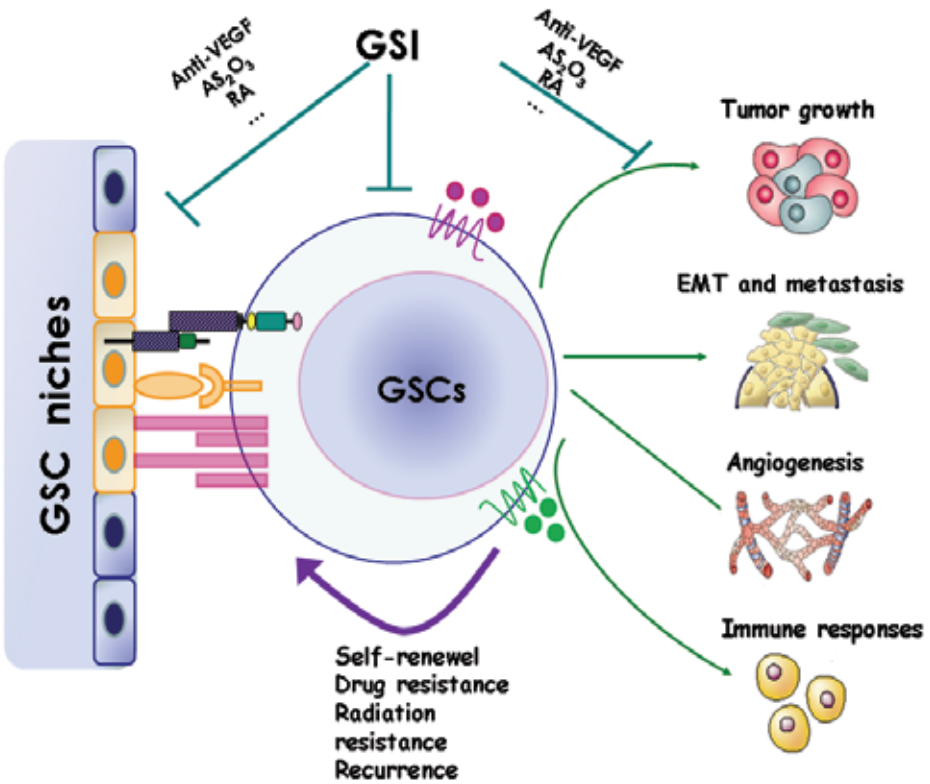

Fig. 2. Roles of Notch signaling in GSCs, and the potential therapeutic strategies targeting Notch signal with GSI in combination with the other drugs, such as anti-VEGF, AS_2O_3, RA, and so on

7. Conclusion

Glioma is the most prevalent and the most aggressive brain tumor resistant to conventional therapies, and as a result, the frequency of recurrence after treatment is definitely very high.

Notch signaling has recently been shown to be responsible for GSCs proliferation, apoptosis inhibition and invasion, specifically for GSCs maintenance, metastasis and chemo-and radio-therapy resistance. To target Notch signaling in GSCs holds a promising treatment strategy against glioma. Although GSI has been applied in clinical trials, considering glioma heterogenicity and tumor microenvironment, combination of Notch signal inhibitor and other therapies will hopefully provide promising ways to improve patient outcome in future (Fig. 2).

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9. References

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Part 4

Signaling Pathways and Markers for Cancer Stem Cells

16

The Hedgehog Signaling Network and the Development of Gastric Cancer

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1. Introduction

The Correa model of gastric cancer reported that atrophy (parietal cell loss) was one of several significant changes that occurred after chronic inflammation (Correa et al., 1975). We now understand that the major cause of chronic inflammation in the normal, acid-secreting stomach is *Helicobacter pylori (H. pylori)* bacterial colonization. It is widely accepted that inflammation that is caused by *H. pylori* infection is a trigger for the development of gastric cancer. An explanation for the causal role of *H. pylori* infection in the pathogenesis of gastric cancer has been described by disruption of differentiation of epithelia as a consequence of elevated pro-inflammatory cytokines such as IFNγ, TNFα and IL-1β (Moss et al., 1994; Padol IT, 2004; Sawai et al., 1999; Smythies et al., 2000; Zavros et al., 2003). However, the question of the mechanism by which inflammatory cytokines induce mucosal damage remains unanswered. Since stomach secretes numerous factors such as TGFβ, Wnt, FGFs and Hedgehog proteins that are known to be responsible for the differentiation of the gastric epithelium, one favored explanation linking inflammation and progression to cancer is due to the loss of these factors (reviewed in (Katoh Y, 2006)). During the progression from inflammation to metaplasia and cancer the cell composition of the stomach changes. In particular, loss of the acid-secreting parietal cells (atrophy) leads to alterations in the cell lineages with the expansion of metaplastic mucous cells.

Emerging evidence shows that Sonic Hedgehog (Shh) signaling is expressed in acid-secreting parietal cells within the adult stomach (van den Brink et al., 2002; Zavros et al., 2008). Since studies have suggested that Shh acts as a morphogen in the adult stomach (Shiotani et al., 2005a; van den Brink et al., 2002), an important hypothesis is that loss of Shh expression during gastric inflammation results in the disruption of epithelial cell differentiation and function leading to cancer. During *H. pylori* infection, the site of chronic inflammation coincides with the secretion of IFNγ and the engraftment of bone marrow-derived mesenchymal stem cells (BM-MSCs) whose progeny populate gastric tumors (Houghton et al., 2004). The mechanism that regulates BM-MSC proliferation and cellular engraftment with host cells during chronic inflammation is unknown.

The permanent engraftment of the BM-MSCs in an area of an IFNγ-rich and abnormal tissue environment results in differentiation of these cells through stages of metaplasia and dysplasia (Li et al., 2006). For this reason these cells behave much like cancer stem cells whereby they have acquired the ability to self-renew and become incorporated into the developing tumor (Li et al., 2006). The mechanism that regulates BM-MSC proliferation and cellular engraftment with host cells during chronic inflammation is unknown, but based on our studies these cells secrete Shh that is responsible for the proliferation and thus may contribute to the differentiation into tumors. In pathological conditions in which immune cells have been implicated, the Hedgehog signaling pathway mediates IFNγ-induced tumor development (Stewart et al., 2003; Wang et al., 2003; Zavros et al., 2005). Given that chronic gastritis is associated with elevated IFNγ expression and the development of cancer (Zavros et al., 2005) a similar mechanism may be occurring in the stomach. *The current chapter focuses on the Shh signaling pathway and its role in the development of gastric cancer, specifically in response to Helicobacter pylori infection. In particular, the chapter presents a comprehensive discussion of the role of the Hedgehog signaling network as a regulatory mechanism within the BM-MSC compartment during the development of gastric cancer.*

2. Role of Shh as a regulator of gastric tissue homeostasis and disease

2.1 Discovery, processing and signaling

Using a saturation mutagenesis screen performed to study the effect of mutations on the patterning of segmented *Drosophila* embryos, Nüsslein-Volhard and Wieschaus first discovered Hedgehog (Nüsslein-Volhard & Wieschaus, 1980). As a result of the mutagenesis screen, Nüsslein-Volhard & Wieschaus identified a group of *Drosophila* mutants that remained covered entirely with denticles (Nüsslein-Volhard & Wieschaus, 1980). The inspiration for the name Hedgehog came from the "spiny" phenotype of the embryos, which resembled a hedgehog. Since the identification of the Hedgehog mutant, three vertebrate Hedgehog homologs have been identified that include Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Of the Hedgehog homologs, Shh has been the most studied in terms of the Hedgehog signaling pathway in vertebrates and in particular gastric function and disease.

In *Drosophila* or zebrafish models (Porter et al., 1995), Shh is synthesized as a 45-kDa precursor protein. The full-length protein subsequently undergoes an autocatalytic cleavage to yield a 26-kDa carboxy-terminal fragment and a 19-kDa amino-terminal fragment (ShhN). ShhC is responsible for catalyzing cleavage of the 45-kDa precursor protein while ShhN is the active signaling fragment. Concomitant with cleavage, ShhC acts as a cholesterol transferase covalently linking a cholesterol moiety to the carboxy-terminus of the 19-kDa fragment (ShhN) (Goetz et al., 2006). The 19-kDa fragment (ShhN) is further modified by a membrane bound O-acyltransferase commonly known as Skinny hedgehog (Ski), which covalently links a molecule of palmitate to the 19- kDa fragment (ShhNp) (Mann et al., 2004; Torroja et al., 2005). The phenotypes of *Drosophila* lacking Ski resemble those of *Drosophila* with Shh knocked out and thus demonstrating the importance of palmitoylation for Shh signaling (Chamoun et al., 2001; Pepinsky et al., 1998). ShhNp can remain anchored to the cell membrane or form secreted, soluble and freely diffusible multimeric units (Goetz et al., 2006). Both the cell-retained and secreted Shh protein fragments are able to activate hedgehog signaling through the hedgehog receptor Ptch (Goetz et al., 2006) (Figure 1A). However, recently it is reported that the full-length precursor Shh protein may also bind to

Ptch and exhibits biological activity (Tokhunts et al., 2009). In an in vivo assay using the developing chick neural tube, full-length Shh induced activation of Shh-dependent *luciferase* reporter gene (Tokhunts et al., 2009). Such findings are relevant when considering the biological activity of Shh with regards to gastric cancer. Prior studies using xenografts of human gastric cancer cell lines show that Hedgehog signaling is required for cancer cell growth. It has been assumed that the Shh ligand mediating the activation was the processed ShhN protein, but the form of Shh produced by the xenografts was not evaluated directly in these studies (Berman et al., 2003). In another study using human gastric tumor samples it was observed that the major form of the Shh ligand present is the 45-kDa peptide (Zavros et al., 2007). However, it remains to be determined whether full-length Shh has any biologic activity and acts as a regulator of tumorigenesis that differs from the processed 19-kDa form.

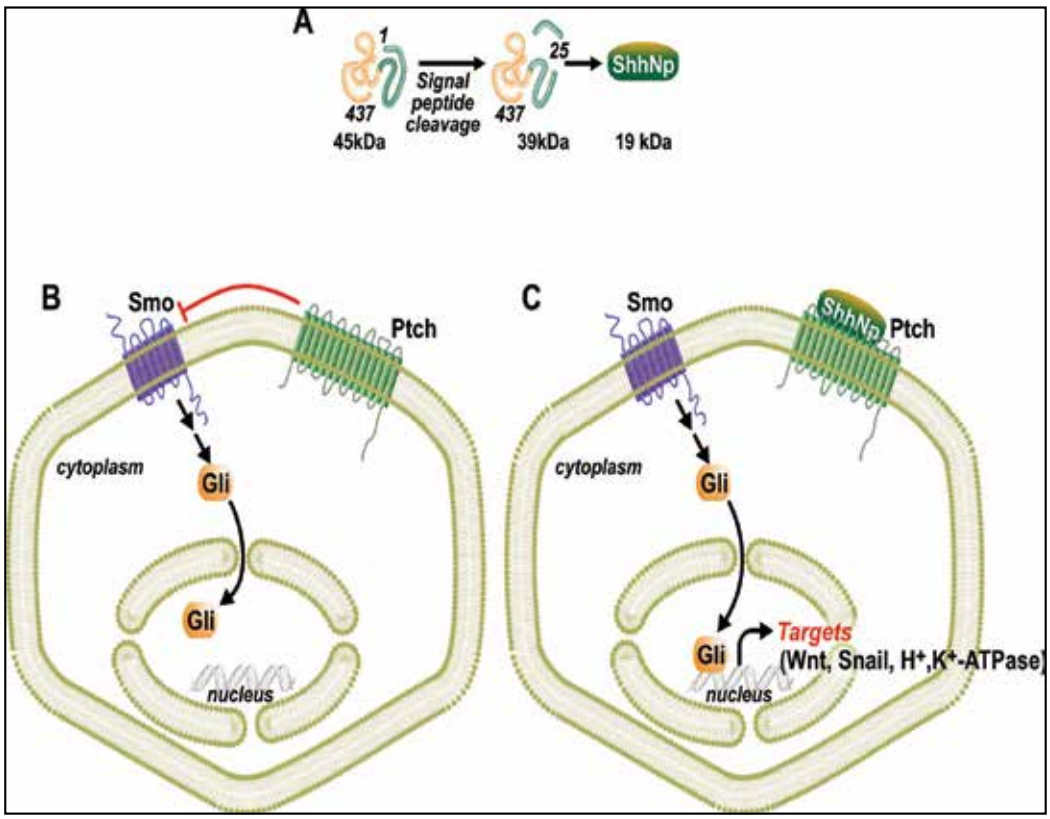

Fig. 1. Schematic diagram of Shh processing and signaling. (A) The cleavage of the 45-kDa full-length precursor generates the signal peptide (39kDa). Autocatalytic or protease-dependent cleavage yields a secreted 19-kDa fragment and a 26-KDa cholesterol modified cell bound protein (ShhNp). (B) In the absence of Shh ligand or unstimulated cells, the activity of the transmembrane protein Smo is suppressed by the Hedgehog receptor Ptch. (C) Binding of Shh to Ptch results in the removal of the inhibitory restraint of Ptch on Smo, consequently activating Smo. Smo then transduces the Shh signal into the cytoplasm. Transduction of the Shh signal into the cytoplasm leads to activation of the Glioblastoma (Gli) family of transcription factors and activation of downstream targets

Shh processing within the gastric mucosa appears to have diverged from the autocatalytic processing originally reported in *Drosophila* and zebrafish models (Porter et al., 1995; Zavros et al., 2007; Zavros et al., 2008). In the mammalian stomach, Shh processing is hormonally regulated and acid dependent (Zavros et al., 2007; Zavros et al., 2008). Changes in acid secretion, that is stimulated by both histamine and gastrin, induces Shh expression and processing (Zavros et al., 2007; Zavros et al., 2008). In particular, intracellular calcium release and protein kinase C activation stimulate Shh gene expression during gastric acid secretion (El-Zaatari et al., 2010). Subsequently, within the acidic environment pepsin A cleaves the 45-kDa precursor protein into the biologically active 19-kDa protein (Zavros et al., 2007). These studies indicate that although autocatalytic processing of Shh may occur in the gastric mucosa, processing of the 45-kDa Shh precursor may predominantly require acidic conditions and the acid-activated protease pepsin A.

Shh signaling in vertebrates is mediated by the seven-span transmembrane receptor Smoothened (Smo) (Goodrich et al., 1996; Taipale et al., 2002). Shh indirectly controls the activity of Smo through binding to the Patched (Ptch) receptor (Goodrich et al., 1996; Taipale et al., 2002). Ptch is a twelve-span transmembrane receptor that catalytically inhibits signaling through Smo in the absence of Hedgehog (Goodrich et al., 1996; Taipale et al., 2002) (Figure 1B). Binding of Hedgehog to Ptch relieves the inhibitory effect of Ptch on Smo and consequently activates Smo (Goodrich et al., 1996; Taipale et al., 2002) (Figure 1C). Transduction of the Hedgehog signal into the cytoplasm leads to activation of the Glioblastoma (Gli) family of transcription factors and target genes that are known to regulate cell cycle, proliferation and differentiation (Hui CC, 1994).

2.2 The role of Shh as a regulator of gastric tissue homeostasis

Evidence for the crucial role of Shh as a regulator of gastrointestinal development comes from the Shh null mouse models ($Shh^{-/-}$ mice), whereby Shh-/- mouse stomachs exhibit an intestinal rather than gastric-type mucosa (Kim et al., 2005; Ramalho-Santos et al., 2000). It is only recently that the direct role of Shh in the adult stomach has been investigated (Waghray et al., 2010; Xiao et al., 2010; Zavros et al., 2008). Shh is believed to regulate epithelial cell differentiation, but its role as a morphogen is based on evidence that correlates the loss of Shh with inflammation of the adult stomach (Shiotani et al., 2005a; Suzuki et al., 2005; van den Brink et al., 2002). In the absence of inflammation, the direct contribution of reduced Shh expression to the disruption of epithelial cell differentiation and cancer progression had never been tested. We have made significant contributions that have advanced the current understanding of not only the role of Hedgehog in the adult stomach, but also the mechanism regulating Shh secretion (Xiao et al., 2010; Zavros et al., 2008). Our laboratory is responsible for discovering that in the mammalian system, Shh secretion from parietal cells is acid- and hormonally-regulated (Zavros, 2007; Zavros et al., 2008). Moreover, we show that Shh from this acid-secreting single cell type has significant biological activity, regulating the differentiation of cell lineages and controlling gastric physiological function (Xiao et al., 2010).

The development of a mouse model expressing a parietal cell-specific deletion of Shh (HKCre/Shh^{KO} mice) has allowed us to assay changes in gastric epithelial cell differentiation and function in the adult stomach (Xiao et al., 2010). The HKCre/Shh^{KO} mouse demonstrated an age-dependent increase in the number of surface pit mucous cells. The surface mucous cell expansion that was observed in the HKCre/Shh^{KO} mice was reminiscent of foveolar hyperplasia observed in the over-expressing TGFα transgenic mice

(Bockman et al., 1995; Goldenring et al., 1996; Nomura S, 2005) and in patients with Menetrier's disease (Larsen et al., 1987; Wolfsen et al., 1993). However, unlike Menetrier's disease and *H. pylori* infected patients, the HKCre/Shh^{KO} mouse model did not develop loss of parietal cells (atrophy). Given that HKCre/Shh^{KO} mice lacked inflammation, this suggests a requirement for additional factors, such as inflammatory cytokines, for parietal cell atrophy to occur. The overproduction of surface mucous cells often occurs at the expense of other cell lineages such as the zymogen cells (Bockman et al., 1995; Goldenring et al., 1996). Consistent with this notion, we observed that the HKCre/Shh^{KO} mice also had delayed differentiation of the zymogen cell lineage from the mucous neck cells in the stomachs of HKCre/Shh^{KO} mice (Xiao et al., 2010). Although we have acquired new knowledge of Hedgehog signaling and gastric differentiation and function, the HKCre/Shh^{KO} mice have experimental limitations.

The HKCre/Shh^{KO} mouse is a constitutive model of parietal cell-expressed Shh. Thus, Shh is deleted during development when the H^+,K^+-ATPase is expressed and is not re-expressed in the gastric parietal cell. In mice, this means Shh would be deleted on embryonic day 19 when H+,K+-ATPase has developed and is expressed within parietal cells (Pettitt et al., 1992). We have created a complimentary experimental approach by developing an advanced mouse model expressing a tamoxifen-inducible parietal cell-specific deletion of Shh (HKCre^{ERT2}/Shh^{KO}). There are two major advantages to using the inducible HKCre^{ERT2}/Shh^{KO} mice for the proposed studies and these include: 1) the inducible model will give us the ability to identify the role of Shh signaling in the adult stomach in a fully differentiated epithelium, and 2) the inducible HKCre^{ERT2}/Shh^{KO} mice is an approach that will allow us to assay changes in epithelial cell differentiation and function in relation to the loss and gain of Shh expression, independent of inflammation. Clinically this is important given that re-expression of Shh in *H. pylori*-infected patients after eradication of bacterial infection results in regeneration of the gastric epithelium and ulcer healing (Kang et al., 2009; Shiotani et al., 2005a; Suzuki et al., 2005).

Observations made in the HKCre/Shh^{KO} mice have allowed us to formulate hypotheses explaining the role of Hedgehog signaling in the stomach. The HKCre/Shh^{KO} mice lacked the ability to secrete acid in response to histamine that was accompanied by severe hypergastrinemia and decreased somatostatin expression (Xiao et al., 2010). Hypergastrinemia was associated with increased Indian Hedgehog (Ihh) consistent with observations made in human stomach where Ihh was predominantly expressed in the pit cells where it induces pit cell differentiation in primary mouse gastric cells (Fukaya et al., 2006). Thus, the phenotype observed with loss of Shh may be attributed to increases in circulating gastrin concentrations due to loss of somatostatin. Besides the proposed role as a morphogen for the gastric epithelium, Shh may also be a fundamental regulator of the gastrin-gastric acid negative feedback mechanism.

Loss of Shh is accompanied by increased Ihh gene expression in the surface pit epithelium (Xiao et al., 2010). As shown in Figure 1, binding of Hedgehog ligand to its receptor Ptch results in removal of the inhibition of Ptch on Smo, and this removal of the inhibition on Smo subsequently results in the activation of the Gli-family of Hedgehog transcription factors. Evidence from Gli1 pathway studies in rat kidney epithelial cells (RK3E) show that Gli1 induces the transcription of the zinc-finger transcription factor, Snail (Li et al., 2006). Snail inhibits transcription of E-cadherin, an integral cell-adhesion protein known to associate with β-catenin at the cell membrane. Suppression of E-cadherin expression is implicated with increased nuclear β-catenin and activation of Wnt pathway targets such as

CD44, MMP-7, c-Myc and Cyclin D1 that have been associated with the progression of gastric cancer (Tanaka M, 2002). In vitro data shows that the Hedgehog signaling pathway is a key regulator of β-catenin (Li et al., 2007), but whether Shh maintains the differentiated phenotype of the stomach by mediating Wnt pathway activation is unknown. Collectively, loss of Shh triggers a number of molecular events, including increased Snail and loss of E cadherin expression, translocation of β catenin and activation of the Wnt pathway, that are consistent with epithelial-to-mesenchymal transition (EMT) of gastric epithelial cells (Li et al., 2006).

Evidence from the HKCre/Shh^{KO} mice demonstrated that loss of Shh triggers epithelial changes consistent with EMT, that included increased Snail accompanied by loss of E cadherin expression (Xiao et al., 2010). While bringing to light that loss of Shh in the stomach contributes to the development of EMT, we turned our attention to tight junctions as a marker of epithelial integrity. In the stomach, the expression pattern of tight-junction scaffolding protein ZO-1 determines epithelial cell organization, differentiation and function, in particular the zymogen cell lineage (Zhu et al., 2009). Disruption of the tight-junction complex is characteristic of a number of diseases including *H. pylori* gastritis (Amieva et al., 2003; Krueger S, 2007). Evidence collected from studies using primary mouse epithelial cell cultures over-expressing Snail demonstrates that Snail directly represses gene expression of claudins/occludin (Ikenouchi et al., 2003). Snail also causes translocation of ZO-1 from the membrane to the cytoplasm in the same isolated mouse epithelial cell cultures (Ikenouchi et al., 2003). In addition, the HKCre/Shh^{KO} mice develop severe hypergastrinemia (Xiao et al., 2010). Besides the contribution of Snail to the disruption of tight-junctions in the stomach, the hypergastrinemia induced in the HKCre/Shh^{KO} mice may also explain the disrupted ZO-1 expression. In support of this notion, progastrin causes the dissociation of tight-junctions by delocalizing ZO-1 and occludin from the membrane to the cytoplasm in IMGE-5 cells (Hollande et al., 2003). Collectively, these studies support that deletion EMT of the gastric epithelium contributes to the dissociation of tight-junction protein ZO-1 and warrants further investigation.

Aside from its role as a regulator of gastric epithelial cell differentiation, Shh may also act to regulate the physiological secretion of acid from the parietal cells. In response to EGF, parietal cells express Shh which positively regulates the expression of the H^+,K^+-ATPase (Stepan et al., 2005). Emerging studies using mouse models in which Shh signaling or expression have been pharmacologically or genetically inhibited suggest that Shh may directly and/or indirectly act as a regulator of the gastrin-gastric acid negative feedback mechanism regulating acid secretion (El-Zaatari et al., 2008; El-Zaatari et al., 2007; El-Zaatari et al., 2010; Xiao et al., 2010). Treatment of mice with cyclopamine, an inhibitor of Hedgehog signaling receptor Smo, results in elevated circulating gastrin levels (El-Zaatari et al., 2008). Loss of Shh may impair acid secretion by decreasing the activity or expression of parietal cell H^+-K^+-ATPase. The reduction in acid secretion would reduce somatostatin release from D-cells of the stomach thus removing the somatostatin-inhibitory effect on gastrin secretion. In support of this hypothesis, we observed that the lack of acid secretion in the HKCre/Shh^{KO} mice was accompanied by significant hypergastrinemia. Treatment of HKCre/Shh^{KO} mice, with the somatostatin analogue octreotide, significantly suppressed hypergastrinemia and subsequently restored differentiation of the zymogen cell lineage and parietal cell function (Xiao et al., 2010). Given that gastrin promotes the growth of gastric adenocarcinomas, the role of Shh as a regulator of gastrin and somatostatin secretion has important implications for the study of gastric cancer.

2.3 Decreased Shh expression during *Helicobacter pylori* infection

Helicobacter pylori (H. pylori) colonizes the stomachs of half the world's population (Bergman et al., 2005). Chronic inflammation caused by persistent *H. pylori* infection is the most consistent lesion that causes the development of gastric cancer (Correa et al., 1975; Correa P, 2007). The gastric mucosal changes of *H. pylori* infection begin with chronic inflammation followed by hyperproliferation, parietal cell atrophy, and metaplastic cell lineage changes including spasmolytic polypeptide-expressing metaplasia (SPEM), intestinal metaplasia and antralization of glands that then proceeds with dysplasia and eventually cancer (Correa P, 2007) (Goldenring JR, 2006). Loss of mature parietal cells from the gastric glands of the stomach plays a central role in the progression of these gastric alterations. Atrophy leads to alterations in the cell lineages with the expansion of metaplastic mucous cells. Since stomach secretes numerous factors such as TGFβ, Wnt, FGFs and including Hedgehog proteins that are responsible for the differentiation of the gastric epithelium, one favored explanation linking inflammation and progression to cancer is due to the loss of Hedgehog as result parietal cell atrophy (reviewed in (Katoh & Katoh, 2006)). In conditions such as gastric atrophy and intestinal metaplasia, where normal gastric morphogenesis is lost, Shh is reduced or absent (Dimmler et al., 2003; Shiotani et al., 2005a; Shiotani et al., 2005b; Suzuki et al., 2005; van den Brink et al., 2002; Van Den Brink et al., 2001). In support of this, in Mongolian gerbils infected with *H. pylori* loss of Shh expression correlates with loss of parietal cells, impaired maturation of the zymogenic chief cells in gastric glands, and intestinal metaplasia (Suzuki et al., 2005). Therefore, loss of Shh signaling may address the impairment of chief cell differentiation and the development of intestinal metaplasia found in late stage *H. pylori* associated gastritis.

It is only until recently that the mechanism responsible for the loss of Shh expression during *H. pylori* infection has been elucidated in vivo (Minegishi Y, 2007; Waghray et al., 2010). As reviewed, acid secretion plays an important role in maintaining Shh expression and secretion in the adult stomach (Minegishi Y, 2007; Waghray et al., 2010; Zavros, 2007; Zavros et al., 2008). Experiments using models of parietal cell dysfunction such as the histamine H(2) receptor-knockout mice in vivo (Minegishi Y, 2007) and isolated rabbit gastric glands and canine parietal cells treated with H^+,K^+-ATPase blocker omeprazole (Zavros, 2007; Zavros et al., 2008), demonstrate that in the absence of acid secretion Shh expression is significantly reduced. Thus hypoacidity would induce the loss of Shh typically found in *H. pylori* infection. However, another group of potential candidates that may inhibit Shh expression are the inflammatory cytokines released in response to *H. pylori* colonization. For example, exogenous infusion of interferon-γ (IFN-γ) alone is sufficient to induce hypergastrinemia and metaplasia in mice, but very little is known about the regulation of Shh by pro-inflammatory cytokines (Zavros et al., 2003). Alternatively, IL-1β correlates with gastric atrophy and gastric cancer (El-Omar et al., 2000; El-Omar et al., 2001) and is a potent inhibitor of gastric acid secretion (El-Omar et al., 2003) making this cytokine also a strong candidate for the causal role of Shh expression. A recent study using Shh-LacZ reporter mice demonstrates that IL-1β produced during *Helicobacter* infection inhibited gastric acid and subsequently Shh expression through IL-1 receptor activation (Waghray et al., 2010). The investigators concluded from this study that proinflammatory cytokine IL-1β reduces Shh expression and function in the gastric mucosa by reducing acid secretion from parietal cells (Waghray et al., 2010). Since Shh induces H^+,K^+-ATPase gene expression in isolated canine parietal cells (Stepan et al., 2005), the investigators rationalized that chronically suppressed

levels of Shh may eventually reduce enzyme expression that is sufficient to induce gastric atrophy and thus, inhibit Shh expression in parietal cells (Waghray et al., 2010). Our study using the HKCre/Shh^{KO} mouse model demonstrates that in the absence of inflammation, although Shh induces foveolar hyperplasia and hypochlorhydria, this was not sufficient to induce atrophy (Xiao et al., 2010). Therefore, there may be a requirement for additional factors, such as inflammatory cytokines, for parietal cell atrophy to develop.

3. Over-expression of sonic hedgehog signaling in gastrointestinal cancers: The role of Shh within the tumor microenvironment

3.1 Over-expression of sonic hedgehog in cancer

The over-expression of Shh signaling components in correlation with the development of gastrointestinal cancers was first recognized through investigation of mRNA expression of Shh and Ihh in tumors throughout the gastrointestinal tract (Berman et al., 2003). Ptch and Gli mRNA transcript levels were measured as indicators of Hedgehog pathway activity, whereby increased Ptch expression was coincident with elevated Shh. In vivo data suggested that the effect of increased Shh was to promote aberrant cell proliferation as tumor growth regressed with treatment of tumor-bearing mice with the Hedgehog pathway inhibitor cyclopamine. These data also confirmed that the tumor growth was indeed stimulated by the Hedgehog autonomous signaling network rather than a result of mutation. Further characterization of Ptch1 and Gli1 expression within the gastric tumor microenvironment was performed using a collection of human biopsies representing matched normal tissue as compared to inflamed tissue, tubular adenocarcinoma, papillary adenocarcinoma and signet-ring cell carcinomas from a series of patients (Ma et al., 2005). In these human samples, elevated Shh corresponded to increased Ptch1 and Gli1 only in cancerous tissue and not in the surrounding normal tissue. Elevated Hedgehog pathway activation was most common in poorly differentiated and high-grade samples, implicating Shh as an inducer of an aggressive phenotype able to evade normal cell cycle control (Ma et al., 2005).

Further work with both intestinal and diffuse gastric cancer-derived cell lines and corresponding human samples, compared to intestinal metaplasias, were used to localize Shh signaling by cell type in the setting of tumor formation and included an examination of the role of Ihh and Dhh (Fukaya et al., 2006). In samples collected from patients with intestinal metaplasia, the mRNA level of Hedgehog signaling pathway components were weakly expressed, while in contrast both Ihh and Shh were increased. Interestingly, the intestinal phenotype expressed low mRNA levels of the downstream targets Smo, Gli1 and Gli2 while the diffuse-type phenotype highly expressed Ptch, Smo, Gli1 and Gli2. The complementary immunohistochemical evaluation of the cells expressing these proteins was crucial, revealing that very little expression of any of the Hedgehog signaling components were detectable in any cell type in intestinal type cancers. However, the diffuse-type samples showed strong Ihh staining throughout the epithelial cancer cells while Shh was expressed in fibroblastic cells co-staining with the markers vimentin, α-actin and desmin (Fukaya et al., 2006). Gastric cancer cell lines used in proliferation assays with cyclopamine treatment confirmed these results implicating Hedgehog activation in increased cell proliferation in cancers representative of the diffuse-type development with high expression of Smo. In contrast to patients with atrophic gastritis that show loss of Shh protein expression, over-expression of Shh appears in gastric carcinoma. The mechanism by which

Hedgehog is first elevated in cancer and then able to act on cancer cells to induce their proliferation remains largely unknown.

3.2 Hedgehog signaling regulates cell-cycle progression in gastric cancer cells

There is overwhelming evidence showing that elevated Hedgehog is capable of promoting cancer cell proliferation and evasion of apoptosis, and recent work has begun to unravel the mechanistic details behind this finding. The treatment of the gastric cancer cell line, SNU16, with cyclopamine consistently induces cell apoptosis and arrest of cells in the G_0/G_1 phase of the cell cycle (Han et al., 2009). Cytochrome c staining within the mitochondria of cyclopamine-treated cells was characteristic of cells entering the apoptotic pathway, with diffuse staining throughout the cell, while in control cells cytochrome c staining co-localized with a marker for mitochondria only. The Bcl-2 protein is one of the key proteins regulating the release of cytochrome c from mitochondria, and the cyclopamine-treated cells exhibited significant decreases in both Bcl-2 and Gli1 by immunoblot. Collectively, these data suggest that the Shh signaling pathway is important in maintaining the level of anti-apoptotic proteins within cancer cells (Han et al., 2009). This study was limited by the use of only one type of gastric cancer cell line, however, a recent study performed a similar analysis on the SNU-16 cell line as well as the AGS, KATO-III, SNU-5, SNU-601 and SNU-638 cell lines (Lee et al., 2010). Cells either over-expressing Shh or having Shh knocked-down were co-cultured with *H. pylori* as the factor initiating cell apoptosis. Serial passage of one of the AGS/N-Shh over-expressing clones exposed to *H. pylori* showed adopted resistance to *H. pylori*-associated apoptosis concomitant with elevations in all Hedgehog signaling components, therefore cell-cycle protein activation was further characterized in this cell line. An immunoblot showed an absence of Bcl-2, confirming the results of Han, et al. (Han et al., 2009), as well as an increase in Cyclin D1 (Lee et al., 2010). These studies demonstrate that the reactivation of the Shh signaling pathway in response to infectious or inflammatory stimuli is critical to the inhibition of programmed cell death. It also provides an interesting hypothesis that mutated gastric cells evade apoptosis and with a proliferative stimulus may repopulate the epithelium and lead to tumor development.

Another interesting update to the role of Hedgehog signaling in the regulation of the cell-cycle comes from a biochemical analysis that demonstrates a direct physical interaction between Ptch and the cyclins (Barnes et al., 2001). Cyclin B1 is a critical regulator of mitotic cell division. During G2 phase, cyclin B1 accumulates in the nucleus as a part of cyclin dependent kinase 1 (CDK1) protein complex and plays a critical role during the G2/M phase transition of the cell cycle (Jenkins, 2009). Interestingly, Shh and Ptch participate in the G2/M phase checkpoint in a 'non-canonical' pathway that may be independent of Smo and Gli (Barnes et al., 2001). In the absence of Hedgehog ligand Ptch1 binds to cyclin B1 and inhibits the translocation to the nucleus. In the presence of Shh ligand, Ptch1 dissociates from cyclin B1 and cyclin B1 is translocated to the nucleus and promotes cell cycle progression.). Given that Ptch functions as a 'tumor suppressor', it is almost intuitive to hypothesize that within the tumor microenvironment, where Shh is elevated, increased proliferation is expected. In support of this hypothesis, mutations in Ptch have been linked to both cancers such as basal cell carcinomas and medulloblastomas (Ruiz i Altaba et al., 2002). Although these examples provide a firm genetic link between mutations of the Hedgehog signaling pathway and the incidence of cancer, there is little biological evidence of the underlying mechanisms linking Hedgehog signaling, cell-cycle progression and its relevance to gastric cancer progression.

4. The role of Bone-marrow derived mesenchymal stem cells (BM-MSCs) in promoting gastric cancer progression

4.1 Bone marrow-derived mesenchymal stem cell (BM-MSCs) recruitment to areas of chronic inflammation

Migration and differentiation of stem or progenitor cells within the stem cell niche of tissues are appropriately regulated to maintain normal organ structure and function. Although stem cells are critical to gastrointestinal development, tissue repair and normal function, the malignant transformation of these cells is critical for initiation of cancer including stomach, colon, liver and pancreas (reviewed in (Merchant & Matsui, 2010)). Traditionally, cancer has been viewed as a disease in which environmental factors induced mutations in critical oncogenes and tumor suppressor genes within a normal cell leading to cancer development. Recently, interest in cancer stem cells has arisen, and evidence has emerged demonstrating that cancer originates from the transformation of tissue stem cells induced by regulatory signals generated within the tumor microenvironment. Such regulatory signals contribute to the cancer stem cell niche by promoting growth of developing tumors through stimulating angiogenesis and the evasion of normal cell death. The intrinsic malignant transformation of stem cells occurs at an accelerated rate under certain environmental pressures that include injury and inflammatory cytokines.

Recruitment of bone marrow-derived mesenchymal stem cells (BM-MSCs) to the local tissue environment is a phenomenon that is traditionally related to the development of an inflammatory response during tumor development in a variety of organs throughout the body (Anjos-Afonso F, 2004; Coffelt et al., 2009; Kidd et al., 2009; Santamaria-Martínez et al., 2009; Shinagawa et al., 2010). The mechanism by which BM-MSCs alter the tissues to which they are recruited is unknown. With chronic inflammation of the stomach bone marrow-derived cells are recruited to the epithelium where, as the inflammatory response progresses, these cells repopulate entire glands and with tumor formation comprise part of the stroma (Houghton J, 2004). Further investigation then demonstrated that mesenchymal stem cells (MSCs) co-expressed gastric markers suggesting they could become incorporated within the gastric epithelium upon recruitment and contribute to the tumor stroma (Houghton J, 2004). Recently, this has been confirmed through an examination of MSC-like cells isolated from human gastric cancer samples which were shown to share many of the same properties as bone marrow-derived MSCs (Cao et al., 2009). To expand on these findings, a further analysis was performed that compared the MSC-like cells isolated from cancer to the same cells from non-cancerous tissue within the same patient, revealing that both populations express similar cells surface markers and genes characteristics of pluriopotent stem cells, mesenchymal cells and factors related to angiogenesis (Xu et al., 2011). Cell cycle analysis also revealed that there was significantly more cancer derived MSC-like cells within the S phase of the cell cycle as compared to the non-cancerous MSC-like cells isolated and normal BM-MSCs (Xu et al., 2011) suggesting that these cells are actively proliferating within the inflamed environment.

To understand how MSCs become carcinogenic, isolated normal BM-MSCs were serially passaged in vitro for over a year and monitored at several stages for malignant transformation by assaying colony formation, growth in soft agar and tumor development in xenografts established in immunocompetent mice (Li et al., 2007). After 12 months of continuous culture, carcinogenic potential was exhibited based on the results of each of these assays and the MSCs were termed “spontaneously transformed”, or stMSCs (Li H,

2007). We have extended these findings by demonstrating that proliferation of stMSCs is dependent on Hedgehog signaling (Figure 2). To examine the role of Hedgehog signaling in stMSC growth in vivo, subcutaneous xenografts using MSCs were established in C57Bl/6 mice. Mice injected with culture media alone were used as controls. Tumors approximately 100-200 mm^3 were measured in mice within 7 days of injection. Media controls showed no tumor growth. After the tumors had grown to approximately 100-200 mm^3, mice bearing these tumors were injected daily with the hedgehog signaling inhibitor cyclopamine ($MSCs^{Cyclopamine}$) or vehicle ($MSCs^{Vehicle}$). While the tumors in the vehicle treated mice ($MSCs^{Vehicle}$) continued to grow over the next 6 days (Figure 2A), the cyclopamine treated animals ($MSCs^{Cyclopamine}$) ceased to grow and began to regress in size (Figure 2A). Interestingly, mice injected with stMSCs expressing knockdown of Shh (MSC^{ShhKO}) cells showed delayed tumor growth and in some animals no tumors at all (Figure 2A). These data show that the Hedgehog signaling pathway is a key component for the growth and proliferation of stMSCs in vivo.

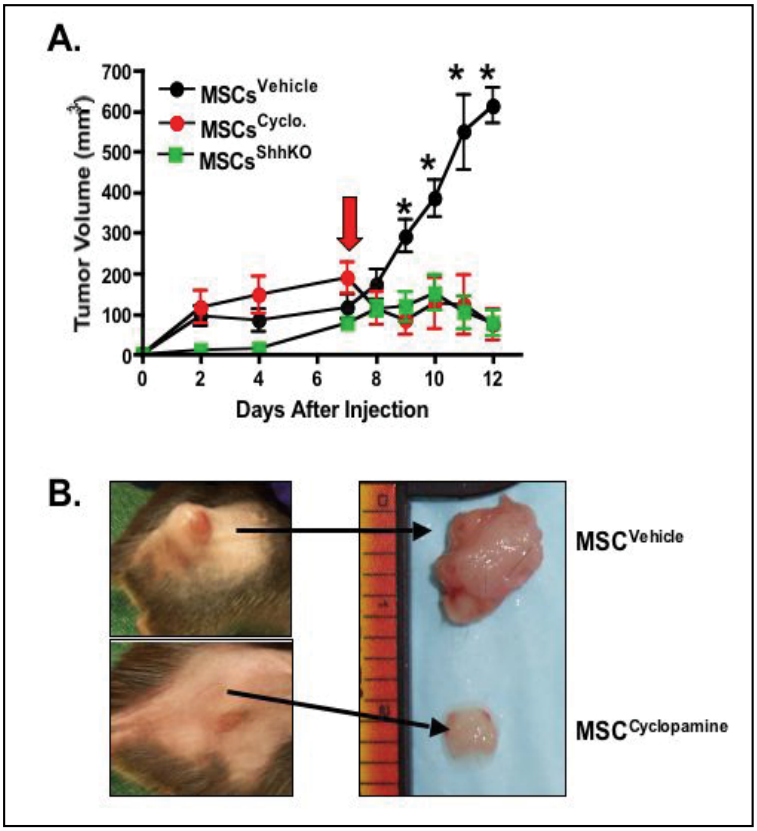

Fig. 2. Hedgehog pathway activity and requirement for growth of stMSCs in vivo. (A) Change in tumor volume (mm^3) in response to either vehicle or cyclopamine treatment, or transduced $MSC^{ShhKO59}$ cells over the 12 day experiment. $P<0.05$ compared to $MSC^{Vehicle}$, n = 3-6 mice per group, data shown as mean ± SEM. Arrow shows start of cyclopamine treatment. (B) Changes in tumor sizes dissected from vehicle-treated and cyclopamine-treated $stMSC^{WT}$ injected mice 12 days after xenograft

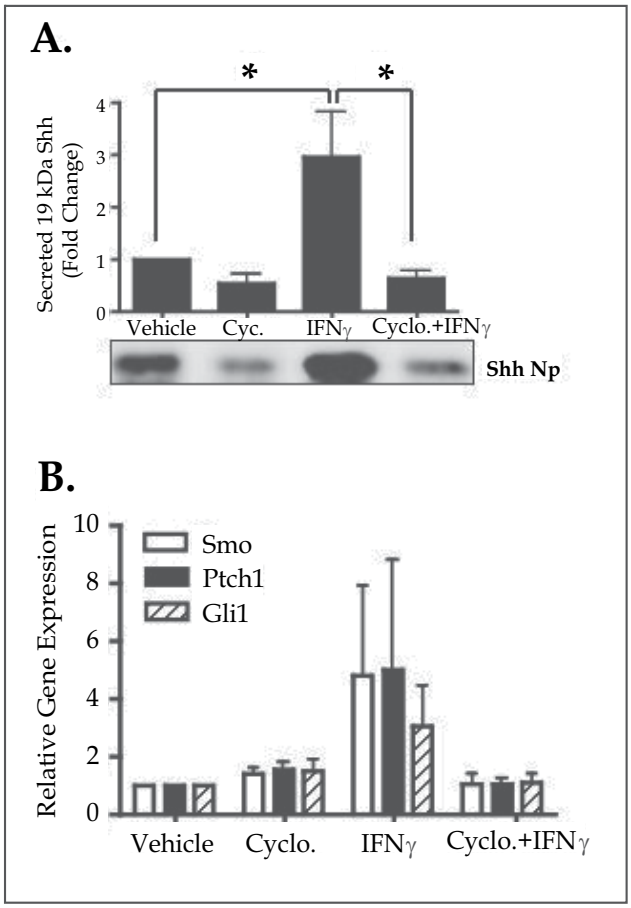

Fig. 3. Changes in Shh secretion and gene expression from IFNγ-treated stMSCs. (A) Western blot analysis of changes in secreted Shh protein (19-kDa, ShhNp) in media collected from stMSCs treated with vehicle, cyclopamine (Cyc.), IFNγ or Cyc. Plus IFNγ. (B) RNA was extracted from stMSCs treated with vehicle, cyclopamine (Cyc.), IFNγ or Cyc. Plus IFNγ and Smo, Ptch and Gli gene expression analyzed by quantitative real-time PCR. *P<0.05 compared to vehicle treated cells, n = 4 individual experiments

The gene expression profile of these cells was compared to early passage BM-MSCs and MSCs isolated from naturally occurring tumors in both aged mice and humans. Carcinogenic MSCs of each type showed uniform increases in expression of factors important in pluripotency and matrix remodeling and metastasis while harboring clinically relevant p53 mutations in addition to alterations in other tumor suppressor genes (Li et al., 2007). These results have since been confirmed in a study of in vivo transformation of MSCs, using BM-MSCs isolated from 2, 8 and 26 month old C57Bl/6 mice in which each set of cell populations were grown in vitro to homogeneity then gene expression compared using the Affymetrix Mouse Genome 430 2.0 GeneChip Array. Of particular interest, between the 8 and 26 month old groups, MSCs displayed significant decreases (> 2 fold) in p53, Cdkn1a, CHEK2 and p21 gene expression, among other apopotic pathway genes (Wilson et al., 2010). Immunoblot in 26 month old MSCs indicated p53 protein expression was essentially absent (Wilson et al.,

2010). While spontaneous transformation of human MSCs (hMSCs) has been more controversial, it has been shown in similar studies that they do undergo transformation in vitro. In fact, data indicate that hMSCs adopt carcinogenic properties as early as 25 days after their initial isolation (Røsland et al., 2009). While these data suggest that the natural process of aging results in mutation and evasion of normal cell death in BM-MSCs, it may not answer the question of what these cells are doing once recruited to a site of inflammation and whether inflammatory insults can also result in modulation of MSC phenotype or transformation.

4.2 Bone marrow-derived mesenchymal stem cells (BM-MSCs) express sonic hedgehog

While there is no doubt that increased Hedgehog is apparent in cancer and plays a critical role in driving cancer progression, the source of its increased expression has yet to be identified. One hypothesis is that remodeling at the DNA level is responsible for increased Shh expression in gastric cancer cells. In studies looking at human gastric cancer samples representative of each clinical stage of gastric cancer progression, elevated Shh was correlated with both loss of methylation within the Shh promoter region and hypermethylation of the promoter for Hedgehog interacting protein, an antagonist of the Hedgehog signaling pathway, changes which synergize to produce more Shh gene transcription (Taniguchi et al., 2007). During gastric cancer development it is shown that both Ihh and Shh are increasing within the cancer promoting cells of the epithelium and within a population of cells recruited to the mesenchyme of the stomach, respectively. An alternative hypothesis may be that in cases in which organs of the gastrointestinal tract are acutely injured or have developed carcinoma, there may be an intimate relationship between the inflammatory response and stimulation of Hedgehog secretion.

There is evidence in medulloblastomas of the cerebellum that expression of the cytokine interferon-gamma (IFNγ) during the immune response leads to a direct elevation of Shh protein (Lin et al., 2004; Sun L, 2010; Wang J, 2004; Wang J, 2003). Our laboratory has investigated this relationship within stMSCs harboring p53 mutations that are aggressively carcinogenic (Houghton et al., 2010; Li H, 2007). Bone marrow-derived mesenchymal stem cells (BM-MSCs) were first recognized as regulators of gastric carcinogenesis with the observation that they are recruited to the site of tumor formation in mice infected with *Helicobacter felis* (*H. felis*) and comprise part of the stroma of developing tumors (Houghton J, 2004). We have observed that treatment of these cells in vitro with recombinant IFN induces a two-fold increase in Shh secretion (Figure 3A) and gene expression of Hedgehog signaling components Smo, Ptch and Gli (Figure 3B). An interesting observation that was made from these data is that cyclopamine pre-treatment of IFNγ-treated cells resulted in an inhibition of Shh secretion compared to the IFNγ treatment alone (Figure 3A). These data would suggest that there is an autocrine feedback mechanism regulating Shh production from the stMSCs in response to IFNγ. Collectively, these data provide compelling evidence that the recruited stMSCs are in fact the source of local Shh, and may be a first step in identifying the historically elusive source of Shh in advanced gastrointestinal tumors.

4.3 Bone marrow-derived mesenchymal stem cells (BM-MSCs) as regulators of cancer stem cells

Recent work with stMSCs using a mouse model of breast cancer has defined the importance of aberrant immune responses in the in vivo transformation and maintenance of these cells

(Houghton et al., 2010). The local tissue environment may be considered carcinogenic, whereby with the ablation of tumor necrosis factor α (TNFα), the progression of neoplasia stimulated by recruited MSCs within epithelia can be halted (Houghton et al., 2010). A study using human MSCs transduced to express TNF-related apoptosis-inducing ligand (TRAIL), are recruited to the site of tumor formation where they produce significant cancer cell apoptosis in a model of squamous and lung cancer cells, enhancing the effects of chemotherapeutic agents (Loebinger et al., 2010). Based on the stromal cell phenotype, investigators suggest that recruited MSCs take on the phenotype of cancer-associated fibroblasts (CAF) (Quante et al., 2011). In IL-1β and *H. felis*-infected mouse models, MSCs are recruited to the gastric epithelium and adopt expression of α–smooth muscle actin, vimentin and FSP1 similar to CAFs (Quante et al., 2011). Differentiated CAFs also express high levels of the chemokines and cytokines such as IL-6, TGF-β, TNFα, and SDF-1α, as compared to wild-type gastric myofibroblasts (Quante et al., 2011). This work suggests several possible roles for these cells in cancer progression, in that they may be involved in regulating the local environment to form a niche for cancer stem cells, impacting the inflammatory response through release of soluble factors and cell-cell interaction or behaving as cancer stem cells themselves.

CD44 is an adhesion molecule expressed in cancer stem cells (Takaishi et al., 2009). The characterization of the resident stem cells of the gastric epithelium is a constantly evolving field of study within gastrointestinal research. While the identification of the CD44 positive cells is unknown, the activation and proliferation within a chronic inflammatory response has indicated that these cells may be the source of the cancer stem cell in the stomach (Ishimoto et al., 2011; Takaishi et al., 2009). The CD44 positive cell population isolated from several human gastric cancer cell lines by flow cytometry displays the phenotype of a cancer stem cell when assayed for spheroid colony formation and in vivo tumorigenicity (Takaishi et al., 2009). Higher CD44 expression was correlated with more aggressive tumor formation when cell lines were transplanted into the skin and stomach of immunodeficient mice, an effect that could be ablated by lentiviral knockdown using shRNA against the CD44 gene (Takaishi et al., 2009). Studies of specific CD44 variants produced by alternative splicing indicate that *H. pylori* infection and inflammation result in upregulation of CD44v6 and CD44v9, while CD44v6 is expressed in the normal gastric mucosa (Fan et al., 1996). Given the recently reported role of Shh as one of the primary stimuli in the induction of cancer stem cell proliferation (Song et al., 2011), we sought to identify the role of Hedgehog as a mediator of stMSC-induced proliferation of CD44 positive cancer stem cells. Figure 4 shows stomach sections collected from mice that were transplanted with stMSC^{Vect} cells tagged with red fluorescent protein (RFP) and injected with either phosphate buffered saline (control, PBS) or IFNγ for 21 days. Stomach sections were collected and immunostained for proliferation marker bromodeoxyuridine (BrdU) and RFP. RFP-tagged stMSC^{Vect} cells were recruited to the gastric mucosa of mice injected with IFNγ(Figure 4B, E) compared to the absence of RFP-tagged MSC^{Vect} cells in the stomachs of PBS-injected mice (Figure 4A). Although IFNγ-treatment appeared to increase the number of proliferating cells within the gastric mucosa (Figure 4B) compared to the PBS-injected mice (Figure 4A), RFP-tagged MSC^{vect} cells stained negative for BrdU (Figure 4B, E). Interestingly, when the same section were immunostained for BrdU and cancer stem cell marker CD44, it appeared as though the proliferating cells were in fact CD44 positive. These results may suggest that BM-MSCs, harboring mutations, are recruited to the sites of inflammation and drive cancer progression through the elevated production of Shh protein that may subsequently induce proliferation of the cancer stem cells.

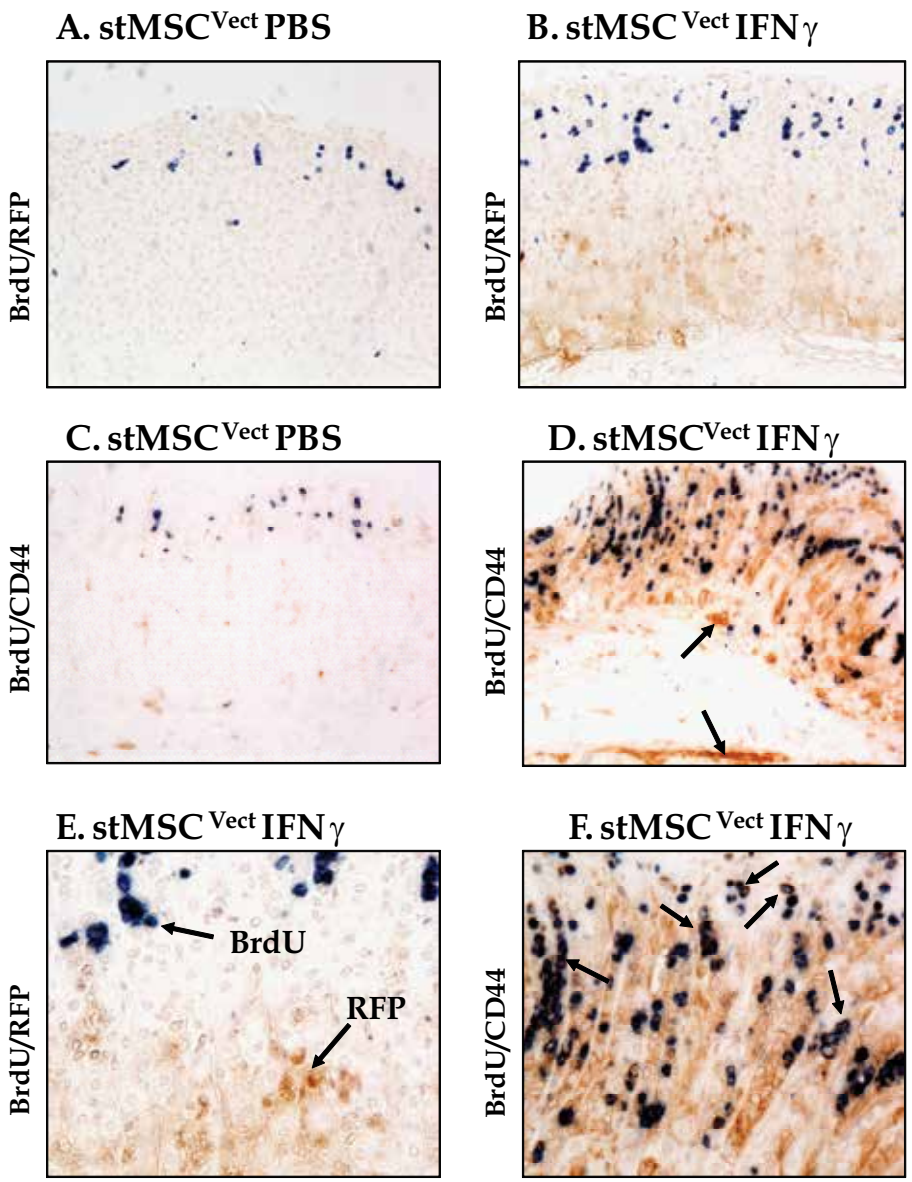

Fig. 4. Proliferation cells within the gastric mucosa of IFNγ-treated mice. Gastric mucosa collected from mice transplanted with stMSC^{vect} cells injected with (A) PBS or (B, E) IFNγ were BrdU labeled (blue) and co-stained with anti-RFP antibody (brown). Higher magnification of image in (B) is shown in (E) where arrows show separate BrdU positive proliferating cells and RFP-tagged stMSCs. Gastric mucosa collected from mice transplanted with stMSC^{vect} cells injected with (C) PBS or (D, F) IFNγ were BrdU labeled (blue) and co-stained with anti-CD44 antibody (brown). Higher magnification of image in (D) is shown in (F) where arrows show BrdU positive proliferating cells co-expressing gastric cancer cell maker CD44. Arrows shown in (D) indicate the expression of CD44 positive cells that are not proliferating. Representative of n = 4-6 mice per group

5. Conclusion: The hedgehog signaling network and the cancer stem cell compartment

While loss of Shh is associated with gastric atrophy, the reemergence and over-expression of Shh protein in gastric cancer is an observation that is well established (Berman et al., 2003). The underlying mechanism(s) regulating Shh re-expression and over-expression in malignant

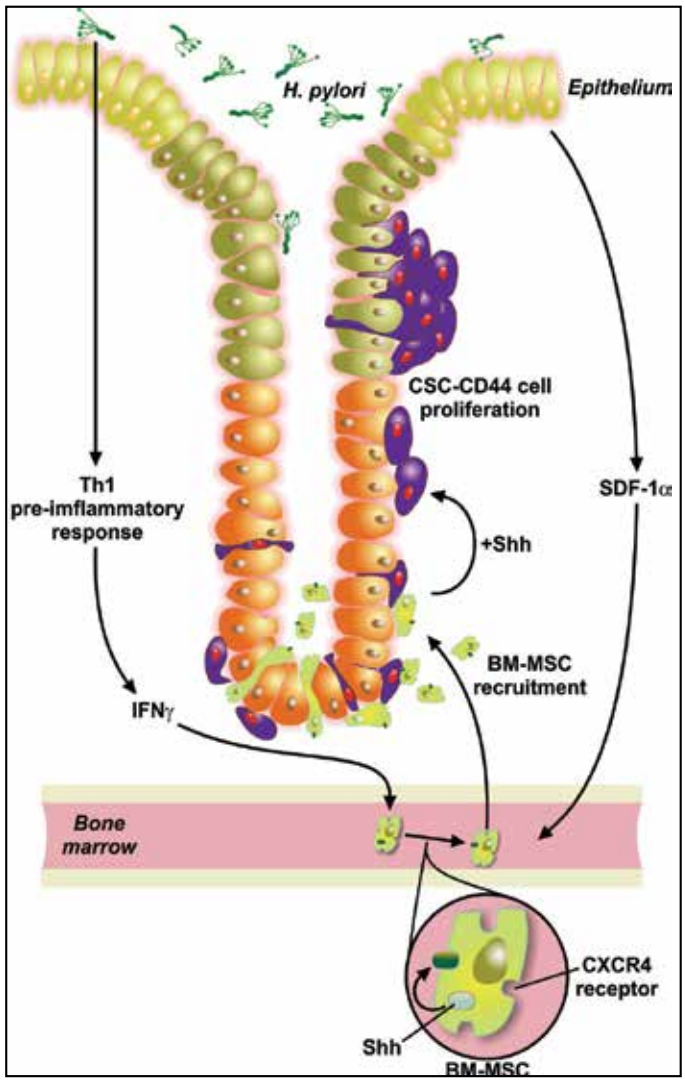

Fig. 5. Proposed model for the role of Shh in the development of gastric cancer. Th1 proinflammatory cytokine IFNg induces Shh expression and secretion from BM-MSCs within the bone marrow compartment. Shh regulates the expression of the CXCR4 that is critical for the recruitment of BM-MSCs to the stomach in response to SDF-1a. The recruitment of BM-MSCs expressing and actively secreting Shh in an environment rich in IFNγ repopulate the damaged gastric epithelium. Shh then induces proliferation of gastric cancer stem cells

that the molecular events begin in the bone marrow compartment in response to inflammation whereby in the stomach is induced by *H. pylori* infection. Several groups have implicated the CXCR4/SDF-1 axis in the recruitment of mesenchymal stem cells to sites of injury as well as to areas of developing carcinoma/tumor stroma (Haider et al., 2008; Kyriakou et al., 2008). What emerges from this body of work is a plausible mechanism for the recruitment of MSCs to the site of developing carcinoma. SDF-1α, that is secreted from the infected epithelium then signals to the BM-MSCs to initiate recruitment to the stomach. The recruitment of BM-MSCs expressing and actively secreting Shh in an environment rich in inflammatory cytokines including IFNγ repopulate the damaged gastric epithelium. Shh then acts on the gastric cancer stem cells to induce proliferation and eventually tumor development (Figure 5). BM-MSCs play a multifaceted role contributing to the cancer stem cell niche but also promote growth of developing tumors through stimulating angiogenesis and the evasion of normal cell death within the cancer stem cell population. Therefore, it is critical to define the mechanisms by which BM-MSCs support these alterations in the setting of cancer development in order to create new therapeutic approaches. The intrinsic transformation into malignant cells, which can occur at an accelerated rate under certain environmental pressures, only highlights the need for more advanced studies.

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17

Differentiation of Cancer Stem Cells

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1. Introduction

Tumors originally develop from normal cells that acquire the ability to grow aberrantly and metastasize to distant organs (Hanahan and Weinberg, 2000). These malignant transformations are considered to be induced by the accumulation of multiple genetic/epigenetic changes (Yamashita et al., 2008b). Although considered monoclonal in origin, cancer is composed of heterogeneous cell populations. This heterogeneity is traditionally explained by the clonal evolution of cancer cells through a series of stochastic genetic events (clonal evolution model) (Fialkow, 1976; Nowell, 1976). In contrast, cancer cells and stem cells have similar capabilities with respect to self-renewal, limitless division, and the generation of heterogeneous cell populations. Recent evidence suggests that tumor cells possess stem cell features (cancer stem cells) to self-renew and give rise to relatively differentiated cells through asymmetric division, thereby forming heterogeneous populations (cancer stem cell model) (Clarke et al., 2006; Jordan et al., 2006). Accumulating evidence supports the notion that cancer stem cells can generate tumors more efficiently in immunodeficient mice than non-cancer stem cells in hematological malignancies and in various solid tumors (Al-Hajj et al., 2003; Bonnet and Dick, 1997; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004).

Cancer stem cells are considered to be resistant to chemotherapy and radiotherapy, which might be associated with the recurrence of the tumor after treatment (Boman and Huang, 2008; Dean et al., 2005; Diehn et al., 2009; Zou, 2008). These findings have led to the proposal of "destemming" cancer stem cells (Hill and Perris, 2007) in order to induce their differentiation into non-cancer stem cells or to eradicate cancer stem cells by inhibiting the signaling pathways responsible for their self-renewal. Recent studies have supported this proposal and suggest the utility of several factors to induce the differentiation of cancer stem cells and facilitate tumor eradication; however, it is still debatable whether the simple differentiation of cancer stem cells effectively eradicates tumors. Here, we summarize current knowledge on the differentiation of cancer stem cells and discuss the utility and limitation of differentiation therapy to eliminate cancer.

2. Cancer stem cell system

The consensus definition of a cancer stem cell is a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that

comprise the tumor, as proposed by the AACR workshop in 2006 (Clarke et al., 2006). Thus, cancer stem cells can only be defined experimentally and their self-renewal ability is generally evaluated by the capacity of serially transplanted cells in immunodeficient mice. A cancer stem cell may give rise to one or two daughter cells that have essentially the same ability to replicate and generate differentiated non-cancer stem cells (Fig. 1 upper and lower left panels).

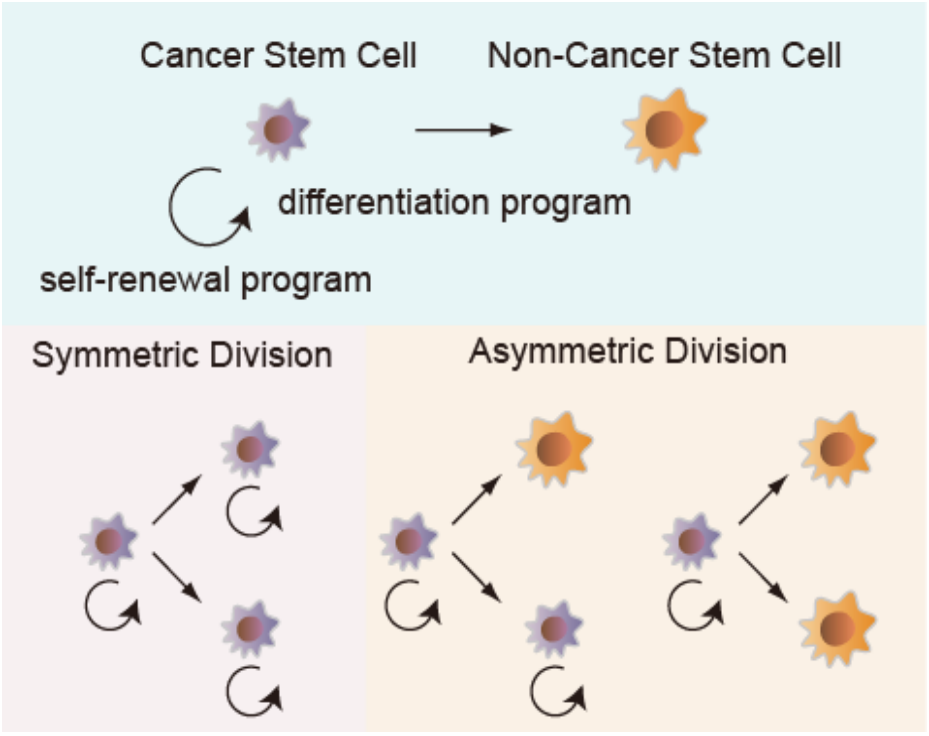

Fig. 1. Symmetric/asymmetric division of a cancer stem cell

Asymmetric cell division could be defined by the generation of one cancer stem cell and one progenitor cell with the loss of self-renewal capacity (Fig. 1 lower right panel). If both progenitors derived from a cancer stem cell lose the capacity of self-renewal by the induction of differentiation, the cancer stem cell population would be depleted and the tumor would subsequently shrink, according to the conventional cancer stem cell model.

2.1 Signaling pathways responsible for the self-renewal of cancer stem cells

A growing body of evidence suggests the similarities of normal stem cells and cancer stem cells in terms of their self-renewal and differentiation programs. Indeed, the self-renewal and differentiation programs in cancer stem cells are considered to be regulated by several signaling pathways that are activated in normal stem cells (Lobo et al., 2007). These signaling pathways seem to be activated during the process of normal organogenesis as well as carcinogenesis in a tissue-dependent manner (Pardal et al., 2003). Therefore, underscoring the significance of these signaling pathways on self-renewal and differentiation is critical for the development of treatment strategies specifically targeting cancer stem cells.

2.1.1 Wnt/β-catenin signaling

Wnt/β-catenin signaling has been studied primarily in developing embryos and was demonstrated to modulate cell proliferation, migration, and differentiation in a cellular context-dependent manner (Decaens et al., 2008; Giles et al., 2003; Moon et al., 2004; Ober et al., 2006). Wnt signaling is involved in the decision of stem cells to self-renew or differentiate during organogenesis, involving, for example, skin, intestine, bone marrow, kidney, and liver development (Moon et al., 2004; Thompson and Monga, 2007). Moreover, mutations of genes involved in Wnt/β-catenin signaling have been reported in a wide variety of human cancers including colorectal cancer, gastric cancer, skin cancer, ovarian cancer, liver cancer, and leukemia (Giles et al., 2003; Merle et al., 2005; Takebe et al., 2010; Tan et al., 2008; Vermeulen et al., 2010; Woodward et al., 2007; Zhao et al., 2007).

Wnt signaling is mediated through a core set of proteins to activate the transcriptional programs responsible for cell proliferation and development (Fig. 2). In the absence of Wnt proteins, β-catenin is phosphorylated and degraded by the Axin-APC-GSK3β complex. Once Wnt proteins bind to their receptor, Frizzled, the degradation complex is inactivated to stabilize β-catenin, which leads to its accumulation in the nucleus and interaction with T-cell factor (TCF) to activate the transcription of target genes (Moon et al., 2004).

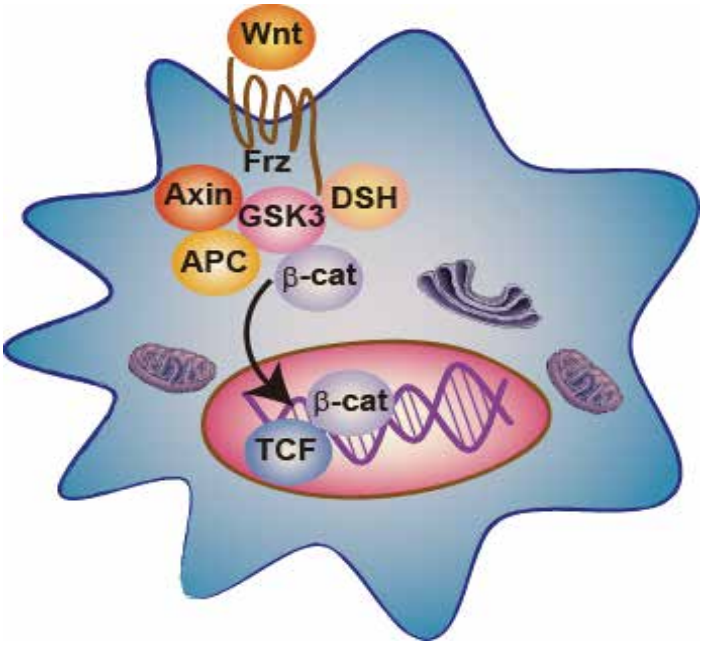

Fig. 2. Wnt/β-catenin signaling. APC, adenomatous polyposis coli; β-cat, β-catenin; DSH, Dishevelled; Frz, Frizzled; GSK3, glycogen synthase kinase 3; TCF, T-cell factor

Recent studies have demonstrated that Wnt/β-catenin signaling also plays a role in the maintenance of cancer stem cells, including colorectal cancer (Vermeulen et al., 2010), breast cancer (Li et al., 2003; Woodward et al., 2007), and liver cancer (Yang et al., 2008). We have recently demonstrated that Wnt/β-catenin signaling augments self-renewal and inhibits the differentiation of liver cancer stem cells by the expression of the stem cell marker EpCAM, which results in the enrichment of the tumor-initiating cell population (Yamashita et al.,

2008a; Yamashita et al., 2009). We have further demonstrated that small molecules, which specifically inhibit the transcriptional activity of the TCF/β-catenin complex, can suppress the cell proliferation of EpCAM-positive liver cancer cell lines, suggesting the utility of these compounds for the eradication of cancers via the inactivation of Wnt/β-catenin signaling (Yamashita et al., 2007).

2.1.2 Hedgehog signaling

The Hedgehog signaling pathway was initially identified as a regulator of segmental patterning in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980). Hedgehog signaling is activated in developing embryos, especially in the skeleton and neural tube, and regulates the cell proliferation, migration, and differentiation of stem cells (Varjosalo and Taipale, 2008). Several types of cancers are reported to have an activated hedgehog signaling pathway, including glioma (Clement et al., 2007), prostate cancer (Sanchez et al., 2005), breast cancer (Liu et al., 2006), pancreatic cancer (Li et al., 2007), and hematological malignancies (Zhao et al., 2009).

Hedgehog signaling is regulated by several proteins, including ligands (Sonic Hedgehog, Desert Hedgehog, and Indian Hedgehog), the Patched (Ptch) receptor, the Smoothened (Smo) transmembrane protein, and the zinc finger transcription factor Gli (Merchant and Matsui, 2010) (Fig. 3). In the absence of ligands, Ptch represses the activity of Smo and the Gli-mediated transcriptional program is constitutively suppressed (Gli- suppressed). Once ligands bind to Ptch, the repression of Smo is released and the Gli-mediated transcriptional program is activated (Gli-activated).

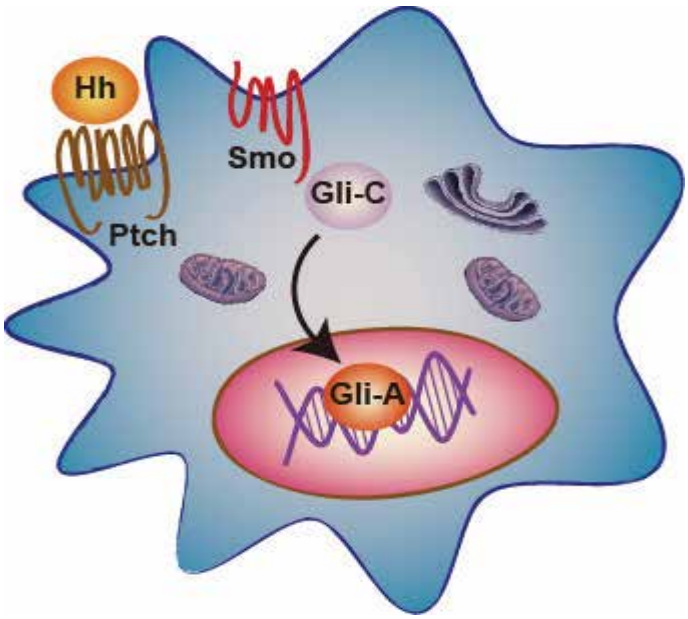

Fig. 3. Hedgehog signaling. Gli-C, Gli complex; Gli-A, Gli-activated; Hh, Hedgehog; Ptch, Patched; Smo, Smoothened

Accumulating evidence suggests that Hedgehog signaling regulates the self-renewal of cancer stem cells in several types of cancer, including glioblastoma and leukemia (Clement

et al., 2007; Zhao et al., 2009). Accordingly, Hedgehog signaling inhibitors have been clinically tested and might be beneficial for patients with advanced medulloblastoma or basal cell carcinoma, although Smo mutations in cancer cells confer resistance against such inhibitors (Rudin et al., 2009; Von Hoff et al., 2009; Yauch et al., 2009).

2.1.3 Notch signaling

Notch signaling has a pivotal role in regulating cell-to-cell communication during embryogenesis (Artavanis-Tsakonas et al., 1999), and is known to regulate stem cell fate in various organs (Androutsellis-Theotokis et al., 2006; Fre et al., 2005). Mammalian Notch ligands consist of the two structurally distinct families Delta-like ligands (DLLs) and Jagged ligands (JAGs), and these ligands are bound to the cell membrane (Fig. 4). The activation of Notch signaling is initiated by the binding of these membrane-bound ligands to Notch receptors, which results in the release of the Notch intracellular domain into the cytoplasm and nucleus by the γ-secretase complex to activate the Notch-specific transcriptional program.

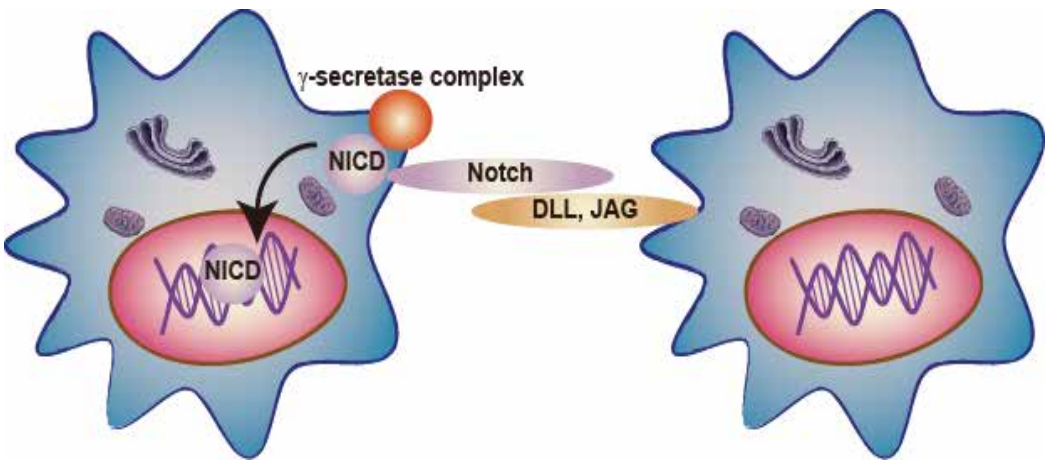

Fig. 4. Notch signaling. DLL, Delta-like ligand; JAG, Jagged; NICD, Notch intracellular domain

Notch signaling has been implicated in various types of cancers, including solid tumors and leukemia (Pannuti et al., 2010). A growing number of recent studies has demonstrated that the activation of the Notch signaling pathway can drive tumor growth via the expansion of the cancer stem cell population (Korkaya and Wicha, 2009; Peacock and Watkins, 2008; Wilson and Radtke, 2006). Indeed, the Notch signaling pathway has been demonstrated to be active in cancer stem cells and to play a critical role in the self-renewal of cancer stem cells (Fan and Eberhart, 2008; Fan et al., 2010; Wang et al., 2009). Thus, Notch signaling is considered to be a good target for pharmacological inhibition to eradicate cancer stem cells, and the effect of Notch inhibitors against Notch, including γ-secretase inhibitors or monoclonal antibodies, have been extensively evaluated (Pannuti et al., 2010).

2.2 Signaling pathways responsible for cancer stem cell differentiation

Although self-renewal pathways are considered to be critical targets for the eradication of cancer stem cells, it is still debatable if differentiation pathways are equally effective for their

eradication. Several recent studies have provided evidence of the utility and limitation of the cancer stem cell differentiation strategy by modulating the signaling pathways responsible for the differentiation of normal stem/progenitor cells.

2.2.1 Bone morphogenic protein signaling

Bone morphogenic protein (BMP) signaling is known to be activated during embryogenesis and to play a pivotal role in the differentiation of neural and intestinal stem cells (Varga and Wrana, 2005). BMPs belong to a subgroup of the transforming growth factor-β superfamily and activate signaling through the BMP-receptor (BMPR)-mediated phosphorylation of Smad proteins. Interestingly, recent studies have suggested the utility of BMPs to induce the differentiation of brain cancer stem cells and facilitate brain tumor eradication (Lee et al., 2008; Piccirillo et al., 2006). More recently, colorectal cancer stem cells have been shown to lack the expression of BMP4, and the administration of BMP4 enhanced the terminal differentiation, apoptosis, and chemosensitization of colorectal cancer stem cells (Lombardo et al., 2011). Interestingly, the effects of BMP4 on the differentiation of colorectal cancer stem cells appeared to be independent of the phosphorylation status of Smad, suggesting the importance of non-canonical signaling pathways activated by BMP4 for the differentiation of these cells.

2.2.2 Oncostatin M signaling

Oncostatin M (OSM) is a pleiotropic cytokine that belongs to the IL-6 family, which includes IL-6, IL-11, and leukemia inhibitory factor (LIF). These cytokines share the gp130 receptor subunit as a common signal transducer, and activate Janus tyrosine kinases and the signal transducer and activator of transcription 3 (STAT3) pathways. However, gp130 forms a heterodimer with a unique partner, for example, the IL6 receptor, LIF receptor, or OSM receptor (OSMR); thus, each cytokine uniquely induces a certain signaling pathway (Heinrich et al., 2003), and OSM is known to exploit distinct signaling in an OSMR-specific manner (Kinoshita and Miyajima, 2002). Of note, OSM is known to activate the hepatocytic differentiation program in hepatoblasts in an OSMR-specific manner (Kamiya et al., 1999; Kinoshita and Miyajima, 2002).

We recently identified that OSMR is expressed in a subset of liver cancer stem cells (Yamashita et al., 2010). Interestingly, OSMR-positive hepatocellular carcinoma (HCC) was characterized by the abundant expression of stem cell markers and poorly differentiated morphology, suggesting that OSMR is more likely to be expressed in HCC with stem/progenitor cell features (Yamashita et al., 2008a). Of note, the OSM-OSMR signaling pathway was maintained in these HCCs, and OSM induced hepatocytic differentiation in liver cancer stem cells (Fig. 5).

Unexpectedly, we identified that the hepatocytic differentiation of liver cancer stem cells by OSM resulted in enhanced cell proliferation *in vitro* and modest anti-tumor activity *in vivo* when administered alone. However, we have further demonstrated that OSM-mediated hepatocytic differentiation of liver cancer stem cells effectively suppresses HCC growth when combined with conventional chemotherapy. It is possible that OSM may boost the anti-tumor activity of 5-FU by "exhausting dormant cancer stem cells" through hepatocytic differentiation and active cell division (Fig. 6). A similar chemosensitization effect was observed in colorectal cancer stem cells differentiated by BMP4 administration (Lombardo et al., 2011).

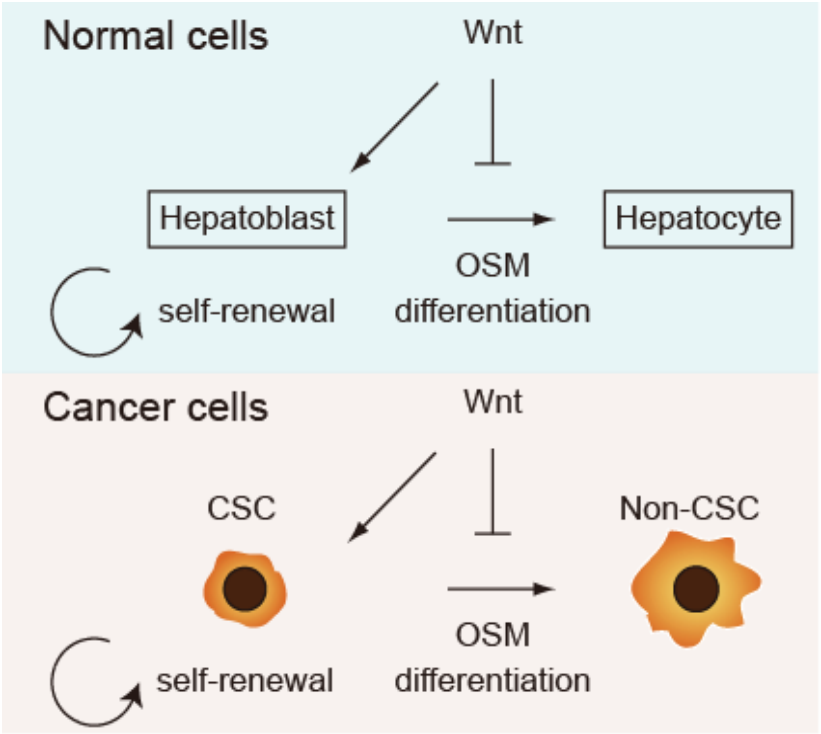

Fig. 5. Signaling pathways responsible for the self-renewal and differentiation of liver cancer stem cells. CSC, cancer stem cell; OSM, oncostatin M

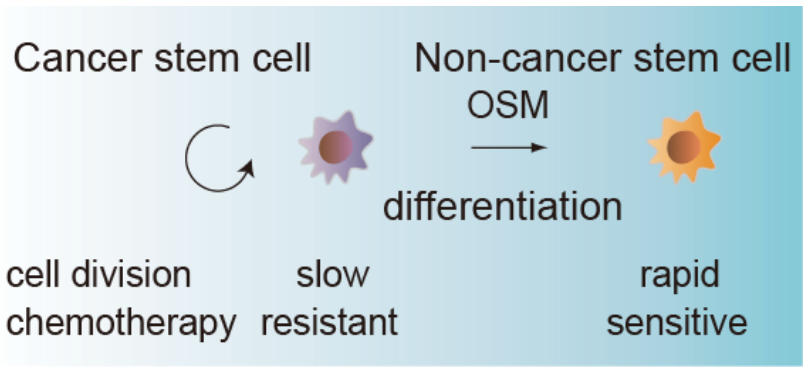

Fig. 6. Effect of oncostatin M (OSM) on exhausting dormant liver cancer stem cells

3. Limitation of cancer stem cell differentiation

As described above, some of the signaling pathways for the differentiation of normal stem cells may be maintained in cancer stem cells. To induce the differentiation of cancer stem cells by specific ligands, the expression of the corresponding receptors bound to ligands is clearly required, suggesting the importance of clarifying the mechanisms for receptor expression regulation. Interestingly, BMPRs and OSMR were detected in colorectal and liver

cancer stem cells, respectively, suggesting the possibility of ligand-induced differentiation therapy in the clinic. However, the expression of these receptors might be transcriptionally suppressed in a subset of cancers through methylation of their promoter regions (Deng et al., 2009; Kim et al., 2009; Lee et al., 2008). Indeed, a recent study suggested that BMP-mediated brain cancer stem cell differentiation failed in a subset of brain tumors in which BMP receptor promoters were methylated and silenced (Lee et al., 2008). Therefore, cancer stem cells may acquire resistance against differentiation therapy by additional epigenetic changes during the differentiation treatment.

It has been postulated that both normal stem cells and cancer stem cells are dormant and show slow cell cycles. Consistently, cancer stem cells are considered to be more resistant to conventional cytotoxic chemotherapeutic agents than non-cancer stem cells, possibly due to slow cell cycles as well as the increased expression of ATP-binding cassette (ABC) transporters, robust DNA damage responses, and activated anti-apoptotic signaling (Bao et al., 2006; Dean et al., 2005; Viale et al., 2009). Therefore, the induction of differentiation programs in cancer stem cells may result in cell proliferation of the tumor. Indeed, we recently demonstrated that differentiation of liver cancer stem cells by OSM increased cell proliferation, at least *in vitro* (Yamashita et al., 2010). Our data clearly suggested the necessity of conventional chemotherapy in addition to differentiation therapy to eradicate non-cancer stem cells originating from cancer stem cells. Furthermore, although the combination of OSM and conventional chemotherapy effectively inhibited tumor growth in our model, we did not observe tumor shrinkage (Yamashita et al., 2010). If both progenitors derived from a cancer stem cell lose their self-renewal capacity by the induction of differentiation, the tumor should subsequently shrink following the depletion of cancer stem cells. However, it is possible that ligand-based differentiation programs cannot completely inhibit the self-renewal programs of target cancer stem cells. Thus, the induction of differentiation in cancer stem cells with the eradication of non-cancer stem cells might not be sufficient for the eradication of the tumor, which may suggest the importance of inhibiting self-renewal as well as stimulating the differentiation of cancer stem cells.

A recent paper suggested that leukemia-initiating cells are composed of genetically diverse, functionally distinct populations (Notta et al., 2011), suggesting the clonal evolution of leukemia-initiating cells. Accordingly, cancer stem cells in solid tumors may also have a distinct tumorigenic/metastatic capacity as well as chemoresistance with certain genetic/epigenetic changes in each subclone as a result of clonal evolution. Thus, the cancer stem cell model and the clonal evolution model are not considered to be mutually exclusive. Therefore, clonal selection of cancer stem cells resistant to differentiation therapy might occur with additional genetic/epigenetic changes during treatment as a result of clonal evolution. The effects of differentiation therapy on the clonal evolution or genetic diversity of cancer stem cells need to be clarified in the future.

4. Conclusion

The recent re-emergence of the cancer stem cell hypothesis has provided novel insights on the effect of differentiation programs on cancer stem cells for the potential eradication of tumors. Although the activation of several signaling pathways by certain cytokines may be effective for the differentiation of cancer stem cells, their utility and limitation for tumor eradication should be clarified in future to provide novel therapeutic opportunities for cancer patients.

5. Acknowledgment

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18

Carbohydrate Antigens as Cancer-Initiating Cell Markers

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1. Introduction

The hypothesis that cancer-initiating cells are a prerequisite for cancer ontogenesis is now widely accepted. The existence of cancer-initiating cells is well documented in brain, lung, and breast cancers (Boman et al., 2008; Takaishi et al., 2009), as well as in other malignancies. Cancer-initiating cells (CIC) exhibit low proliferative rates, self-renewing capacity, a propensity to differentiate into actively proliferating tumour cells, and show resistance to chemotherapy or radiation (Vander Griend et al., 2008; Sell & Leffert, 2008). Since cancer-initiating cells clearly differ from the majority of cells of the tumour mass, studying the expression and function of surface molecules on cancer-initiating cells is an important aspect of tumor biology.

A great number of more or less specific surface molecules of cancer-initiating cells have been described during recent years. Among the markers most widely accepted is CD44 (Shipitsin et al., 2007; Ponnusamy & Batra, 2008). CD44 is a cell surface type I transmembrane glycoprotein involved in cell-cell interactions, cell adhesion, and migration. It is a receptor for hyaluronic acid, but can also interact with other ligands (Marhaba & Zöller, 2004). CD44 was found to be expressed on cancer-initiating cells in gastric cancer (Takaishi et al., 2009). A single $CD44^+$ cell from a colorectal tumour could form a sphere in vitro and was able to generate a xenograft tumour resembling the properties of the primary tumour (Du et al., 2008). CD133 is also a widely recognized marker of cancer-initiating cells. CD133 was initially described as a surface antigen specific for human haematopoietic stem cells and as a marker for murine neuroepithelial and several other embryonic epithelia (Singh et al., 2004). In a number of recent studies, CD133 alone or in combination with other markers was used for the isolation of CIC from malignant tumours of colon, lung and liver (Haraguchi et al., 2008). $CD133^+$ tumour cells repair radiation-induced DNA damage more effectively than $CD133^-$ tumour cells (Bao et al., 2006). CD133 is an independent prognostic marker that correlates with poor overall survival in patients with malignancies (Horst et al., 2008).

2. Carbohydrate antigens as cancer-initiating cell markers

Almost all CIC markers described so far are proteins. Carbohydrate CIC markers have been rarely reported (Son et al., 2009). This is surprising, because some of them are known as "stage-specific embryonic antigens" for a long time (Solter & Knowles, 1978), and because many carbohydrate epitopes are known as tumour markers (Hakomori, 1989). We consider carbohydrate antigens as biologically active entities, which may indicate or even be actively involved in the fundamental functional changes during the transformation of an ordinary epithelial cell into a carcinoma cell, as well as in the process of tumour progression (Cao et al., 1997). Changes in glycosylation may provide diagnostic markers and therapeutic targets. CD173 (Blood group antigen H type 2, H2), CD174 (Lewis Y, LeY) and CD176 (Thomsen-Friedenreich antigen, core-1) are known to be developmentally regulated carbohydrate antigens which are expressed to a varying degree on many human carcinomas. We have found that CD173, CD174 and CD176 were expressed on $CD34^+$ malignant human hematopoietic cells (Cao et al., 2001; 2008). In current studies, we found the expression of CD173, CD174 and CD176 on cancer-initiating cells of several epithelial cancers (Lin et al. 2010a; 2010b).

2.1 CD173 and CD174 as markers of cancer-initiating cells

ABH and Lewis (Le) blood group antigens are cell surface carbohydrate structures. Besides their expression on erythrocytes, they are also widely distributed in body fluids and normal tissues, especially on epithelia of glandular tissues. H2 (Fucα1-2Gal-β1-4GlcNAcβ1-) and LeY (Fucα1-2Gal-β1-4[Fucα1-3]GlcNAcβ1-) are fucosylated derivatives of the same type 2 precursor carbohydrate chain found on glycoproteins. H2 is synthesized by the addition of a fucose to the type 2 precursor chain mediated by α1-2 fucosyltransferase encoded by the H gene. LeY results from the additional action of α1-3 fucosyltransferase encoded by the LeX gene. Histo-blood group antigens H2 and LeY were assigned as CD173 and CD174, respectively, during the Seventh Workshop and Conference on Human Leucocyte Differentiation Antigens in Harrogate in June 2000 (Cao et al., 2002a). In this chapter we use the new designations for these oligosaccharide structures (Figure 1).

A number of studies have shown that abnormal expression of CD173 and CD174 occurs in epithelial malignancies (Fujitani et al., 2000; Baldus et al., 2006). We have found that CD173 and CD174 were expressed on $CD34^+$ haematopoietic progenitor cells (Cao et al., 2001). Moreover, fucosylated histo-blood group antigens (H antigens) were co-expressed on CD44v6 after transfection of α(1-2)fucosyltransferase concomitant with an enhanced tumorigenicity in rat colon adenocarcinoma cells (Goupille et al., 1997). In our current study, immunocytological staining and flow cytometric analysis were performed to investigate the co-expression of CD173 or CD174 with CD44 on breast cancer cells. We observed that CD44 together with CD173 or CD174 are located at the cell surface and reveal co-expression in a significant proportion of cultured breast cancer cells and in tissue specimens taken from breast cancer.

Tamoxifen (4-OHT) which is an oestrogen receptor ligand, was reported to induce G0/G1 growth arrest and to inhibit the proliferation of breast cancer cells. A recent study showed that 4-OHT treatment increased the number of mammary cancer stem cell-like cells (Mani et al., 2008). To assess whether CD44, CD173, and CD174 expression is simultaneously affected after exogenous treatment, we treated the breast cancer culture cells with 4-OHT. In semiquantitative flow cytometric analysis, the number of $CD44^+/CD173^+$ or $CD44^+/CD174^+$ breast cancer cells could be enhanced in cultured cells after 4-OHT treatment (Figure 1). Therefore, we conclude that CD44 is co-expressed with CD173 and CD174 in breast cancer.

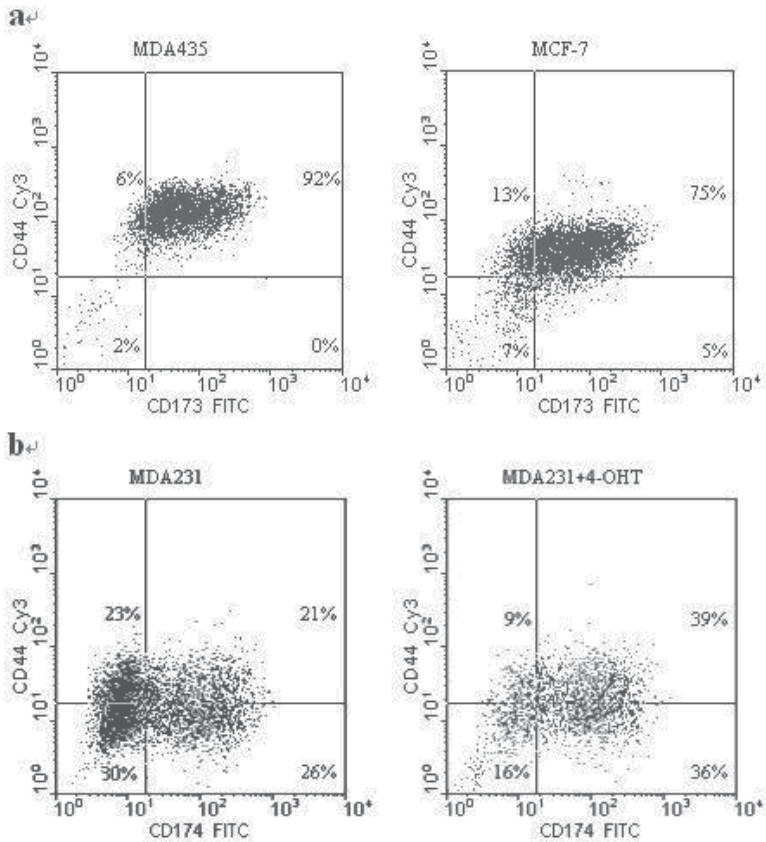

Fig. 1. a: Flow-cytometric analysis of $CD44^+/CD173^+$ expression in the breast adenocarcinoma cell lines MDA-MB-435 and MCF-7. Cancer cells were incubated with anti-CD44 (IgG2b) and anti-CD174 (IgM) antibodies, respectively, followed by incubation with anti-IgG-Cy3 (γ chain-specific) and anti-IgM-FITC (μ chain-specific). Values are taken from one of three similar experiments. Large proportions of both cell lines are positive for both markers (CD44 and H2). b: Flow-cytometric analysis of $CD44^+/CD174^+$ expression on MDA-MB-231 cells before and after 4-OHT treatment. 4-OHT treatment results in an increase in the proportion of cells expressing both CD44 and CD174 (LeY). Values are taken from one of three similar experiments (from Lin et al. 2010a)

CD133 is also a marker of cancer-initiating cells. We found that CD133 was indeed co-expressed with CD173 and CD174, although at a lower percentage (<5% of the total cancer cells). The manifestation of the lower percentage was due to the smaller subpopulation of cancer cells possessing CD133 (<5%). In addition, we found that cases with increased CD173 and CD174 expression correlated with raised CD133 expression.

An interesting observation was the strong staining for CD173 of myoepithelial/basal cells in cases of intraductal breast carcinomas (Figure 2.). In normal ducts of transitional tissues of the same sections, the basal cell layer was only occasionally positive for CD173 (Karsten et al., 1993). At present, we cannot explain the cause and significance of this observation. Since H and LeY antigens are developmentally regulated antigens, this phenomenon might also be indicative of an ongoing epithelial-mesenchymal transition of these cells.

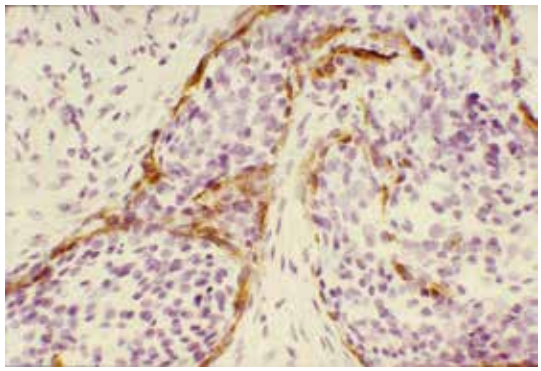

Fig. 2. Immunohistochemistry of an intraductal carcinoma section stained with the CD173 antibody A46-B/B10. Basal cells (stem cell-like cells) of the remaining duct walls are strongly stained.

Type 2-based ABH oligosaccharides are carried on several different glycoproteins and glycolipids (Hakomori et al., 1981). In epithelial ovarian cancer, the major carrier proteins of CD174 are CA125 and MUC1 (Yin et al., 1996). In $CD34^+$ hematopoietic stem cells, the major carrier of CD173 and CD174 is a 170-kDa glycoprotein (Cao et al., 2001). CD44 is also a carrier of H antigens in the rat PRO cell line (Rapoport et al., 1999). In our studies, potential glycoproteins carrying CD173 or CD174 were analyzed in three breast carcinoma cell lines by immunoprecipitation and in a sandwich ELISA. The CD44 immunoprecipitate from the lysates of the three cell lines was subjected to immunoblot analysis using CD173 and CD174 antibodies. Both antibodies stained the CD44 band (Figure 3). In a new sandwich solid-phase enzyme-linked immunosorbent assay (ELISA) with anti-CD44 as capture antibody, followed by CD173 or CD174 antibodies, respectively, both antibodies scored positive in all three cell lines examined, indicating that CD173 and CD174 epitopes are expressed on the CD44 molecule.

It is believed that CD173 and CD174 structures on glycoprotein expressed by carcinomas contribute to adhesion, cell aggregation, invasion, and metastasis. CD174 is involved in early cell-cell contacts during tumor-associated angiogenesis (Moehler et al., 2008). CD173 and CD174 are apparent markers of the degree of malignancy in cancer patients (Fujitani et al., 2000; Steplewska-Mazur et al., 2000). Higher expression of CD173 and CD174 was more often found in patients with high grade tumours and poor prognosis compared to those with better prognosis (Baldus et al., 2006). In lymph node negative breast carcinomas, over-expression of CD174 was associated with significantly decreased patient survival (Madjd et al., 2005). Increased tumorigenicity mediated by α1-2 fucosylation is associated with increased resistance to apoptosis and escape from immune control (Rapoport et al., 1999; Goupille et al., 2000). All these phenomena may be associated with the expression of CD173 and CD174 on cancer-initiating cells.

Failure of current cancer therapies may be ascribed to the inefficacy of drugs on potentially quiescent cancer-initiating cells. Treatment strategies therefore need to consider the presence of cancer-initiating cells. The high expression of CD173 and especially of CD174 on the surface of cancer-initiating cells in breast carcinomas suggests that these antigens could be potential targets for antibody-mediated diagnosis and therapy. Anti-LeY antibodies have already been tried in adjuvant cancer therapy (Stahel et al., 1992). More recent studies have demonstrated that the administration of low doses of anti-CD174 antibodies may lead to an

effective anti-tumour response, even without induction of TNF-α release (Dettke et al., 2000), and anti-CD174 antibody conjugated with doxorubicin is presently under evaluation in the therapy of epithelial tumours (Tolcher et al., 1999).

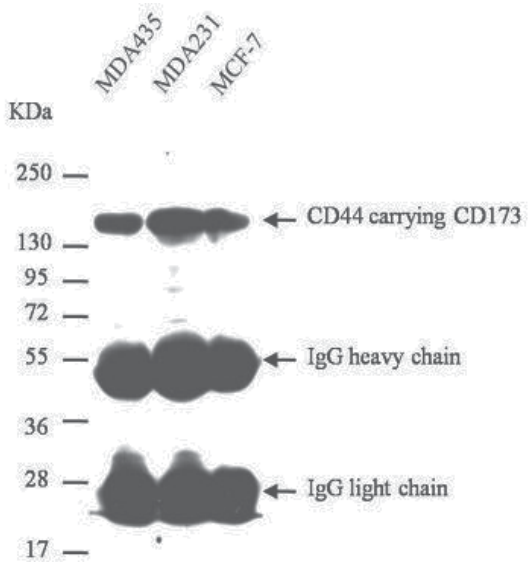

Fig. 3. Immunoprecipitation of lysates from breast adenocarcinoma cell lines MDA-MB-231, MDA-MB-435, and MCF. The CD44-immunoprecipitated material was resolved by SDS-PAGE and analyzed by immunoblotting using mAb CD173. The data show that in breast carcinomas CD44 is carrying CD173 (H2) (from Lin et al. 2010a)

2.2 CD176 as a marker of cancer-initiating cells

The Thomsen-Friedenreich antigen (TF, or CD176) is a tumour-associated carbohydrate epitope with the structure Galb1-3GalNAca1-O-. While this disaccharide is a ubiquitous core structure (core-1) found in a cryptic manner on many membrane glycoproteins of normal cells, its exposure on tumour cells is obviously restricted to a few specific carrier proteins. TF was assigned as CD176 during the Seventh Workshop and Conference on Human Leucocyte Differentiation Antigens in Harrogate in June 2000 (Cao et al., 2002b). In this chapter we use the new designation for this oligosaccharide structure. It has been demonstrated that CD176 is expressed on the surface of various cancer cells, such as breast carcinomas (Springer 1997; Imai et al., 2001; Goletz et al., 2003), colorectal carcinomas (Cao et al., 1995), hepatocellular carcinomas (HCC) (Cao et al., 1999), several leukaemias (Cao et al., 2008), and other types of cancer, but absent from almost all normal adult cell types (Cao et al., 1996). As a functional moiety, CD176 on the surface of cancer cells is involved in the invasive and metastatic properties of the cells (Cao et al., 1995). An anti-CD176 antibody could induce apoptosis of leukaemic cells (Cao et al., 2008). As CD176 is strongly expressed on the surface of cancer cells and virtually absent from normal tissues, it appears reasonable to assume that this carbohydrate structure is a suitable target for cancer biotherapy (Springer, 1997; Goletz et al., 2003; Franco, 2005).

In addition to its presence on tumour cells, CD176 is known as a differentiation antigen that is generally expressed in human foetal epithelia (Barr et al., 1989). We examined the co-

expression of these two markers of cancer-initiating cells, CD44 and CD133, with CD176 (Lin et al., 2010b). Double immunofluorescence staining experiments with lung, breast and liver cancer cell lines demonstrated that CD44 and CD176 were located at the cellular surface and exhibited co-expression of single cells or cell clusters (Figure 4). In the examined cancer tissues, cells co-expressing CD176 with CD44 and CD133 were also found. Furthermore, when we added 4-OHT to breast cancer cells (20 nM for 24 h), the $CD44^+/CD176^+$ phenotype in one of three cell lines (MDA-MB-435) was enhanced after this treatment. We consider this result as additional evidence for the assumption that this phenotype identifies cancer-initiating cells.

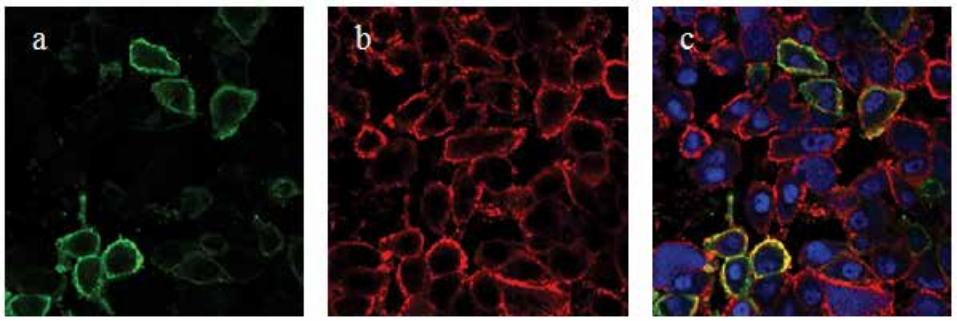

Fig. 4. Confocal microscopy analysis performed with the lung cancer cell line NCI-H446 (magnification: 400x). Cells were stained with monoclonal antibodies specific for CD44 (red) and CD176 (green). Nuclei were counterstained with DAPI (blue). CD44 is strongly expressed in most NCI-H446 cells (a). CD176 expression could be seen at the membrane of cell clusters (b). The mixed picture (c) demonstrates co-localization of CD44 and CD176 in these cell clusters (yellow) (from Lin et al., 2010b)

According to the cancer stem cell hypothesis, recurrences and metastases of cancer depend on cancer-initiating cells (Dalerba et al., 2007). The existence of a population of such cells with properties different from the tumour mass may explain why conventional therapies, e.g. treatment with tamoxifen, are only able to suppress cancer but often cannot completely eradicate it. On the contrary, this treatment may even enhance the number of cancer-initiating cells (Mani et al., 2008). On the other side, the elimination of cancer-initiating cells could actually prevent recrudescences of tumours. The development of new therapeutic approaches to target cancer-initiating cells may therefore have a profound impact on cancer therapy. Our current study demonstrated that CD176 is not only expressed on mature cancer cells but obviously also or even preferably on cancer-initiating cells of solid tumours. The identification of CD176 on cancer-initiating cells of solid tumours is an important argument for the development of CD176-based immunotherapies, and may explain the success of Georg Springer's early vaccination attempts (Springer, 1997). Demasking of CD176 seems to be a selective process that involves only a few among all possible candidate glycoproteins present at the cell membrane. The most prominent carrier molecule of CD176 identified in epithelial cells so far is the polymorphic epithelial mucin MUC-1, for example in breast and colorectal carcinoma (Barr et al., 1989; Cao et al., 1997; Baldus et al., 1998). In $CD34^+$ malignant human hematopoietic stem cells, we have observed that the major carrier of CD176 is a 150-kDa glycoprotein which is CD34 (Cao et al., 2008). Another study showed that a splice variant of CD44 is a carrier of CD176 on colorectal carcinomas (Singh et al.,

2001). In our study, we applied a special sandwich ELISA and examined whether CD44 might also be the carrier molecule for core-1 in lung, breast and liver carcinoma cells. Our data suggest that CD176 is indeed carried by CD44 in tumours other than colorectal carcinomas (Lin et al., 2010b). In other words, it is a more general phenomenon.

3. Conclusion

CD173 (Blood group antigen H type 2, H2), CD174 (Lewis Y, LeY) and CD176 (Thomsen-Friedenreich antigen, core-1) expression were observed in human lung, breast and liver carcinomas and in cell lines derived from these malignancies. Co-expression of CD173, CD174 and CD176 with CD44, as well as CD133 was found in vitro and in vivo. Evidence is provided through immunoprecipitation and in a new sandwich ELISA suggesting that CD44 is a carrier molecule for CD173, CD174 and CD176 not only in colorectal cancer as previously reported, but also in lung, breast and liver cancer. The identification of CD173, CD174, and CD176 on cancer-initiating cells may offer new opportunities in the design of therapies that target cancer-initiating cells in the prevention of relapse. More importantly, these data make CD176, which is almost absent on normal and benign adult human tissues, an even more promising target for tumour therapies.

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19

Influence of Culture Environment and *Mollicutes* Contaminations on CD133 Modulation in Cancer Stem Cells

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1. Introduction

During last years great effort has been addressed towards the research area of cancer stem cells (CSC), also called tumour-initiating cells. The intriguing hierarchical model underlying the CSC theory, has been extensively described: it implies that only a very small cellular compartment within a neoplastic tissue is able to maintain indefinitely tumour growth (Bomken, 2010; Sottoriva, 2010). This cellular nucleus is made up of self-renewing stem cells, connoted by a more aggressive phenotype, that give rise to all differentiated populations forming the tumour (O'Brien, 2010; Zhou, 2009).
Increasing experimental evidences highlight the key role of CD133 (prominin-1) as CSC marker in several human cancers, such as colon carcinoma as well as neural and hepatic tumours (Vermeulen et al., 2008).
The aim of this section is to describe, in details, how environmental factors, culture conditions and mycoplasma infections can play a relevant role in CD133 modulation.
It was clearly demonstrated that environmental variables can determine a dramatic increase of CD133 expression in several human cellular models such as primary culture of tumor cells, neurosphere cultures and continuous cancer cell lines. In particular, it has been shown that a reduction in oxygen tension, during the culture, both in neurosphere cultures (Bar et al., 2010) and in human glioma cell lines (Soeda et al., 2009) can be promptly translated in a reversible over-expression of the stemness marker.
Recently, the involvement of transforming growth factor β (TGFβ) in up-regulation of CD133 expression has been clearly demonstrated in human hepatoma, suggesting the epigenetic mechanism through TGFβ influences CD133 modulation (You et al., 2010).
Additionally, we found evidence that the percentage of CD133+ cells in human colorectal cancer cell lines is considerably increased, in reversible manner, in presence of *Mycoplasma hyorhinis* infection (Mariotti et al., 2010).

In conclusion, all these observations suggest, the need to maintain CSC cultures in a suitable microenvironment, faithful to the hypoxic condition that is "physiologically" found in tumors. In addition, it is fundamental to handle cells in good tissue culture practices in order to prevent dangerous mycoplasma infections. In this respect, it is essential to constantly monitor contamination status of the cultures, by specific detection methods.

2. Cancer stem cells and CD133

Stem cells represent the cell population within a tissue, characterized by a longer lifespan compared to its more differentiated progeny. This feature makes progenitor cells more exposed to genotoxic damage, leading more easily to the induction of oncogenetic mutations, and so to cancer development (Pardal, 2003; Reya, 2001).
The CSC theory implies that cells in tumour have the same hierarchical organization that is generally present in the normal tissue counterpart. Therefore, CSC, can be considered the population responsible for maintaining and growing the tumour, while other cancer cells can only contribute to tumour bulk (Lobo et al., 2007).
The CSC population within a tumour, due to its "auto-protective" nature, is frequently resistant to chemotherapy and radiotherapy and thus it can be responsible for disease relapse, or for metastasis generation; for these reasons the targeting and the eradication of CSC represent the next frontier in cancer therapy (Dean et al., 2005).
Currently, there are increasing evidences that CSC, constitute a small compartment of distinct cells, characterized by a peculiar phenotype in comparison with other cells that form the tumour. Presently, one of the most investigated CSC markers is the CD133. This molecule, also called prominin-1, was the first to be identified in the family of prominin membrane proteins, both in humans and mice and it was initially classified as a marker of primitive haematopoietic and neural stem cells (Mizrak et al. 2008). From a structural point of view, CD133 is a cell surface glycoprotein, formed by five trans-membrane domains, two cytoplasmic loops, two glycosylated extracellular domains, and a cytoplasmic C-terminal domain (Corbeil, 2000; Miraglia, 1997; Yin, 1997). Biological function of CD133 has not yet been elucidated, but its peculiar localization suggests its involvement in the organization of plasma membrane protrusion (Maw et al., 2000). Recently, it has been confirmed that CD133 antigen plays a key role as CSC marker in several human cancers, such as colon (O'Brain, 2007; Ricci-Vitiani, 2007) and hepatocellular (Yin, 2007; Zhang, 2011) carcinoma as well as neural (Pallini, 2011; Singh, 2004) and renal (Bruno, 2006; Florek, 2005) tumours. In particular, it has been demonstrated the ability of CD133 positive cellular fraction, isolated from human tumours, (i) to proliferate *in vitro*, (ii) to differentiate *in vitro* and (iii) to develop a neoplasia with the original phenotype, when it is transplanted in immunodeficent mice, unlike its CD133 negative counterpart (Neuzil, 2007; Singh, 2003).
In conclusion, CSC can be purified on the basis of their specific cell membrane immunophenotypes and in this context, the correct individuation of a peculiar antigenic paradigm can become a fundamental target for CSC detection and isolation, aimed at the development of future innovative therapeutic strategies.

3. Culture environment and CD133 modulation sustained by *Mollicutes*

The employment of human continuous cell lines is essential for studying biological and functional alterations of tumour cells (Hayashi, 2011; van Staveren, 2009). In the previous

paragraph, the relevance of CD133 marker as well as its role in detecting and isolating CSC from several kinds of human solid tumour has been elucidated. Unfortunately, the correct determination of CD133 expression intensity, on cellular surface, can be prejudiced by several environmental factors. Our aim is to describe how modifications of culture microenvironment, induced by a strong reduction in oxygen tension or growth factors exposure or even by *Mollicutes* infections, can play an important role resulting in a dramatic increase of CD133 marker expression. This occurrence has been shown in primary culture of tumour cells (Sheehan, 2011; Soeda, 2009), neurosphere cultures (Bar et al., 2010) and in several human cellular models such as continuous cancer cell lines.

3.1 Culture environment

In standard culture conditions, cancer cells are exposed to the ambient oxygen tension, that is of about 20%. This percentage is far from the mean oxygen tension generally described for *in vivo* tissues, that ranges from 2% to 9% (Brahimi-Horn, 2007; Studer, 2004). Thus, in conventional cell cultures, the oxygenation parameter does not reflect a physiological condition and it can be responsible for function alterations, decrease of proliferation and loss of stem cells undiffentiated state (Ezashi, 2005; Mohyeldin, 2010).

These negative effects of hyperoxygenation on stem cells can be explained by the generation of dangerous reactive oxygen species, that in turn can cause aberration in DNA structure (Busuttil et al., 2003); on the other hand, it has been demonstrated the involvement of hypoxia in the activation of the molecular pathways for the regulation of two fundamental modulators of stemness, such as Oct-4 and Nocht (Simon & Keith, 2008).

Interestingly, the hyperoxic condition can reversibly affect the CD133 intensity of expression in neurosphere (Bar et al., 2010) and human glioma cell lines cultures (Soeda et al., 2009). In particular, human primary tumor neurosphere culture exhibited higher CD133 percentage at 3% of oxygen compared to control culture maintained at 20% of oxygen (Platet et al., 2007); in addition, in hypoxia that is about 1% of oxygen, the human U251MG glioma cells showed a time dependent CD133 increase, that was reversed when cells were exposed to standard normoxic conditions that is about 21% of oxygen (Griguer et al., 2008).

These important data have methodological and conceptual implications. Firstly, the standard culture condition, under atmospheric oxygen, could represent one of the variables involved in the decrease of CD133 cellular phenotype; secondly, CD133 up-regulation could be interpreted as an event occurring when tumor cells are exposed to a non-physiological condition or rather as a stress response.

The environment necessary to obtain stem cells cultures requires several cytokines and growth factors use. In this regard, it was demonstrated that TGFβ is involved in the up-regulation of CD133 expression in the Huh7 continuous cell line, a model for studying human hepatocellular carcinoma. Unexpectedly, TGFβ influenced the marker expression in a dose- and time-dependent manner, triggering specific epigenetic events such as the inhibition of DNA methyltransferase 1 and DNA methyltransferase3β expression, and subsequent demethylation of promoter-1 (You et al., 2010).

Finally, it was also established that epidermal growth factor (EGF) heightened the CD133-positive subpopulation in primary brain tumor stem cell cultures, determining an increase of the stemness marker in a growth factor concentration-related manner (Soeda et al., 2008).

3.2 *Mollicutes* contaminations

Mollicutes contamination still remains one of the major problems tightly correlated to the manipulation of human continuous cell lines. *Mollicutes* infections are extremely dangerous for two main aspects. Firstly, the contaminations are not evident and they can be revealed exclusively by the application of specific detection techniques, such as bioluminescence assays (Mariotti et al., 2008), Polymerase Chain Reaction (PCR), by using specific primer sequences (Uphof, 2002; Shahhosseiny, 2010) and aerobic and anaerobic microbiological agar culture (Uphoff et al., 1992). Secondly, the chronic infections can determine several negative consequences on the cultures, including significant morphological changes, induction of chromosomal aberrations, modification in protein concentration and alterations in DNA and RNA synthesis (Drexler, 2002; Uphoff & Drexler, 2005). In the most serious events *Mollicutes* contaminations lead to the production of unreliable data or to the irreversible lost of the culture itself (Derxler & Uphoff, 2002).

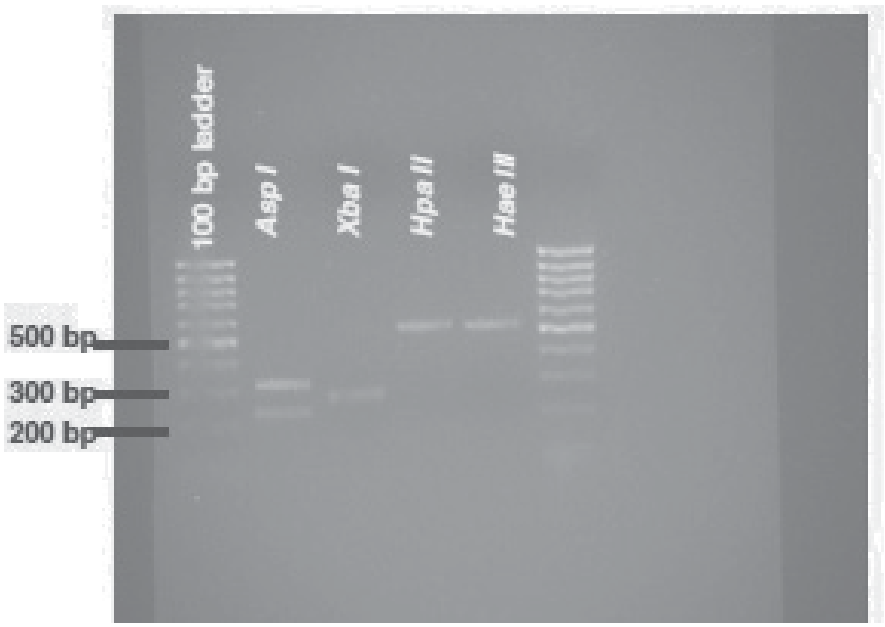

Fig. 1. Agarose gel with the pattern of restriction fragment length polymorphisms that demonstrates the presence of *Mycoplasma hyorhinis* species in cell culture supernatants

In this context, our group demonstrated the heavy influence of *Mycoplasma hyorhinis* contamination on CD133 expression in the CSC colon carcinoma compartment (Mariotti et al., 2010). In particular, our research project was focused on the isolation and characterization of CD133 positive CSC fraction, belonging to the three human colorectal cancer continuous cell lines such as GEO, SW480 and HT-29. All cell lines, as suggested by good tissue culture practice (UKCCR, 2000), were tested for *Mollicutes* contaminations by two independent methods: the biochemical assay MycoAlert® (Cheong, 2010; Mariotti, 2008) and the aerobic and anaerobic microbiological agar culture (Young et al., 2010). All supernatants, derived from the three tested cultures, resulted unequivocally *Mollicutes* positive to the above mentioned applied assays. With the aim of establishing the species of

the *Mollicutes* contaminant, we performed on every continuous cell line culture the extraction of mycoplasmatic DNA, the amplification by PCR using specific primer sequences and the digestion of the amplicons with the restriction endonucleases Asp I, Xba I, Hpa II and Hae III. Finally, the analysis of modified restriction fragment length polymorphisms (Uphoff & Drexler, 2005) demonstrated that GEO, SW480 and HT-29 cell lines were infected by *Mycoplasma hyorhinis,* as shown in figure 1.

It is well known that this mycoplasma species is one of the most common infectious agent found in continuos cell lines, with a frequency of contamination ranging from 10% to 40%. *Mycoplasma hyorhinis* is a frequent pathogenic agent, isolated from the upper swine respiratory tract (Friis & Feenstra, 1994) even if it is thought to derive from bovine source. In fact, as swine and cattle are processed through the same abattoirs, the swine strain of *Mycoplasma hyorhinis* may be introduced into bovine serum, commonly used in cell culture practice (Drexler & Uphoff, 2000).

All cell lines for CD133 cytometric expression were then characterized, in confluent culture of *Mycoplasma hyorhinis* GEO, SW480 and HT-29 infected cells, after adequate detachment with trypsin/EDTA. The monoclonal antibody used in the study was the anti-CD133/1-PE, AC133 clone purchased from Miltenyi Biotec company; an unstained control was prepared for each cell line in order to establish the degree of background cellular autofluorescence (Figure 2, panel A). All samples were analyzed by a FACSAria flow cytometer with the FACS-Diva software.

As shown in panel B of figure 2, we observed the marked separation of the CD133 positive CSC population that was equal to 5.7%, 52.5% and 92.5% in case of GEO, SW480 and HT-29 mycoplasma infected cell lines, respectively.

After mycoplasma decontamination, according to the United Kingdom Coordinating Committee on Cancer Research guidelines (UKCCR, 2000), CD133 expression was re-evaluated in the rescue cell lines, in another set of cytometric experiments. As evidenced by panel C of figure 2, a noteworthy decrease in CD133 expression occurred, resulting in 2.3%, 0.2% and 75.6% in case of GEO, SW480 and HT-29, respectively. These interesting data confirmed the reversibility of the surprising antigenic variation.

In order to confirm the exclusive *Mycoplasma hyorhinis* ability to increase the percentage of CD133 positive cells in human colon carcinoma cell lines , we re-infected GEO model by aliquots of the same contaminant purchased from the American Type Culture Collection (ATCC) international cell bank.

On the day of the inoculation, the medium was completely replaced with fresh medium containing an established dilution of viable *Mycoplasma hyorhinis,* at a concentration of about 10 Colony Forming Unit/ml. The infected cells were then incubated at 37°C and 5%CO_2 and tested with MycoAlert® every seven days. Only in case of positive results they were detached with trypsin/EDTA and processed by flow cytometry, for the evaluation of CD133. The contamination was detectable only after 3 weeks of culture exposition to the specific infective agent.

This experiment set confirmed that the increase of CD133 expression was closely related to *Mycoplasma hyorhinis* presence in GEO cells, and demonstrated that this "side effect" became more severe with the chronicization of the infection. In particular, 3.4%, 4.3%, 26.5% and 37.2% CD133 positive cells were found on days 21, 28, 49 and 56 post infection respectively, wheras 0.3%, 0.7%, 0.8% and 0.9% were detected on days 21, 28, 49 and 56 respectively, in *Mycoplasma hyorhinis* free GEO cells (figure 3 panel C and B).

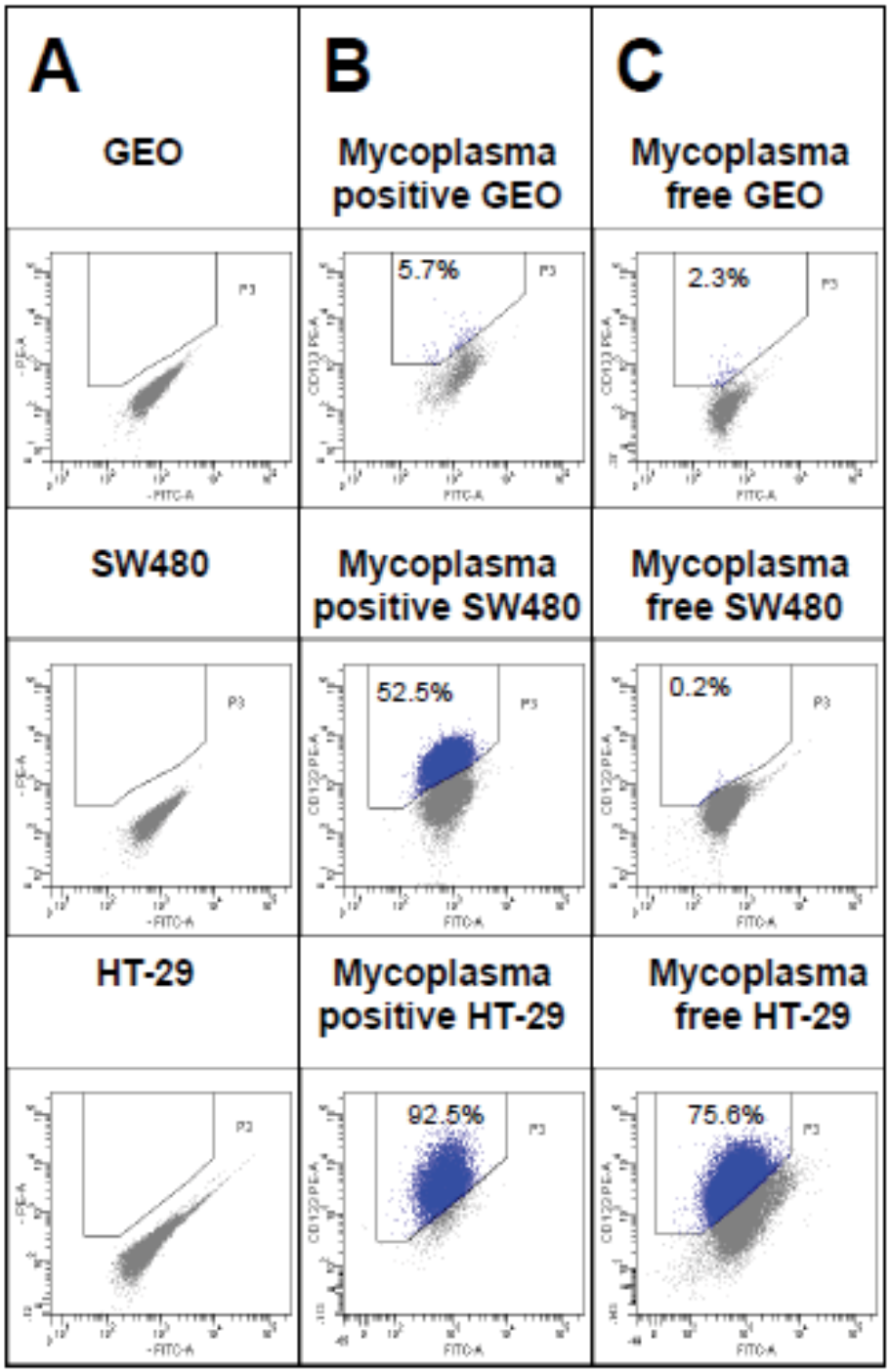

Fig. 2. Cytometric evaluation of CD133 expression in GEO, SW480 and HT-29 cell lines in presence of *Mycoplasma hyorhinis* (panel B), or decontaminated (panel C). Panel A represents the negative control

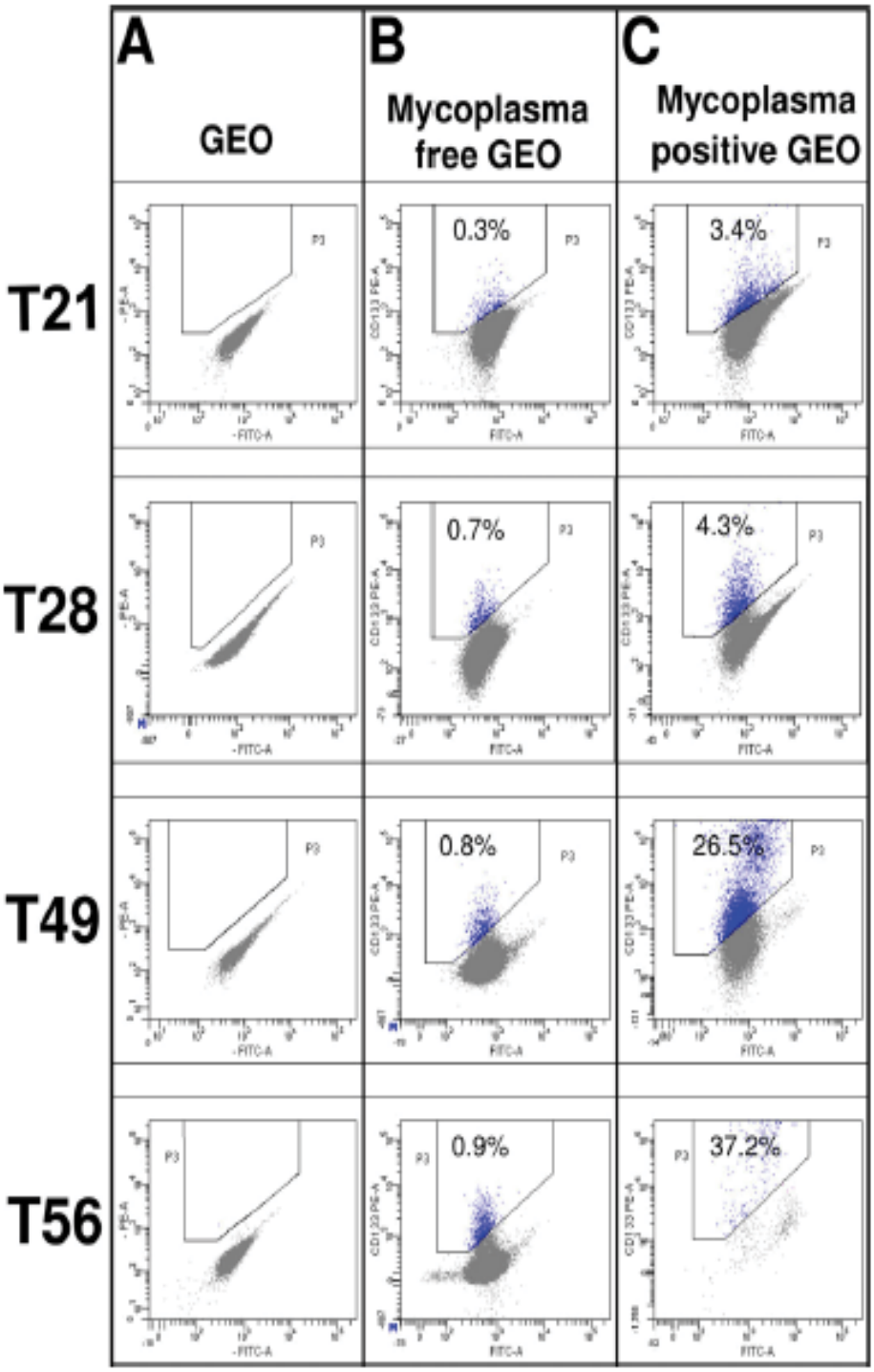

Fig. 3. Cytometric evaluation of CD133 expression in mycoplasma free GEO cell lines (panel B), and (panel C) infected. Panel A represents the negative control

In recent times, the direct *Mycoplasma hyorhinis* involvement in carcinogenesis of human prostatic cells has also been shown (Namiki et al., 2009) as well as its significant role in the increase of mesenchyal stromal cells anti-proliferative action in standard lymphocyte proliferation assay (Zinocker et al., 2011).

4. Conclusion

The aim of this section is to describe the relevant role of environmental factors, culture conditions and *Mollicutes* contamination in CD133-positive CSC compartment.

First of all, even thought standard culture condition implies the use of about 20% of oxygen tension, this "non-physiological condition" seems to be involved in the decrease of CD133-positive CSC cellular fraction. It is important to highlight the need to maintain CSC cultures in a more suitable microenvironment, faithful to the hypoxic condition that is "physiologically" found in tumors. This is desirable in order to work with an *in vitro* cellular model that is, as similar as possible to the original tumor from a biologic and physiologic point of view.

In addition, growth factors as TGFβ and EGF are able to increase CD133 expression in CSC population in a time- and concentration-dependent manner. It is evident that a culture medium containing much more appropriate concentrations of cytokines and growth factors could be another fundamental tool that enables CSC obtained *in vitro* to show a phenotype which can be better compared to the fresh tumor.

Finally, it is interesting to notice the effects exerted by *Mycoplasma hyorhinis* infection on the up-regulation of CD133 positive CSC fraction. This occurrence may be justified by: i) the influence of the contaminant on monoclonal antibody binding sites, or ii) the selective pressure on the CD133-positive CSC fraction, in response to mycoplasma infection. We further demonstrated that the extent of CD133 modulation grew with the increase of the exposure time to the specific infectious agent.

We are confident that a tighter control of microenvironmental variables combined with prevention measures, detection methods and eradication of *Mollicutes* infections, will be an integral part of the basic panel of CSC manipulation techniques.

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Part 5

Cancer Stem Cells in Targeted Therapeutics: New Perspectives

20

Therapeutic Strategies Targeting Cancer Stem Cells

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1. Introduction

The classical clonal evolution theory of neoplastic development supports the notion that cellular transformation results from random mutations and subsequent clonal selection. However, recent advancements in identification of the malignant populations responsible for tumor maintenance and recurrence have lent support to the cancer stem cell (CSC) hypothesis as a model of carcinogenesis, and posit that tumor growth is driven by a rare subpopulation of cells with stem cell-like properties. Cancer stem cells are commonly thought of as derivatives of a normal tissue stem cell that undergoes genetic alterations, which allow for sustained aggressive and clonal characteristics, self-renewal capacity and the ability to differentiate into all populations within a malignancy. Cancer stem cells alone, as compared to the bulk of cells in a tumor, are considered to be responsible for tumor initiation, metastasis and resistance to treatment. Moreover, CSCs are believed to share many properties with normal stem cells providing them with an overall insensitivity to conventional radio- and chemotherapy. Therefore, successful targeting of such a highly tumorigenic yet rare population must be considered in order to improve the therapeutic efficacy of the currently available anti-cancer therapy. Here, we provide a summary of recent progress towards development of biomarkers that identify CSCs, the molecular mechanisms behind the conventional anti-cancer therapy resistance, and the development of therapeutic strategies to selectively target CSC populations.

2. Normal stem/progenitor cells and CSCs

In the developing embryo, stem cells are located in the inner mass of the blastocyst where they are known as the embryonic stem cells and can give rise to the majority of cell types in the body, a characteristic referred to as pluripotency. At later developmental stages, embryonic stem cells differentiate into adult stem cells, which are multipotent (i.e., they can give rise to a restricted number of cell types) and form different tissues and organs (Fuchs and Segre 2000). One of the most important features of stem cells is their ability to undergo asymmetric cell division, a process whereby a progenitor cell gives rise to a new stem cell that can maintain its own progeny through self-renewal.

CSCs reportedly share many properties with normal stem cells. It is still not clear whether CSCs derive from mutated tissue specific stem cells or more differentiated cells that have re-

initiated a self-renewal program as part of or following transformation. Regardless of their origin, CSCs have been found to resemble the normal stem or progenitor cells of their tumor's derivative tissue (Calabrese et al. 2007). Microscopic analysis of many malignancies reveals a complex heterogeneous picture composed of significant phenotypic diversity. Even though CSCs make up only a minor fraction of a tumor, they are defined by their ability to self-renew, producing tumorigenic daughter cells, and their ability to give rise to different nontumorigenic cancer cell phenotypes. Collectively, CSC-descendents contribute to the cellular heterogeneity of the tumor.

3. Molecular signature for cancer stem cell

Cancer stem cells are commonly characterized on the basis of the expression of specific- or combinations of molecules (e.g. CD133 and CD44), but they can also be distinguished from their progeny by functional attributes such as high expression of cytoprotective enzymes (e.g. aldehyde dehydrogenese, ALDH) and drug-efflux pumps (e.g. ABC transporters). Advancements in cell sorting technology via flow cytometry and fluorescent antibodies have enabled researchers to reproducibly isolate phenotypically defined rare cell populations. Utilizing these tools, John Dick's laboratory first isolated CSCs from acute myeloid leukemia (AML) as early as 1997 (Bonnet and Dick 1997). In this pioneering work, Dick and colleagues showed that in human AML, a rare subset of tumor cells with the $CD34^+/CD38^-$ signature possessed the ability to recapitulate the entire original disease over several transplantations. These findings suggest that self-renewal and pluripotency are characteristic of this small subpopulation, and are absent within the broader $CD34^+/CD38^+$ population (Lapidot et al. 1994; Bonnet and Dick 1997). The consensus view of hematological malignancies is that the $CD34^+/CD38^-$ signature does not identify most CSCs (Alison et al., 2011). However, it should be noted that other markers have also been used to characterize CSCs in these cancers (Alison et al. 2011). In contrast, there is little agreement regarding the molecular identity of CSCs in solid tumors. The first solid tumor associated CSCs were isolated from breast cancer in 2003 (Al-Hajj et al. 2003). Since then, CSCs have been identified in brain- (Hemmati et al. 2003; Singh et al. 2003), colon- (O'Brien et al. 2007), melanoma- (Fang et al. 2005), pancreatic- (Hermann et al. 2007), prostate- (Collins et al. 2005), ovarian (Bapat et al. 2005), lung- (Eramo et al. 2008) and gastric-cancers (Fukuda et al. 2009). The isolation of many solid CSCs has been carried out using a number of adhesion markers including CD44 and CD24, or other CSC- associated functional markers such as multidrug efflux proteins ABC transporter and Prominin1 (CD133), an apical plasma membrane protein predominantly found on embryonal epithelial structures. In glioblastoma and medulloblastoma, CD133 is routinely used to enrich for CSCs (Singh et al. 2003). CD133 expression has also been used as a CSC phenotypic marker for colon cancer (O'Brien et al. 2007). However, although CD133 has continued to identify tumor cells with self-renewal capacity in a number of other solid tumors, there is an ongoing debate as to how robust a universal marker it actually is within the solid tumor CSCs (Wu and Wu 2009). Most of the cell surface markers used to distinguish stem cells in normal and cancerous tissues have thus far not been expressed exclusively by stem cells alone. Additionally, the same markers used for isolation of CSCs in one organ cannot directly be used for identification in other organs. Such situations underlie the importance of combining phenotypic markers with functional markers as a signature to identify tissue specific CSCs.

4. Cancer stem cell and patient prognosis

According to the CSC hypothesis, these cells are not only responsible for the unlimited growth of a tumor, but also for the maintenance of the minimal residual disease and constitutive recurrences following therapy and metastasis. Therefore, quantification of the presence of this rare population within a disease burden may serve as a prognostic indicator. Generally, it is believed that a high proportion of stem cells signify a worse prognosis. For example, in breast cancer, the most poorly differentiated tumors have the highest burden of CSCs (Pece et al. 2010). Similarly, elevated immunoreactivity of nestin and CD133 in tumor specimens is associated with a poor prognosis in patients with brain tumors (Laks et al. 2009). Furthermore, others have reported that high CD133 expression in brain tumors is a dismal prognostic marker for progression free- and overall-survival (Zhang et al. 2008). Contrary to these findings, Kim and colleagues recently examined the three established stem cell markers, nestin, CD133 and CD15 in 88 cases of glioblastoma by immunohistochemical analysis and reported that there was no correlation between stem cell marker expression and the clinical outcome of these patients (Kim et al. 2011). Elevated expression of CD133 in colon cancer is also an independent marker of poor prognosis and is associated with liver metastasis (Horst et al. 2009). In pancreatic cancer, high CD133 expression is an independent adverse prognostic factor for 5-years survival and is associated with lymph node metastasis (Maeda et al. 2008). Furthermore, the expression of the CSC specific functional markers, such as ALDH, is directly correlated with a poor prognosis in a number of tumors including AML (Cheung et al. 2007), breast (Morimoto et al. 2009), head and neck squamous cell carcinoma (Chen et al. 2009) and prostate cancer (Rasheed et al. 2010). Another potential CSC associated marker is the ABC transporter, which has also been reported to be a sign of poor prognosis in AML patients, whereby there is a reduced overall 4-year survival associated with elevated expression of ABCC11 (Guo et al. 2009). In summary, patients with tumors that express elevated levels of molecular markers related to CSCs tend to have a poorer prognosis than patients with tumors that express low levels of these makers. However, considering the inconsistency between individual stem cell markers, there is a need to define a CSC signature more precisely before it can be considered as a predictor of clinical outcome.

5. Mechanisms of resistance and stem cell biology

Like normal stem cells, CSCs are considered to be relatively quiescent. This characteristic leads to their relative resistance towards conventional radio- and chemotherapy, which predominantly targets rapidly dividing cells (Rich and Bao 2007). During therapy, the tumor burden may decrease significantly following treatment with the superficial appearance of tumor regression. However, quiescent CSCs can survive this therapy and eventually give rise to new daughter tumor cells and thereby re-initiate disease resulting in recurrence. In chronic myeloid leukemia (CML), a rare population of cells with the hematopoietic stem cell (HSC)-like phenotype harbor the definitive genetic aberration, t(9;22)(q34;q11) – the Philadelphia chromosome. This fusion product, a tyrosine kinase, is sufficient to initiate CML. Despite the immense success of Imatinib, a tyrosine kinase inhibitor, in controlling disease burden, a subpopulation of quiescent stem cells remains inherently resistant to therapy (Elrick et al. 2005).

DNA damage repair pathways are considered to be the guardians of genomic and chromosomal stability. Because stem cells are at the basis of tissue homeostasis, they appear

to have a very efficient DNA repair capacity (Maynard et al. 2008). Moreover, it has been demonstrated that central nervous system (Smith et al. 2000) and hematopoietic stem cells contain lower levels of reactive oxygen species (ROS) as compared to their mature counterparts due to increased antioxidant defenses (Ito et al. 2004). In glioblastoma, increased DNA repair capacity in $CD133^+$ CSCs appears to be related to the resistance to treatment (Bao et al. 2006). Other contributions to CSC radioresistance may arise from the fact that they may reside in hypoxic niches (Baumann et al. 2008). An increased expression of drug transporters, such as the ABC transporters, has been reported in many different types of normal stem cells (Zhou et al. 2001) and also in CSCs (Ginestier et al. 2007). Some studies suggest that the expression of such pumps on the cell surface represents a class of CSCs with high drug efflux capacity and an inherently increased resistance to chemotherapeutic agents (Hirschmann-Jax et al. 2004). Thus, it seems that CSCs may resist standard anti-cancer therapies via a combination of molecular mechanisms associated with normal stem cell biology. It is widely believed that in order to prevent relapse, effective targeting of CSCs is likely to be essential.

6. Therapeutic strategies targeting cancer stem cells

Most currently available cancer therapies are designed to target cells that are highly mitotic and rapidly dividing. However, nearly all malignancies are heterogeneous in nature. Many different types of cells have been shown to be present in tumors, including CSCs. These cells, which appear to provide the tumor initiating capacity in many cancers, are further capable of evading conventional chemo- and/or radiotherapy due to their inherent stem cell characteristics. After therapy, tumors have a higher population of therapy resistant stem cells; therefore specific targeting of this population is of utmost importance. Here, we highlight the latest strategies to successfully target and eliminate CSCs, the roots of cancer.

6.1 Pharmacological targeting

Small molecule inhibitors have shown promising results when used alone or in combination to target slow growing chemo- and radio-resistant CSCs. Several strategies have been employed including targeting signaling pathways that impart chemo- and radio-resistance to CSCs, thereby increasing their susceptibility to conventional therapy. Examples include targeting protective vascular niches that shield CSCs from environmental insults (Gilbertson and Rich 2007) and directly targeting apoptotic pathways, hence inducing programmed cell death in CSCs.

In malignant brain tumor models, Bao et al. demonstrated that following conventional radiation therapy, a fraction of CD133+ brain tumor stem cells is enriched, which essentially contributes to the resistance of glioma through preferential activation of the DNA damage checkpoint response and to an increase in DNA repair capacity. Using specific pharmacological inhibitors of the Chk1 and Chk2 DNA checkpoint kinases, the radioresistance of $CD133^+$ brain tumor stem cells can be reversed, making them susceptible to ionizing radiation (Bao et al. 2006). Reactive oxygen species (ROS), critical mediators of ionizing radiation-induced cell killing, are shown to be present at lower levels in some subsets of the CSC population in breast cancer. Lower ROS levels in CSCs are associated with an increased expression of free radical scavenging systems predisposing CSCs to develop less DNA damage and preferential sparing after irradiation. Pharmacological depletion of ROS scavengers in CSCs significantly decreases their clonogenicity and results

in radiosensitization (Diehn et al. 2009). Similarly, several pharmacological agents have shown promising results in the reversal of chemoresistance to the CSC population. In the setting of malignant glioma, the therapeutic efficacy of temozolomide (TMZ), an alkylating agent, on chemoresistant glioma stem cells was shown to be enhanced by Notch and SHH pathway inhibition with GSI-I and Cyclopamine (Ulasov et al. 2011). In CML, the combination of tyrosine kinase inhibitors (TKIs), i.e., imatinib, nilotinib, and/or dasatinib, with inhibitors of autophagy resulted in a near complete elimination of phenotypically and functionally defined CML stem cells (Bellodi et al. 2009). Pharmacological inhibition of Hedgehog (Hh) signaling, an essential stem cell maintenance pathway, has been found to impair not only the propagation of CML driven by wild type BCR-ABL1, but also the growth of imatinib-resistant CML in both mouse models and in humans (Zhao et al. 2009). BMS-214662, a cytotoxic farnesyltransferase inhibitor, alone or in combination with the tyrosine kinase inhibitors, imatinib mesylate and/or dasatinib, induces a selective apoptosis of proliferating and quiescent CML stem/progenitor cells while sparing normal stem/progenitor cells. BMS-214662 was also found to be cytotoxic even against CML blast crisis stem/progenitor cells, particularly in combination with a tyrosine kinase inhibitor and equally effective in cell lines harboring wild-type vs. mutant BCR-ABL (Copland et al. 2008). In AML, Zeng et al. demonstrated that SDF-1alpha/CXCR4 interactions contribute to the resistance of leukemic cells to signal transduction inhibitors and chemotherapy-induced apoptosis (Zeng et al. 2009). Therefore, it is not surprising that the disruption of these interactions with pharmacological inhibitors of CXCR4 is an effective strategy for sensitizing these leukemic stem cells to chemotherapy, thus targeting CSCs within the protective bone marrow microenvironment (Zeng et al. 2009).

CSCs are situated in vascular niches or microenvironments that tightly regulate the supply of oxygen and nutrients to CSCs and thus control their self renewal and differentiation of CSCs (Gilbertson and Rich 2007). In glioma xenograft models, the depletion of vascular endothelial cells by ERBB2 inhibitors (erythroblastic leukemia viral homolog 2, also known as human epidermal growth factor receptor 2) or VEGF signaling inhibitor has been shown to ablate self-renewing cells (Calabrese et al. 2007). Various small molecules have been shown to directly induce apoptosis in CSCs. In leukemia, TDZD-8 (4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5 dione) induces cell death by rapid loss of membrane integrity, depletion of free thiols and inhibition of both the PKC and FLT3 signaling pathways in phenotypically primitive cells, *in vitro* colony-forming progenitors, and leukemia stem cells, while sparing the physiology in the hematopoietic stem cell compartment (Guzman et al. 2007). The mTOR inhibitor rapamycin also effectively targets leukemia stem cells via modulation of the phosphoinositide 3-kinase/phosphatase and tensin homolog (PI3K/PTEN) pathway (Yilmaz et al. 2006). In AML, combinational treatment with the proteasome inhibitors carbobenzoxyl-l-leucyl-l-leucyl-l-leucinal (MG-132) and anthracycline idarubicin produces rapid and extensive apoptosis in the CSC population via inhibition of nuclear factor kappaB (NF-κB) and activation of p53-regulated genes, while leaving the normal hematopoietic stem cells viable (Guzman et al. 2007). In the setting of prostate cancer, inhibition of PI3K activity by the dual PI3K/mTOR inhibitor, NVP-BEZ235, inhibited the growth of $CD133^{+}/CD44^{+}$ prostate cancer progenitors (Dubrovska et al. 2009). Treatment with Lapatinib, an epidermal growth factor receptor [EGFR]/HER2 pathway inhibitor, in conjunction with conventional therapy, decreases the relative frequency of chemoresistant $CD44^{+}/CD24^{-/low}$ breast cancer stem cells (Li and Ren 2008). In a phase III randomized trial for metastatic breast cancer using doxorubicin with or without N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine

(DPPE; tesmilifene), apoptosis was preferentially induced in $CD44^+/CD24^{-/low}$ breast cancer stem cells. The increase in apoptosis attributed to the addition of the latter agent resulted in a significant improvement in the overall survival and a trend towards progression-free survival (Deng et al. 2009).

Most of the anti-cancer drugs target proliferating tumor cells, which dramatically reduce tumor burden but to achieve durable cures may require successful elimination of CSCs that are proven to be responsible for tumor initiation, metastases and relapse. In the search for drugs that selectively target CSCs, we need to make sure that identified compounds do not affect normal stem cells with which CSCs share many properties.

6.2 Immunotherapy

Cancer stem cells (CSCs) have been associated with immunosuppressive properties (Di Tomaso et al. 2010), which are likely a critical part of the mechanism which endows the cells with tumor-promoting and immunomodulatory characteristics. CSC lines have also been shown to be deficient or expressing low levels of MHC-I, MHC-II, antigen processing machinery and NKG2D. Accordingly, recent work has demonstrated that the culture supernatant of adipose tissue stem cells isolated from breast cancer tissues upregulates the immunosuppressive cytokines (IL-10, TGF-β and induces conversion of conventional T cells into FoxP3-expressing regulatory T cells (Razmkhah et al. 2011), a potently immunosuppressive cell type of the adaptive immune system. Furthermore, CD200 has recently been recognized to be expressed by CSCs (Kawasaki et al. 2007). CD200-deficient mice exhibit gross autoimmune pathology when challenged in various models of experimental autoimmune encephalomyelitis and collagen-induced arthritis (Hoek et al. 2000), implicating the important contribution of CD200 to normal tolerance and immunosuppression.

Understanding the many different immunosuppressive pathways in CSCs allows for a more effective design of therapeutic elimination strategies. Among them, a combinatorial approach incorporating immunotherapy has been given serious consideration. One of the tenants of immunotherapy for CSCs is that while chemotherapy, surgical debulking and/or radiotherapy destroy the majority of cancer cells, the survival of small populations of cells with cancer-initiating potential persists, which can be targeted by the activated immune system. Accordingly, recent work has demonstrated that dendritic cells CSCs-fusion resulted in the activation of T cells expressing elevated levels of IFN-γ, with an enhanced killing-property against CSCs (Weng et al. 2010). In glioblastoma, CSCs growth and survival is supported by interleukin 6 (IL6) signaling based on data demonstrating that IL6 blockade contributes to CSC clearance (Wang et al. 2009). In patients with glioblastoma, elevated IL-6 and its receptor expression are associated with reduced survival indicating potential clinical utility of the IL-6 signaling cascade as a target for therapy of primary tumors of the CNS (Wang et al. 2009). Glioma specific CSCs can also be targeted and killed by cytoloxic T lymphocytes (CTL) through a perforin-mediated mechanism. This is supported by recent work by Brown and colleagues demonstrating that CSCs derived from high-grade glioma may be recognized and eliminated by $CD8^+$ CTLs (Brown et al. 2009).

6.3 Genetic targeting

6.3.1 miRNA

Given that different types of cancer cells have a specific microRNA expression profile, utilization of microRNA-based therapeutic tools to target CSCs is being increasingly

exploited. The potential for CSCs self-renewal depends on a complex interplay of gene expression and environmental stimuli. Gene expression is controlled at different levels and can also be regulated by small micro-RNA molecules (miRNAs). miRNA are non-coding short nucleotide sequences (≈22 nucleotides in length) that block translation of transcripts with complementary mRNA sequences (Bartel 2004). Since miRNAs regulate differentiation and can function as tumor suppressors or oncogenes, the field of miRNA biology has become a subject of great interest for understanding stem cell self-renewal, immunosuppressor status and long lifespan. The number of miRNAs associated with cancer stem cells is continuously expanding. RNA interference strategies are at the frontline of targeting the aberrant expression of different genes utilizing miRNAs. Yu et al. (Yu et al. 2007) using breast cancer stem cell were able to increase the expression of let-7 miRNA, accomplished via a lentiviral vector, leading to reduced proportion of stem cells and resulting in delayed tumor formation and metastasis. Similar results can be achieved not only in solid tumors, but also in leukemia. miRNA-17-92 was found to be up-regulated in leukemia stem cells (Jiang et al. 2010). However, targeting such CSC specific miRNA through RNA interference is yet to be tested in other major types of malignancies.

6.3.2 Oncolytic virus

Virotherapy, a therapeutic approach utilizing conditionally replicative viruses, may hold a potential to directly target self-renewing CSCs. In pre-clinical trials these viruses have demonstrated that these vectors function independent of common resistance pathways that exist for chemotherapeutic agents. Moreover, it is possible to construct viruses that would preferentially target cancer stem cells or other drug-resistant cells. Here, we summarize the recent efforts to develop oncolytic virus in order to target CSCs.

Herpes simplex virus

Attenuated herpes simplex virus (HSV) was used as one of the first gene therapy vectors to target glioma cells. To reduce neurotoxicity, HSV was deleted for RL1, which encodes the ICP34.5 and allows for virus replication even in the presence of the interferon (IFN) response. Later generations of the HSV construct restored the gene under the control of specific enhancers expressed in cancer cells. To preferentially target glioma stem cells that expressed high levels of nestin, the ICP34.5 was restored under the control of nestin, creating rQnestin34.5 (Kambara et al. 2005). Glioma stem cells shown to be resistant to conventional therapies were susceptible to the virus. Interestingly, IFNβ treatment inhibited viral replication only in neurospheres, known to be enriched of stem cells (Kurozumi et al. 2008). This is important in light of the general view that cancer cells have a deficient interferon response and viruses such as VSV may not be able to target CSCs.

Adenovirus

Adenoviruses (Ad) are the most commonly used gene therapy vectors. After entry into the cell, the adenoviral early transcription region E1A binds with the cell cycle regulating protein retinoblastoma (Rb). Rb happens to be frequently mutated in various human cancers. Oncolytic adenoviruses were created by deleting a 24 nucleotide (Δ24)-specific region in E1A that binds Rb: as a result, the virus replicates only in cells that contain mutant Rb cells. Another approach has been to create adenoviruses that express E1A under the regulation of specific promoters found only in cancer cells. Since not all target cells express the necessary coxsackie-adenovirus receptor (CAR) to improve viral entry, adenoviruses

have undergone further surface modifications, such as replacing the fiber with serotype 3 adenovirus (Ad5/3), or by adding polylysine (Ad.pk7) and RGD (Ad.RGD). Breast cancer stem cells were shown by Eriksson et al. to be successfully targeted by mutated Ads, Ad5/3-Δ24 and Ad5.pk7-Δ24 (Eriksson et al. 2007). These viruses were able to kill not only $CD44^{+}/CD24^{-/low}$ breast cancer stem cells, but also more differentiated cells. By using stem cell specific promoters, Cox-2, hTERT and mdr, Bauerschmitz et al. (Bauerschmitz et al. 2008) from the same group was able to show a reduction in breast cancer stem cell population after treatment with Ad5/3-mdr-Δ24. Adenoviruses have shown similar efficacy in gliomas, esophageal and other CSC models.

Reovirus

Not all oncolytic viruses are affected by stem cell properties of cancer cells or target preferentially a sub-population within the tumor. Reovirus relies on abnormal activation of the Ras signaling pathway and Ras levels are equally upregulated in cancer stem cells and in the more differentiated populations (Marcato et al. 2009). As a result, Reovirus induces rapid oncolysis of colon-, breast-, lymphoma-, brain- and spinal-cancer cells without changing the cancer stem cell frequency. Since it causes a restriction of the cancer stem cell pool, it can also prove of benefit in prolonging remissions.

The major benefit of gene targeting in cancer stem cells is the opportunity it offers in discovering new therapies. Although most of genetic approaches remain to be proven in clinical trials, they appear promising. Their safety profile and no cross-resistance with current therapies make them formidable candidates to combine with the conventional anti-cancer therapy.

7. Conclusion

The identification of CSCs and the recent knowledge about the ability of CSCs to resist conventional chemo- and radiotherapy have prompted a number of novel targeting strategies to attack this rare but extremely important tumor subpopulation in order to eradicate cancer. Functional characterization of CSCs in disease progression and therapeutic resistance has altered our understanding of malignancy, which has led to a re-evaluation of conventional therapies for cancer. Although the jury is still out regarding the molecular signature used for the definitive isolation and characterization of the CSCs as well as defining the exact role that CSCs play in malignant disease progression, there is little doubt regarding the extraordinary capacity of CSCs to promote tumor growth, angiogenesis, invasion, therapeutic resistance and disease relapse post-therapy. These characteristics make CSCs a vital cell population that should be targeted effectively in order to develop successful anti-cancer therapy. Novel and effective therapies directed against CSCs may significantly improve the therapeutic efficacy of the currently available conventional therapies. Recent advancements in stem cell biology have allowed us to gain remarkable insight into the molecular mechanisms or signaling pathways that are differentially present in CSCs and non-stem cell tumor burdens. In this chapter, we have discussed several key molecular and signaling targets that have the potential to be utilized for future development of anti-CSC therapeutics. Most of these targets are still a long way from clinical application. However, the CSC paradigm has provided exciting new venues to improve cancer treatment by reducing recurrence and metastasis, which are considered to be the main cause of most cancer fatalities.

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Latest Therapeutic Approaches Based on Cancer Stem Cells

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1. Introduction

A tumor is a caricature of normal tissue and appears undifferentiated because of the preponderance of undifferentiated proliferating stem cells in relationship to the number of cells that have differentiated and become benign. The hypothesis that cancer arises from de-differentiation suggests that cancer is a multigenic complex disease that broadly represents uncontrolled proliferation, blockage in cellular differentiation and metastasis [1]. Recently, cancer stem cells (CSCs) theory hypothesizes the presence of a hierarchically organized, relatively rare population of cells that possess self-renewal capacity and pluripotency, and can drive tumor initiation and maintenance, and the hypothesis has been generally accepted by most basic and clinical scientists.

In recent years the putative CSCs have become the focus of intense research, with the understanding the biological characteristics of the CSCs that have now been identified in several tumor types might prove useful in new therapeutic targets [2]. It is known that tumor ablation, hormonal therapy, radiotherapy, antiangiogenic, and chemotherapy, individually or in combination with, are currently the mostly applied therapies for treating patients diagnosed with cancers including leukemias and malignant solid tumors. Although these therapies are effective in an initial phase of treatment, the progression of cancer to locally invasive and metastatic cancer is often associated with intrinsic or acquired resistance to routine treatment, and the prognosis is poor. The CSC model has favored the conceptual development of targeted therapies towards molecular pathways relevant in CSC maintenance and progression, independent of their ability to induce cell death of non-CSC [3]. However, how to achieve CSC eradication is emergency to overcome the major barrier. It is thus evident that latest therapeutic approaches based on CSCs have suggested that developing novel strategies by focusing biological characteristics on the CSCs may allow for the discovery of effective methods to eradicate seeds of malignant tumor cells by specifically targeted therapy. In this article, we describe here latest therapeutic approaches based on CSCs over the past years, of which includes targeted therapy directed toward CSCs by

surface specific markers, CSC survival niche, blocking signal pathways, manipulation of miRNA and siRNA, screening directly drug-resistant CSCs in three dimensional cell culture and dendritic cell vaccination.

2. Therapeutic strategies to target CSCs

2.1 Targeted therapy directed toward CSCs

CSCs have been clearly identified in leukemias and some solid tumors. The major barrier to therapy is the CSCs with constitutive multidrug resistance (MDR). The approaches can be developed to eliminate the CSCs without excessive toxicity to normal stem cells if successful therapy awaits the discernment of biological and immunologic differences between the tumor and normal stem cells. Increasing evidence suggests that the treatment approaches may be acquired successfully by the targeted therapy directed toward CSCs.

2.1.1 Oncolytic viruses-based CSC therapies

Oncolytic viruses have undergone many developments and through multiple generations offer an effective way to specifically target and eradicate CSCs without damaging normal tissue and stem cells. Therefore, use of oncolytic adenoviruses or oncolytic reovirus presents an attractive antitumor approach for eradication of CSCs. For instances, the $CD44^{+}CD24^{-/low}$ cells that were identified as CSCs isolated from breast tumor patients could be effectively killed by oncolytic adenoviruses Ad5/3-Delta24 and Ad5pk7-Delta 24. In mice, $CD44^{+}CD24^{-/low}$ cells formed orthotopic breast tumors but Ad5/3-Delta24 and Ad5 pk7-Delta24 were effective against advanced orthotopic $CD44^{+}CD24^{-/low}$-derived tumors. This suggested that Ad5/3-Delta24 and Ad5pk7- Delta24 could kill $CD44^{+}CD24^{-/low}$ breast CSCs as well as committed breast cancer cells, making them promising agents against CSCs [4,5]. More recently, to test the efficacy of oncolytic reovirus to target and kill breast CSCs that were identified based on $CD44^{+}$ $CD24^{-/low}$cell surface expression and overexpression of aldehyde dehydrogenase *in vivo*, Marcato and his colleagues established palpable tumors in the mammary fat pads of immunodeficient NOD/SCID mice using a core biopsy sample of a primary infiltrating ductal carcinoma obtained from a breast cancer patient at the time of her primary surgery. The result showed that the oncolytic reovirus that induces inhibition of human breast cancer primary tumor samples xenografted in immunodeficient NOD/SCID mice also effectively targets and kills CSCs in these tumors. The experiments indicate that oncolytic reovirus has the potential to induce tumor regression in breast cancer patients. More important, the CSC population was equally reduced and was as susceptible to reovirus treatment as the non-CSC population [6].

2.1.2 Telomerase-based CSC therapies

Telomerase is the enzyme that synthesizes telomeric DNA, the terminal DNA at chromosome ends which, together with telomere-binding proteins, confers stability to hromosomes [7, 8]. Telomerase is expressed in almost all cancer cells and is required for long-term maintenance of telomeres and replicative immortality but is inactive in most normal somatic cells. The telomerase properties suggests that tumors are less likely to develop resistance to telomerase-based therapies than they are to other cancer targets that are members of a family of genes such as growth factor receptors or signal transduction enzymes. Putative CSCs are telomerase-positive and require telomerase to proliferate,

therefore, telomerase is an important drug target for CSCs. Imetelstat (GRN163L), a specificinhibitor of the reverse transcriptase activity of telomerase, in combination with effective tumor de-bulking agents, might help meet a major unmet need: that is notoriously resistant to standard chemotherapy and radiation [9]. The study indicated that the telomerase activities in established radioresistant (R) esophageal carcinoma cell lines (Seg-1R Seg-1R, Seg-1R side population (SP), and TE-2R) were significantly higher than in Seg-1, Seg-1R non-SP, and TE-2 cells, respectively. Using the telomerase-specific oncolytic adenoviral vector carrying apoptotic tumor necrosis factor-related apoptosis-inducing ligand and E1A gene (Ad/TRAIL-E1) may preferentially target CSCs because Seg-1R and TE-2R CSCs were more sensitive to Ad/ TRAIL-E1 than parental cells. Increased coxsackie-adenovirus receptor and elevated transgene expressions were found in the Seg-1R and TE-2R CSCs. Ad/TRAIL-E1 caused marked tumor growth suppression and longer survival in Seg-1R-bearing mice with no obvious toxicity [10]. In breast and pancreatic cancer cell lines, telomerase activity in the bulk tumor cells and CSC subpopulations were inhibited when these cells were treated with GRN163L *in vitro*. Additionally, *in vitro* treatment with GRN163L, but not control oligonucleotides, the breast and pancreatic cell lines also reduced the proliferation and self-renewal potential and resulted in cell death after less than 4 weeks of treatment. *In vitro* treatment of PANC1 cells showed reduced tumor engraftment in nude mice, concomitant with a reduction in the CSC levels. Significantly, the differences between telomerase activity expression levels or telomere length of CSCs and bulk tumor cells in these cell lines did not correlate with the increased sensitivity of CSCs to GRN163L, suggesting a mechanism of action independent of telomere shortening for the effects of GRN163L on the CSC subpopulations [11]. However, in human multiple myeloma CSCs derived from cell lines and primary clinical specimens, telomerase inhibition targets clonogenic myeloma CSCs through telomere length-dependent and independent mechanisms. This is because two weeks of exposure to GRN163L resulted in a significant reduction in telomere length and the inhibition of clonogenic myeloma growth both *in vitro* and *in vivo* ^{125}I-TSH binding inhibitor immunoglobuli.

No matter what mechanisms, through telomere length-dependent and independent mechanisms or mechanism of action independent of telomere shortening, these results suggest that GRN163L-mediated depletion of CSCs may offer an alternative way by which telomerase inhibition may be exploited for CSCs therapy [12].

2.2 Targeted therapy toward CSC by surface specific markers

CSC markers are expressed in the different cancers with the different patterns seen for the different histological types and degrees of differentiation. These markers provide the means to effectively target and eradicate CSCs.

2.2.1 ABCB5 and ABCG2 markers

ABCB5 molecule is one of ATP-binding cassette (ABC) transporters that have been identified in a variety of mammalian cells and represents a novel molecular marker for a distinct subset of chemoresistant, stem cell phenotype-expressing tumor cells among melanoma bulk populations, whereas, none of the benign nevi of non-melanoma patients demonstrated expression of ABCB5. One study indicated that human malignant melanoma CSCs were defined by the expression of the chemoresistance mediator ABCB5$^+$ molecule that the specific targeting of this tumorigenic minority population inhibited tumor growth.

ABCB5$^+$tumor cells showed a primitive molecular phenotype and correlated with clinical melanoma progression. In the serial human-to-mouse xenotransplantation experiments, ABCB5$^+$melanoma cells possess greater tumorigenic capacity than ABCB5$^-$bulk populations. Systemic administration of a monoclonal antibody directed at ABCB5 molecule was shown to be capable of inducing antibody-dependent cell-mediated cytotoxicity in ABCB5$^+$CSCs or ABCB5 blockade significantly reversed resistance of G3361 melanoma cells to doxorubicin [13, 14].

ABCG2, also called BCRP1 (breast cancer resistance protein 1) and another ABC transporters, often overexpress in therapeutics-resistant CSCs. More recent study showed that using ABCG2- molecule-expressing side population cells may identify cancer stem-like cells in a human ovarian A2780 cell line. The up-regulation of ABCG2 in cancer stem-like cells is closely associated with resistance to anticancer drugs vincristine, and after the ABCG2$^+$ cancer stem-like cells were incubated with the anti-ABCG2 monoclonal antibody, the cell survival rate was remarkably decreased compared with the ABCG2$^+$ cancer stem-like cells without monoclonal antibody incubation. The data suggest that the selective anti-ABCG2 monoclonal antibody can effectively targets and inhibits ovarian cancer stem-like cell growth in the A2780 cell line [15].

2.2.2 CD133 markers

CD133 molecule has been proposed as a surface marker of CSCs in a variety of solid primary tumors such as medulloblastomas, glioblastomas and subsequent CSCs in the cancers of epithelial tissues [16]. For noninvasive imaging of CSCs as well as CSC-specific therapies, Tsurumi et al used the CD133-specific monoclonal antibody AC133.1 for quantitative fluorescence-based optical imaging of mouse xenograft models based on isogenic pairs of CD133 positive and negative cell lines. They selected lentivirally transduced CD133-overexpressing U251 glioblastoma cells and HCT116 colon carcinoma cells that uniformly express CD133 at levels comparable to primary glioblastoma stem cells. The results indicated that the visualization and quantification of CD133 in overexpressing U251 xenografts was set up. The binding of i.v.- injected AC133.1 antibodies to CD133$^+$CSCs isolated from xenografts made efficient targeting and elimination of CSCs. This data showed that CD133 antibody-based CSC targeting is feasible [17]. In addition, a murine anti-human CD133 antibody (AC133) conjugated to a potent cytotoxic drug, monomethyl auristatin F, effectively inhibited the growth of Hep3B hepatocellular and KATO III gastric cancer cells by induced cell apoptosis in the cancer cells *in vitro*. Simultaneously, anti- CD133-drug conjugate treatment also resulted in significant delay of Hep3B tumour growth in SCID mice [15]. Certainly, it is pivotal to how select and develop the specific antibodies to CSCs as well as the targets they bind to and the drugs used in combination with them.

2.3 Targeted therapy toward CSC survival niche

In all tissues, stem cells are located in a specialized vascular microenvironment, the niche; intrinsic and extrinsic signals from the niche regulate self-renewal and differentiation. The hypothesis that CSCs may exist in microenvironmental niches is also important in understanding the potential dynamic state of CSCs [19, 20]. As many properties of stem cells are shared by at least a subset of cancer cells or CSCs, targeting the CSC survival niche may disconnect intrinsic and extrinsic signals from the niche that maintains and governs CSCs in their division and differentiation.

2.3.1 Vascular niche

Oncogenic transformation and aberrant cellular differentiation are regarded as key processes leading to CSCs. Intracellular events involved in these changes profoundly impact the extracellular and systemic constituents of cancer progression, including the angiogenesis, vasculogenesis, activation of the coagulation system and formation of CSC-related and premetastatic niches. It is known that CSCs initiate tumor neovascularization and promote invasion by the secretion of vascular endothelial growth factor (VEGF) and recruit bone marrow-derived cells (BMDC), which play a critical role during the progression of cancer in tumor-bearing mice and cancer patients, to what is now referred to as the pre-metastatic niche. Although the mechanisms of CSC neovascularization is not clear an interfering with BMDC induction by blocking secretion of VEGF virtually eliminates metastasis in these cancers. For example, Bevacizumab, a monoclonal antibody against VEGF, and poly-ADP- ribose polymerase inhibitors, is currently in clinical trials for its antiangiogenic properties that have shown promising preclinical and clinical activities against metastatic colorectal cancer, particularly in combination with chemotherapy [21, 22]. Herbert et al treated the 402 patients with previously untreated metastatic colorectal cancer with irinotecan, bolus fluorouracil, and leucovorin (IFL) plus bevacizumab (5 mg per kilogram of body weight every two weeks), and the 411 patients by IFL plus placebo, respectively. The overall survival, the response rate, the duration of the response, safety were evaluated. The results showed that the median duration of survival was 20.3 months in the group given IFL plus bevacizumab, as compared with 15.6 months in the patients treated with IFL plus placebo, corresponding to a hazard ratio for death of 0.66 ($P<0.001$). The median duration of the response was 10.4 months in the patients treated with IFL plus bevacizumab, as compared with 7.1 months in the patients treated with IFL plus placebo ($P=0.001$).The data showed that the addition of bevacizumab to fluorouracil-based combination chemotherapy results in meaningful improvement in survival among patients with metastatic colorectal cancer[21].

Investigation of the role of CSCs in tumor vascularization and the interaction between CSCs and their vascular niches provides new insight into our understanding for tumorigenesis. A growing body of literature shows that CSCs preferentially produce higher levels of angiogenic factors, for instance, VEGF and interleukin 8 (IL-8, CXCL8). In an established breast carcinoma cell line MCF-7, an identified subpopulation of sphere-forming cells with CSC properties expressed higher levels of VEGF mRNA and detected higher amounts of VEGF protein in culture medium, which suggested CSCs might possess stronger proangiogenic capability than more differentiated tumor cells [23]. Evidence from the studies has indicated that CSCs might generate or transdifferentiate into endothelial cells (ECs) for neovascularization. 20%-78% of the ECs identified by CD105 expression exhibited amplification of the oncogene MYCN in the origin of the nephroblastoma, all of which strongly implicates the possible cancer cell origin of ECs in MYCN amplification [24] nephroblastoma. These studies suggested that CSCs might initiate and promote neovascularization at the early stage of tumor tumorigenesis and progression. The fact that vascular niches support self-renewal, proliferation and differentiation of CSCs suggests the combination of targeting CSCs and their vascular niche will provide more effective therapy for tumor treatment [25].

Tissue factor (TF) is a unique cell-associated receptor for coagulation factor VIIa, initiator of blood coagulation, and mediator of cellular signalling, all of which influence vascular homeostasis. TF pathway may play an important role in formation of the vascular niche for

tumor initiating CSCs, through its procoagulant and signal effects. It was noted recently that in human glioma cells, a transforming mutant of the epidermal growth factor receptor (EGFRvIII) triggers not only the expression of TF, but also of its ligand (factor VII) and protease activated receptors (PAR-1 and PAR-2). The tumor cells expressing EGFRvIII become hypersensitive to contact with blood borne proteases (VIIa, thrombin), which upregulate their production of angiogenic factors (VEGF and IL-8), and result in formation of the growth promoting microenvironment (niche). If TF was overexpression the change could accompany the features of cellular aggressiveness such as markers of CSCs (CD133), epithelial-to-mesenchymal transition (EMT) and expression of the angiogenic and prometastatic phenotype. If TF was blocked with blocking antibodies the change could inhibit tumor growth, angiogenesis, and especially tumor initiation upon injection of threshold numbers of tumourigenic cells. These observations suggest that both cancer cells and their adjacent host stroma contribute TF activity to the tumor microenvironment. Therefore, the TF pathway may play an important role in formation of the vascular niche for CSCs, through its procoagulant and signaling effects. Therapeutic blockade of these mechanisms could hamper CSC processes [26-28].

2.3.2 Tumor-associated ECM

An increasing body of evidence has shown that the host microenvironment undergoes extensive change during the evolution and progression of CSCs. This involves the generation of cancer-associated fibroblasts (CAFs), which lead to enhanced angiogenesis, increased tumor growth and invasion by release of growth factors and cytokines that activate the adjacent extracellular matrix (ECM) and induce the selection and expansion of CSCs. The tumor- associated ECM may be modified and lead to altered signaling in tumor cells. This activated ECM also conferred chemoresistance mediated by β_1-integrins that adhere to fibronectin, leading to the activation of β_1-integrin [29,30]. Thus, anti-ECM agents may have a place in overcoming resistance to chemotherapy. The expression of 'tumor specific' ECM proteins has been exploited to target delivery of bioactive molecules to tumors: these ECM components are highly abundant in tumors and are often more stable than antigens located on the cell surface of tumor cells. Radiolabelled antibodies specific to fibronectin and tenascin-C domain C (TNC) domains A1 and D have been used successfully in the clinic to treat glioma and lymphoma [31]. However, the ECM in solid tumors affects the effectiveness of therapeutics through blocking of intratumoral diffusion and/or physical masking of target receptors on malignant cells. Based on the situation, the researchers used monoclonal antibodies towards tumor-associated ECM isoforms such as TNC to target ECM. Using antibody phage technology, a human monoclonal antibody to the C domain of TNC has been generated, and this scFv protein shows a highly selective uptake in gliomas, making it a promising tool for the future [32]. In immunohistochemical studies of tumor sections from breast cancer patients and xenografts, Beyer's group observed colocalization of ECM proteins and Her2/neu, a tumor-associated antigen that is the target for the widely used monoclonal antibody trastuzumab (Herceptin). They tested whether intratumoral expression of the peptide hormone relaxin (Rlx) would result in ECM degradation and the improvement of trastuzumab therapy by a hematopoietic stem cell (HSC)-based approach to deliver the Rlx gene to the tumor. HSC-mediated intratumoral Rlx expression resulted in a decrease of ECM proteins and enabled control of tumor growth, in mouse models with syngeneic breast cancer tumors. If trastuzumab therapy was combined with Rlx expression

a significant delay of tumor growth emerged in a model with Her2/neu-positive BT474-M1 tumors as well as more treatment- refractory tumors derived from HCC1954 cells [33].
These studies above-mentioned suggested that the CAFs in tumor-associated ECM plays a critical role in the regulation of tumor behavior—that of modulating the CSC phenotype. The researchers try to find the methods to specifically kill CAFs. For example, Loeffler et al constructed an oral DNA vaccine targeting fibroblast activation protein, which is specifically overexpressed by fibroblasts in the tumor stroma. Through $CD8^+$T cell-mediated killing of CAFs, the vaccine successfully suppressed primary tumor cell growth and metastasis of multidrug-resistant murine colon and breast carcinoma. Furthermore, tumor tissue of fibroblast activation protein -vaccinated mice revealed markedly decreased collagen type I expression and up to 70% greater uptake of chemotherapeutic drugs. This strategy opens a new venue for the combination of immuno-and chemotherapies [30]. Another study indicated that the co-culture of colon cancer cells with myofibroblasts or myofibroblast conditioned medium resulted in enhanced nuclear β-catenin, increased Wnt activity and enhanced clonogenic activity, with enhanced tumorigenicity when coinjected into mice. These studies suggest that the stem cell phenotype is plastic and is dependent on the tumor-associated ECM, and that targeting the CSC-tumor- associated ECM interface may be the most effective approach to overcome stem cell resistance to current therapies[34].Elucidating the nature of the interactions between the tumor and the multiple facets of the microenvironment will allow us to harness this relationship for clinical benefit.

2.4 Targeted therapy toward CSCs by blocking signal pathways

In order to effectively target CSCs, a detailed understanding of the signal pathways regulating the growth and self-renewal of CSCs is needed. It is now clear that most CSCs depend on more than one signal pathway for their growth, survival, invasion and metastasis. Moreover, multiple cell signal pathways may control a given step in tumorigenesis. Thus agent or drug that inhibits multiple pathways or their combination is needed for CSC treatment [35, 36].

2.4.1 Targeting Hh signal pathway

The Hh signal pathway plays a critical role in development and is usurped by transformed cells for tumor initiation, progression, and metastasis. The hedgehog proteins are secreted signal proteins that were initially identified in *Drosophila* as segment polarity genes. Hh can function both as a morphogen and a mitogen. As a morphogen, Hh induces cell differentiation in a concentration-dependent manner. As a mitogen, it drives the proliferation of precursor cells and mediates the interaction between the epithelial and mesenchymal compartments [37, 38]. Hh signal pathway activation is noted in the neighboring stromal cells but not in the epithelial cells consistent with a paracrine signal mechanism [39], and only the stromal compartment is competent to activate Hh signaling [40]. This observation is consistent with Hh signaling in CSCs that do not express markers of epithelial differentiation. In order to determine whether increased Hh activity is a property of all pancreatic tumor cells or is selective for the CSCs, Li et al. demonstrated that Hh transcripts were 4-fold upregulated in the bulk pancreatic xenograft cells and 43-fold upregulated in the $CD44^+CD24^+ESA^+$ pancreatic CSCs as compared with normal pancreatic epithelial cells using quantitative real time-PCR [41]. Aberrant activation of the Hh pathway is common in basal cell carcinoma, medulloblastoma, a tumor of cerebellar granule neuron

progenitor cells, and rhabdomyosarcoma, a muscle tumor and breast tumors. The modulation of Hh pathway activity in these cell types results in decreased tumorigenic potential and depletion of the CSC compartment [42].
As the Hh pathway represents an attractive target for drug development, it has shown promise in Phase I clinical trials of advanced basal cell carcinoma and medulloblastoma with GDC-0449, an Hh pathway inhibitor [43, 44]. In addition, a role for Hh signaling in CSCs was demonstrated by specific deletion of smoothened in BCR-ABL positive chronic myeloid leukemia stem cells, which prevented tumor-initiation, and treatment with cyclopamine increased survival of mice transplanted with BCR-ABL leukemia cells [45-47]. Aldehyde dehydrogenase has been identified as one of putative markers of pancreatic CSCs. The initial study suggests that inhibition of the Hh pathway results in depletion of the pancreatic CSC compartment and is a putative mechanism for metastasis. Mueller et al further extended these findings by demonstrating that dual inhibition of the Hh pathway and the mTOR pathway in combination with gemcitabine is required to completely eliminates both $CD133^+$ and $CD24^+CD44^+EpCAM^+$ pancreatic CSC populations *in vitro*. In contrast, inhibition of Hh pathway alone in combination with gemcitabine abolished the $CD133^+CXCR4^+$ migratory CSC population. Similarly, in a mouse model of orthotopic pancreatic cancer, treatment with cyclopamine, rapamycin (mTOR inhibitor) and gemcitabine was required to fully inhibit growth at the primary site and resulted in a significant overall survival benefit. However, treatment with cyclopamine and gemcitabine resulted in complete inhibition of metastatic activity. Thus, they concluded that dual pathway inhibition is required for complete abrogation of tumorigenic potential [38, 48].

2.4.2 Targeting Notch-1 signaling pathway

Notch-1 and its ligands (Delta and Jagged) control an ancestral pathway of cell division and organ formation which is conserved in humans [20]. Notch-1 controls cell cycle progression from G0 to G1. The Notch-1 pathway is important as it precedes activation by oncogenes. Notch-1 activates *c-myc* and *hypoxia* genes, which in turn activate expression of embryonic stem cell (ESC) genes. Notch activation starts from a signal from Delta and Jagged on the neighboring cell. The signal 'pulls and breaks' the Notch heterodimer. The Notch-1 extracellular domain, N1ECD, is endocytosed together with Delta/Jagged in neighboring cells. The intracellular domain of Notch-1 protein (N1ICD) binds its suppressor, Numb, and pushes it for degradation. Signals from Notch-1 co-activator family, Mastermind-like-1 members, enhance Notch-1 signaling [49,50]. Currently, the Notch pathway is one of the most intensively studied putative therapeutic targets in CSCs, and several investigational Notch inhibitors are being developed. However, successful targeting of Notch signaling in CSCs will require a thorough understanding of Notch regulation and the context-dependent interactions between Notch and other therapeutically relevant pathways. Understanding these interactions will increase our ability to design rational combination regimens
that are more likely to prove safe and effective. Additionally, to determine which patients are most likely to benefit from treatment with Notch-targeting therapeutics, reliable biomarkers to measure pathway activity in CSCs from specific tumors will have to be identified and validated [51,52].

2.4.3 Targeting Wnt/β-catenin signaling pathway

Wnt/β-Catenin is an essential component of both intercellular junctions and the canonical Wnt signaling pathway and the aberrant activation of Wnt signaling is involved in tumor

development and progression. Therefore, the Wnt signaling pathway is clearly important for the self-renewal and maintenance of stem cells, as seen by the effectiveness of inhibiting Wnt/β-catenin signaling in blocking human U251 glioma cells [53, 54]. The study showed that Wnt2, Wnt5a, frizzled2, and β-catenin were overexpressed in gliomas. Knockdown of Wnt2 and its key mediator β-catenin in the canonical Wnt pathway by siRNA in human U251 glioma cells inhibited cell proliferation and invasive ability, and induced apoptotic cell death. Furthermore, treating the nude mice carrying established subcutaneous U251 gliomas with siRNA targeting Wnt2 and β-catenin intratumorally also delayed the tumor growth. The acetaminophen is an anti-inflammatory, antipyretic and analgesic drug that is already in clinical use for drug and induces differentiation of CSCs, such as a human breast cancer cell line (MDA-MB-231 cells that contains cancer stem cell-like cells). In 2011 Takehara et al demonstrated that the increased susceptibility of MDA-MB-231 cells to acetaminophen seems to involve suppression of expression of multidrug efflux pumps, which suggests that this induction of differentiation is mediated by inhibition of a Wnt/β-catenin canonical signal pathway. Treatment of MDA-MB-231 cells with acetaminophen *in vitro* resulted in the loss of their tumorigenic ability in nude mice. Furthermore, administration of acetaminophen inhibited the growth of tumor xenografts of MDA-MB-231 cells in both the presence and absence of simultaneous administration of doxorubicine, a typical anti-tumor drug for breast cancer. The result indicates that acetaminophen may be beneficial for breast cancer chemotherapy by inducing the differentiation of CSCs [55]. It is thus evident that the Wnt/β-catenin pathway might provide a new therapeutic approach against CSCs in the malignant gliomas. Similarly, β-catenin signaling is essential in sustaining the epidermal tumor CSC phenotype. Ablation of the β-catenin gene resulted in the loss of CSCs and complete epithelial tumor regression [56].

2.5 Targeted therapy toward CSCs by manipulation of miRNA and siRNA therapy

2.5.1 Manipulation of miRNA therapy

MicroRNAs (miRNA) constitute a large family of small, approximately 21-nucleotide long, non-coding RNAs that have emerged as key post-transcriptional regulators of gene expression in metazoans and plants. In mammals, miRNAs are predicted to control the activity of approximately 30% of all protein coding genes, and have been shown to participate in the regulation of almost every cellular process investigated and to play an important role in many developmental processes. Although a relatively new field, there is already a clear and definitive role for miRNA that function as tumor suppressors were found to be markedly down regulated in malignant transformation and tumor progression. Thus, miRNAs have spurred studies to investigate whether miRNAs play an important role in the CSC phenotype.

Recently, a study demonstrated that certain miRNA that regulate the critical promoter of stem cell self-renewal factor BMI1 was downregulated in purified populations of normal mammary epithelial stem cells and CSCs. The data showed that the mRNA encoding BMI1 was specifically targeted by miR-200c, miR-200b, and miR-183. The three miRNA clusters had decreased expression in freshly isolated $CD44^{+}CD24^{-/low}$ breast CSCs compared to cells in the tumor bulk. It is possible that miR-200c blocked stem cell self-renewal by targeting the 3′UTR of the self- renewal gene BMI1, resulting in the loss of BMI1 protein, and the attenuation of the ability of CSCs to self-renew and form tumors [57,58].

Prostate CSCs with enhanced clonogenic and tumor-initiating and metastatic capacities are enriched in the $CD44^{+}$ cell population. If miR-34a was overexpressed in bulk or purified

$CD44^+$ prostate cancer cells purified from xenograft and primary tumors the clonogenic expansion, tumor regeneration, and metastasis were inhibited. However, the expression of miR-34a antagomirs in $CD44^-$ prostate cancer cells facilitated tumor development and metastasis. Administratively delivered miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice. The identified $CD44^+$ prostate cancer cells may be as a direct and functional target of miR-34a and the CD44 knockdown phenocopied miR-34a overexpression in inhibiting prostate cancer regeneration and metastasis. The study suggests that miR-34a is underexpressed in tumorigenic $CD44^+$ prostate cancer cells, and that it has potent antitumor and antimetastasis effects and is a novel therapeutic agent against prostate CSCs [59]

The symmetric division of CSCs is one mechanism enabling expansion in their numbers as tumor grow, while EMT is an increasingly recognized mechanism to generate further CSCs endowed with a more invasive and metastatic phenotype. Since the EMT of ovarian cancer cells located at the periphery of primary tumors is essential to this process, molecular interventions that can block EMT are of potential clinical significance [60]. It was found that the members of the miR200 family of microRNAs have been implicated in EMT in other cancers. In 2011 Chen et al [61] tested gene expression profiles of two ovarian cancer cell lines with different metastatic potentials by using quantitative reverse transcription polymerase chain reaction. The results showed that molecular profiling of two ovarian cancer cell lines with differing metastatic potentials identified significant differences in previously established epithelial and mesenchymal cell biomarkers, such as E-cadherin, ZEB1, ZEB2, miR-205 and miR-200 family microRNAs. They demonstrated that ectopic overexpression of miR-200 family microRNA (miR-429), in mesenchymal-like ovarian cancer cells resulted in reversal of the mesenchymal phenotype (mesenchymal- epithelial transition, MET). The data indicate that miR-429 may not only be a useful biomarker of EMT in ovarian cancer, but also of potential therapeutic value in abating ovarian cancer metastasis.

More recently, Strauss et al [62] developed a subpopulations of ovarian cancer cells that simultaneously express epithelial and mesenchymal markers, and the subpopulations are not homogenous, however, the subsets that can be distinguished based on a number of phenotypic features including the subcellular localization of E-cadherin, and the expression levels of Tie2, CD133, and CD44. A cellular subset (E/M-MP) (membrane E-cadherin^{low}/cytoplasmic E-cadherin^{high}/ CD133^{high}, CD44^{high}, Tie2^{low} is highly enriched for CSCs. The group demonstrated that E/M-MP cells are able to differentiate into different lineages under certain conditions, and have the capacity for self-renewal. Trans-differentiation of E/M-MP cells into mesenchymal or epithelial cells is associated with a loss of stem cell markers and tumorigenicity. Xenograft tumor growth *in vivo* is driven by E/M-MP cells, which give rise to epithelial ovarian cancer cells. In contrast, E/M-MP cells differentiate into mesenchymal cells *in vitro*, which involves pathways associated with the EMT. The study provides a better understanding of the phenotypic complexity of ovarian cancer and has implications for ovarian cancer or ovarian CSC therapy. In addition, miRNAs might play important roles in stemness maintenance of ovarian or colon CSCs, and targeting miRNA may provide a new strategy for CSC therapy by impairing resistance to chemotherapy [63-65].

2.5.2 Manipulation of siRNA therapy

RNA interference (RNAi) is triggered by short interfering RNAs (siRNAs) of between 19 and 21 nucleotides in length, which induces the targeted cleavage of mRNA with sequences

of homology to the siRNA. Because of its high degree of specificity and efficacy, the potential for RNAi-based therapeutics have been employed as transcriptional inhibitors of oncogene and growth factor signaling [66]. To this end, several groups are attempting to develop effective vehicle for delivering siRNA to *in vivo* growing tumors. Octamer 4 (Oct4), a member of the POU family of transcription factors, plays a key role in the maintenance of pluripotency and proliferation potential of embryonic stem cells. Continuous Oct4 expression in epithelial tissues is observed to lead to dysplastic disorders by inhibiting cellular differentiation in a manner similar to that in embryonic cells. Oct4 has also been reported to be an oncogenic fate determinant. High levels of Oct4 increase the malignant potential of embryonic stem cells-derived tumors whereas inactivation of Oct4 induces a regression of the malignant component [67]. The role of Oct4 in CSC-like cells (CSCLC) was recently evaluated by Hu et al [68]. They found that almost all murine Lewis lung carcinoma 3LL cells and human breast cancer MCF7 cells express Oct4 at high levels *in vitro*. This expression of Oct4 is effectively reduced by siRNA that finely causes cell apoptosis. The signal pathway Oct4/Tcl1/Akt1 is involved in this process. The repression of Oct4 reduces Tcl1 expression and further down-regulates the level of p-Ser.473-Akt1. Only approximately 5% of tumor cells were detected to express Oct4 in established 3LL and MCF7 tumor models, respectively, *in vivo*. siRNA against Oct4 successfully reduces the CSCLCs and remarkably inhibits tumor growth. Oct4 might maintain the survival of CSCLCs partly through Oct4/Tcl1/ Akt1 by inhibiting apoptosis. The results strongly suggestes that targeting Oct4 may have important clinical applications in cancer and CSC therapy.

Emerging data indicate that transglutaminase 2 (TG2) has closely relationship among the EMT, and CSCs in inflammation and cancer. TG2 is a structurally and functionally complex protein implicated in such diverse processes as tissue fibrosis, wound healing, apoptosis, neurodegenerative disorders, celiac disease, atherosclerosis and cancer. Depending on the cellular context, TG2 can either promote or inhibit cell death. Increased expression of TG2 in several types of cancer cells or CSCs have been associated with increased cell invasiveness, cell survival and decreased survival of patients with cancer. Down-regulation of TG2 by siRNA or its inhibition by small molecule inhibitors has been shown to significantly enhances the therapeutic efficacy of anticancer drugs and inhibit metastatic spread [69].

2.6 Targeted directly drug-resistant CSCs in 3D cell culture

Tissues and organs are three dimensional (3D). Cell behavior, which includes survival, motility, and differentiation, mainly depends on its 3D growth environment. Cells growing on flat two-dimensional (2D) tissue culture substrates can differ considerably in their morphology, cell–cell, and cell–matrix interactions, and differentiation from those growing in more physiological 3D environments that display enhanced cell biological activities and promote normal cell polarity and differentiation. In addition, signal pathway and other cellular functions also differ in 3D compared with 2D systems [70, 71].

Cukierman and his colleagues revealed their approach that an *in vivo*-like ovarian stromal 3D system would enable researchers to study ovarian stromal progression and to uncover mechanisms that promote ovarian tumor development, progression, and metastasis. Interactions between cancer cells and stroma in the 3D environment were considered critical for growth and invasiveness of epithelial tumors [72]. Because the vast majority of studies to identify cancer-associated genes and therapeutic targets use adherent cells grown in 2D on a plastic substrate, the multicellular composition of these 3D tumor spheroids presents both challenges and opportunities for their imaging and characterization. Robertson et al

described approaches to image 3D breast CSC spheroid structures, allowing for characterization of specific traits of CSCs, including self-renewal as assessed by their clonogenic growth, their ability to retain a nucleoside analog label such as 5-ethynyl-2′-deoxyuridine, and their expression of specific surface markers. They explored the major challenges of imaging and analyzing the activities of breast CSCs when cultured as 3D tumor spheroids and provide insight into potential solutions that allow multicellular tumor spheroids to be imaged and analyzed to further characterize signatures and therapeutic targets of CSCs [73]. Botchkina et al reported that the selected CSC phenotype was isolated from three independent invasive colon cancer cell lines, HCT116, HT29 and DLD-1. In 3D culture, the colonospheres induced by purified $CD133^{high}/CD44^{high}$ expressing cells contained some minority cell populations with high levels of expression of Oct4, Sox2, Nanog and c-Myc, which are essential for stem cell pluripotency and self-renewal. Using the new-generation taxoid SB-T-1214 at concentration 100 nM^{-1} microM for 48 hr not only induced growth inhibition and cell apoptosis in these three types of colon cancer spheroids, but also mediated massive inhibition of the stem cell-related genes and significant down-regulation of the pluripotency gene expression. Importantly, viable cells that survived this treatment regimen were no longer able to induce secondary floating spheroids and exhibited significant morphological abnormalities [74]. Although emerging reports indicate that *in vitro* 3D culture system was also used for cardiac stem cells and ovarian cancer cell lines[75, 76], we know very little about cell-based assays used for screening target stem cell or CSC drugs in 3D cultures at present. In order to develop such drug screening systems, it is essential that novel methods be explored to use the 3D culture system *in vitro* for development of specific drugs that target chemoresistant CSCs, and at the same time to minimize unwanted side effects of the drugs to host tissues [77].

2.7 Targeted therapy toward CSCs by dendritic cell vaccination

There is growing evidence that were based on the use of tumor-homogenate pulsed dendritic cells (DC) for patients with recurrent gliomas (GBM) and provided encouraging results. Xu's study showed that CSCs express high levels of tumor-associated antigens as well as major histocompatibility complex molecules and they explored the suitability of CSCs as sources of antigens for DC vaccination again human GBM. This DC vaccination elicited antigen-specific T cell responses against CSCs. DC vaccination induced interferon-γ production is positively correlated with the number of antigen- specific T cells generated. In using a 9L CSC brain tumor model, vaccination with DCs loaded with 9L CSCs, but not daughter cells or conventionally cultured 9L cells, induced cytotoxic T lymphocytes against CSCs, and prolonged survival in animals bearing 9L CSC tumors [78]. Murine brain tumor GL261 GBM cell line may mimic the growth of human GBM-CSC because the characterization i*n vivo* and *in vitro* demonstrates that GL261-NS (neurospheres) satisfy criteria used to identify CSCs and are more immunogenic than GL261-AC (Adherent Cells). DC from the bone marrow of syngeneic mice were then used for immunotherapy of GL261-NS and GL261-AC tumors. The results showed that DC loaded with GL261-NS (DC-NS) lysates protected mice against tumors from both GL261-NS (cured 80%) and GL261-AC(cured 60%), respectively, whereas DC-AC cured only 50% of GL261-AC tumors. The study also indicated that GL261-NS expressed higher levels of MHC and costimulatory molecules (CD80 and CD86) than GL261-AC and that DC-NS splenocytes had higher lytic activity than DC-AC splenocytes on both GL261-NS and GL261-AC. Immunohistochemistry showed that DC-NS vaccination was associated with robust tumor infiltration by $CD8^+$ and

$CD4^+$ T lymphocytes. The results indicate that only DC vaccination against neurospheres can restrain the growth of a highly infiltrating and aggressive model of GBM and may have implications for the design of novel, more effective immunotherapy trials for malignant GBM and possibly other malignancies. These data suggest that DC targeting of CSC provides a higher level of protection against GL261 GBM, a finding with potential implications for the design of clinical trials based on DC vaccination [79, 80]. These findings suggest that understanding how immunization with CSCs as sources of antigens for DC vaccination generates superior antitumor immunity may accelerate development of CSC-specific immuno-therapies and CSC vaccines [77, 81].

3. Conclusions

We are still in the early days of clinically validating CSCs as a cancer target. We do not fully understand the role of CSC biology and cancer pathophysiology. Undoubtedly, proper characterization and refinement of the tools used for the identification, isolation, and propagation of CSCs may lead to a better understanding of how these cells initiate and sustain tumor growth.

Currently, novel therapeutic approaches to eliminate CSCs are imperative because CSCs may escape standard therapies and cause disease recurrences and/or metastasis after apparently complete remissions. Developing effective cancer treatment by focusing therapy on the relatively more malignant and quiescent cells could be a direct result of the application of CSC hypothesis to tumor growth. In order to achieve this goal it is important to determine which cancers possess a CSC traits and which do not, and to address technical issues related to tumorigenesis assays [3, 82]. It is known that CSC targeting is essential, but recently it has been speculated that non-CSCs in a tumor need to be targeted as well. These non-CSCs could form CSCs and might even sustain the tumor even after CSCs have been destroyed [83]. Furthermore, innate immunity including natural killer cells and gammadelta T cells and adaptive immunity (cytotoxic T lymphocyte-based cellular immunity and antibody-based humoral immunity) can recognize CSCs *in vitro* efficiently, of which CSC-specific monoclonal antibody therapies are also efficient *in vivo*[84]. Taken together, targeted therapies on the CSC compartment could provide cancer curability through targeted therapy directed toward CSCs, and along with the conventional treatments, gene therapy and nanotechnology as well as new technologies could provide sophisticated multifunctional agents to target CSCs, all of which has begun to revolutionize approaches for simultaneous drug design, targeting, imaging, and therapy of CSCs [2, 77,85].

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5. References

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22

Potential Application of Natural Dietary Components to Target Cancer Stem Cells

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1. Introduction

The emergence of cancer stem cell (CSC) theory has profound implications for cancer prevention and therapy. Although a large majority of chemotherapeutic drugs can considerably shrink tumor sizes (Reya et al. 2001), they often fail to eradicate tumors due to the inability to effectively kill CSCs (Reya et al. 2001; Hambardzumyan et al. 2006; Shafee et al. 2008; Korkaya et al. 2009). The cancer may eventually develop drug resistance and recurrence (Williams et al. 1987; Lippman 2000; Stockler et al. 2000; Reya et al. 2001; Zhou, B. B. et al. 2009). Therefore, the CSC population has become a promising target for cancer prevention and therapy (Zhou, B. B. et al. 2009).

Since a large number of epidemiological studies have demonstrated an association between consumption of fruits and vegetables and the reduced risk of various cancers, naturally-occurring dietary components have received considerable attention for their effects in cancer chemoprevention (Smith-Warner et al. 2003). The anti-cancer activities of many dietary components against various types of cancer have been reported for both *in vitro* and *in vivo* studies (Chinni et al. 2001; Mukhopadhyay et al. 2001; Choudhuri et al. 2002; Lamartiniere et al. 2002; Li and Sarkar 2002; Gupta, S. et al. 2003; Hastak et al. 2003; Li et al. 2003). Recently, a number of studies have found that several dietary components can directly or indirectly affect CSC self-renewal pathways (Kawasaki et al. 2008), and thus may have potential impact on CSCs. This chapter reviews current attempts to target CSCs with bioactive dietary components, with a special emphasis on our work.

2. Self-renewal pathways of CSCs

CSCs produce the tumor mass through continuous self-renewal and differentiation, which may be regulated by similar signaling pathways occurring in normal stem cells (Reya et al. 2001; Liu, S. et al. 2005). Understanding the mechanisms that underlie the self-renewal behavior of CSCs is of greatest importance for discovery and development of agents targeting CSCs. So far, several major pathways including Wnt/β-catenin, Hedgehog, and Notch have been identified to play pivotal roles in CSC self-renewal (Smalley and Dale 1999; Dontu et al. 2004; Liu, S. et al. 2006).

2.1 Wnt/β-catenin pathway

Wnt/β-catenin pathway was demonstrated to modulate cell proliferation, migration, apoptosis, differentiation, and CSC self-renewal (Akiyama 2000; Polakis 2000; Yamaguchi 2001; Turashvili et al. 2006). It has been shown that Wnt/β-catenin signaling is implicated in the maintenance of CSCs of leukemia (Ysebaert et al. 2006; Khan et al. 2007; Kawaguchi-Ihara et al. 2008), melanoma (Chien et al. 2009), breast (Li et al. 2003; Woodward et al. 2007), colon (Schulenburg et al. 2007), liver (Yang, W. et al. 2008), lung (Teng et al. 2010) cancers. For example, over-expression of β-catenin in stem cell survival pathway was shown to mediate the resistance of mouse mammary stem/progenitor cells to radiation (Woodward et al. 2007). Yang and his colleagues reported that Wnt/β-catenin signaling promoted expansion of the hepatic progenitor cell population when it is over-expressed in transplanted rat oval cells and when it is transiently expressed in adult mice (Yang, W. et al. 2008). Elimination of β-catenin abrogated the chemo-resistant cell population endowed with progenitor-like features (Yang, W. et al. 2008).

β-Catenin, the essential mediator of canonical Wnt signaling, participates in two distinct functions in the cell, depending on its cellular localization. Membrane-localized β-catenin is sequestered by the epithelial cell-cell adhesion protein E-cadherin to maintain cell-cell adhesion (Nelson and Nusse 2004). On the other hand, cytoplasmic accumulation of β-catenin and its subsequent nuclear translocation, followed by cooperation with the transcription factors T cell factor/lymphoid enhancer factor (TCF/LEF) as a transcription activator, eventually leads to activation of Wnt target genes such as *c-Jun*, *c-Myc*, *fibronectin*, and *cyclin D1* (He et al. 1998; Mann et al. 1999; Orsulic et al. 1999; Tetsu and McCormick 1999; Lin, S. Y. et al. 2000; Liu, S. et al. 2005; Clevers 2006). Binding of Wnt proteins, a family of secreted proteins, to Frizzled receptors results in the cytoplasmic accumulation of β-catenin (Schweizer and Varmus 2003). In the absence of Wnt signaling, β-catenin forms a multi-protein complex with glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli, casein kinase1α, and axin (Takahashi-Yanaga and Sasaguri 2008). When β-catenin is phosphorylated at Ser33/Ser37/Thr41 by GSK3β, it is immediately subject to ubiquitin-proteasome degradation (Liu, C. et al. 2002; Takahashi-Yanaga and Sasaguri 2008).

The link between Wnt/β-catenin and PI3K/Akt pathway has been established by several studies. Activated Akt (i.e., phospho-Akt Ser473) was shown to be able to phosphorylate Ser9 on GSK3β, which may decrease the activity of GSK3β, thereby stabilizing β-catenin (Yost et al. 1996; Pap and Cooper 1998; Cohen, P. and Frame 2001). Furthermore, Korkaya et al. demonstrated that PI3K/Akt pathway is important in regulating the mammary stem/progenitor cells by promoting β-catenin downstream events through phosphorylation of GSK3β (Korkaya et al. 2009).

2.2 Hedgehog pathway

Another critical pathway that is involved in CSC self-renewal is hedgehog signaling pathway (Cohen, M. M., Jr. 2003; Liu, S. et al. 2006; Clement et al. 2007; Charafe-Jauffret et al. 2008). For instance, Liu et al. have demonstrated that the hedgehog pathway plays a crucial role in regulating self-renewal of normal and malignant human mammary stem cells by utilizing both *in vitro* and mouse model systems (Liu, S. et al. 2006). Another recent study revealed the essential role of hedgehog-Gli signaling in controlling the self-renewal behavior of human glioma CSCs and tumorigenicity (Clement et al. 2007).

In the absence of hedgehog ligands (Sonic Hedgehog, Desert Hedgehog, and Indian Hedgehog), their transmembrane receptor Patched (Ptch) associates with Smoothened (Smo) and blocks Smo function (Cohen, M. M., Jr. 2003; Lewis and Veltmaat 2004; Liu, S. et al. 2005). When secreted hedgehog ligands bind to Ptch, Smo is released, triggering dissociation of transcription factors, Gli1, Gli2, and Gli3 from Fused (Fu) and suppressor of Fused (SuFu), leading to transcription of an array of genes, such as *cyclin D*, *cyclin E*, *Myc*, and elements of EGF pathway (Cohen, M. M., Jr. 2003; Pasca di Magliano and Hebrok 2003; Lewis and Veltmaat 2004; Liu, S. et al. 2005).

Sonic hedgehog pathway is also linked to transcription factor NF-κB signaling. NF-κB was suggested to be a prominent factor in controlling tumor growth and apoptosis resistance of pancreatic CSCs (Kallifatidis et al. 2009). Over-expression of sonic hedgehog is activated by NF-κB in pancreatic cancer and pancreatic cancer cell proliferation is accelerated by NF-κB in part through sonic hedgehog over-expression (Nakashima et al. 2006). Kasperczyk et al. further characterized sonic hedgehog as a novel NF-κB target gene and mapped minimal NF-κB consensus site to position +139 of sonic hedgehog promoter (Kasperczyk et al. 2009).

2.3 Notch pathway

Notch signaling is known to control cell proliferation and apoptosis to modulate the development of many organs (Wang, Z. et al. 2009). A number of recent studies have demonstrated that Notch-activated genes and pathways can drive tumor growth through the expansion of CSCs (Wilson and Radtke 2006; Charafe-Jauffret et al. 2008; Fan and Eberhart 2008; Kakarala and Wicha 2008; Peacock and Watkins 2008; Scoville et al. 2008; Wang, Z. et al. 2009). Notch pathway is believed to be dysregulated in CSCs, ultimately leading to uncontrolled CSC self-renewal (Wang, Z. et al. 2009). For example, Notch pathway was shown to play an important role in the self-renewal function of malignant breast cancer CSCs (Dontu et al. 2004; Farnie and Clarke 2007).

Five Notch proteins, Notch-1 to Notch-4, have been identified to express as transmembrane receptors in a variety of stem/progenitor cells (Mumm and Kopan 2000). Binding of surface-bound ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4) triggers serial cleavage events at the Notch proteins by ADAM protease family and γ-secretase (Wu, J. Y. and Rao 1999; Mumm and Kopan 2000; Borggrefe and Oswald 2009). Subsequently, the intracellular domain of Notch is released and translocates into the nucleus, where it acts as a transcription co-activator of recombination signal sequence-binding protein Jκ (RBP-J) to activate downstream target genes, e.g., *c-Myc*, *cyclin D1*, *p21*, *NF-κB* (Oswald et al. 1998; Rangarajan et al. 2001; Ronchini and Capobianco 2001; Satoh et al. 2004; Palomero et al. 2006; Weng et al. 2006; Borggrefe and Oswald 2009).

Notch1 has been reported to be the upstream regulator of NF-κB pathway in diverse cellular situations (Oswald et al. 1998; Wang, J. et al. 2001; Nickoloff et al. 2002; Dontu et al. 2004; Jang et al. 2004; Wang, Y. et al. 2004; Shin et al. 2006; Wang, Z. et al. 2006; Chen et al. 2007). Specifically, Notch-1 is necessary for expression of several NF-κB subunits (Cheng et al. 2001; Jang et al. 2004) and stimulates NF-κB promoter activity (Jang et al. 2004).

3. Sulforaphane

Numerous studies have substantiated the chemopreventive properties of high consumption of cruciferous vegetables, especially broccoli and broccoli sprouts, against various types of

cancer (Clarke et al. 2008). Cruciferous vegetables are characterized by their high content of glucosinolates (Herr and Buchler 2010; Fahey et al. 2001), which are converted to isothiocyanates by the action of myrosinase. The chemopreventive effects have been mostly attributed to the activity of these isothiocyanates (Zhang et al. 1992; Clarke et al. 2008). In particular, sulforaphane (Figure 1) is converted from glucoraphanin, the principal glucosinolate in broccoli and broccoli sprouts (Fahey et al. 1997). Sulforaphane has been shown to be not only effective in preventing chemically induced cancers in animal models, including colon, lung, breast, pancreatic, skin and stomach cancer (Zhang et al. 1994; Fahey et al. 1997; Chung et al. 2000; Fahey et al. 2002; Conaway et al. 2005; Gills et al. 2006; Kuroiwa et al. 2006; Xu et al. 2006), but also inhibit the growth of established tumors (Jackson and Singletary 2004; Singh, A. V. et al. 2004).

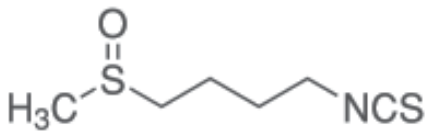

Fig. 1. Chemical structure of sulforaphane

Early research focused on inhibition of Phase 1 metabolism enzymes that convert procarcinogens to carcinogens and induction of Phase 2 metabolism enzymes that enhance elimination and excretion of carcinogens by sulforaphane (Clarke et al. 2008), which enhances the detoxification of carcinogens and "blocks" carcinogenesis at the initiation stage of cancer (Juge et al. 2007; Clarke et al. 2008).

Subsequent studies suggest that sulforaphane provides protection against tumor development during the "post-initiation" phase by modulating diverse cellular activities including apoptosis, cell cycle, angiogenesis and metastasis (Zhang and Tang 2007; Clarke et al. 2008). Sulforaphane affects classical molecular targets involved in the apoptosis pathways such as down-regulation of anti-apoptotic Bcl-2 and Bcl-$_{XL}$, up-regulation of pro-apoptotic Bax expression, proteolytic activation of caspase-3, and the degradation/cleavage of poly(ADP-ribose) polymerase, induction of apoptotic protease activating factor-1 (Choi et al. 2007; Park, S. Y. et al. 2007). The ability of sulforaphane to induce cell cycle arrest is associated with regulation of many molecules including cyclins, Cdks, and p21 (Singh, S. V. et al. 2004; Herman-Antosiewicz et al. 2007; Zhang and Tang 2007). More recent studies demonstrate that sulforaphane is also capable of suppressing angiogenesis and metastasis, which are associated with transcriptional down-regulation of vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1α (HIF-1α), c-Myc and matrix metalloproteinase-2 (MMP-2), and MMP-9 (Bertl et al. 2006; Zhang and Tang 2007).

Our studies have shown that sulforaphane is effective in targeting breast CSCs *in vitro* and *in vivo* (Li et al. 2010). Mammosphere culture was first used to isolate and expand mammary stem/progenitor cells by Dontu et al., based on the ability of stem/progenitor cells to grow in serum-free, non-adherent suspension as spherical clusters of cells while differentiated cells fail to survive under the same condition (Dontu et al. 2003). By employing this technique, we demonstrated that sulforaphane (0.5-5 μM) significantly suppressed the mammosphere formation of both SUM159 and MCF7 cells. A decrease in the number of sphere-forming cells in the 2nd and 3rd passages indicated a reduced self-renewal capacity of these stem/progenitor cells (Dontu et al. 2003). In breast carcinomas, a cell population

with high aldehyde dehydrogenase (ALDH) activity as assessed by the Aldefluor assay has been demonstrated to enrich tumorigenic stem/progenitor cells (Ginestier et al. 2007). This cell population is capable of self-renewal and generating tumors resembling the parental tumor (Ginestier et al. 2007). We found that sulforaphane (1-5 μM) was able to significantly decrease the tumor-initiating ALDH-positive cell population of SUM159 by 65% to 80% *in vitro*. Of special note, concentrations of sulforaphane which inhibit stem/progenitor cells in both mammosphere formation assay and Aldefluor assay had only minimal effects on the bulk population of breast cancer cell lines, implying that sulforaphane is likely to preferentially target stem/progenitor cells compared to the differentiated cancer cells.

We further demonstrated that sulforaphane can inhibit breast CSCs *in vivo* (Li et al. 2010). The injection of human breast cancer cells into the mammary fat pad of immune-deficient NOD/SCID mice provides a reliable and sensitive *in vivo* system for studying human breast cancer (Al-Hajj et al. 2003; Dick 2003). Daily injection of sulforaphane for two weeks suppressed tumor growth in primary NOD/SCID mice and reduced ALDH-positive cell population of the tumors by more than 50%. Most importantly, we found that recipient NOD/SCID mice inoculated with tumor cells derived from sulforaphane-treated primary xenografts largely failed to develop tumor re-growth up to 33 days, whereas control tumor cells quickly initiated new tumors upon re-implantation. These results suggest that sulforaphane is able to eliminate breast CSCs *in vivo*, thereby abrogating tumor re-growth after re-implantation of primary tumor cells into the secondary mice.

We also observed a down-regulation of Wnt/β-catenin self-renewal pathway in sulforaphane-treated breast cancer cells (Li et al. 2010). Park et al. previously reported that β-catenin was down-regulated by sulforaphane in human cervical carcinoma HeLa and hepatocarcinoma HepG2 cells (Park, S. Y. et al. 2007). In consistent with their study, we showed that sulforaphane was able to down-regulate Wnt/β-catenin self-renewal pathway in breast cancer cells, and sulforaphane-induced β-catenin phosphorylation (Ser33/Ser37/Thr41) and proteasome degradation was possibly through activation of GSK3β. The down-regulation of Wnt/β-catenin self-renewal pathway might contribute to the inhibitory effects of sulforaphane on breast CSCs. Further studies are warranted to establish the conclusive role of this down-regulation in inhibition of breast CSCs by sulforaphane.

In addition, our recent work has revealed a new molecular target of sulforaphane. Sulforaphane inhibits heat shock protein 90 (Hsp90) function by blocking the interaction of Hsp90 with it cochaperone $p50^{Cdc37}$, and we traced this activity to a novel interaction site of Hsp90, which fundamentally differs from the mechanism of other Hsp90 inhibitors (unpublished data). LC-MS peptide mapping identified a covalent adduct of sulforaphane with a short peptide IDIIPNPQER in Hsp90 N-terminal domain. NMR experiment with full-length Hsp90 revealed sulforaphane interaction in sheet 2 and the adjacent loop in Hsp90 N-terminal domain, in which this short peptide resides. Akt is a well-known Hsp90 client protein. Our and several other studies have reported the activity of sulforaphane to down-regulate the protein level of Akt and Akt pathway in ovarian, prostate, and colorectal cancers (Chaudhuri et al. 2007; Shen et al. 2007; Shankar et al. 2008). PI3K/Akt pathway was recently demonstrated to play an important role in regulating breast stem/progenitor cells by promoting β-catenin down-stream events through phosphorylation of GSK3β (Korkaya et al. 2009). Therefore, inhibition of Hsp90 chaperone function by sulforaphane may contribute to the effect on Akt/GSK3β/β-catenin pathway.

The resistance of pancreatic cancer toward TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is due to TRAIL-activated NF-κB signaling (Ibrahim et al. 2001). Kallifatidis et al. demonstrated that sulforaphane (10 μM) was able to deplete pancreatic CSCs ($CD44^+CD24^-$) by interfering with NF-κB binding and abrogating apoptosis resistance (Kallifatidis et al. 2009). They found that the presence of pancreatic TICs correlated with apoptosis resistance towards TRAIL due to the enhanced binding of NF-κB complexes to DNA. Sulforaphane alone or in combined with TRAIL reduced growth of TIC^{high} tumors *in vivo* without toxicity to normal tissue.

Like TRAIL, sorafenib was also observed to strongly up-regulate NF-κB activity (Rausch et al. 2010). Sulforaphane (10 μM) completely abolished sorafenib-induced NF-κB binding in CSC^{high} cells, thereby synergistically inhibiting pancreatic CSC ($CD44^+CD24^-$) (Rausch et al. 2010). The growth of pancreatic CSC^{high} tumor xenografts was synergistically inhibited by combination of sulforaphane and sorafenib, which involved induction of apoptosis, inhibition of proliferation and angiogenesis, as well as down-regulation of epithelial-mesenchymal transition (EMT) related proteins (vimentin, Zeb-1, and Twist-2). EMT induction in cancer cells results in the acquisition of invasive and metastatic properties (Singh, A. and Settleman 2010; Gupta, P. B. et al. 2009; Klarmann et al. 2009; Sarkar et al. 2009). CSCs undergoing metastasis usually express EMT markers (Tang et al. 2010).

This same group then combined sulforaphane (5 μM) with several chemotherapeutic drugs and observed increased cytotoxicity toward pancreatic CSCs ($CD44^+CD24^-$) (Kallifatidis et al. 2011). Sulforaphane not only down-regulated basal Notch-1 expression in CSC^{high} cells, but also prevented the gemcitabine-induced Notch-1 up-regulation. There was no tumor growth in mice re-implanted with tumor cells derived from sulforaphane-treated or combination-treated xenografts.

Another recent study also examined the molecular mechanisms by which sulforaphane inhibits growth and induces apoptosis of pancreatic CSCs (Srivastava et al. 2011). They demonstrated that sulforaphane (5-10 μM) inhibited self-renewal capacity of pancreatic CSCs ($CD44^+CD24^+ESA^+$). Sulforaphane induced apoptosis by inhibiting the expression of Bcl-2 and XIAP, phosphorylation of FKHR, and activating caspase-3. Moreover, sulforaphane inhibited expression of EMT markers (β-catenin, vimentin, Twist-1, and Zeb-1), suggesting the blockade of early metastasis signaling.

In summary, all of these findings strongly support that combination of sulforaphane or even broccoli/broccoli sprout preparations with chemotherapy may be a promising strategy to eradicate tumors and improve patient survival in different types of cancer.

4. Curcumin

Curcumin (Figure 2) is a dietary polyphenol present in the Indian spice turmeric, which is produced from rhizome of the plant *Curcuma longa* and usually used in preparation of mustard and curry (Park, C. H. et al. 2005). Curcumin has been studied as a chemoprevention agent in several cancer models (Mukhopadhyay et al. 2001; Shao et al. 2002; Lin, J. K. 2007; Anand et al. 2008; Kunnumakkara et al. 2008; Strimpakos and Sharma 2008).

Curcumin has been shown to regulate many cellular pathways (Lin, J. K. 2007; Hatcher et al. 2008; Kunnumakkara et al. 2008; Sa and Das 2008; Ravindran et al. 2009), some of which are associated with self-renewal signaling. Curcumin was suggested to induce caspase-3-mediated cleavage of β-catenin, leading to inactivation of Wnt/β-catenin signaling in

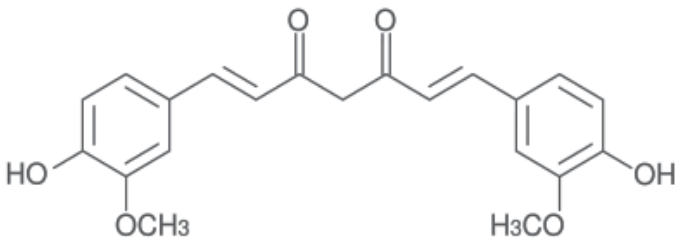

Fig. 2. Chemical structure of curcumin

HCT116 intestinal cancer cells (Jaiswal et al. 2002). The work of Park et al. strengthened the point that curcumin decreased β-catenin/TCF transcription activity in all tested cancer cell lines, including gastric, colon, and intestinal cancer cells, which was attributed to the reduced amount of nuclear β-catenin and TCF-4 proteins (Park, C. H. et al. 2005). Moreover, analysis of gene transcription profile revealed that the expression of Wnt receptor Frizzled-1 was potently suppressed by curcumin (Yan et al. 2005). Curcumin was also shown to be able to attenuate response of β-catenin to Wnt-3a in colon cancer cells through down-regulation of p300, a positive regulator of Wnt/β-catenin signaling (Ryu et al. 2008). In addition, Wang and his colleagues demonstrated that curcumin down-regulated Notch-1 mRNA level in pancreatic cancer cells, indicating a transcriptional inactivation of Notch-1 by curcumin (Wang, Z. et al. 2006). AP-1 and NF-κB signaling pathways were shown to be inhibited by curcumin in glioblastoma cells (Dhandapani et al. 2007). Curcumin-induced inactivation of NF-κB DNA-binding activity was potentially mediated by Notch-1 signaling pathway (Wang, Z. et al. 2006).

Kakarala et al. demonstrated that curcumin (5-10 μM) was able to target breast stem/progenitor cells, as evidenced by suppressed mammosphere formation along serial passage and a decrease in the percentage of ALDH1-positive cells (Kakarala et al. 2009). Similar to sulforaphane, the concentrations of curcumin inhibiting mammosphere formation was much lower compared to the concentrations of curcumin having impact on differentiated cells. Results from serial passaging suggest that curcumin interferes with breast CSC self-renewal. By utilizing a TCF-LEF reporter assay system in MCF7 cells, the authors confirmed that the effect of curcumin on breast cancer stem/progenitor cells was mediated through its potent inhibitory effect on Wnt/β-catenin signaling (Kakarala et al. 2009). These results support the work in other systems showing the ability of curcumin to inhibit Wnt signaling (Jaiswal et al. 2002; Ryu et al. 2008; Prasad et al. 2009). The effects of curcumin was further potentiated by piperine, another dietary polyphenol isolated from back and long peppers (Kakarala et al. 2009). Piperine was suggested to enhance the bioavailability of curcumin through inhibition of P glycoprotein-mediated efflux of curcumin (Shoba et al. 1998; Chearwae et al. 2004; Anand et al. 2007; Limtrakul et al. 2007). Curcumin and piperine, alone or in combination did not cause toxicity to differentiated cells (Kakarala et al. 2009).

Side population (SP) cells, first identified for isolation of murine hematopoietic stem cells from bone marrow (Goodell et al. 1996; Zhou, S. et al. 2001; Hirschmann-Jax et al. 2004), can be used to enrich CSCs (Hadnagy et al. 2006; Wu, C. and Alman 2008). Curcumin inhibited SP of the rat C6 glioma at low concentration (5 μM) that had minimal effect on proliferation of C6 cells (Fong et al. 2010). Very recently, a polymeric nanoparticle formulation of curcumin (5-20 μM) was shown to significantly inhibit clonogenicity and depleted the $CD133^+$ stem-

like cell population from brain tumor cultures (Lim et al. 2011). They also found that Gli1 and Ptch1B, two key components of hedgehog signaling, were significantly reduced in embryonal tumor derived cell line DAOY after curcumin treatment.

5. Epigallocatechin-3-gallate (EGCG)

Green tea is one of the most widely consumed beverages in the world. Epidemiological studies suggest an association between green tea consumption and chemopreventive effects against skin, lung, breast, colon, liver, stomach, and prostate cancers (Yang, C. S. et al. 2002; Landis-Piwowar et al. 2007). The various polyphenolic catechins contained in green tea are thought to contribute to its chemoprevention activity.

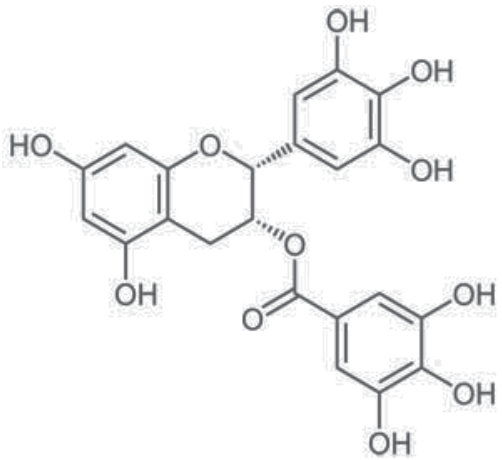

Fig. 3. Chemical structure of EGCG

In particular, numerous studies indicate that EGCG (Figure 3), the most abundant catechin in green tea, is the primary component for these activities (Fujiki 1999; Nagle et al. 2006). *In vitro* and *in vivo* studies have shown that EGCG modulates a wide array of molecular pathways, resulting in induction of apoptosis and cell cycle arrest, and inhibition of invasion, angiogenesis, and metastasis (Shankar et al. 2007; Shankar et al. 2008).

Some studies have found that EGCG may directly or indirectly affect CSC self-renewal pathways. The basal NF-κB activity and ATP- or IL-1β induced activation of NF-κB were negatively regulated by EGCG (Ahmad et al. 2000; Afaq et al. 2003; Guo, S. et al. 2006; Kim, S. J. et al. 2007; Sarkar et al. 2009). EGCG suppressed Akt activation in both colon cancer cell lines and *in vivo* mouse models (Ju et al. 2005; Shimizu et al. 2005; Peng et al. 2006; Bose et al. 2007). In our previous study, EGCG was shown to inhibit the chaperoning function of Hsp90 by impairing the interaction between Hsp90 with its co-chaperones in pancreatic cancer cells, thereby down-regulating Hsp90 client proteins including Akt (Li et al. 2009). EGCG blocked Wnt signaling by stabilizing mRNA of HBP1, a suppressor of Wnt signaling, thereby reducing breast cancer cell tumorigenic proliferation as well as invasiveness (Kim, J. et al. 2006; Kawasaki et al. 2008). The nuclear import of β-catenin was decreased in adenomas isolated from EGCG-treated $Apc^{Min/+}$ mice, a widely used transgenic model recapitulating human colon cancer that bears an Adenomatous Polyposis Coli (APC) gene mutation (Ju et al. 2005; Bose et al. 2007).

Combination of EGCG and doxorubicin was suggested to eradicate putative prostate CSCs ($CD44^+$) (Stearns et al. 2010). EGCG (30 and 60 μM), either alone or in combination with doxorubicin, reduced the colony-forming capability of human prostate cancer cell line PC-3ML. Relatively low dose of EGCG (57 mg/kg) in combination with nontoxic, sub-therapeutic dosages of doxorubicin can eradicate established prostate tumors derived from $CD44^{high}$ tumor-initiating cells isolated from PCa-20a cells in NOD/SCID mice.

Tang et al. have shown that EGCG either alone or in combination with quercetin can eliminate prostate CSC characteristics (Tang et al. 2010). EGCG inhibited the growth and self-renewal capacity of $CD44^+CD133^+$ CSCs contained in human prostate cancer cell lines and $CD44^+\alpha2\beta1^+CD133^+$ CSCs isolated from human primary prostate tumors, as measured by spheroid and colony formation assay. They also suggested that EGCG was able to induce apoptosis in prostate CSCs. In addition, EGCG was found to suppress EMT by inhibiting the expression of vimentin and nuclear β-catenin, as well as the transcription factors slug and snail which are required for EMT induction. The inhibition of EMT markers by EGCG could retard early metastasis of prostate CSCs.

6. Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) (Figure 4) is a ubiquitous plant polyphenol, naturally occurring in most edible fruits and vegetables, with the high levels being found in apples, cranberries, and blueberries (Androutsopoulos et al. 2010; Guo, W. et al. 2009). Many studies have demonstrated that quercetin possess anti-oxidant, anti-inflammatory and anti-cancer activities (Williamson and Manach 2005; Guo, W. et al. 2009). Quercetin has also been shown to enhance the anti-cancer effects of several chemotherapeutic drugs (Borska et al. 2010; Du et al. 2010; Shih et al. 2010; Wong and Chiu 2010; Limtrakul et al. 2005; Du et al. 2009).

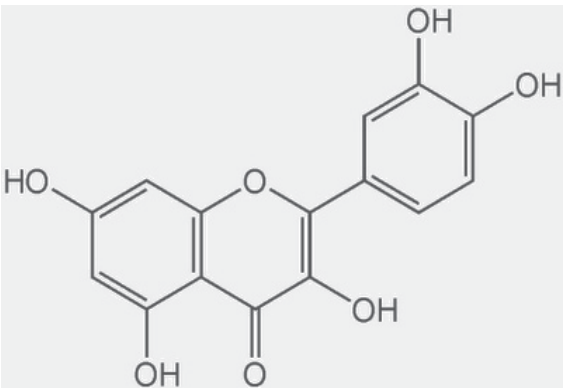

Fig. 4. Chemical structure of quercetin

Several studies have indicated that quercetin may modulate self-renewal pathways. Quercetin was suggested to be a potent inhibitor of β-catenin/TCF signaling in SW480 colon cancer cells, and the reduced β-catenin/TCF transcriptional activity was due to the decreased nuclear β-catenin and TCF-4 proteins (Park, C. H. et al. 2005). The inhibition of colon cancer cell growth by quercetin was related to the inhibition of cyclin D1 and surviving expression through Wnt/β-catenin signaling pathway (Shan et al. 2009).

Zhou et al. demonstrated that quercetin (100-400 μM) mediated reduction of self-renewal capacity of pancreatic CSCs, decreased ALDH1 activity, overcame apoptosis resistance of pancreatic CSCs ($CD44^+CD24^-$), and diminished the expression of proteins involved in the EMT (vimentin and Twist-2) in CSC^{high} cells (Zhou, W. et al. 2010). Quercetin strongly reduced rapid growth of CSC-enriched xenografts, while no toxic side effects were observed (Zhou, W. et al. 2010). They further demonstrated that combination of quercetin with sulforaphane led to a synergistic reduction in self-renewal capacity and a complete abrogation of tumor growth in xenograft mouse model. Similarly, in another recent study, quercetin enhanced the inhibitory effects of sulforaphane on self-renewal capacity of pancreatic CSCs ($CD44^+CD24^+ESA^+$) (Srivastava et al. 2011). Moreover, quercetin (20 μM) was found to synergize with green tea EGCG in inhibiting prostate CSCs (Tang et al. 2010). Quercetin not only potentiated the inhibitory effects of EGCG on self-renewal, migration and invasion capacities of prostate CSCs isolated from primary tumors, but also synergized with EGCG to induce apoptosis (Tang et al. 2010).

Natural Dietary Compound	Food Origins	Cancer Stem Cells	Potential Molecular Targets
Sulforaphane	Cruciferous vegetables	Pancreatic cancer Breast cancer	Wnt/β-catenin; NF-κB binding; EMT markers; Notch-1
Curcumin	Turmeric	Breast cancer Brain tumor	Wnt/β-catenin; Gli1 and Ptch1B
EGCG	Green tea	Prostate cancer	β-catenin; EMT markers; slug and snail
Quercetin	Ubiquitous, e.g., apple, cranberry, blueberry	Pancreatic cancer Prostate cancer Lung cancer	EMT markers
Piperine	Black and long pepper	Breast cancer	Wnt/β-catenin; NF-κB
Genistein	Soy		GSK3β, β-catenin, Wnt-5a; Notch-2
Resveratrol	Grapes, berries, plums, and peanuts		β-catenin, GSK3β; Notch-1
Lycopene	Tomatoes, watermelon, papaya, pink grapefruit		β-catenin
Vitamin D3	Fish, egg yolk, beef liver		TCF-4, E-cadherin

Table 1. Natural dietary components that potentially regulate self-renewal pathways and inhibit CSCs

7. Conclusion

Naturally-occurring dietary components are advantageous in several aspects as chemoprevention agents: (1) they are present in commonly consumed food, which is readily available to most people in daily life; (2) they usually have very low or no toxicity, in contrast to most chemotherapy drugs; (3) many of these compounds have shown potential as an adjunct to chemotherapy drugs in some clinical trials. Although the reports were very limited for dietary components to inhibit CSCs, many of them have been shown to be directly or indirectly involved in modulation of CSC self-renewal pathways. All of these studies stress the need for investigating the efficacy of dietary components against CSCs and elucidating the mechanisms of action. In Table 1, we summarize the compounds discussed in this chapter as well as some others dietary components that may affect the element(s) of self-renewal pathways.

Since CSCs are more resistant to conventional therapies in comparison with differentiated cells constituting the tumor bulk, these studies will provide strong rationale for preclinical and clinical evaluation of the dietary components or potentially their native food extracts combined with chemotherapy. Combination of dietary intervention that are directed against CSCs and conventional chemotherapy would have the potential to eliminate CSCs, overcome tumor resistance, reduce recurrence, and eventually improve patient survival.

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23

Towards New Anticancer Strategies by Targeting Cancer Stem Cells with Phytochemical Compounds

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1. Introduction

Cancer stem cells (CSCs) are believed to be responsible for tumor initiation and development, metastasis and resistance to radio-therapy and *a priori* to numerous natural or synthetic chemical compounds. A large body of observations support now the 100 year old hypothesis which predicted a clonal genetic background of the heterogeneous cell population found in a tumor outgrowth [Paget, 1889]. Accordingly, since Dick's laboratory pioneering work in 1994 [Lapidot et al., 1994], growing realizations suggest that CSCs arise from embryonic, fetal or adult stem cells (SCs) or closely related dedifferentiated descendants. Interestingly, the concept that CSCs give rise to the bulk cancer cells is in accordance with the germ theory of disease developed by Koch in the 19th century [Garcion et al., 2009]. This theory points out that any disease has a unique causative agent. Although Koch's dogma suggests that tumors should arise from CSCs, it must also be borne in mind that their descendant cancer cells can generated dedifferentiated cells with a parental phenotype and therefore can be involved in the outburst of a secondary cancer.

On the basis of epidemiological data, it has been recurrently reported that diet rich in fruits and vegetables has cancer-protective properties; this suggests that plant-derived compounds are able to restrict the expansion of CSCs and even to kill them. The chemotherapeutic benefits of different natural or synthetic phytochemical agents on cancer cells are well documented. However their effects on CSCs are poorly understood, to a large extent because on the absence of well characterized experimental models. The objective of this chapter is therefore to recapitulate some aspects of the biology of CSCs and to propose different cellular tools and molecular preys for thorough pharmacological studies on CSCs, on the basis of the most recent data concerning the stemness factor Oct4. After reviewing known effects of specific phytochemicals on CSCs, we will focus on related promising strategies which could target the Achilles' heel of CSCs, in particular those harboring a selective sensitivity to oxidative stress and/or present in weakly differentiated Oct-4 expressing cancers.

2. Overview of cancer stem cell biology

2.1 Properties of cancer stem cells

CSCs share many characteristics with SCs and can be defined by their capacity to undergo self-renewal and to differentiate into more or less restricted cell types (from pluripotency to monopotency), depending on their embryological origins. The ability to self-renew allows the expansion of either the SCs or CSCs pool, in response to controlled or uncontrolled systemic and local signals respectively. Cell self-renewal involves either an asymetric or a symetric division process and allows the production of two daughter cells, one being identical to the mother cell and the second being expected to lose some of its lineage-specific competencies [Morrison & Kimble, 2006]. Actually differentiation from the SC or CSC compartment involves a sequential production of cells with more and more tissue-specific spezialization [Lobo et al., 2007; Sell, 2004]. Interestingly the level of aggressiveness of the CSCs seems to be related to their state of differentiation; poorly differentiated cells are highly aggressive while nearly terminally differentiated cells only give rise to benign tumors (Fig. 1). Since the fine balance between proliferation and differentiation is expected to be corrupted in CSC, it can be hypothesized that the biological chaos will be even more pronounced when a CSC exhibits a higher proliferation rate for a longer retention time until its final differentiation.

In view of its properties, a CSC can be firmly distinguished from a cancer cell by its unique capacity to undergo differentiation. However both of these cell types have enhanced growth ability which can be closely correlated with elevated levels of glycolysis and increasing metabolic activity. This property, previously described by Warburg, is considered as one of the most fundamental alterations occurring during malignant transformation [Warburg, 1924]. Adversely it has been postulated that a given CSC could originate from a cancer cell (or a CSC with lower lineage-specific competencies) which dedifferentiates into a stem-like cell (or into a CSC with higher lineage-specific competencies). Although dedifferentiation has not yet been identified as a naturally occurring process, an increasing number of reports assume the concept of such oncogene-induced plasticity [Rapp et al., 2008; Visvader, 2011], recently validated by mathematical modelings [Leder et al., 2010].

2.2 Regulatory networks of cancer stem cells in the niche

Accumulating evidences have shown that CSCs, like SCs, are regulated by common molecular pathways. Wnt/beta-catenin, Notch and Hedgehog pathways have been shown to be involved in the self-renewal regulation of both SCs and CSCs [Blank et al., 2008; Lobo et al., 2007]. In particular, Wnt signaling is known to promote proliferation of SCs when it binds its receptor Frizzled; a negative signal is then sent to inhibit the activity of APC (Adenomatous Polyposis Coli) which controls the degradation of beta-catenin. Increased amount of stabilized cytoplasmic and consequently nuclear catenin triggers then cell growth. Accordingly, accumulation of beta-catenin has also been frequently reported in various cancer cell types [Reguart et al., 2005]. Mice expressing constitutively activated beta-catenin showed highly proliferative tumors. Nevertheless apart their incidence on SC/CSC proliferation, an impaired activity of Wnt, Notch and/or Hedgehog pathways should also interfere with SC/CSC competency and its differentiation capacity. Indeed the balance between proliferation and differentiation for a proper self-renewal is difficult to determinate, as observed for example by the dual role of Wnt in both cell processes. A growing body of reports suggests that Wnt signaling can provide instructive signals that change the

commitment of SCs [Angers & Moon, 2009]. Given that different Wnt and Frizzled proteins can lead to the activation of either a catenin-dependent or -independent pathways, it seems obvious that these latter could somehow influence the cell fate of both SCs and CSCs.

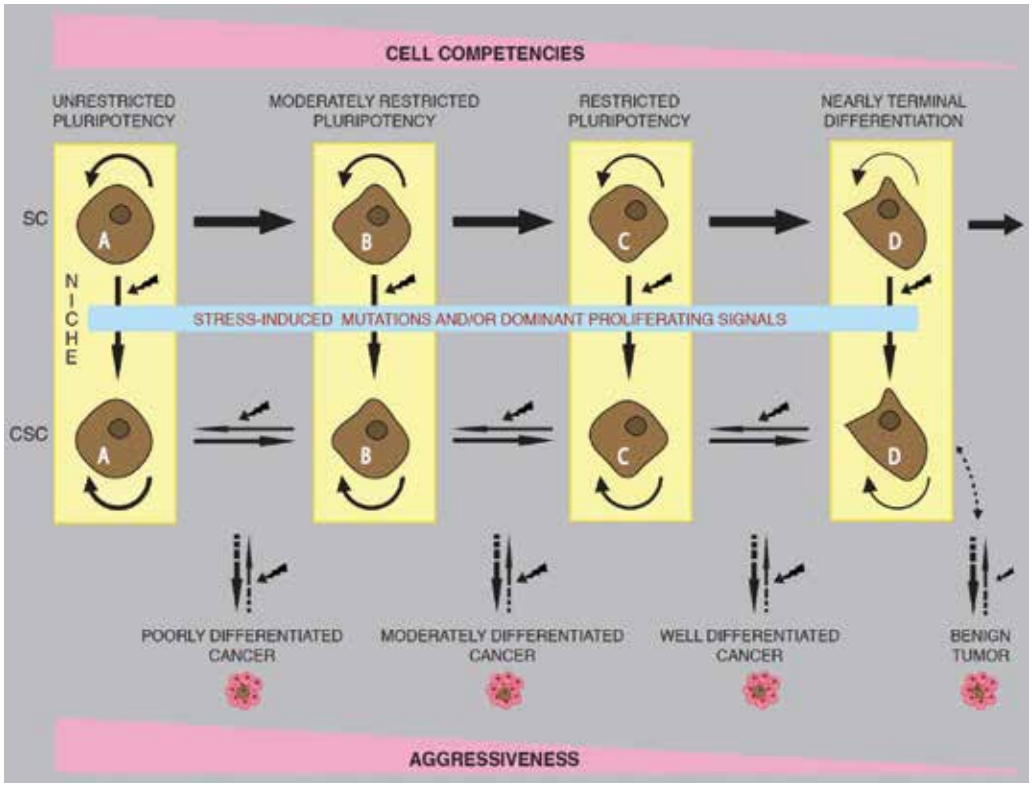

Fig. 1. Hierarchical and dynamical relationships between SCs, CSCs and their descendants. By successive differentiation waves, SCs lose step by step their cell competencies and become highly specialized cells. Stress-induced mutation and/or dominant proliferating signals trigger the transformation of SCs, committed to a specific fate, into CSCs which share the same differentiation profile. Uncontrolled proliferation of their homeless descendants leads to the outburst of a tumor which malignancy depends upon the differentiation stage of the tumor-initiating cells. Additional oncogenic hit likely allows CSC descendants to dedifferentiate into cells which possess higher lineage-specific competencies. The different types of SCs (and their corresponding CSCs) harbor distinct phenotypic markers (depicted by A to D) and show differential self-renewal capacity (depicted by faint to thick semi-circular arrows)

CSCs, in contrast to SCs, are not able to control their own population size. This suggests that the homeostatic regulation of CSCs by the niche is impaired. Indeed SCs are known to be anchored to niches found in a limited and specialized microenvironment of the different organs in the body (Fig. 1). Usually these cells are quiescent and are devoted for replenishing dead cells and repairing damaged tissues ; however SCs may be transformed in activated CSCs when exposed to repetitive mutation-inducing stress injuries without any ability to escape throughout the cell flux or to undergo apoptosis. Leaving their dormant

state, CSCs poised by failures of the self-renewal system and unable to respond in an appropriate manner to Wnt, Notch or Hedgehog signals, may then proliferate without restraint and escape from the niche, leading to the outburst of the tumor. However it has also been hypothesized that alteration of the niche by dominant proliferation-promoting signals could explain why SCs lost the dependance for limited expansion and become uncontrolled with a higher risk of oncogenic drift [L. Li & Neaves, 2006]. Thus restoring the regulatory signaling pathways in the niche might be a promising strategy to keep CSCs in check.

2.3 Models for studying cancer chemoprevention: identification of CSC markers

A direct consequence of the existence of CSCs assumes that future anticancer treatments should target this cell population. It is therefore critical to better characterize them. Some markers, like the cell surface antigen CD133, have been recommended for a prospective isolation of CSCs (Table 1). However recent studies have indicated that CD133 is also expressed in differentiated normal cells of various organs and CD133-negative cancer cells can also initiate tumors [Salnikov et al., 2009]. In this point of view, a very convincing report has clearly shown that a hierarchy of self-renewing CSC types expressing or not CD133 can be identify in glioblastoma tumors [Chen et al., 2010]. Therefore the relative reliability of such markers in CSCs, and consequently the absence of *bona fide* CSCs lines, remains a main barrier for studying the effects of potential cancer chemopreventive agents. For that purpose, commonly used chemical carcinogens were also used to initiate tumors of specific cell types; moreover the question of interlaboratory variability and standardization still remains

Tissue	Marker	Description	Reference
Brain	CD133 (Prominin 1)	Transmembranic	Prestegarden & Enger, 2010
Breast	CD44 (Homing Cell Adhesion Molecule)	Transmembranic	Garvalov & Acker, 2011
Colon	CD24 (Heat-Stable Antigen)	GPI-anchored	Todaro et al., 2010
Ovary	CD117 (c-Kit)	Transmembranic	Garvalov & Acker, 2011
Pancreas	CD326 (Epithelial-Specific Antigen –ESA-)	Transmembranic	C. Li et al., 2009
Prostate	Prostate Stem Cell Antigen (PSCA)	GPI-anchored	Saeki et al., 2010
Testis	CD9 (Tetraspanin 29)	Transmembranic	Biermann et al., 2007

Table 1. Frequently recommended cell surface marker for the detection and isolation of CSCs in various tissues. Some additional markers are routinely used to purify CSCs to homogeneity (Keysar & Jimeno, 2010), like CD44 for colorectal cancer or CD133 for breast carcinoma. It should be noticed that CD44 is recurrently mentioned as a reliable marker of any type of CSCs. Moreover the marker expressed in tumor-initiating cell denotes more precisely the embryonic origin of the cell than the tissue where it developped [Visvader, 2011]. GPI: glycosylphosphatidylinositol. (Prestegarden & Enger, 2010)

to be solved [Rosenberg et al., 2009]. Within the framework in this debate, embryonic stem cell lines (and their malignant counterparts, the embryonal carcinoma stem cell lines) are expected to be suitable models of CSCs and can be used as surrogated investigational tools for thorough evaluation of potential anticancer chemopreventive agents. Accordingly, numerous available teratocarcinomal cell lines were obtained after serial xenotransplantation and cultivation; such experimental design allows the recapture of the malignant phenotype and is widely used to isolate CSCs from any tumor tissue [Sell, 2004].

2.4 The Oct4 mystery

2.4.1 Regulation of Oct4 expression in stem cells

Oct4 (also known as POU5F1), a member of the POU-domain family of transcription factors, plays an essential role in the maintenance of embryonic stem cell potency and the establishment of the germ cell lineage. In embryonic stem cells, an Oct4 expression level between 50% and 150% of the endogenous amount appears to be permissive for self-renewal and maintenance of cell potency. Oct4 is downregulated during gastrulation when SCs differentiate, and eventually its expression is confined to the germ cell lineage. Consistent with its expression profile, it has been shown that Oct4 is active in embryonic stem cells, embryonal carcinoma cells and embryonic germ cells (Fig. 2). Upon treatment with retinoic acid (RA), these cells differentiate and Oct4 is rapidly downregulated [Pesce & Schöler, 2001].

Finely tuned functional Oct4 levels are crucial for phenotype stability and it is believed that the induction or repression of Oct4 is heavily regulated in order to avoid any deleterious effect of a transient dysfunction. It has been shown that the regulation of Oct4 expression involves different members of the nuclear receptor superfamily, including SF-1 (Steroidogenic Factor 1), LRH-1 (Liver Receptor Homolog-1) and GCNF (Germ Cell Nuclear Factor) [Kellner & Kikyo, 2010]. By means of genetic, molecular, and pharmacological studies, a recent report has demonstrated that a catenin-dependent LRH-1 regulation is required for maintaining steady-state levels of Oct4 [Wagner et al., 2010]. This means that the balance between proliferation and differentiation of pluripotent SCs involves, at least in part, a Wnt/beta-catenin control which can specifically target the upstream regulators of the stemness factor Oct4. Moreover it has been argued that GCNF is able to recruit different MBD (Methylated CpG Binding Domain) proteins to the Oct4 promoter, suggesting a link between Oct4 gene repression and its epigenetic locking [Gu et al., 2006]. A cascade of events from the binding of extracellular signaling molecules to Oct4 gene silencing can therefore be outlined. However additional as yet unknown mechanisms of regulation might also emerge in the future.

MicroRNAs (miRNAs) are known to regulate posttranscriptionally a target, by pairing with a short antisense stretch located in the 3′-untranslated region of its mRNA, in order to affect its stability and/or translation. A recent report has shown that miR-145 binds to Oct4 mRNA, represses its expression and induces lineage-restricted differentiation of embryonic SCs [N. Xu et al., 2009]. Intriguingly, three other miRNAS, *e.g.* miR-134, miR-296 and miR-470, have been described to target in the amino acid coding sequence of Oct4 mRNA, leading to transcriptional and morphological changes of the pluripotent SCs [Tay et al., 2008]. Theses observations demonstrate therefore that the levels of Oct4 can be indirectly regulated by different naturally occurring miRNAs and that these latter can induce a phenotypic switch of SCs from a highly pluripotent to a more restricted state.

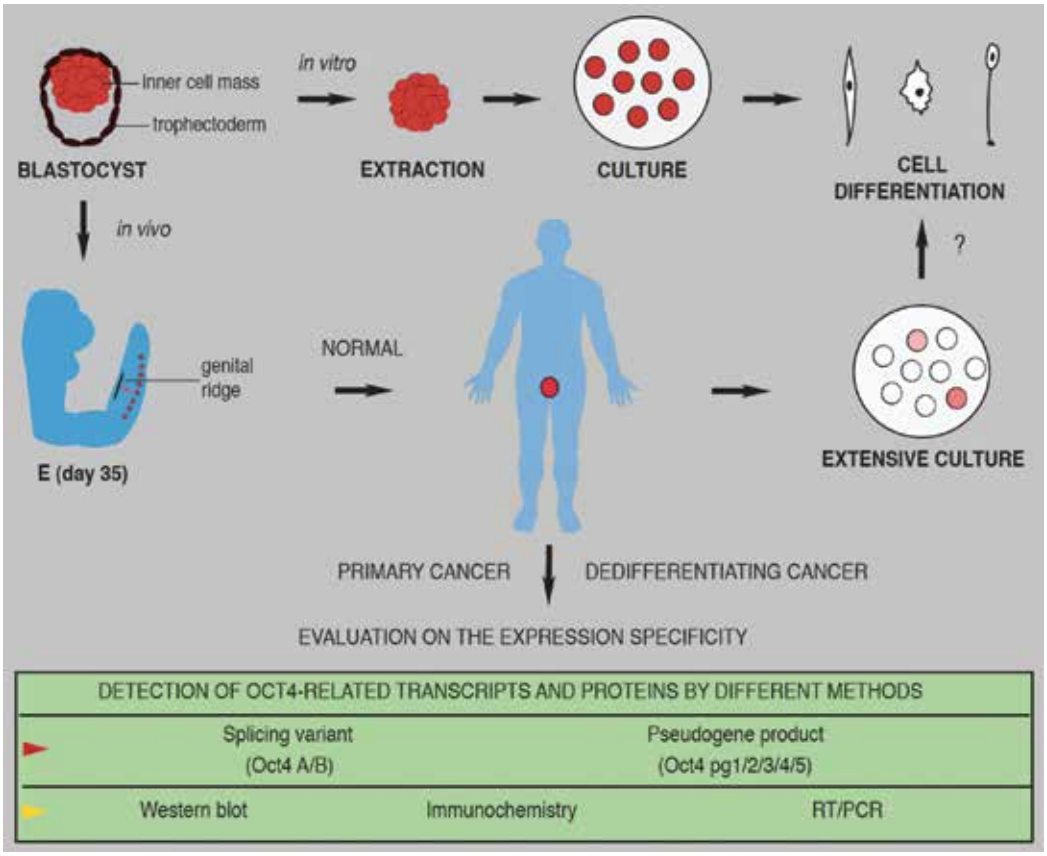

Fig. 2. Recapitulation of *in vivo* and *in vitro* expression of Oct4-related proteins in human SCs and CSCs. Oct4 is highly expressed in the inner cell mass of the blastocyst; at day 35 of the embryonic development, the expresion of the protein is confined in the germ cells, which will later on invade the genital ridges. Cultivated embryonic stem cells from the blastocyst give rise to different cell types which do not express Oct4. Extensively cultivated somatic cells might express Oct4 to a limited extent, but their ability to acquire stem-like properties remains questionable. An Oct4 signature can be identified in numerous cancer cells; depending upon the experimental method used, the detectable expression of Oct4 splicing variants and/or pseudogene products might be artifactitious or specific of some types of CSCs, and therefore could be involved in the etiology of the neoplasia. The role of Oct4 homologs (*e.g.* Oct1) should also be considered. Oct4 is depicted in red; the brightness of the color represents the amount of expression

Oct-4 transcriptional activity is regulated at the posttranslational level by different mechanisms. Sumoylation by SUMO-1 (Small Ubiquitin-related Modifier, 1) increases the stability of the protein and its transactivation potential [Wei et al., 2007]. In contrary, its ubiquitination by the E3 ubiquitin-protein ligase WWP2 (WW domain-containing Protein, 2) promotes its degradation [H. Xu et al., 2009]. Finally the repression of Oct4 expression, at the transcriptional, posttranscriptional and now posttranslational level [Shi & Jin, 2010], implicates multiple regulators which might be potential targets for a selective ablation of Oct4 function. This issue will be specifically addressed in the last section.

2.4.2 Oct4-dependent transcriptional networks in stem cells

As a major guardian of early stemness preservation, Oct4 regulates the transcription of numerous genes to maintain the self-renewal and pluripotency properties of the embryonic stem cells (Table 2). The POU factor interacts via its two domains POU and Hox with the octamer motif ATGCAAAT (or certain variants) located at the promoter(s) and/or the regulatory regions of the different target genes [Pesce & Schöler, 2001]. By this way, Oct4 activates or represses genes which are associated with proliferation and differentiation processes. Through ChIP-on-chip analysis, more than 900 putative direct downstream targets of Oct4 have been identified [Jung et al., 2010].

Target gene	Protein function	Cell process	Reference
CDX2 (caudal type homeobox transcription factor 2)	Transcription factor	Differentiation induction	Babaie et al., 2007
FGF4 (fibroblast growth factor-4)	Signaling molecule	Differentiation repression	Chew et al., 2005
nanog	Transcription factor	Stem cell identity	Rodda et al., 2005
NDUFA3 (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 3)	Mitochondrial electron carrier	Cell metabolism	X. Chen et al., 2008
Oct4	Transcription factor	Stem cell identity	Chew et al., 2005
p21 (WAF1 ; CIP1)	Cyclin-dependent kinase inhibitor	Proliferation inhibition	Lee et al., 2010
SOX2	Transcription factor	Stem cell identity	Chew et al., 2005
TP53 (p53)	Tumor suppressor	Cell death	Campbell et al., 2007
SUZ12 (suppressor of zeste 12 homolog)	Polycomb repressive complex 2	Chromatin remodeling	Sharov et al., 2008

Table 2. Representative set of genes targeted by Oct4. The above mentioned downstream effectors of Oct4 were selected on the basis of experimental evidences, mainly involving functional genomic analysis after loss of Oct4 function. Note that *nanog*, *Oct4* and *SOX2* are also transcriptionally regulated by Oct4, pointing out the crucial role of the POU factor in the maintenance of a stemness profile in embryonic stem cells. By targeting genes involved in cell growth and differentiation, Oct4 enables an efficient and proper self-renewal of undifferentiated cells

To achieve a higher specificity, Oct4 may form protein complexes with other transcriptional regulators, including the homeobox protein nanog and the SRY-related HMG-box protein SOX2. Indeed large-scale mapping studies interrogating the binding sites of these three transcription factors showed their co-occupancy on distinct sets of target genes, suggesting

that their assembly in multiprotein complexes could serve as a mechanism for directing specificity by regulating stem cell-related gene expression [Boyer et al., 2005].
Interestingly, a growing body of observations highlights new insights about Oct4 activity in SCs and particularly in embryonic stem cells. Some candidates transcriptionally regulated by Oct4 are miRNAs. As an example, the POU factor binds to the promoter region of the miR-302 cluster in pluripotent cells, inducing an transcriptional activation of the miR-302s and the translational repression of its corresponding targets, such as the cell cycle regulator cyclin D1 [Card et al., 2008]. Interestingly miR-302 members are predicted to target many cell cycle regulators, suggesting that Oct4 could indirectly be implicated in the control of the different cell cycle checkpoints. More recently, it has been shown that Oct4 regulates the expression of miR-106b family members which target *p21* mRNA at its 3′ untranslated region and thus indirectly p21 levels [Koster et al., 2010]. These observations first suggest an unconventional supplementary link between Oct4 and cell cycle regulation in highly undifferentiated SCs. It can also be expected that in the future an increasing number of miRNA genes might be identified as targets of Oct4.

2.4.3 Oct4-related proteins in stem cells and cancer stem cells

The human *Oct4* gene comprises five exons. Two isoforms generated by alternative splicing, namely *Oct4A* and *Oct4B*, have been identified. Both have identical DNA-binding domains and C-terminal transactivation domains. Oct4A (*i.e.* Oct4) is localized in the nucleus while Oct4B is mainly localized in the cytoplasm and therefore should not be able to sustain self-renewal [Cauffman et al., 2006; Lee et al., 2006]. On the other hand, five different pseudogenes (numbered *Oct4-pg1* to *Oct4-pg5*) have been evidenced by whole-genome analysis and are highly homologous to the parental *Oct4* gene [Pain et al., 2005]. In view of their structure, these pseudogenes can theoretically be transcribed and translated and therefore could participate unexpectedly in some physiological or physiopathological processes. The plethora of Oct4 isoforms and *Oct4* pseudogene products should therefore be carefully taken in account when a putative Oct4 signature is expected to be detected in a specific type of SCs or CSCs (Fig. 2). Recently some *in vivo* studies have reported the detection of the stemness factor Oct4 in a variety of somatic tissue-derived cells, but these observations seem to be related to experimental pitfalls. However it cannot be excluded that somatic cells cultured for extensive periods of time could reactivate Oct4 function [Lengner et al., 2008]. The discussion that this reflects or not physiological processes to maintain somatic SCs in a self-renewal mechanism is still open.
Although the transcription factor Oct4 is known to be essential for pluripotency maintenance and self-renewal, its expression in putative CSCs, like that of CD133, remained controversial in the past years [Liedtke et al., 2008]. However growing body of evidences support now the idea that Oct4 could be expressed in CSCs from diverse tumor origin [Kang et al., 2009]. Oct-4 expression is clearly associated with bladder carcinogenesis [Atlasi et al., 2007] and germ cell malignancy [Cheng et al., 2007]. It should be noted that, as nongerminomatous germ cell tumors, embryonal carcinomas and their derived cell lines, are therefore expected to be suitable experimental models for studying the biology of Oct4-positive CSCs. Similarly a pluripotency gene expression signature has been evidenced in poorly differentiated and highly aggressive cancers [Ben-Porath et al., 2008]. Since this stemness identity involves Oct4 and its two coregulators, *e.g.* nanog and SOX2, it can be assumed that the regulatory networks controlling the activity of SCs are also functional in some cancers. Interestingly, the three proteins seem to be present even in far developmentally

related adult tumors, suggesting that the bulk cancer cells were able to dedifferentiate to a less restricted competency state.
Intriguingly, it has been shown that breast carcinoma and glioma can express *Oct4* pseudogenes; however it seems that their products lack Oct4-like activity on the basis of their absence of transcriptional activation potential on known Oct4-responsive luciferase constructs [Zhao et al., 2011]. This suggests that the detection of *Oct4* pseudogenes could have led to misinterpretation of some previous studies claiming the presence of Oct4 in CSC subtypes. Further investigations are thus necessary in order to address this issue and to solve whether Oct4 splicing variants and pseudogene products might be involve in SC identity and in the etiology of certain cancers (Fig. 2). Moreover a possible role of some Oct4 homologs in the induction of the neoplasic process has been recently emphasized; Oct1, like Oct4, binds to the same DNA sequences, regulates common target genes and are under the control of identical upstream regulators. It is therefore hypothesized that Oct1 or other Oct proteins might carry out similar malignancy functions as Oct4 [Kang et al., 2009].

3. Phytochemicals and cancer stem cells

Epidemiological studies have consistently linked the intake of fruits and vegetables with reduced risk of initiation and development of cancer [Steinmetz & Potter, 1996]. Adversely, recent reports based on large propective studies downgraded the previous conclusions; the potential chemopreventive effects of diets rich in fruits and vegetables seem to be rather associated with healthy nutritional principles [Key, 2011]. However it is still believed that particular constituents in certain fruits and vegetables could have benefit effects. More than three-fourths of the anticancer compounds are either derived substances from natural products or the natural products themselves, mostly originating from herbal medicinal and dietary plants or from microbial sources. This section will therefore only focus on the cancer chemoprotective effects of some plant-derived compounds which chemical structure is known.
In a strict sense, phytochemicals with chemopreventive properties hinder the (re)appearance of a cancer by targeting CSCs, whereas phytochemicals with chemotherapeutic properties destroy a preexisting cancer by targeting cancer cells. However these latter can conceptually be considered, at least in part, as potential CSCs with very limited cell competencies (see Fig. 1). For that reason, chemotherapy and chemoprevention become hard to distinguish to each other, since they can theoretically target the cancer cell as well as the CSC. It is therefore not surprising that numerous plant-derived compounds might act on both cell types and have therapeutic and preventive effects [Aggarwal et al., 2004].

3.1 Targeting cancer cells by phytochemicals

3.1.1 Generalities

There is a plethora of *in vivo* and *in vitro* studies which have highlighted the benefits of phytochemicals against distinct cancer types. An exhaustive list of plant-derived compounds with known chemotherapeutic properties can be found elsewhere [Kawasaki et al., 2008; Shu et al., 2010]. By modulating multiple signaling pathways, they can target various cell processes, including induction of apoptosis as well as inhibition of cell survival, metastasis and angiogenesis. Such pleiotropic activity is for instance displayed by curcumin [Das et al., 2010]; this potent polyphenol antioxidant was originally extracted from tumeric, a spice made from the root of the plant *Curcuma longa* and which is widely consumed in the Indian

subcontinent countries. Curcumin is one of the most studied phytochemical compound and will be used as a referential model for the next issues.
It is worth noting that several plant-derived compounds are able to reverse the multidrug resistance (MDR) phenotype, usually observed in aggressive subpopulations of cancer cells. This pathological phenomena results from an intrinsic dysfunction of different energy-dependent transporter proteins (for example P-glycoprotein or member of the multidrug resistant-associated proteins) which are involved in drug entry and efflux [Molnár et al., 2010]. By down-regulating the expression of transporter proteins, some phytochemicals, like curcumin, can restore the chemosensitization in drug-resistant cancer cells [Limtrakul, 2007].

3.1.2 Molecular targets of apoptosis-inducing chemotherapeutic phytochemicals

Acquired resistance towards apoptosis is the key hallmark of all types of cancer [Hanahan & Weinberg, 2000]. Apoptosis is induced by both intrinsic (mitochondrial) and extrinsic (death receptor) pathways (Fig. 3). It is accompanied by successive biochemical events and morphological changes, like DNA condensation and fragmentation, cell shrinkage, membrane blebbing and membrane-associated apoptotic bodies [Saraste & Pulkki, 2000]. Curcumin is a very potent inducer of apoptosis and interferes with both the intrinsic and extrinsic proapoptotic signaling pathways; this phytochemical can therefore kill a wide variety of cancer cells, even if they exhibit some mutation(s)-induced failures in several steps of their proapoptotic machinery [Ravindran et al., 2009].
Apoptosis and cell survival are tightly associated in order to maintain cell population in a healthy homeostatic state. There are several points of crosstalk between the two operating systems. As a consequence, prosurvival signals increase the expression and/or the activity of antiapoptotic regulatory proteins, while repressing the expression and/or the activity of proapoptotic factors. At the opposite, proapoptotic signals activate the function of antisurvival molecules and inhibit the function of prosurvival factors. Such duality of action can be achieved because the two regulatory networks share common molecular targets. One of the most studied crosstalk between life and death signaling pathways is illustrated by the nuclear factor-κB (NF-κB). This DNA-binding protein participates in a dual role, wherein it mediates both prosurvival and proapoptotic signals. Actually the NF-κB pathway targets antiapoptotic and proapoptotic factors; depending upon the cellular context, its transcriptional competencies are modulated by specific upstream activators, like the serine/threonine protein kinase AKT or the FasL/TNF/TRAIL (Fas Ligand/Tumor Necrosis Factor/Tumor necrosis factor-Related Apoptosis-Inducing Ligand) death receptors [Jin & El-Deiry, 2005]. Such yin and yang connection can also be observed between the survival factor AKT and the tumor suppressor p53, or between NF-κB and p53 which competitively interact with the nuclear coactivators CBP/p300 (CREB-Binding Protein, related p300) and therefore reciprocally repress their activity [Dey et al., 2008].
Heat-shock proteins (HSP) are the significant integrators of the interconnective activity of the proapoptotic and prosurvival signaling networks. For instance, the chaperone protein HSP90 interferes with the function of several factors (*e.g.* p53) of the intrinsic and extrinsic apoptosis pathways, leading to cell death inhibition [Walerych et al., 2004]; HSP90 also promotes cell survival through its involvement in the formation of active NF-κB and the maintenance of AKT in its active phosphorylated form [Arya et al., 2007]. Taken together, all these findings might ultimately result in the development of highly efficient chemotherapeutic candidates which are able to target the upstream integrators, as well as several nodal points

of the pro-apoptotic and pro-survival machineries. In view of its widespread biological properties, curcumin should be considered; it is capable to disrupt HSP90 function [Wu et al., 2006], to upregulate p53 expression and to inhibit NF-κB and AKT activities [Ravindran et al., 2009]. However additional studies are needed to gain the full insights of the multifocal activity of curcumin and to identify the different proapoptotic and prosurvival crosstalk mechanisms that it can target.

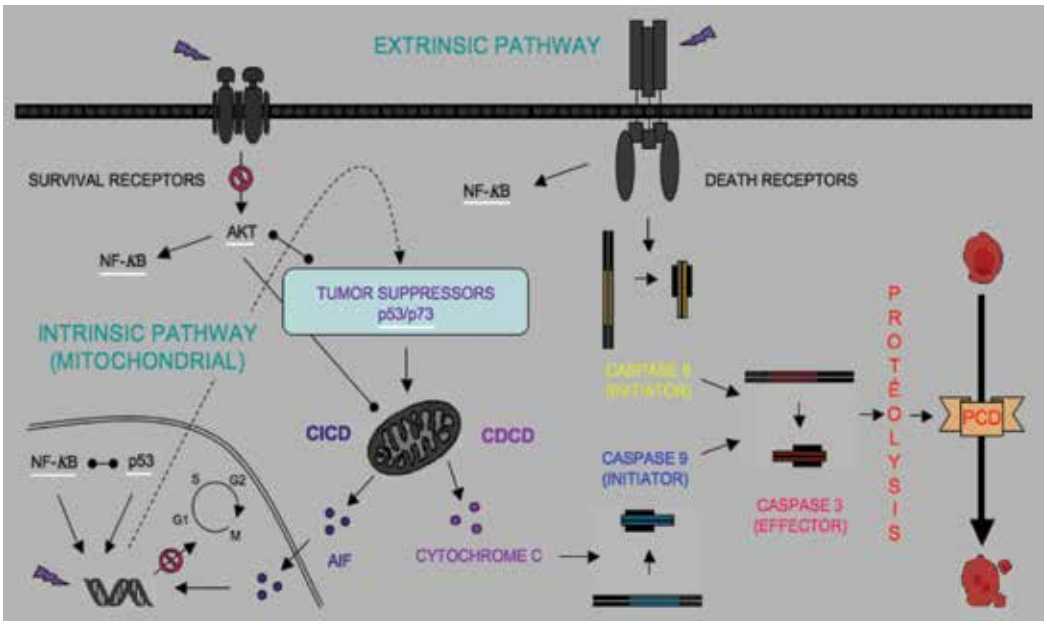

Fig. 3. Simplified scheme of the activity of a generic phytochemical on the different pro-apoptotic and cell survival-associated pathways. In response to a cellular stress generated by a phytochemical, two inter-connected proapoptotic pathways can be induced. The extrinsic pathway is initiated by an activation of the death receptors (Fas, TNF-R, TRAIL-R) and triggers a caspase 8-dependent apoptosis. The intrinsic signaling pathway is driven by the tumor suppressor p53 (or the p53-homolog p73), a main regulator of cell cycle progression; the DNA-damage induced activation of p53 initiates a caspase-independent (CICD) or caspase 9-dependent (CDCD) cell death process, associated with a mitochondrial release of either the chromatinolytic factor AIF (Apoptosis-Inducing Factor) or cytochrome C respectively. Finally the cleavage of pro-caspase 3 in its active subunits leads to proteolysis and programmed cell death (PCD). The functional repression of the survival receptors and their effectors, *e.g.* AKT and NF-κB, can also trigger apoptosis. It is worth noting that the death receptors are also implicated in the regulation of NF-κB pathway. HSP90, as an integrator of both cell survival and apoptotic activities, targets various factors (including those underlined in white); some of these factors repress each other's function (depicted by left right circle-headed arrow). The proposed phytochemical could be for instance curcumin (depicted by a zigzag arrow). Detailed relationships between the different factors can be found elsewhere [Jin & El-Deiry, 2005; Ravindran et al., 2009; Sarkar et al., 2009]. Note that failures of the intrinsic proapoptotic pathway can lead to an accumulation of DNA damages which in turn leads to incorrect DNA repair and mutations

Interestingly, induction of apoptosis triggers the suppression of the angiogenic process and the inhibition of tumor growth [Wang & Sun, 2010]. Moreover the signals that allow a migratory cancer cell to invade normal tissue, promote also cell survival by suppressing apoptosis [Stupack, 2007]. Therefore targeting cancer cells by apoptosis-inducing phytochemicals indirectly interrupt tumor neovascularization and metastasis. Accordingly curcumin has been shown to prevent angiogenesis and cancer cell invasion, thanks to its antiproliferative and proapoptotic activity [Kunnumakkara et al., 2008]. Finally, this phytochemical, by modulating the expression and activity of a wide variety of proteins, exhibits a strong therapeutic efficacy and could play an important role by cutting down cancer incidence. It should also be noted that this reductionist approach which only focuses onto two interconnected processes, namely apoptosis and proliferation and understates the other processes is theoretically valuable. Indeed it is expected that a normal cell should be corrupted by at least six different mutations to be convert into a cancer cell; these mutations are associated with self-sufficiency towards proliferative signals, insensitivity to growth suppressors, resistance for cell death, ability for limitless replication, angiogenesis induction and metastasis activation [Hanahan & Weinberg, 2000]. However the concomitant occurrence of multiple mutations in any cell is statistically rare if not impossible. This suggests that only one or two mutations, affecting selectively the proapoptotic or/and growth inhibitory potential, are necessary for the normal cell to initiate tumorigenesis [L, Li & Neaves, 2006]. As a fact, tumor-associated mutations in a single gene, *i.e. TP53*, are the main hallmark of most human cancers [Whibley et al., 2009]. In the light of the above considerations, it becomes clear that targeting the main actors of crucial processes, like apoptosis or cell survival, by a pharmacological agent remains one of the most effective strategy in anticancer treatment.

3.2 Targeting cancer stem cells by phytochemicals

3.2.1 Generalities

CSCs, like cancer cells, exhibit uncontrolled growth and therefore show quite similar susceptibility to plant-derived compounds, which should target, through their antiproliferative properties, common molecular pathways [Aggarwal et al., 2004]. Nevertheless it is expected that differentiation-inducing phytochemicals are able to counteract two different cell processes, namely the self-renewal maintenance which is specific of CSCs and the dedifferentiation drift which can affect both CSCs and cancer cells (see Fig. 1).

3.2.2 Targeting selectively cancer stem cells by apoptosis-inducing phytochemicals

A growing body of studies suggests that phytochemicals can trigger a proapoptotic response of potential or full-blown CSCs. For example, curcumin induces a decrease of the stem-like side population of the rat C6 glioma cell line, likely through a proapoptotic process [Fong et al., 2010]. Accordingly, curcumin activates the caspases of both the extrinsic and intrinsic pathways of apoptosis and represses AKT function (see Fig. 3) in various ovarian carcinoma cell lines [Watson et al., 2009], including the SKOV3 cell line from which a side population of CD133/CD117-positive cells (see Table 1) with cancer stem-like properties can be isolated [Ma et al., 2010]. The broccoli compound sulforaphane, a member of the isothiocyanate family of phytochemicals, represses NF-κB-dependent prosurvival activity of pancreatic CD44-positive tumour-initiating cells, leading to the downregulation of antiapoptotic proteins and induction of caspase activity followed by apoptosis [Kallifatidis et al., 2009].

The polyphenolic compound resveratrol triggers apoptosis by activating caspase-3/7 in pancreatic CD133/CD44/CD24/ESA-positive CSCs isolated from human primary cancer, suggesting that this pharmacological agent could be used for the prevention and treatment of pancreatic malignant tumor [Shankar et al., 2011]. In view of these examples, it can be expected that numerous phytochemicals, identified as proapoptotic agents on cancer cells, will also be recognized in the future as killers of CSCs. The main reason for this assumption is that various types of tumors and cancer cell lines, previously analyzed for their reactivity to plant-derived compounds, contain CSCs which self-renew and express SC markers [Kondo, 2007]. Therefore it is likely that the described sequence of pro-apoptotic events induced by a specific phytochemical in a given cancer cell should also be observed in its corresponding initiator. Such paradigm should notably be validated for plant-derived compounds, like polyphenols, which act as DNA damage inducers; the canonical molecular cascade usually implicates an activation of the p53 or p73-dependent cell cycle checkpoint signaling pathway and consequently an initiation of a caspase-mediated protein degradation and DNA fragmentation, leading to an irreversible growth inhibition by enhanced apoptosis [Narayanan, 2006]. Such sequence of events, shown in cancer cells, might also be evidenced in CSCs. If this extrapolation is confirmed, this would finally mean that apoptosis-inducing phytochemicals remain very powerful weapons against cancer since they target both CSCs and their descendants, hereby by common pathways.

Intriguingly some phytochemicals, in a reasonable range of concentrations, kill cancer cells without having any toxic effects on normal cells. This selectivity is poorly understood. Several explanations have been put forward. Most of tumor cells, in contrast to normal cells, constitutively express active NF-κB which mediates their survival [Prasad et al., 2010]. Phytochemicals with differential cytotoxic properties, like curcumin or the flavone wogonin, are known to repress the activity of NF-κB downstream targets [Li-Weber, 2009; Shishodia et al., 2005], thereby normalizing the exaggerated proliferation capacity of cancer cells and inducing their death. It is therefore tempting to think that all cancer cells exhibit a deregulation of NF-κB expression which could be selectively targeted by proapoptotic plant-derived compounds. However such cause-effect relationship has to be clearly demonstrated. Different mechanisms which lead to a constitutive expression of active NF-κB in cancer cells have been suggested, including dysregulation of cytokine receptors [Prasad et al., 2010]. Interestingly, it is known since the early 1990s that NF-κB is a redox-sensitive transcription factor; its activity is upregulated by enhanced levels of ROS (Reactive Oxygen Species) which are tighly associated with malignant initiation and progression. However several studies have shown that ROS has paradoxic effects on NF-κB activity, depending upon its levels. Mild increase of free radicals often induces NF-κB activation and sustained cell survival, while a drastic increase of free radicals leads to a repression of NF-κB function and cell death [Trachootham et al., 2008; Trachootham et al., 2009]. Actually, intracellular ROS production result from several processes, including the mitochondrial oxidative phosphorylation which involves a set of enzymatic complexes (*e.g.* NADH dehydrogenase, succinate dehydrogenase) constituting the respiratory chain. At the opposite, enzymatic antioxidants (*e.g.* superoxide dismutases, glutathione peroxidase) or scavengers (*e.g.* cystein, albumin) contribute to regulate the levels of oxygen-free radicals in order to protect the cells from oxidative damage and prevent mutation-induced malignancy. According to the oxidant and antioxidant mechanisms involved, changes in ROS levels can either directly impair the DNA binding capacity of NF-κB or trigger its transcriptional activity by promoting its nuclear translocation [Pani et al., 2010].

The redox status plays a crucial role in maintaining the cell activity under normal conditions. To reduce the risk of oxidative-induced mitochondrial apoptosis due to high proliferative-linked metabolic activity, cancer cells « adopt » a glycolytic state to the detriment of an oxidative state. This Warburg's effect leads cancer cells to maintain high levels of free radicals, in contrast to normal cells. Although CSCs similarly show enhanced ROS content compared to normal SCs, these two cell types produce only a limited amount of oxygen radicals, likely because they reside in a low oxygen microenvironment [Diehn et al., 2009]. This explains why some CSCs are resistant to radiotherapy and chemotherapic phytochemicals which require the availability of local oxygen to develop their cytotoxic activity. However the redox status and adaptation displayed by the CSCs and their descendants, as well as by their normal counterparts is a key mechanism that, to a certain extent, might explain the selective cytotoxic effect of a ROS-producing plant-derived compound. In regard to the crucial influence of redox homeostasis on the life-and-death processes, it was suggested that an additional and robust ROS-producing stress could kill the adapted but easily overwhelmed cancer cells, without having toxic side effects on normal cells [Fruehauf & Meyskens, 2007; Trachootham et al., 2008]. It can be postulated by extrapolation that CSCs, in contrast to normal SCs, harbor a similar weak adaptation capacity of the redox machineries when exposed to oxidative stress, induced for instance by a cytotoxic phytochemical. A hypothetical sequence of events can then be set forth to explain the selective effects of a plant-derived compound on cancer cells and CSCs (Fig. 4). However the molecular mechanism which leads to the quenching of the ROS buffering capacity has still to be identified. Limited adaptability of the redox homeostasis might be linked to some steric hindrance of factors involved in mitochondrial activiy. The absolute level of the redox balance in the normal and cancer cells (or their progenitors) might be the key parameter which needs to be considered for expecting a selective cytotoxic effect of a ROS-inducing pharmacological agent. Finally the higher sensitivity of some SCs to DNA damage-induced mutations has lead to cancer transformation and consequently to an adaptative redox response which might be easily and selectively overwhelmed by a prooxidant phytochemical. A redox-modulating strategy which targets the Achilles' heel of CSCs and their descendants could therefore have major implications in cancer treatment.

It is worth noting that a notable body of studies has highlighted the dual effects of some phytochemicals as antioxidants and prooxidants. As antioxidant agents, they are believed to protect DNA integrity by quenching oxygen-free radicals produced by a pathogenic oxidative stress and thus should impede the transformation of injured SCs. As prooxidant agents, they are able to kill CSCs and their descendants by triggering an intracellular production of ROS. These compounds, such as the polyphenolic flavonoids, are therefore acting as double-edged swords by targeting the redox regulatory system. The antioxydant or prooxidant effect of a particular phytochemical seems mostly to be dose- and time-dependent [Procházková et al, 2011; Schwartz, 1996]. The reason for this paradoxical activity of some plant-derived compound is still poorly understood. They could initially target a same ROS-sensing molecule which is involved in both antioxidant and prooxidant cell processes. The mitochondrial coenzyme Q could be such a candidate; this electron carrier is known to contribute to mitochondrial oxidative damage and antioxidant defenses [James et al, 2004]. However its precise role in the phytochemical-induced redox response of SCs, CSCs and their offsprings requires further studies.

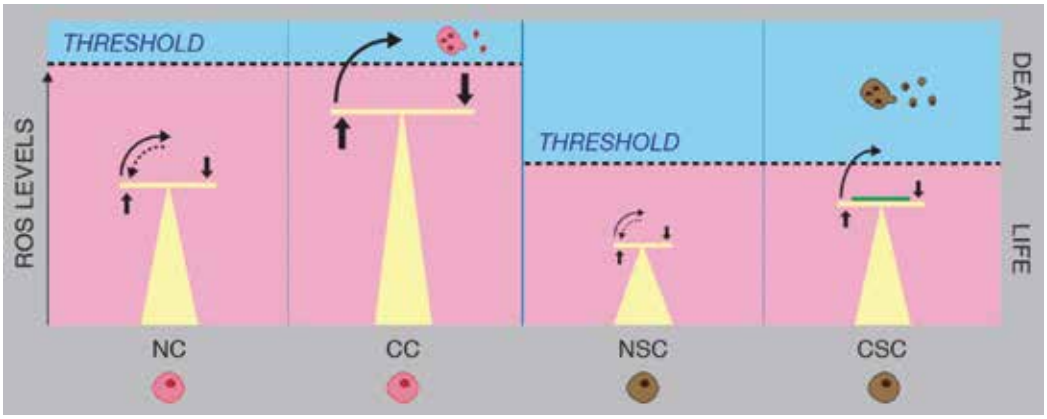

Fig. 4. Hypothetical effects of a selective cytotoxic prooxidant phytochemical on the redox homeostasis of normal cells, cancer cells and their respective progenitors. Normal cell (NC) and normal stem cell (NSC) are able to maintain low levels of intracellular ROS by controlling the steady-state activity of the oxidant and antioxidant machineries (depicted respectively by a left and right arm of a balance); both cell types can tolerate, in a reasonable range of concentrations, an oxidative stress (represented as a clockwise semicircle arrow) induced by a phytochemical (depicted by an upwards and/or downwards thick arrow pointing either one or the two arms of the balance) through adaptative antioxidant responses (represented as an dotted anticlockwise semicircle arrow). Cancer cell (CC) and cancer stem cell (CSC) show increased basal levels of ROS due to exaggered metabolic activity and are more vulnerable to further oxidative stress induced by a ROS-generating phytochemical. Enhanced production of oxidants overwhelms the antioxidant capacity of both cancer cells and CSCs which reaches a toxic biological limit (represented as a dotted horizontal line), leading to death. It is assumed that the death threshold is lower in NSC and CSC than in their corresponding counterpart, since they reside in an autarkic microenvironment which does not allow detoxication exchanges. CSC chemoresistance might be associated with a reorientation of the oxidant and antioxidant activities towards its initial levels (depicted by a green line on the top of the balance). Adapted from a previous review [Trachootham et al., 2009]

3.2.3 Targeting cancer stem cells by differentiation-inducing phytochemicals

3.2.3.1 Targeting the self-renewal pathways of cancer stem cells by phytochemicals

The aggressiveness of a CSC is proportional to its lineage-specific competencies (Fig. 1). Moreover several studies have reported that the differentiation level of a specific type of CSC is inversely correlated with its resistance capacity to radiotherapy and chemotherapy [Al-Hajj et al., 2004]. Therefore disrupting the molecular pathways which regulate CSC self-renewal is an attractive alternative for reducing the aggressiveness and MDR phenotype of the tumor bulk. By targeting these pathways, it is assumed that the CSC switches from a highly proliferative and undifferentiated state to a harmless low-growing and mature state. Accordingly, genes encoding proteins involved in Wnt/beta-catenin, Notch and Hedgehog signalings, are frequently mutated or aberrantly expressed in several fulminant cancers [Blank et al., 2008; Lobo et al., 2007]. Through direct or indirect modulation of the impaired

signaling pathway activities, plant-derived compounds are therefore expected to affect CSC self-renewal, leading to cancer regression and reduced risk of relapse. The most exhaustively studied differentiation-inducing pharmacological compounds are retinoids, including vitamin A and its derivatives. As an adjunct to clinical therapy, RA treatment allows complete remission of about 90% of patients with acute promyelocytic leukemia [Freemantle et al., 2003]. The anticancer activities of retinoids have been attributed, at least in part, to increased proteasomal degradation of beta-catenin, herein normalizing the aberrant activation of the Wnt signaling observed in some leukemias and solid tumors [Dillard & Lane, 2007; Mikesch et al., 2007]. This example highlights the strong positive impact of the differentiation strategy which is able to block the tumor burden. Actually, an increasing number of *in vitro* and *in vivo* studies show that various plant-derived compounds are potential anticancer agents since they can specifically target the self-renewal properties of CSCs [Kawasaki et al., 2008; Y. Li et al., 2011]. Moreover, links between different molecular components involved in the prosurvival and the self-renewal signaling pathways have been described in several reports, pointing out the fine-tuned balance which controls cell proliferation and differentiation [Konopleva & Jordan, 2011]. As a consequence, it is not surprising that some phytochemicals could act as multi-target agents by modulating the activity of specific nodal points of the prosurvival and self-renewal machineries [Sarkar et al., 2009]. All these issues will be discussed in details elsewhere (see Chapters 20 and 22).

Reprogramming of gene expression through epigenetic modifications could explain the prodifferentiating anticancer activity of plant-derived compounds. The Polycomb and Trithorax groups of proteins are known to reverse respectively active or repressed transcription states of developmentally important genes during SC fate commitment [Ringrose & Paro, 2004]. It is therefore expected that certain differentiation-inducing phytochemicals could disturb the activity of these epigenetic chromatin modifiers, leading transiently to an active resetting of the histone code and an erasure of DNA methylation. This assumption is based on the fact that at least two components of the Polycomb multiprotein complex, namely Bmi1 polycomb ring finger oncogene and SUZ12, have been shown to be direct effectors of Hedgehog and Wnt signaling respectively. As such, both proteins are implicated in SC self-renewal and known to be upregulated in different cancers [Galmozzi et al., 2006]. Although some naturally-occurring inhibitors of the hedgehog and beta-catenin signalings can regulate CSC proliferation and differentiation by modulating *a priori* the expression of Bmi1 and SUZ12, the discussion of a direct effect of a specific plant-derived compound on epigenetic chromatin modifiers is still open and needs further investigation. However several phytochemicals, including polyphenols, are able to target specifically several epigenetic alterations which might have led to cancer development. For instance, curcumin can reverse DNA hypermethylation and is suspected to reactivate methylation-silenced tumor suppressor genes in several colon cancer cell lines. Moreover curcumin, as a potent histone modifying compound, promotes the proteasome-dependent degradation of the histone acetyltransferase (HAT) p300/CBP in cell extracts from different cancer types. Such inhibitory effects is known to be associated with histone H3/H4 hypoacetylation and repression of HAT-dependent chromatin transcription, a hallmark of a highly proliferative and indifferentiated cell state [Link et al., 2010]. Plant-derived compounds seem therefore to be promising weapons against the epigenetic disorders which could affect cancer cells and CSCs. However due to data scarcity, an epigenetic reorientation strategy for an alternative anticancer therapy remains difficult to evaluate.

3.2.3.2 Targeting Oct4 function in cancer stem cells by phytochemicals

Targeting Oct4 network in poorly and aggressive Oct4-expressing cancers by phytochemical compounds is a very promising approach, in regard to its crucial role in stemness maintenance in both SCs and CSCs. On the other side, induced pluripotent stem (iPS) cell research could provide new insights into the mechanisms engaged during CSC differentiation. Indeed the current state of our knowledge in the field of cancer therapy and tissue engineering seems to indicate that CSC reactivity and somatic cell dedifferentiation share common molecular pathways in which Oct-4 could play a pivotal role (Fig. 5).

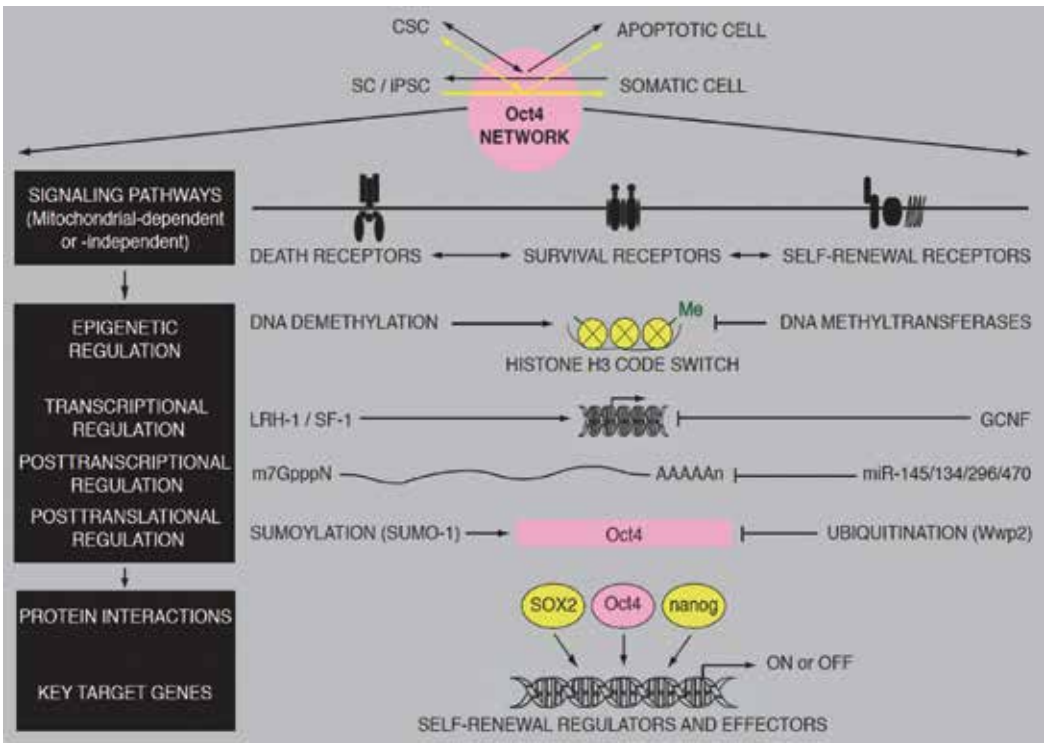

Fig. 5. Oct4 network. Several nodes of the Oct4 pathway, active in stem cells (SC), induced pluripotent stem cells (iPSC) or cancer stem cells (CSC), are potential targets for plant-derived compounds. Details relationships between the different actors can be found in section 2.4 of this chapter and in previous reviews [Kang et al., 2009; Shi & Jin, 2010]. Methylation of Oct4 promoter and specific modifications in the histone H3 code leads to a transcriptional repression of *Oct4*

Since the pioneering work of Yamanaka's laboratory in 2006, an exploding number of studies have unequivocally shown that ectopic expression of at least two transcription factors, namely Oct4 and SOX2, can reset the epigenome of somatic cells with restricted lineage-specific competencies to an highly undifferentiated pluripotent state [Feng et al., 2009; Takahashi & Yamanaka, 2006]. However the efficiency of the reprogramming process still remains very low, albeit a lot of effort has been put to improve the methods. Interestingly, *TP53* inactivation seems to facilitate significantly the embryonic SC switch, suggesting that a p53-mediated proapoptotic DNA damage response limits iPS cell

production [Marion et al., 2009]. Recently, it has been observed that reprogrammed pluripotent cells show genomic aberrations [Pasi et al., 2011]; this could explain why they can form malignant tumors when injected in donor mice [Sarig et al., 2010]. Induced Oct4-expressing somatic cells seem therefore to adopt a SC or CSC phenotype or to undergo apoptosis in a stochastical manner; this firstly suggests that Oct4 is only one of the key decision-makers in stemness and carcinogenic behavior (see top of Fig. 5). Elucidation of the precise molecular mechanisms through which Oct4 maintains and reinitiates pluripotency is thereby necessary before planning cell therapy using iPS. An improved understanding of Oct4 biology will also provide a number of novel targets for the design of specific phytochemical therapy that aims to eradicate poorly differentiated CSCs. Different sets of proteins located either upstream or downstream from the Oct4 pathway have already been identified (Fig. 5); however the mechanisms of action of certain pharmacological agents, such RA, capable to target specifically Oct4-centered protein interactomes have still to be clearly established. For that purpose, the evaluation of a selective action of a specific plant-derived compound on Oct4 network in CSCs has also to be considered, in regard to its potential side effects on normal SCs.

4. Conclusions and future perspectives

Conclusions of experimental data suggesting a potential biological activity of a plant-derived compound on a specific cancer and CSC type should be carefully analyzed. In view of the present chapter, it seems that only phytochemicals which can selectively target the ROS-induced proapoptotic and/or differentiation processes, have some promising therapeutic values. The main reason for this assumption is that CSCs, as well as cancer cells, share a nearly satured adaptability of the redox capacity and escape from the signals emitted by the well embedded and protective niche. In regard to their susceptibility to prooxidant stresses and, to a certain extent, to prodifferentiation inducers, it is expected that only CSC and their descendants could be selectively targeted by natural or synthetic plant-derived compounds. One of the best example is retinoic acid which is known to be the most powerful anticancer agent and is used with success in chemotherapy. However dosing schedules of a considered phytochemical is a critical point which has to be taken in account in order to minimize and, if possible, to avoid toxic side effects on normal cells and their progenitors.

Promising anticancer strategies have recently be developed by targeting CSCs. Although this chapter supports the anticancer benefits of phytochemical compounds, only future studies, likely using comparative well-defined CSC lines, will determine if they can reasonably act as selective chemopreventive agents in the key steps of the carcinogenic process. In this point of view, understanding the role of Oct4 in cancer stem cell biology is crucial for two main reasons. As an initial protein in the cancer cell hierarchy, and therefore as a reliable marker of cancer aggressiveness, its detection, as well as the available tools to repress its function, have strong prognostic and diagnostic values. Secondarily, as a key protein for somatic cell reprogramming, its controlled activity is a prerequisite for save immunocompetent cell regenerative therapy, without any harmful tumorigenic drift.

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Part 6

Techniques and Technical Details

24

Isolation of Liver Cancer Stem-Like Cells by Hoe33342 or Rhodamine123 Efflux

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1. Introduction

Already 150 years ago, the German pathologist Rudolf Virchow postulated in his theory of the cellular pathology that cancer initiates from immature cells. But it still took 100 years until Sajiro Makino introduced the term "tumor stem cells" for a small subpopulation of cells that were insensitive to chemotherapy and had chromosomal features different from the bulk of cells (Makino, 1959). Although the existence of cancer stem cells (CSCs) was proposed over decades ago, only in the past decade increasing evidence supports that cancers contain a small subset of their own stem-like cells (Dalerba et al., 2007; Pardal et al., 2003; Wicha et al., 2006). At present, these cells have been identified in human leukemias (Konopleva & Jordan, 2011), and more recently in solid tumors that include liver (Ma et al., 2007), breast (Dontu et al., 2003), prostate (Lukacs et al., 2010), brain (Huang et al., 2010), colon (Kemper et al., 2010) and lung cancers (Sullivan & Minna, 2010). These studies suggest that, like normal stem cells, CSCs should be rare, quiescent, and capable of self-renewing and maintaining tumor growth and heterogeneity. Therefore, development of effective therapies that will specifically target CSCs may become a promising therapeutic option to fight cancer.

The researches of CSCs are based on successful viable CSCs sorting. Universally accepted markers of CSCs are very important to cell sorting. In the last decade several molecular properties have been utilized to identify and characterize CSCs from different hematopoietic and solid tumors. The first markers used were cell surface proteins already known to define stem and progenitor cells, e.g. CD133 and CD166. Meanwhile, some surface markers that are associated with human and mouse stem cells are also found on cancer stem cells, such as CD34, CD117, Sca-1, and other markers, such as CD44, CD24, CD20 CD105, and CD326 (EpCAM) have been found on cancer stem cells (Keysar & Jimeno, 2010). Furthermore, molecules that facilitate drug resistance in cancer cells like ABCB1 and ABCG2 were added to the list of putative CSC markers (Calcagno et al., 2010) as well as proteins for which no

involvement in stemness or cancerogenesis was known, e.g. CD20. Although CSCs have been isolated by cell surface markers such as CD24, CD44, and CD133 as well as on the basis of sphere formation after in vitro cultivation (Jordan et al., 2006), the identification of a putative cancer stem cell subpopulation with validated methods and markers for each tumor entity remains controversial. Because of this, suitable cells are needed for the analysis of CSCs biology.

While the cells are stained with Hoe33342 (Hoe) vital dye, a cell-permeable DNA-specific bisbenzimidazole dye, the display of Hoe fluorescence simultaneously at two emission wavelengths localizes a distinct, small, non-stained cell population that is designated as side population (SP) cells. Because SP cells are known to have highly efficient pumps for the dyes, they are considered to be resistant to multi-chemotherapeutic drugs and to confer malignant phenotypes to tumors (Wu & Alman, 2008). More importantly, the SP cells express high levels of stem cell markers and low levels of differentiating markers (Haraguchi et al., 2006). Hence, the characterization of SP cells might be a useful tool for analysis of CSCs, especially when specific CSC surface markers are unknown.

As an alternative approach, the SP phenomenon has been used to identify and isolate stem cell populations from a variety of tissues including bone marrow (Goodell, 2002), mammary gland (Welm et al., 2003), skin (Montanaro et al., 2003), liver (Wulf et al., 2003), lung (Majka et al., 2005), skeletal muscle (Meeson et al., 2004), limb (Umemoto et al., 2006), heart (Martin et al., 2004), and brain (Kim & Morshead, 2003). This approach overcomes the barrier of phenotypical markers and replaces it by more direct functional markers. The most widely accepted assays to obtain SP cells are efflux analysis of Hoe as well as further detection of known stem cell markers in cancer cells and verification by xenotransplantations. Such specific dye can only be excluded out of cells by the ATP-Binding Cassette (ABC) transporters, which is a stem-like characteristic. That is to say, only the cells with ABC transporters expression can exclude such dye. Thus, dye exclusion is a valuable technique to indirectly identify stem-like cells.

The dye Hoe exclusion has been successfully applied in many tumors and corresponding cell lines. The blue fluorescent Hoe is a cell permeable bisbenzimidazole derivative that binds to the minor groove of DNA. After excitation of Hoe its emission can be measured simultaneously in the blue and red spectrum. But although Hoe enters viable cells, it is also actively pumped out by ABC transporters of the cell membrane (Scharenberg et al., 2002). There is mounting speculation that ABC transporters repress the maturation and differentiation of stem cells (Spangrude & Johnson, 1990). Although several subfamilies of genes coding for these transporters have been identified (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG), the best-known are: members of the ABCB and ABCG subfamilies, such as ABCB1 (MDR1, P-gp) and ABCG2 (Bcrp1) in humans (Choi, 2005). It has been demonstrated that the exclusion of Hoe by SP cells is an active process mainly involving Bcrp1 (Uchida et al., 2002). To determine the size of the SP, Verapamil, an L-type calcium channel blocking agent serves as an important control. Blocking the calcium channels inhibits the efflux of Hoe-dye from these cells, so it is then possible to gate for the side population, which is suspected to consist of cancer stem cells. However, the toxicity of Hoe should be addressed and always kept in mind when applying this dye to isolate putative CSCs. As Hoe binds to DNA, it can disrupt DNA replication during cell division. Consequently, it is potentially mutagenic and carcinogenic. This toxicity suggests a potentially severe limitation for the use of Hoe dye in combination with fluorescence activated cell sorting (FACS).

Rhodamine123 (Rho) is always used as a substrate of ABCB1/P-gp transporter to evaluate the toxicity of drugs (Vautier et al., 2008) and to examine the functional activity of P-gp in cultured cells (He & Ji, 2008). Like Hoe, it is actively pumped out of the cells by ABC transporters, e.g. ABCB1/P-gp protein (Zhou et al., 2001). Our study found that Rho was shown to be non-toxic to cells even at high concentrations, it can be an alternative to the use of Hoe.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third most frequent cause of cancer deaths worldwide. It has been ranked the second leading cause of cancer death in China since the 1990s. The prognosis of HCC patients remains extremely poor, with a 5-year survival rate of less than 5%. HCC respond poorly to standard chemotherapy treatment regimens. The reasons for this clinical phenotype are believed to be based on cellular heterogeneity of the tumor and the presence of multidrug resistance genes, which encode for pumps that actively expel the cytotoxic substances. Because the Hoe and Rho efflux capacity of SP cells is also dependent on the presence of membrane pumps, we hypothesized that identification of the SP cells in HCC could potentially represent a suitable isolation method to evaluate stem cell-like tumor characteristics in HCC. It is widely accepted that the MHCC97 cell line has heterogeneity, but there are few methods to separate SP cells from this cell line. Thus, we investigate the method to isolate liver cancer stem like cells by Hoe or Rho efflux from MHCC97 cell line and detect the stem cell properties of the obtained SP cells.

In this chapter, we will present the method that isolation of liver cancer stem like cells by Hoe or Rho efflux and discuss the advantages and disadvantages of the two methods.

2. Methods and analysis

We firstly examined in vitro cytotoxicity of Hoe and Rho, and then used both Hoe/FACS and Rho/FACS to enrich CSCs from the MHCC97 cell line (Key Laboratory for Carcinogenesis and Cancer Invasion, Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China). We tried different conditions detailed in methods to determine the optimal dye concentration. After cells were successfully sorted by the two methods, we obtained four typed cells: SP cells (low staining with Hoe) and NSP cells (high staining with Hoe), Rho^{low} subpopulation cells (low Rho fluorescence and Verapamil negative) and Rho^{high} subpopulation cells (high Rho fluorescence). Among them, SP and Rho^{low} cells were CSC like cells. We compared the effects of Hoe and Rho on CSCs sorting and found the ability of Rho staining to separate subpopulations from the MHCC97 cell line is similar to Hoe staining. Based on the analysis of several CSCs' characteristics, we found the Rho^{low} cells had similar characters as the SP cells, such as high proliferative ability, high expression of stem cell markers (early hepatic marker AFP and CSCs marker CD133) and strong tumorigenicity in vivo. Specific methods of operation are as follows.

2.1 Agents test

Each compound was screened for possible cytotoxic effect with the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). This is a colorimetric assay for the quantification of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant. Cytotoxicity was calculated as a percentage of the effect obtained

with the positive control (total cell lysis) (Storch et al., 2007). To guarantee the specificity of subpopulations, Verapamil (Sigma Chemical Co., St. Louis, MO) was used for second check. The influence of Verapamil on the fluorescence of the ABC transporter substrates was evaluated by adding increasing concentrations to Rho or Hoe solution. Among the tested compounds, only 8 μg/ml Hoe caused cytotoxic effect >50%. The cytotoxic effect caused by 6 μg/ml Hoe was 30.8%. Besides, when the concentration of Hoe was<4 μg/ml, it caused cytotoxic effect <30%. Through statistical analysis, the IC50 of Hoe was 7.52 μg/ml. For all Rho patterns (the concentrations ranged from 0.05 to 1 μg/ml), no cytotoxic effect was found. There was no quenching effect caused by Verapamil to both Hoe and Rho solutions.

2.2 Cells culture and grouping

The cells from the MHCC97 cell line were maintained in DMEM culture (Invitrogen, Karlsruhe, Germany), which was supplemented with 10% FCS, 2 mM glutamine, 1 mM HEPES, 100 U/ml penicillin G and 100 g/ml streptomycin (Invitrogen, Karlsruhe, Germany). These cells (at logarithmic growth phase) were randomly divided into four groups: group A (Hoe trial group), group B (Hoe control group), group C (Rho trial group) and group D (Rho control group). All the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2 Hoe/FACS

2.2.1 Cells sorting by Hoe/FACS

As a standard control, the cultured cells were sorted by Hoe/FACS. The single-cell suspension was prepared by passing through a 50 μm mesh filter and diluted to a working concentration of 1×10^6 cells/ml with ice-cold PBS containing 2% FCS. In group A the cells were stained with the Hoe dye (Sigma Chemical Co., St. Louis, MO) at a concentration of 6 μg/ml (37 °C for 90 min) under mild shaking. In group B the cells were incubated in the presence of Verapamil (50 μM) and Hoe for 90 min at 37 °C. The Varapamil, which blocks the transporters responsible for Hoe exclusion, is applied to check the purity of the SP cells. After washed twice by PBS/2%FBS, the cells were incubated with 10 μg/ml propidiumiodide (PI) (Sigma Chemical Co., St. Louis, MO) for exclusion of dead cells. After excitation of the Hoe dye at 350 nmby ultra-violet (UV) laser on MoFlow (Cytomation Inc., Fort Collins CO., USA) and measurement of the fluorescence profile in two wave-lengths analysis (450 and 695 nm), both SP and non side population (NSP) cells were collected. SP cells showed low staining with Hoe and NSP cells were more brightly stained. A second 488 nm argon laser (100 mW) was used to excite PI fluorescence for excluding dead cells. Results were analyzed using the Summit v3.1 software (Cytomation, Dako, Glostrup, Denmark).

2.2.2 Profiles of isolated subpopulations by Hoe/FACS

SP cells should be present in the low forward scatter (FSC) and the low side scatter (SSC) fractions. There are two scatter plots reflected the distribution of cells, and a very low profile of SP (Fig. 1A) cells was shown. According to PI tests, there were about 30.8% dead cells in the Hoe stained cells (Fig. 1C). In group A, the percentage of SP was 1.4 ± 0.01% (Fig. 1B). When exclusion of the dye was inhibited by Verapamil in group B, the SP cells almost could not be discriminated from the MHCC97 cell line (Fig. 1D).

2.3 Rho/FACS

2.3.1 Cells sorting by Rho/FACS

The cells in group C were stained with Rho (Sigma Chemical Co., St. Louis, MO) at a concentration of 0.1 μg/ml (37 °C for 30 min) and washed with PBS/2%FBS twice. The cells in group D, which were used to define fluorescence threshold, were co-incubated in the presence of Verapamil (50 μM) and Rho for 30 min at 37 °C. All the above cells were centrifuged and suspended in PBS/2%FBS containing 10 μg/ml PI to extrude dead cells. Flow cytometric analysis of Rho fluorescence at 485 nm was carried out with Aria flow cytometer (Becton Dickinson cooperation, Mountain View, CA). Similar to Hoe/FACS, two filters were selected to discriminate cell subpopulations. As the Rho dye is activated through the FITC filter (Wang et al., 2006), the combination of FITC and PerCP-Cy5.5 filters was found optimal. The low Rho fluorescence and Verapamil negative was designated as Rho^{low} subpopulation, and the remaining cells were Rho^{high} subpopulation.

2.3.2 Profiles of isolated subpopulations by Rho/FACS

Rho^{low} cells should be present in the low FSC and the low SSC fractions. There are two scatter plots reflected the distribution of cells, and a very low profile of Rho^{low} (Fig. 1E) cells was shown. According to PI tests, there were about 30.8% dead cells in the Rho stained cells (Fig. 1G), much fewer dead cells than in the Hoe stained cells. In group C, the percentage of Rho^{low} in total cells was 2.1 ± 0.02% (Fig. 1F). When the exclusion of Rho dye was inhibited by Verapamil, the Rho^{low} cells could not be discriminated from the MHCC97 cell line either (Fig. 1H).

2.4 Cross detection of cell phenotypes

To further compare the effects of Hoe and Rho on CSCs sorting, the cross detection of SP and Rho^{low} cells was done. After the SP cells were isolated, they were immediately incubated with 0.1 μg/ml Rho (at 37 °C for 30 min) for Rho fluorescence analyzing. Meanwhile, the freshly isolated Rho^{low} cells were also evaluated by Hoe dyeing. The Rho^{low} (Fig. 1I) and SP (Fig. 1K) cells contained much fewer cells than the unsorted cells. The SP cells in Rho^{low} subpopulation was 52.5 ± 0.29% (Fig. 1J), in contrast, the Rho^{low} cells in SP subpopulation was 72.7 ± 0.36% (Fig. 1L). Based on the above results, the ability of Rho staining to separate subpopulations from the MHCC97 cell line is similar to Hoe staining.

2.5 Comparison of the proliferative and self-renewal ability of SP and Rho^{low} cells

The cells were adjusted to 1×10^7/ml and seeded in six-well plates (5×10^5 cells per well). Each kind of cells had 7 parallel samples. The culture media was changed every two days. During 7 days period, in each group, the cells of one parallel sample were trypsinized at 8 o'clock each day. Then the cells were counted under an inverted microscope (BX50-32E01, Olympus, Tokyo, Japan). As Fig. 2A shows the Rho^{low} cells with stronger ability of self-renewal and shorter doubling time than the Rho^{high} cells ($P < 0.05$). The Rho^{low} cells doubled their number in 16.9 ± 0.24 h; the Rho^{high} cells doubled in 25.4 ± 0.28 h. Besides, the Rho^{low} cells had a bigger multiplication number than the Rho^{high} cells ($P < 0.05$). The biggest multiple of the Rho^{low} cells was 15.2 ± 0.15 and that of the Rho^{high} cells was 12.3 ± 0.18. Thus, the Rho^{low} (Fig. 2B) and Rho^{high} (Fig. 2C) cells had greatly different densities 3 days after cultivation. Through statistical assay, the SP cells had little shorter doubling time (16.2 ± 0.23

h) and a slightly bigger multiplication number (15.7 ± 0.21) than the Rho^{low} cells ($P > 0.05$). Taken together, the Rho^{low} cells had similar ability to the SP cells for self -renewal.

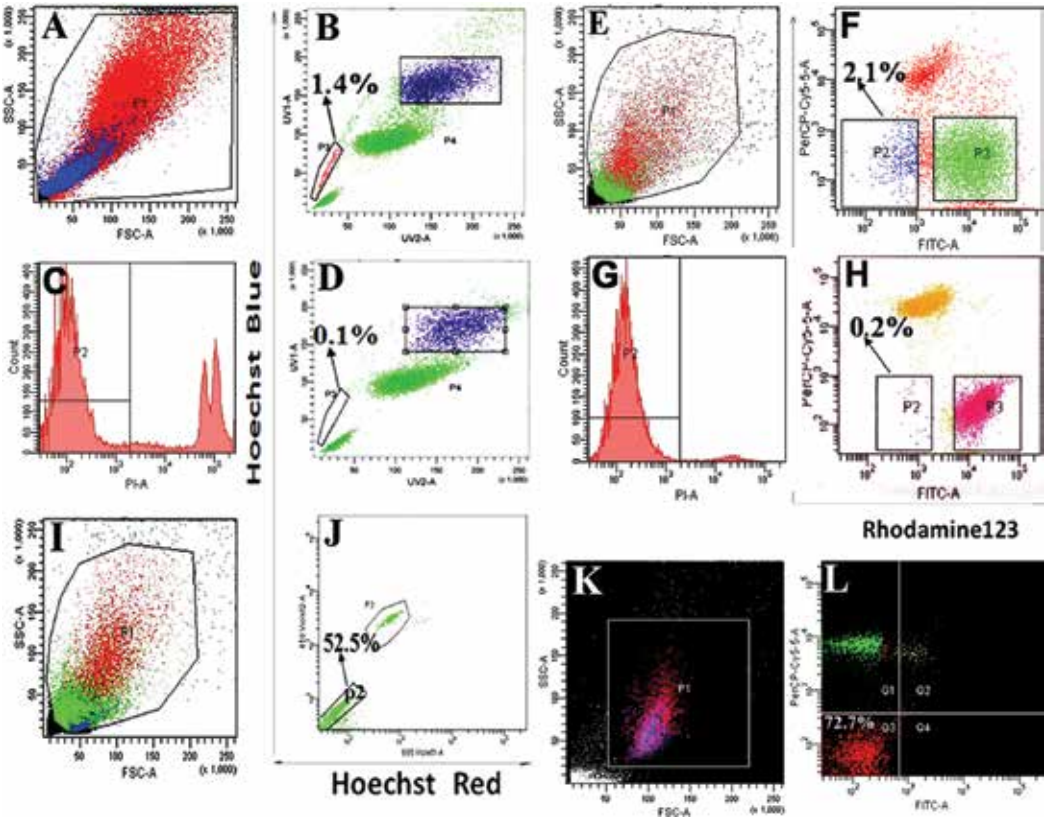

Fig. 1. The profiles of cell subpopulations in the MHCC97 cell line

(A), when total cells were stained with Hoe (p1), the blue population indicated possible SP cells with low fluorescence. (B), parts of them were demonstrated to be true SP cells (p3), parts were non SP cells (p4). (C), the viable and dead cells were determined by PI. (D), the profile of SP population (p3) decreased in the presence of Verapamil. (E), the distribution of total cells stained with Rho. (F), the Rho^{low} cells were shown as a low Rho fluorescence (p2) and the Rho^{high} cells were with high fluorescence (p3). (G), the most cells were viable. (H), when the cells were treated with Verapamil, the fluorescence of the Rho^{low} cells was shifted to a higher level (p3). The distributions of (I) the Rho^{low} cells stained with Hoe (p1) and (K) the SP cells stained with Rho (p1). (J), the percentage of the SP cells in Rho^{low} (p2). (L), the percentage of Rho^{low} cells in SP (Q3).

We also designed soft agar clone formation test to compare the self-renewal capacity of cells from each subpopulation. The 2×DMEM (containing 200 ml/L FCS) were mixed with equal volume agarose (12 mg/ml) (Sigma Chemical Co., St. Louis, MO) as the bottom-layer agar. The cells from SP, NSP, Rho^{low} and Rho^{high} were diluted to 1×10^4 cells/ml and put on the bottom-layer agars, respectively. Immediately, the cells were covered with the top-layer

agars, which consisted of agarose (7 mg/ml) and equal volume 2×DMEM. Culture for 14 days, the number of cell clones was counted. After culture for 14 days, most of the SP and Rho^{low} cells had formed cell clones. The rate of clone formation in the SP cells was 73.5 ± 0.12%, and that in the Rho^{low} cells (Fig. 2D) was 70 ± 0.11%. In contrast with the SP and Rho^{low} cells, the NSP and Rho^{high} cells had much lower clone formation rates ($P < 0.01$). Such as the clone formation rate in the NSP cells was 25.2 ± 0.06%, and that in the Rho^{high} cells (Fig. 2E) was 30.4 ± 0.07%.

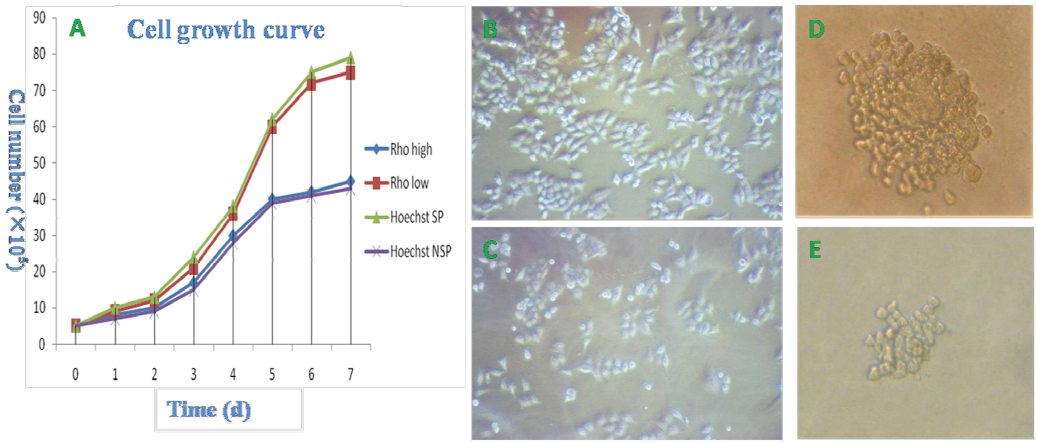

Fig. 2. The proliferative abilities of different typed cells

(A), cell growth curve. (B) the Rho^{low} and SP cells from the MHCC97 cell line had a stronger capacity to proliferate than (C) the Rho^{high} and NSP cells, respectively. The cell clones formed by (D) the Rho^{low} cells was much bigger than that by (E) the Rho^{high} cells. Magnification: panels B-D, ×100.

2.6 Cell marker expression

2.6.1 Immunocytochemical assay

One early hepatic marker was selected to evaluate the maturity of the cells from different subpopulations. First-anti AFP mAb (Zhong-Shan Co., Beijing, China) (1μl) was diluted with PBS (99 μl) to a working density. The cells from different subpopulations ($1×10^4$/ml) were incubated with mAb, coloured by DAB (Zhong-Shan Co., Beijing, China), and stained by hematoxylin. In negative control groups, the PBS was adopted instead of first antibody. These cells were periodically viewed under an inverted microscope. When the appearance of Buffy colour particles showed in the cytoplasm, the result was identified positive. The positive cells were counted and their percentage was calculated. The percent of AFP positive cells<10% was defined as negative (-), 10–25% as positive (+), 25–50% as moderate positive (++), >50% as strong positive (+++). We collected and incorporated the same immunocytochemical results. The detailed data have been enumerated in Table 1. The Rho^{low} (Fig. 3A) and SP cells (Fig. 3C) expressed higher AFP than the Rho^{high} (Fig. 3B) and NSP (Fig. 3D) cells, respectively ($P < 0.01$). Besides, the SP cells had similar expression level of AFP to the Rho^{low} cells ($P > 0.05$) (Fig. 3I). These results demonstrated the cells were immature and from hepatic carcinoma.

cell subpopulation	sample number	AFP-	AFP+	AFP++	AFP+++
Rho^{low} a	35	1±0.14	6±0.28	20±0.86	8±0.28
Rho^{high}	35	10±0.43	16±0.56	6 ±0.28	3±0.14
SP b	35	1±0.14	4±0.14	21±0.86	9±0.43
NSP	35	12±0.43	15±0.56	5±0.14	3±0.14

Table 1. The expression of AFP in cell subpopulation

All AFP immnocytochemical data were viewed by three different researchers and expressed as mean±SD. a Rho^{low} **VS** Rho^{high} , N=35, P<0.05; b SP **VS** Rho^{low}, N=35, P>0.05.

2.6.2 Immuno-flow cytometric analysis

One CSCs marker was applied to identify the characters of CSCs in each subpopulation. The freshly isolated Rho^{low}, SP, NSP and Rho^{high} cells were prepared as single-cell suspensions at a density of 1×10^6 cells/ml, incubated with 0.075 ml CD133 mAb (Zhong-Shan Co., Beijing, China) for 30 min at 4 °C, washed in PBS twice, and fixed in 0.1% formaldehyde for flow cytometric analysis. Irrelevant isotype matched mAb was used as negative controls. Dead cells were excluded by PI gating. The percentage of CD133 positive cells in Rho^{low} (Fig. 3E) was 51.84 ± 0.33%, in Rho^{high} (Fig. 3F) was 13.36 ± 0.11%, in SP (Fig. 3G) was 53.24 ±0.42%, and in NSP (Fig. 3H) was 12.70 ± 0.10%. The CD133 expressed significantly different among these subpopulations ($P < 0.01$) (Fig. 3J).

AFP expressed in (A) the Rho^{low} cells was strongly higher than that in (B) the Rho^{high} cells; AFP expressed higher in (C) the SP cells than in (D) the NSP cells. For figures (E)-(H), the black histogram was the isotype control, and the red histogram was the trial. A fluorescent value was set as the regional marker (brunneus bar), which must be just bigger than the fluorescent values of cells in control group. Then the fluorescent value was remembered and used as the regional marker (brunneus bar) in trial group. The positive region was where the fluorescent values were bigger than the set fluorescent value in trial group. The percentages of the CD133 positive cells in (E) Rho^{low} and (G) SP were also higher than those in (F) Rho^{high} and (H) NSP, respectively. The detailed expressions of (I) AFP and (J) CD133 are reflected by column charts. Magnification: panels A-D, ×400.

2.7 Tumor formation in nude mice

The gold standard for measuring tumor initiating potential is to assess the capacity of cells to form tumors when injected into immune-deficient mice. Using this model, we tended to check the differentiating ability of the cells from each subpopulation. Thirty-two male BALB/C nude mice (Fourth Military Medical University, Xi'an, China) were maintained under standard conditions according to the College's guidelines. The freshly isolated SP, NSP, Rho^{low} and Rho^{high} cells (1×10^6) were injected into the right back of mice, respectively. When the xenograft tumors grew to proper size, the mice were sacrificed and the tumor tissues were collected, fixed in 4% formaldehyde, and embedded in paraffin for H&E staining to assess tumors histology. To avoid subjective bias, each chip was marked with

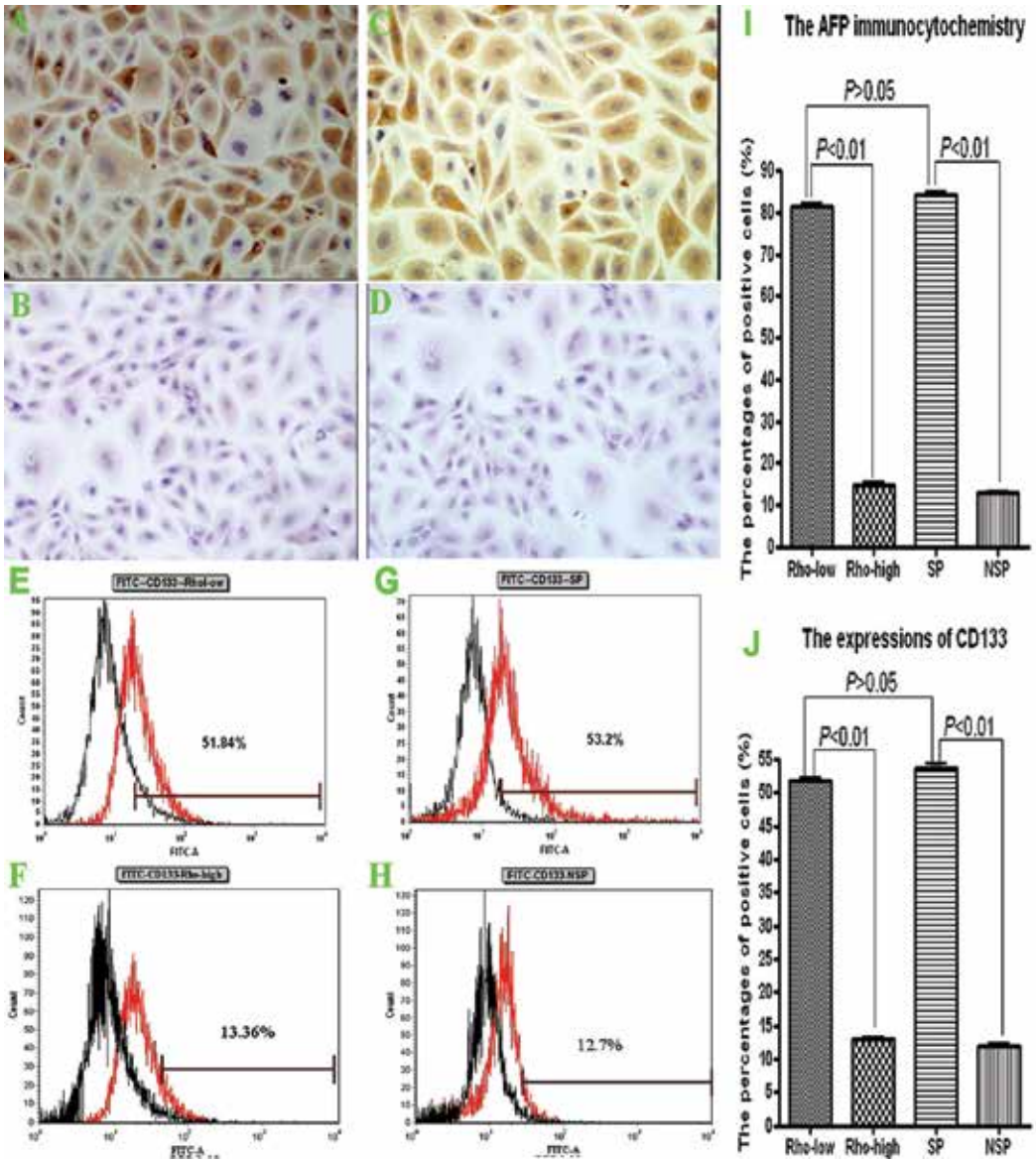

Fig. 3. The expressions of CSCs markers

one number within 1–1 to 1–7 (SP), 2–1 to 2–7 (NSP), 3–1 to 3–7 (Rho^{low}) and 4–1 to 4–7 (Rho^{high}), and viewed by three different pathologists. Based on the tumor size, no macroscopic tumor was defined as negative (-), the diameter of tumor <0.2 cm as positive (+), 0.2–0.5 cm as moderate positive (++), >0.5 cm as strong positive (+++).Since the mice were injected with cells from different subpopulations, they were checked each day. The xenograft tumors grew to proper size 5 weeks later, within the mice which were injected with SP or Rho^{low} cells, however, we couldn't find obvious tumor in some mice injected with NSP or Rho^{high} cells. We counted the number of tumors in each mouse, measured the size of each tumor and summarized those data in Table 2. The tumor formation rate in mice with

SP cells injected was 78.0 ± 1.21%, and that with Rho^{low} cells injected was 66.0 ± 1.03%. In addition, the rate in mice with Rho^{high} cells injected was <15.0%. This indicates that the Rho^{low} cells had stronger ability to form tumors in vivo than the Rho^{high} cells ($P < 0.01$); however, the Rho^{low} cells (Fig. 4A) had slightly lower ability to form tumors than the SP cells ($P > 0.05$) (Fig. 4B). Part of each tumor was made into chips and stained by H&E (Fig. 4C–F) to assess tumor pathology. According to three different pathologists' judgements, the tumors were all hepatic tumor genesis, which could help us discriminate the neoplasms from other kinds of tumors. After the cells from some tumors were isolated and cultured, we used flow cytometry to analyze the phenotype of those cells. Through our analysis, the tumors generated by the injected cells, were mainly from the SP or Rho^{low} cells.

cell subpopulation	sample number	tumor-	tumor+	tumor++	tumor+++
Rho^{low} a	28	1±0.11	12±0.51	11±0.28	4±0.14
Rho^{high}	28	17±0.36	7±0.21	4±0.15	0±0.00
SP b	28	1±0.11	10±0.23	8±0.24	9±0.21
NSP	28	19±0.36	6±0.20	3 ±0.12	0±0.11

Table 2. The tumor formation rate of cell subpopulation in nude mice

All the mice were checked carefully and wholly for tumors, and the diameter of each tumor was measured. a Rho^{low} VS Rho^{high}, N=28, $P<0.01$; b SP VS Rho^{low}, N=28, $P<0.05$.

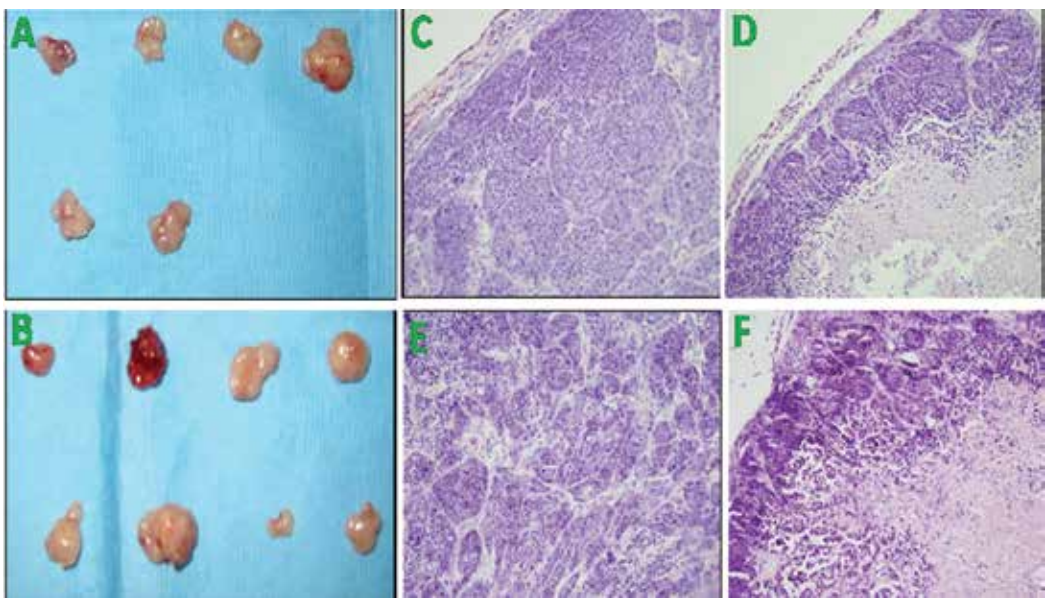

Fig. 4. Tumor formation capacity of each typed cells

Both (A) the Rho^{low} and (B) SP cells formed different sized tumors. H & E dyeing revealed the tumors formed by (C) the Rho^{low}, (D) Rho^{high}, (E) SP and (F) NSP cells were all hepatic carcinoma genesis. Magnification: C-F,×100.

2.3 Comparison of two methods

The cells that exclude Hoe dye are SP cells. Because the SP cells express high levels of stem cell markers and low levels of differentiating markers (Haraguchi et al., 2006), the SP subpopulation is widely used to enrich stem cells. Early in 1993, with a sequential Hoe/Rho sorting system, the subfractions of cells that stained most weakly with both dyes were demonstrated to be long-term repopulating primitive hematopoietic cells (Wolf et al., 1993). Again in 2004, Bertoncello and Williams found that the supravital dyes Rho and Hoe have proven to be remarkably powerful probes for the characterization, resolution, isolation and purification of primitive hematopoietic stem cells (PHSCs) (Bertoncello & Williams, 2004). However, since the cytotoxicity of Hoe is confirmed in recent years (after 2004) (Adamski et al., 2007), the combination of the two dyes is rare.

There are two major limitations in the SP research. First one is the toxicity of Hoe (Kondo et al., 2004). It is demonstrated that Hoe interferes with C2C12 cell fusion as long as the dye is present in the nucleus (Adamski et al., 2007), more and more researchers have found that Hoe staining can affect on cell differentiation. For each cell line, the Hoe concentration should be optimised to minimize cytotoxicity. Second one is the requirement of an UV laser to excite the Hoe dye. The flow cytometer equipped with UV sources is expensive to own and operate and is not readily available to many laboratories or institutions. To solve this problem, some researchers (Cabana et al., 2006) tried to design a minimum UV excitation implement for measuring Hoe. At present, the related endeavor is still undergoing. However, the major toxic drawback of Hoe remains unsolved. Thus, it may be better to find a replacement of Hoe in isolating stem-like cells. Like Hoe, Rho is actively pumped out of the cells by ABC transporters. Some researches have also shown that no toxicity on cells is found by Rho, even at large dose (1–10 mM) (Ribou et al., 2003). In our study, we tested the cytotoxic effects of the two dyes and found Hoe was much more harmful to cells than Rho. That is to say, the Rho dye avoided one main disadvantage of the Hoe dye for CSCs isolation. Besides, to perform Rho/FACS, it only needs a common flow cytometer, in which the fluorescence is activated by argon-ion or helium–cadmiumlaser. The common flow cytometer is a lower cost (both in terms of purchase and maintenance) than cytometers offering UV excitation. Our FACS results indicate the percentage of SP was slightly lower than that of Rho^{low} in the MHCC97 cell line. It does not mean that Rho can accumulate more CSCs than Hoe. The reason may be that the purity of CSCs in Rho^{low} is lower. Based on the cross detections of SP and Rho^{low} cells, the percentage of CSCs in total cells may be 1% (72.7% Rho^{low} cells in 1.4% SP subpopulation; 52.5% SP cells in 2.1% Rho^{low} subpopulation). In other words, the combination of Rho/FACS and Hoe/FACS may enrich much purer CSCs, which is consistent with others.

Based on the analysis of several CSCs characteristics, we found the Rho^{low} cells had similar characters as the SP cells, such as high proliferative ability and high expression of CD133. The use of CD133 has proven to be very successful in the identification of CSCs in both human brain and colon carcinomas (O'Brien et al et al., 2007). Interestingly, about 5 days in culture, Rho^{high} and NSP subpopulations reached a plateau in the growth curve. The reasons

maybe parts of the cells in NSP or Rho^{high} became older and older and finally died. Meanwhile, a few of stem cells gave birth to new cells. The number of dead cells was similar to that of the new-born cells. However, the cells from SP and Rho^{low} were mostly stem cells, which were long lived and could continually differentiate into different typed cells. These cells would continue proliferating for a long time. In addition, we found that the Rho^{low} and SP cells had similar tumor formation rates and the H&E staining confirmed the tissues hepatic genesis. In one word, the Rho^{low} and SP cells shared similar characters both in vivo an in vitro. That is to say, although Rho/FACS is not as effective as Hoe/FACS to isolate stem cells, given its low cytotoxicity and low cost, it may replace Hoe/FACS in isolating some typed CSCs.

2.4 Limitations

Although many findings support the isolation of SP cells via Hoe and Rho staining as an identification method for CSCs, some controversial results were obtained regarding the expressions of specific stem cell/CSC markers on SP cells. Burkert et al found that SP cells of several gastrointestinal cancer cell lines showed no increased expressions of stem cell markers like CD133, CD44, Musashi-1, Oct-4 and CD117 compared to non-SP cells. Both fractions were similarly clonogenic in vitro, tumorigenic in vivo, and displayed similar differentiation potential in vitro and in vivo (Burkert et al., 2008). Due to these controversial findings, dyes efflux and isolation of SP cells can not be applied to identify and isolate CSCs, at least for some tumor entities. Various cell types needs different optimized protocols, such as single cell suspension levels, optimal dye concentration, concentration of Verapamil, the density of cells in culture and so on. Optimized and standardized protocols for each cell type as well as stringent cell culture and isolation settings are required to eliminate the risk of analyzing different SP cells. These standards will help to abolish skepticism and uncertainty about the general validity of the technique and potential of SP cells.

3. Conclusion

Based on the results, we get these conclusions: firstly, to isolate CSCs from the MHCC97 cell line, the effectiveness of Rho/FACS in enriching CSCs is similar to that of Hoe/FACS. Secondly, the Rho^{low} cells had similar characters as the SP cells; lastly, the Rho/FACS is less toxic and expensive than the Hoe/FACS. To conclude, when the Hoe/FACS is too toxic or expensive to do biological researches of some typed stem cells, the Rho/FACS is still another elective method. To conclude, in circumstances where suspected cancer stem-like cells may be susceptible to Hoe toxicity and/or where the cost of UV-FACS is prohibitive, the application of Rho/FACS is a credible method of isolation.

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Large-Scale Production of Human Glioblastoma-Derived Cancer Stem Cell Tissue in Suspension Bioreactors to Facilitate the Development of Novel Oncolytic Therapeutics

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1. Introduction

Glioblastoma multiforme (GBM, World Health Organization grade IV glioma) is the most common and aggressive form of brain cancer in adults and accounts for 17% of all childhood cancers (Cheng et al., 2010; Dirks, 2008). Even with conventional cancer treatment, the median survival time is between 12-15 months, with a 2-year median survival rate between 8% and 12% (Stupp et al., 2002; Stupp et al., 2005). This grim prognosis is partly due to GBM cells infiltrating the brain and spinal cord thus preventing complete surgical resection. These invasive tumour cells appear to be more resistant to cytotoxic therapy and have a higher proliferative potential (Furnari et al., 2007). Additionally, molecular profiling of GBMs have indicated that there is great heterogeneity within these tumours which may be due to the presence of a sparse population of cancer cells that exhibit stem-cell-like characteristics (Mischel et al., 2003; Parsons et al., 2008; Paugh et al., 2010; Verhaak et al., 2010). Specifically, these cells are able to self-renew and retain the capacity to differentiate but lack proliferative control. These stem-like cancer cells are referred to as either tumour-initiating cells or cancer stem cells (CSCs), and have been identified in a number of hematopoietic malignancies and solid tumours (Hope et al., 2004; C. Li et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004; Stingl & Caldas, 2007). CSCs have also been shown to be more resistant to conventional cancer drugs and have been implicated in the more aggressive nature of tumour drug resistance after remission (Bao et al., 2006; Dean et al., 2005). Hence, by effectively targeting these CSCs the prognostic outcome of GBM patients may be improved. However, the development of novel oncolytic therapeutics to target CSCs is currently hindered by the scarcity of CSCs found within these solid

tumours. Thereby, the development of novel drug treatments may rely on a continuous and reproducible supply of tissue for genomic analysis and high-throughput drug screening.

In order to address this issue, there is a major need for a large-scale cell expansion platform that allows for the generation of a large number of GBM-derived tissue containing cancer stem cells (GBM-CSCs). It has been shown that neurosphere culture can isolate and enrich GBM-CSCs (Singh et al., 2003; Yuan et al., 2004). There has been some controversy with this method of derivation, and recently data was presented by Dirks and colleagues who argued that GBM cell populations expanded in adherent culture, in comparison to neurosphere culture, have uniform access to growth factors and that adherent culture hampers the ability of short-lived progenitor cells to proliferate (Pollard et al., 2009). By comparing expression of differentiation markers on glioma cells grown in both adherent and sphere-forming culture, they found that increased numbers of cells grown as spheres expressed these differentiation markers. However, a source of contention is that the authors only compared one type of serum-free medium for the expansion of these glioma cells as spheres and also grew them in static vessels where the cells did not experience any shear forces. In a contrasting study, De Witt Hammer et al. (2008) showed that the genomic profiles of GBM-derived cells grown in spheroid culture were more representative of the parental tumor profiles, whereas in adherent cultures, genetic changes developed that led to genetic deviations from the parental tumors. Based on our studies with neural precursor cells from multiple regions of the brain and with GBM-derived CSC tissue, we have shown that we can expand these cells reproducibly in suspension culture (Baghbaderani et al., 2008; Baghbaderani et al., 2010; Panchalingam et al., 2010). Additionally, we have shown that the bioreactor-expansion of GBM-derived CSC tissue as spheroids conserves the basic nature of the tissue (Panchalingam et al., 2000). This was achieved by using a serum-free medium, PPRF-h2, which was developed in our laboratory for neural cell isolation and expansion.

Typically, GBM-CSCs are expanded in static culture vessels prior to use in basic cancer research. However, these conventional culture systems for expanding GBM-CSCs are inefficient at generating large numbers of cells and are labour intensive and non-homogenous. Therefore, we looked to develop protocols using suspension bioreactors which can lead to better process control. Shear fields in our suspension bioreactors were controlled with a central impeller that was able to eliminate concentration heterogeneities and lead to homogenous environmental conditions. Moreover, the shear generated by the impeller influenced tissue aggregate size, therefore allowing for the expansion of the generation of homogenous GBM-CSC spheroids. We adapted large-scale suspension bioreactor culture protocols for the expansion of human neural precursor cells to human GBM-derived CSC tissue. Studies were conducted to investigate the role of different bioengineering parameters on the expansion of GBM-derived CSC tissue. Through the refinement of experimental procedures, we were able to expand successfully our cell populations. Additionally, we were able to show that these bioreactor-expanded GBM-derived CSC tissue retained similar genetic characteristics to the initial cell population (Panchalingam et al., 2010). Through the use of a serum-free medium, PPRF-h2 (Baghbaderani et al., 2010), and the development of successful bioprocess cell expansion conditions, we can alleviate the bottleneck in GBM-CSC supply for use in research and potentially for the development of new brain cancer therapies through small molecule screening.

2. Human glioblastoma multiforme (hGBM)

Brain tumours are the leading cause of death resulting from solid cancers in children under the age of 20, and the third leading cause of death in young adults age 20-39 (Jemal et al., 2007). Moreover, brain tumours are diversely defined based on their histological characteristic and tissue origin (Vescovi et al., 2006). In general, brain tumours arise from brain cells (primary brain tumours) or from metastatic tumours originating from other areas of the body (secondary brain tumours) which continue to proliferate in the brain. As the cancer cells proliferate and invade surrounding brain tissue, symptoms may result including hemiparesis, aphasia, and seizures. Additionally, as the tumour grows intracranial pressure also increases due to the space constraints in the skull. The increase in pressure causes secondary complications by damaging or killing healthy brain cells, resulting in symptoms such as headaches, nausea, and changes in mental status (Patkar et al., 2000).

Astrocytic tumours, the most common type of gliomas, are typically classified based on their histological features and fall into two categories: low-grade or high-grade tumours. Low-grade astrocytic tumours (World Health Organization (WHO) Grade I and II) such as pilocytic astrocytomas, have an appearance similar to normal cells and tend to grow slowly. High-grade astrocytic tumours (WHO Grade III and IV), are tumours that tend to grow quickly, invade nearby tissue and the tumour cells have a different appearance than normal cells. GBMs are classified as grade IV astrocytic tumours (Siebzehnrubl et al., 2011). Histopathological features of GBMs usually include cellular anaplasia (undifferentiated cell growth), nuclear atypia, increased mitotic activity, vascular proliferation and extensive necrosis. Additionally, GBMs can arise as primary tumours or from the transformation of lower grade tumours into secondary tumours (Denysenko et al., 2010). Risk factors for gliomas remain elusive, although there have been some factors such as occupation, environmental carcinogens and diet which has been reported to be associated with the development of gliomas (Verma, 2009).

The majority of brain tumours are diagnosed usually when the patient experiences seizures or neurological symptoms (such as cognitive or motor deficits) without a clear cause (DeAngelis, 2001). Currently, there is no method that has been developed for screening or preventing brain cancer. Upon diagnosis, the treatments for brain tumours are multimodal involving surgical resection, radiation and chemotherapy. In children younger than three years of age, it is standard practice to employ chemotherapy instead of radiation due to its negative effect on the developing nervous system (Pollack, 1994). However, in both children and adults, these treatment modalities are not curative and invariably result in relapse and treatment failure. Also, since these treatments are invasive in nature, they usually result in cognitive impairment among other side-effects (Taphoorn & Klein, 2004). Due to the lack of effective therapeutic options, the effective treatment of GBMs represents a major challenge to clinicians.

3. Cancer stem cells

3.1 Cancer stem cell hypothesis

The cancer stem cell hypothesis proposes that tumours arise from stem cell-like tumour-initiating cells (TICs) or cancer stem cells (CSCs), capable of self-renewal but lacking proliferative control, which are the instigators of tumour formation and the precursors of malignancy (Reya et al., 2001; Singh et al., 2004). Under normal physiological conditions,

stem cells would either self-renew or differentiate into proliferating progenitor cells. These proliferating progenitor cells would then be able to differentiate and give rise to the cell populations of that tissue type (i.e. oligodendrocytes, neurons, or astrocytes) [Figure 1]. According to the cancer stem cell hypothesis, a mutagenic agent (e.g. environmental stresses) would cause the oncogenic transformation of the stem cell or a down-stream progenitor cell. These transformed cancer stem cells would retain some stem cell properties, such as self-renewal, but uncontrollably produce differentiated non-tumourigenic cells that would form the bulk of the tumour. This hypothesis has lead to the identification of CSCs in numerous malignancies, including leukemia, breast cancer, brain cancer, pancreatic cancer, and colon cancer (Hope et al., 2004; C. Li et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004; Stingl & Caldas, 2007).

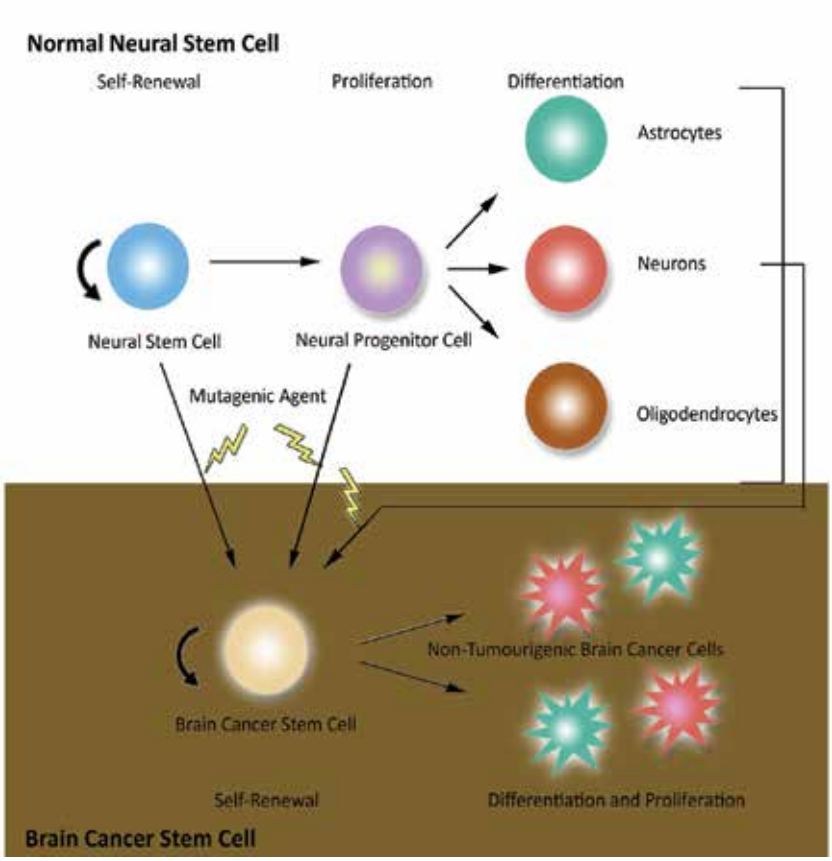

Fig. 1. Proposed mechanism of tumourigenesis through the formation of CSCs. Stem cells are able to self-renew and produce more differentiated progenies. Due to their long-lived, slow-dividing nature, they are exposed to damaging environmental factors over long periods of time, that may lead to the development of oncogenic mutations. The result of these mutations give rise to CSCs (also known as tumour-initiating cells). It has also been hypothesized that CSCs can be formed from the dedifferentiation of committed cells which also acquire oncogenic mutations. These CSCs uncontrollably produce large quantities of non-tumourigenic cells that result in a tumour mass

3.2 Alternate view of the cancer stem cell hypothesis

Quintana et al. (2008) questioned the existence of CSCs within human melanomas. The authors argued that the original assessment of the scarcity of CSCs in human high-grade melanoma (0.001%), was an artefact of a xenotransplant tumour-initiation assay, caused by residual innate immunity of the recipient mouse. Dissociated melanoma cells were transplanted in non-obese diabetic/severe compromised immunodeficient (NOD/SCID) mice at dilutions of 10^2-10^7, and found that only four of the seven samples generated palpable tumours after 8 weeks. Performing a limiting dilution assay, only 1 in 10^6 cells were observed to be tumour-initiating cells. However, by increasing the observation time to 32 weeks, the CSC frequency increased to 1 in 10^5 cells (Quintana et al., 2008). Because NOD/SCID mice retain some innate immunity, Quintana et al. (2008), utilized a mouse model depleted of natural killer cells (NOD/SCID/*Il2rg-/-*). By utilizing this model along with Matrigel to provide the cells with a more supportive environment, it was found that 15-25% of the tumour cells exhibited CSC activity, thereby creating uncertainty on the existence of a cancer cell hierarchy within human high-grade melanomas (Quintana et al., 2008). The results of this study show that within human melanomas, CSCs might not be as scarce as once thought. However, these results cannot be directly correlated with other solid tumours in the body, since even in mouse models of leukemia there exists wide variations in the frequency of CSCs from higher than 1 in 10^6 cells (*c-myc and bcr/abl/CDKN2*) to 1 in 10^4 cells (*MOZ-TIF and Pten-/-*) (Dick, 2009). From the results presented by Quintana et al. (2008), inferences may be made concerning the population of cancer stem cells within solid tumours. First, there may be human cancers that have a high-frequency of cancer stem cells that do not follow the cell hierarchy model (Eaves, 2008). Second, the optimization of assays, specifically xenotransplant models devoid of immunity, to correctly identify tumour cells that possess any stem cell function need to be further investigated. Third, it is important to develop tumour endpoints to discriminate between CSCs (or progenitors) that have limited self-renewal capacity and CSCs that possess extensive capacity for self-renewal (Dick, 2009).

3.3 Brain cancer stem cells

Brain tumours are phenotypically and morphologically diverse. Additionally, the tumours mass is highly heterogeneous and the CSC hypothesis reasons that this observed heterogeneity is due to the existence of a hierarchical organization, in which CSCs are the parental cell for the other cells present in the tumour (Siebzehnrubl et al., 2011). Consequently, the development of an assay that identifies the cells responsible for maintaining tumour growth may lead to the development of novel effective therapies to treat brain tumours. Also, Singh et al. (2003) showed that human brain cancer cells expressing the surface marker CD133 are capable of tumourigenesis in NOD/SCID mice with as few as 100 cells, whereas the brain cancer cells lacking this expression appear to have no proliferative ability. In addition, they obtained human brain tumours and enzymatically dissociated them to generate a single cell suspension. The cells were inoculated into conditions shown to be conducive for the isolation of neural stem cells. In culture, a minority of the cells formed neurosphere-like clusters (termed tumourspheres), which showed immunoreactivity for Nestin (a marker of neural stem cells) and CD133 (a putative marker of neural stem cells) (Singh et al., 2003). A sphere formation assay was carried out to assess the frequency of the stem cell population which yielded a frequency between 0.3% to 25.1%. They demonstrated that the $CD133^+$ population of cells differentiated in culture into tumour cells that phenotypically resembled the tumour from the patient

(Singh et al., 2003). However, not every $CD133^+$ brain cancer cell was able to form spheres *in vitro*, indicating that the $CD133^+$ brain cancer cells contain a subpopulation that are the 'true' brain cancer stem cells (Singh et al., 2003; Ward & Dirks, 2007).
In 2004, Galli and colleagues (Galli et al., 2004) isolated tumourigenic brain cancer cells from human GBMs that exhibited characteristics consistent with neural stem cells. The cells were found to be unipotent *in vivo* and multipotent *in vitro*, which may have resulted from the influence of the microenvironment on cellular signaling. Also, the cells were capable of generating tumours, through serial transplantations, that had similar histopathological features as the original tumour. As the cells were grown *in vitro*, the growth rate of the cells increased for the later passages, which could be attributed to an increase in the amount of proliferative cells. Interestingly, although all the cell lines studied were identified histopathologically as GBMs, they displayed varying growth kinetics and differentiation profiles. As stated previously, histopathological diagnosis of brain tumours is very subjective and may be unable to identify tumour variability between patients. This study suggests that the variability seen between GBM-cell lines might explain the differential response of tumour patients to treatment modalities (Galli et al., 2004).
Since the seminal paper by Singh et al. (2004), it has been shown by multiple groups, that CD133 is not a definitive CSC marker (Beier et al., 2007; Joo et al., 2008). Not only do CD133+ cells form tumours in an immunodeficient animal model, but also CD133- cells have been shown to form tumours. From this, many new markers for CSCs have been proposed such as stage-specific embryonic antigen 1 (SSEA-1), A2B5, SRY-like high mobility group 2 (Sox2), Sox4 and many others (Fang et al., 2011; Phi et al., 2008; Son et al., 2009; Tchoghandjian et al., 2010).

3.4 Therapeutic implications of the cancer stem cell hypothesis

Ideally, untransformed adult stem cells should remain functional throughout an individual's lifetime and retain the capacity to produce large quantities of undifferentiated progeny. In order to preserve this ability, stem cells have developed mechanisms to protect themselves from external factors that may potentially have an adverse effect on their function. Specifically, one such mechanism is the activation of ATP binding cassette (ABC) transporters, which are integral to DNA repair and inhibit apoptosis (Dean et al., 2005; S. Zhou et al., 2001).
It has been suggested that CSCs can develop from either stem cells or progenitor cells, or dedifferentiate from somatic differentiated cells, upon mutation of cancer-critical genes (B. B. Zhou et al., 2009). If CSCs are derived from stem/progenitor cells, it has been suggested that they would acquire the drug resistance mechanisms found in stem/progenitor cells. In GBMs this may explain the drug resistance observed for these tumours, as well as the high probability of recurrence. Four viewpoints have been developed on the implications of this drug resistance:

- CSCs, through their derivation from normal adult stem cells, acquire natural drug resistance thereby aiding their survival during chemotherapy, and preventing their full elimination from the body (Dean et al., 2005).
- Upon chemotherapy, some cancer cells acquire drug resistance that permits their survival. Following chemotherapy, the surviving cells pass on their drug resistance to their progeny, thereby rendering the original treatment of limited value (Dean et al., 2005).

- Chemotherapy selects for drug-resistant CSC variants that then repopulate the tumour with progenies that are also drug-resistant after chemotherapy (Dean et al., 2005).
- CSCs and their resulting progenies are inherently drug-resistant and therefore therapies have little or no effect on the tumour (Dean et al., 2005).

Presently, it is not clear which viewpoint represents the correct tumour model. However with the discovery of CSCs and their role in the formation of tumour drug-resistance, it is clear that the development of effective cancer therapeutics must be focused on this cell population. The use of surgery, radiation therapy and chemotherapy are not only invasive and ineffective, but are also standardized for the histopathology of the tumour without regards to its molecular basis. Hence, a systems biology approach to analyze *in vitro* expanded CSCs within hGBMs can lead to the development of oncolytic drugs that specifically target the molecular pathways within the GBM-CSC population (Foltz et al., 2006; Foltz et al., 2009; Shah et al., 2011; Yan et al., 2011). In order to generate a clinically-relevant number of patient-specific GBM-CSCs, it is necessary to optimize the *in vitro* culture environment, such that the basic genetic nature of the GBM-CSCs does not significantly change.

4. Culture conditions

Initially, GBM-derived CSCs were isolated and expanded in neurosphere culture based on protocols developed for the isolation and expansion of murine and human neural precursor cells (NPCs). However, a recent publication evaluated the derivation and expansion of GBM-derived CSC-lines in adherent culture. In this section both neurosphere and adherent culture will be discussed and evaluated.

4.1 Neurosphere cultures

In a landmark paper, Reynolds and Weiss (Reynolds & Weiss, 1992) were the first researchers to isolate neural stem cells (herein referred to as neural precursor cells (NPCs)) from the adult mouse brain using the neurosphere formation assay. They generated a single cell suspension of cells from the striatial tissue (including the subventricular zone), and inoculated this cell suspension into culture dishes void of attachment substrates. In these conditions, free floating spheres (termed neurospheres) formed from NPCs present in the murine brain. This was confirmed by analyzing the cells for defining stem cell characteristics, namely (1) self-renewal, and (2) differentiation into astrocytes and neurons (unipotent). Their methods were adapted over the years by key research groups for the isolation and expansion of human NPCs and GBM-derived CSC lines (Baghbaderani et al., 2008; Baghbaderani et al., 2010; Galli et al., 2004; Kelly et al., 2009; Panchalingam et al., 2010; Singh et al., 2003; Svendsen et al., 1998; Vescovi et al., 1999).

Conventionally, GBM-derived CSC lines are derived from a single cell suspension of dissociated GBM-derived cells inoculated into suspension conditions (either ultra-low attachment vessels or uncoated tissue culture vessels), and allowed to expand as neurospheres in serum-free culture medium. GBM-derived neurospheres are heterogeneous by nature and contain both stem and progenitor cells. However, it has been shown that the genomic changes in GBM cells expanded as neurospheres resemble the same changes detected in the primary tumours of the corresponding patients (Ernst et al., 2009). Additionally, in contrast to adherent culture, cells in neurosphere cultures maintain these genomics over the culture period (De Witt Hamer et al., 2008).

4.2 Adherent cultures

Recently, Dirks and colleagues (2009) reported on work which involved modifying the original protocol by Reynolds and Weiss (1992), by adding laminin as an attachment factor and culturing the cells as a monolayer of adherent cells. The authors expanded GBM-derived CSC lines in adherent and neurosphere culture and showed the cells in neurospheres expressed higher levels of differentiation markers. Also, higher levels of cell death were found within the neurospheres (Pollard et al., 2009). Using copy-number gains on the chromosomes and differentiation propensity, Pollard et al. (2009) showed that differentiated adherent cells were similar to parental tumours and that the adherent cells exhibited genomic characteristics commonly associated with glioblastomas. However, as mentioned in the previous section, there have been many groups that have shown that cells expanded in neurosphere culture exhibit similar genomics as seen in the parental tumour. Pollard et al. (2009) also did not mention the size of spheres that were analyzed. It is critical to consider the size of spheres in culture since mass transfer limitations in large spheres can result in the formation of necrotic centers (due to an inadequate supply of oxygen and nutrients to cells in the center of the spheres). This could have biased their comparison of neurospheres and adherent cells [for a comprehensive review please refer to Reynolds and Vescovi (2009)]. Based on our extensive work with neurosphere culture, and the work that has been done by numerous other groups, we chose to isolate and expand our GBM-derived CSC lines as neurospheres.

5. Development of PPRF-h2 medium

PPRF-h2 medium was developed to support the long-term expansion of human NPCs (hNPCs) in culture while retaining the multipotency of the cells (Baghbaderani et al., 2010). This medium was developed from an existing medium utilized for the expansion of murine NPCs (mNPCs) called PPRF-m4 (Sen et al., 2001). Modifications to this pre-existing medium focused on the dose-dependent effect of basic fibroblast growth factor (bFGF) and human leukemia inhibitory factor (hLIF) on the expansion and differentiation of hNPCs in culture. Additionally, the effect of dehydroepiandrosterone (DHEA) on the proliferation and differentiation of hNPCs was investigated. The different medium modifications studied are summarized in Table 1.

Growth Medium	Base Medium	Growth supplements added to the base medium
Control	PPRF-m4	10 μg/L bFGF
PPRF-h1	PPRF-m4	20 μg/L bFGF + 10 μg/L hLIF
PPRF-h2	PPRF-m4	20 μg/L bFGF + 10 μg/L hLIF + 1.0 μM DHEA

Table 1. The formulations of three different growth media that were developed by adding growth supplements (i.e. bFGF, hLIF and DHEA) to an existing serum-free medium (PPRF-m4) (Baghbaderani et al., 2010).

Fetal cortical-derived hNPCs were expanded in static suspension culture in the three different culture modifications outlined in Table 1. Cells expanded in PPRF-h2 resulted in a higher viable cells density over five passages. The cumulative cell-fold expansion over five passages in PPRF-h2 medium was $2.17\pm0.5x10^4$, which was significantly higher than cells expanded in PPRF-h1 ($0.59\pm0.1x10^4$) or control medium ($0.07\pm0.03x10^4$) [Figure 2A and 2B]

(Baghbaderani et al., 2010). Cells in PPRF-h2 and PPRF-h1 medium also formed larger aggregates (approximately 500 μm). The aggregates were morphologically different compared to aggregates expanded in PPRF-m4 medium [Figure 2C] (Baghbaderani et al., 2010) which adhered to the surface of the culture flask. This altered the phenotype of the cells as differentiation analysis showed a significantly higher number of neurons were derived from hNPCs expanded in PPRF-h2 medium (43.6±2%) compared to the control - PPRF-m4 medium (22.9±2%) (Baghbaderani et al., 2010).

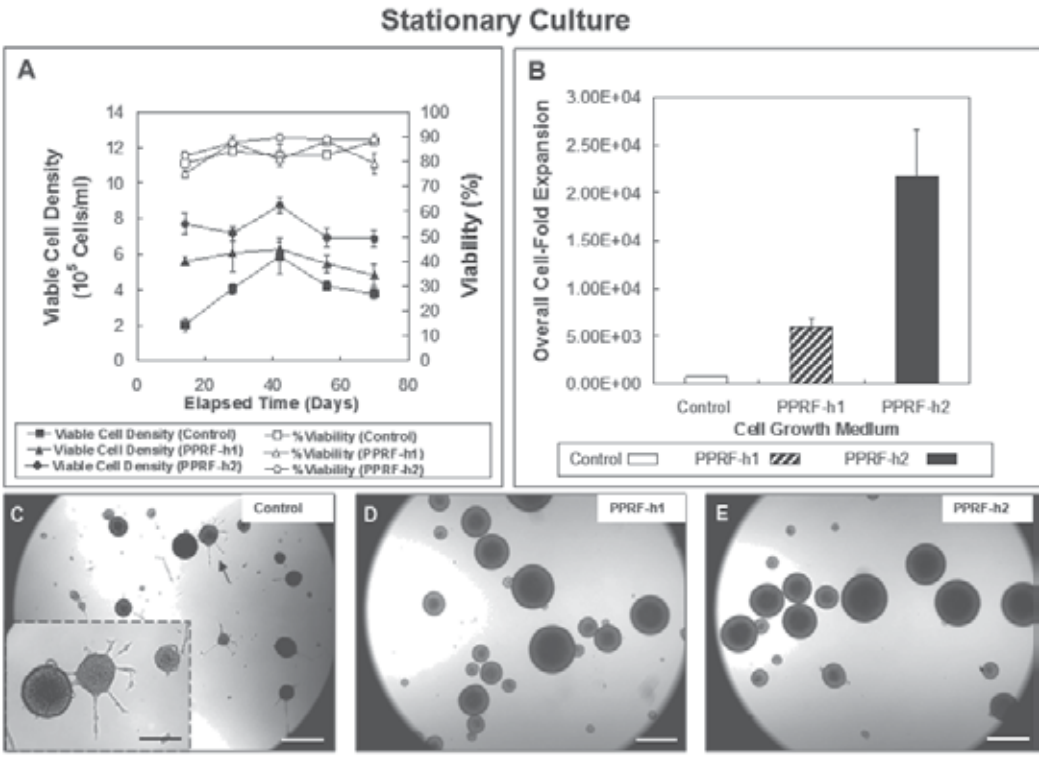

Fig. 2. Stationary culture of fetal cortex-derived hNPCs in three different growth medium: control medium, PPRF-h1 and PPRF-h2. Shown are (A) viable cell density and viability and, (B) overall cell-fold expansion of hNPCs from P10 to P14 over 70 days in the three different growth media. Also shown are photomicrographs of cells grown in (C) control medium, (D) PPRF-h1 medium and (E) PPRF-h2 medium at 14 days post-inoculation (P14). The inset shows a higher magnification of the aggregates (shown by arrow) adhered to the surface of the culture flask in the control medium. Scale bars represent 500 μm (scale bar in the inset is 250 μm) (Baghbaderani et al., 2010)

Since the presence of bFGF and hLIF in the growth medium exhibited a potent effect on the expansion and differentiation of hNPCs, the dose-dependent effect of bFGF and hLIF on the expansion of hNPCs was also investigated. Levels of bFGF were varied between 0-40 μg/L in PPRF-h2 medium. While there was a dose-dependent effect of bFGF on the proliferation of hNPCs (i.e. cell-fold expansion of hNPCs increased with increasing levels of bFGF), the addition of bFGF above 20 μg/L did not provide any significant beneficial effect on the

proliferation of hNPCs (Baghbaderani et al., 2010). Additionally, hLIF was varied between 0-20 μg/L. We observed that above 10 μg/L that there was no added beneficial effect on the growth of the hNPCs (Baghbaderani et al., 2010). Based on this study, a final concentration of bFGF and hLIF in PPRF-h2 medium was maintained at 20 μg/L and 10 μg/L, respectively (Baghbaderani et al., 2010).

6. Large-scale production of human glioblastoma-derived cancer stem cell tissue

The development of therapies to target CSCs is hindered by the scarcity of the CSCs within solid tumours. In brain cancers, the frequency of cells expressing CD133 varied with pathological subtype and grade from 1% to as high as 45% (Joo et al., 2008). As discussed previously, not every $CD133^+$ cell is capable of forming a sphere *in vitro* (an assay for CSC identification), and therefore a subpopulation within the $CD133^+$ exists that contains the scarce 'true' CSCs or TICs (Ward & Dirks, 2007). It is this lack of pure populations of CSCs for research that hinders the development of effective cancer therapies. In developing effective cancer therapeutics, it will be necessary to observe and quantify the interactions within brain tumours which require drug regimes and therapeutics to be individually tailored. This would require a large number of cells obtained from each individual, so that numerous treatment modalities could be tested on it. The availability of CSCs *in vivo* limits the type of analyses that can be performed, restricts the knowledge that can be gained about the basic cancer stem cell biology and hinders the development of effective, individualized cancer therapeutics. Thus it is necessary to develop a bioprocess that is capable of producing a reproducible, clinically-relevant number of cells.

Conventionally, the *in vitro* expansion of stem/progenitor cells has been performed in stationary tissue culture flasks. Although large cell numbers could be produced using this method of culture, it becomes increasingly laborious as the number of tissue culture flasks increases. Additionally, with handling a large number of tissue culture flasks, the probability of introducing cell culture variability between flasks becomes high, thereby reducing reproducibility between cultures. Such issues would be detrimental to the development of therapeutics since this culture variability would lead to increased phenotypic variability in cells expanded in different flasks, thereby impairing subsequent analysis to determine therapeutic targets. In order to generate large numbers of stem cells reproducibly, suspension bioreactors have been used for the expansion of a number of adult stem cell/progenitor lines. Figure 3 shows a comparison of the large-scale production of CSCs using tissue culture flasks and suspension bioreactors. A 1.0 L suspension bioreactor, for example, is equivalent to 200 T-25 tissue culture flasks using a 5.0 mL volume. The use of suspension bioreactors can generate a reproducible, large number of cells with well defined characteristics, while enabling control and maintenance of a homogenous environment.

Suspension bioreactors to expand a population of adult and embryonic precursor cells have been discussed in detail in literature. We have expanded human and murine neural precursor cells, murine mammary epithelial stem cells and breast cancer stem cells as aggregates in suspension culture (Baghbaderani et al., 2008; Baghbaderani et al., 2010; Youn et al., 2006; Youn et al., 2005). We have shown that protocols developed for these cell types can be adapted to other cell types, such as hGBM-derived CSC tissue. hNPCs bioreactor expansion protocols were adapted for the expansion of hGBM-derived CSC tissue (Panchalingam et al., 2010). Baghbaderani et al. (2008) found that inoculating hNPCs at

$1.0x10^5$ cells/mL was conducive for cell growth. Operating in a semi-fed-batch mode resulted in a maximum cell density of $3.0x10^6$ cells/mL over 18 days, corresponding to a 36-fold expansion (Baghbaderani et al., 2008). From these protocols, we investigated the effect of key culture parameters on the proliferation and phenotype of hGBM-derived CSC tissue expanded in suspension bioreactors. These key bioengineering parameters were: (1) liquid shear, (2) inoculation density, (3) feeding strategy, (4) medium composition and, (5) hypoxia.

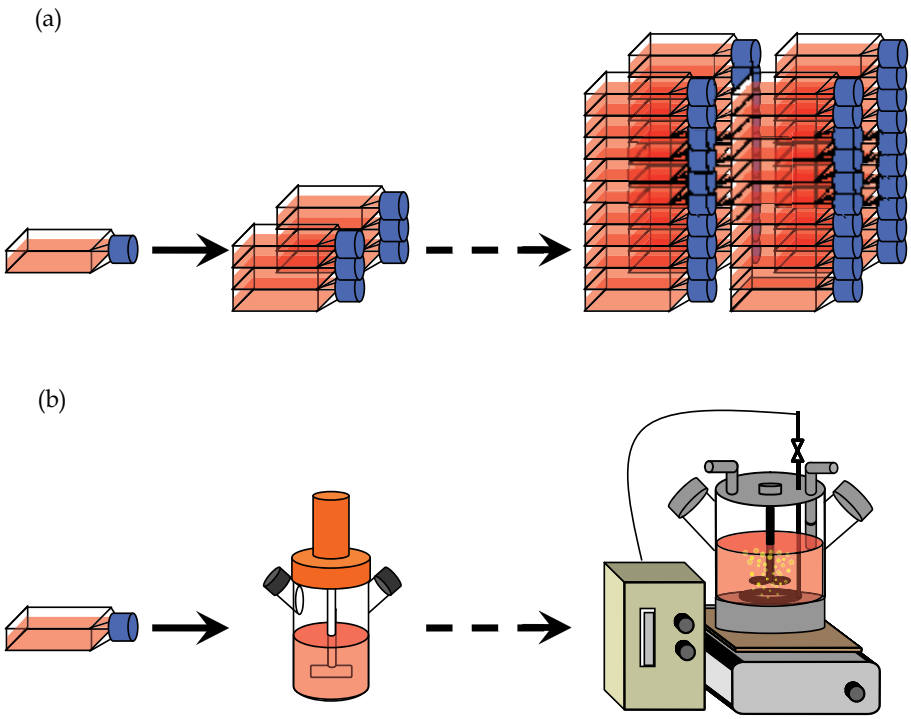

Fig. 3. Comparison of large-scale CSC cultures using tissue culture flasks and bioreactors. Two methods of scale-up to larger volumes are available: (a) large number of T-flasks, and (b) a single bioreactor. Bioreactors are a significantly better alternative for anchorage independent cells due to their reproducible, homogeneous, controllable environment

6.1 Bioengineering issues

6.1.1 Bioreactor liquid shear

Within suspension bioreactors, liquid shear control ensures adequate oxygen mass transfer and bulk fluid mixing. However, it is also important to make sure that hydrodynamic shear associated with the level of turbulence is within acceptable limits. The shear that is seen in stirred suspension bioreactors can have a negative impact on shear sensitive mammalian cells, but can also maintain aggregate size within acceptable limits (Kallos & Behie, 1999; Kallos et al., 1999; Sen et al., 2001). Also, the maximum shear stress occurs near the tip of the impeller, where turbulence is at its greatest (Cherry & Kwon, 1990). Briefly, Cherry and Kwon (1990) developed a relationship for maximum shear stress that is experienced by a cell on the surface of an aggregate. This relationship is represented by:

$$\tau_{max} = 5.33\rho(\varepsilon\nu)^{1/2} \quad (1)$$

where τ_{max} is the maximum shear stress (Pa), ρ is the medium density (kg/m^3), and ν is the kinematic viscosity (m^2/s). The power dissipation per unit mass, ε, can be calculated using the vessel working volume (V, m^3) and density:

$$\varepsilon = \frac{P}{V\rho} \quad (2)$$

where P is the power input to the system (W), which is estimated by the Nagata (1975) correlation using the power number (N_p). Since the power number is correlated with Reynolds number (and therefore with agitation rate), shear stress can be controlled by adjusting the agitation rate of the culture vessel. For hNPCs, we found that a maximum shear stress between 0.35 to 0.80 Pa may sustain the expansion of these cells in small-scale suspension culture (Baghbaderani et al., 2008). Within our 125 mL suspension bioreactors this corresponds to an agitation speed of 70 - 130 rpm.

We inoculated hGBM-derived CSC tissue at 7.5x10^4 cells/mL and operated at either low shear (60 rpm) or high shear (100 rpm) in 125 mL suspension bioreactors under batch conditions and grown as neurospheres. hGBM-derived CSC tissue inoculated in high-shear conditions reached a maximum cell density of 9.30x10^5 cells/mL compared to cells inoculated in low-shear conditions which reached a maximum cell density of 6.15x10^5 cells/mL (p-value = 0.046). This corresponded with a cell-fold expansion of 14.4 and 7.0 for the high- and low-shear condition, respectively. Moreover, we observed that the mean neurosphere diameter on day 22 was around 300 μm for the low-shear condition. Whereas, in the high-shear condition the mean neurosphere diameter did not exceed 200 μm. Over the entire course of the study, the average neurosphere size in the low-shear condition was higher than for the cells expanded in the high-shear condition. Additionally, the standard deviation of the diameters of neurospheres expanded in the high-shear condition was lower than the neurospheres expanded in the high-shear condition. This implies our high-shear expansion of hGBM-derived CSC tissue can lead to more homogenous culture morphology.

6.1.2 Inoculation density

Inoculation density is an important parameter in cell culture. Inoculating at too low a cell density may not be sufficient to support cell growth or cause a considerably prolonged lag phase. Such a low cell density will lead to a lack of cell-to-cell contact, which has been shown to be crucial to initiate cell expansion. Moreover, the release of autocrine factors released by the cells inoculated at a low density may not be sufficient for cell growth. In contrast, inoculating the cells at too high a cell density can result in (1) a lower cell-fold expansion and, (2) the need to sparge oxygen into the culture in order to maintain adequate oxygen transfer. To successfully grow hNPCs a sufficiently high inoculation density was required for their expansion (1.0x10^5 cells/mL) (Baghbaderani et al., 2008). Additionally, preliminary experiments on the expansion of hGBM-derived CSC tissue in static suspension culture experiments found that inoculating hGBM-derived CSC tissue at 5.0x10^4 cells/mL resulted in the highest cell-fold expansion (Baghbaderani et al., 2008). We further investigated the influence of inoculation density on the expansion of hGBM-derived CSC tissue in our 125 mL stirred suspension bioreactors. Cells were inoculated at a density of 5.0x10^4 cells/mL and 1.0x10^5 cells/mL. An inoculation density of 5.0x10^4 cells/mL resulted

in the highest cell fold expansion (19.9) with the cells attaining a maximum viable cell density of $1.12x10^6$ cells/mL. Additionally, we observed that at the higher cell density, that the consumption of key nutrients, namely glutamine and glucose, was higher compared to the cells inoculated at the lower cell density. Additionally, the nutrient uptake of bioreactor-expanded hGBM-derived CSC tissue to hNPCs, was higher, even when comparing hNPCs inoculated at $1.0x10^5$ cells/mL (compared to $5.0x10^4$ cells/mL for hGBM-derived CSC tissue). The rapid loss of nutrients from the culture medium may be mitigated by using a semi-fed-batch approach.

6.1.3 Feeding strategy

As cells proliferate in culture, they metabolize key nutrients in the medium (i.e. glutamine and glucose) and produce toxic by-products (i.e. ammonium and lactate) (Butler, 2004). Within tumours, the rate of glycolysis and glutaminolysis are higher than their untransformed counterparts, and nutrient limitations have been shown to be an important factor on cancer tissue expansion (DeBerardinis et al., 2008; Elstrom et al., 2004; Youn et al., 2006). The 'Warbug' effect states that in tumour cells the increased rate of glycolysis is accompanied with an increase in the lactate production of the tumour cells (Kim & Dang, 2006; Vander Heiden et al., 2009). This contrasts with their untransformed counterparts which - (1) have comparatively low rates of glycolysis and, (2) metabolize glucose to pyruvate (part of aerobic respiration) within the mitochondria. This 'effect' is a metabolic hallmark of aggressive tumours, as the cells utilize anaerobic respiration in order to maintain their need for large amounts of energy. Additionally, within cell culture glutamine serves as not only an energy source for the cells, but also as a source of reduced nitrogen for maintenance of nucleotide biosynthesis and non-essential amino acids (Newsholme et al., 2003). From this information and our previously discussed studies, it was surmised that periodic medium replacement within our stirred suspension bioreactor hGBM-derived CSC tissue cultures may be beneficial. Baghbaderani et al. (2008) replaced 40% of the culture medium every 6 days for the expansion of hNPCs in suspension bioreactors. However, hGBM-derived CSC tissues utilized their nutrients at a higher rate than hNPCs and also produce the associated metabolic by-products at a higher rate. Therefore, in our work we examined the effect that a 40% medium replacement every 6 days as well as every 2 days had on the expansion and phenotype of bioreactor-expanded cells. Total medium replacement was not considered, since it has been suggested that cells in culture secrete endogenous factors that support cell proliferation (Zandstra & Nagy, 2001). It was found that hGBM-derived CSC tissue expanded in 125 mL suspension bioreactors in a 2-day fed-batch mode (40% medium replacement every 2 days) resulted in the highest expansion (90 cell-fold expansion) [Figure 4] (Panchalingam et al., 2010). Additionally, it was seen that as the cultures progressed, significantly larger aggregates were seen in the 2-day fed-batch conditions, resulting in average aggregate sizes around 600 μm compared with 350 and 450 μm in the batch and 6-day fed-batch conditions, respectively, on day 24. Baghbaderani et al. (2008) found that average aggregate diameters of < 600 μm would not compromise cell viability and would maintain the phenotypic characteristics of the hNPCs. Nutrient and metabolite analysis was performed for all the culture conditions, and it was observed that in the fed-batch conditions there was an increase in the specific consumption rate of glucose and glutamine. This resulted in an increase in the specific production rate of lactate and ammonium in the fed-batch culture conditions compared to the batch conditions. The level

of lactate and ammonium however did not rise above 2.0 g/L and 2.0 mM, which is generally considered detrimental for mammalian cell growth (Butler, 2004). The difference in nutrient uptake between batch and fed-batch conditions illustrates the activation of different metabolic pathways that may affect the phenotype bioreactor-expanded hGBM-derived CSC tissue.

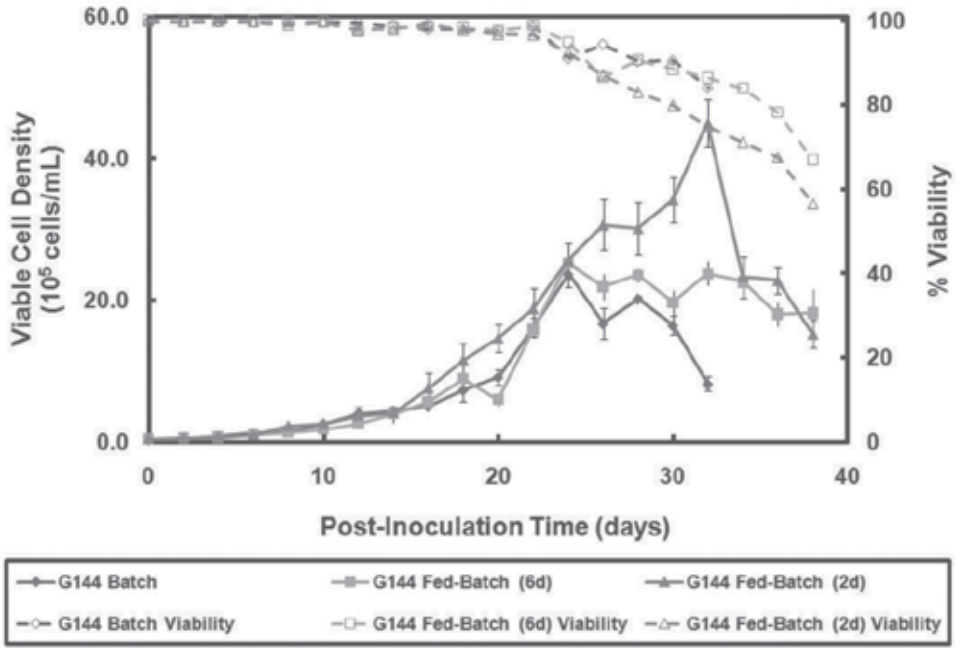

Fig. 4. Viable cell density and percent viability of hGBM-derived CSC tissue grown in batch, 6-day fed-batch, and 2-day fed-batch culture modes. All data points represent the average of duplicate bioreactors ± S.D. (Panchalingam et al., 2010)

Cell phenotype was assessed by (1) using flow cytometry to quantify the expression of the surface antigen CD133, (2) differentiation analysis to observe the propensity of cells to differentiate into the three neural lineages and, (3) microarray analysis to identify whether the bioreactor expansion process changed the basic nature of the hGBM-derived CSC tissue. The hGBM-derived CSC tissue in the fed-batch conditions maintained their CD133 expression greater than 90% for the entire course of the experiment. In comparison, cells expanded in the batch conditions had a CD133 expression of 95% on day 24 which decreased rapidly as cell viability decreased (Panchalingam et al., 2010). Immunocytochemical analysis of undifferentiated cells revealed that the hGBM-derived CSC tissue were positive for GFAP (GFAP+, a marker of astrocytes) and negative for Nestin (Nestin-, a marker of neural stem cells) (Panchalingam et al., 2010). It has been shown that $GFAP^+/Nestin^-$ cells are a subset of cells derived from glial progenitor cells (Miyaguchi, 1997). Upon differentiation, the cells spontaneously differentiated into primarily $GFAP^+/\beta\text{-}tub^+$ (a marker of neurons) cells. Genomic analysis of the hGBM-derived bioreactor-expanded CSC tissue revealed that there were more genomic changes within the 2-day fed-batch condition compared to the batch condition. However, Gene Ontology (GO)

enrichment analysis revealed that key genomic constructs were less affected than in the other culture conditions [Figure 5]. These findings show that the bioreactor-expansion of our hGBM-derived CSC tissue using a 2-day fed-batch methodology results in cells maintaining their phenotypic and genomic characteristics.

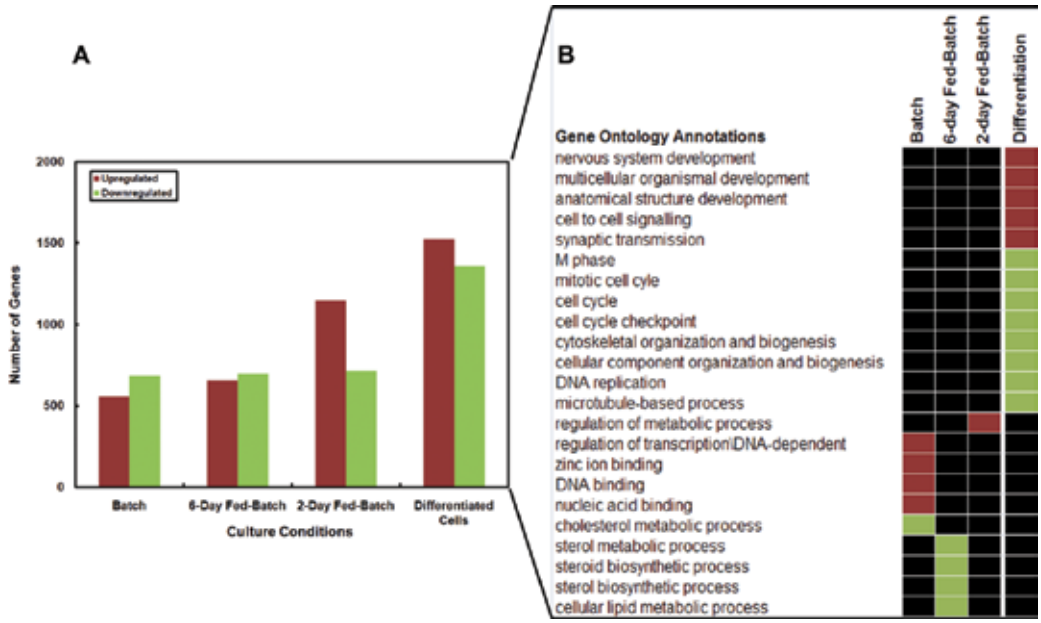

Fig. 5. Differentially-expressed genes of bioreactor-expanded GBM-derived CSC tissue grown in batch, 6-day-fed-batch and 2-day fed-batch culture modes. Shown are (A) the number of genes that showed a fold changed greater than 2 with a p-value < 0.007 and, (B) gene ontology (GO) annotations for the differentially-expressed genes with a p-value < 0.007 for the three different culture conditions on day 24 compared to day 0. Red represents above-average expression level, green represents below-average expression level, and black represents no change in the gene expression with cells before inoculation into the bioreactors (Panchalingam et al., 2010)

6.1.3 Culture medium

Within our laboratory, the influence of different culture components on the growth kinetics and phenotype of different stem/progenitor lines has been evaluated (Baghbaderani et al., 2010; Jung et al., 2010). As was mentioned in previous sections, studies were done to elucidate the influence of bFGF, hLIF and DHEA on the proliferation and phenotype of hNPCs (Baghbaderani et al., 2010). We observed that these chemical mitogens had a significant effect on the growth kinetics and phenotype of hNPCs. Moreover, we evaluated the effect our growth medium, PPRF-h2, had on the growth kinetics of hGBM-derived CSC tissue in suspension culture compared to another growth medium used extensively by other research groups, Neurocult NSA (STEMCELL Technologies, Vancouver, Canada). Two glioma cell lines (SN184-grade II glioma-derived and SN143-hGBM-derived) were inoculated at 5.0×10^4 cells/mL into 125 mL stirred suspension bioreactors in either PPRF-h2 or NSA medium. Here, the SN184 and SN143 cells in PPRF-h2 medium reached a maximum

viable cell density of $2.7x10^6$ cells/mL and $3.4x10^6$ cells/mL (80.6 and 73.2 cell-fold expansion), respectively. In NSA medium, the SN143 and SN184 cells reached a maximum viable cell density of $2.2x10^6$ cells/mL and $3.1x10^6$ cells/mL (data not shown). Also, we saw that the cells in both media had similar aggregate size distributions. Although the growth kinetics of the cells were similar in both media, it will be important to evaluate the genomics of the cells as they may have been altered through the culture period. This is currently being evaluated within our laboratory.

6.1.4 Hypoxia

The expansion of hNPC and GBM-derived CSC tissue *in vitro* has often been done at atmospheric oxygen levels of 21% (Baghbaderani et al., 2010; Panchalingam et al., 2010). However, the physiological levels of O_2 in the brain vary between 2.5% to 12.5%, and in solid tumours it has been reported that the oxygen tension can be below 0.1% in necrotic regions (Bar, 2011; Panchision, 2009). hGBM tumours were shown to experience mild to moderate/severe hypoxia, with oxygen concentrations between 0.5% to 2.5% for mild hypoxia and 0.5% to 0.1% for moderate/severe hypoxia (Bar, 2011). This agrees with the observations by many researchers who note that hypoxia tends to correlate with increased tumour aggressiveness (Evans et al., 2004; Helczynska et al., 2003; Jogi et al., 2002; Kunz & Ibrahim, 2003). This may be due to the different genes that are upregulated in hypoxic conditions, which are primarily controlled by hypoxia-inducible factors 1α, 2α, and 3α (HIF-1-3α) (Bar, 2011). HIF-1α has been shown to repress p53, which is a major effector of mitotic arrest and apoptosis and can also block normal CNS precursor differentiation (Gustafsson et al., 2005; Hammond & Giaccia, 2005; Panchision, 2009). Also, it has been shown that HIF-1α and HIF-2α can result in an increase in the expression of CD133 (Z. Li et al., 2009; Soeda et al., 2009). Expanding hGBM-derived CSC tissue *in vitro* under physiological oxygen concentrations (1-7%), results in the tumour stem cell signature genes being overexpressed and also enhances the stem-cell like phenotype of the expanded GBM cells (McCord et al., 2009; Seidel et al., 2010). Additionally, Pistollato et al. (2009), cultured Grade III and IV (GBM)-derived precursor cells (referred to as high-grade gliomas - HGG) which proliferated to a higher extent in a hypoxic environment (Pistollato et al., 2009). In their study, they compared the expansion of these HGG cells at 2%, 5% and 20% O_2 tension and found that HGG cell expansion was significantly higher at 2% and 5% O_2 tension than at 20% O_2 tension (Pistollato et al., 2009). At these lower oxygen tensions, there was also a selective suppression of mitotic arrest and apoptosis. Also, at these low oxygen tensions, HIF1α expression repressed BMP signalling (BMP under high oxygen tension promotes differentiation) thereby conferring resistance to differentiation and death (Pistollato et al., 2009). In using *in vitro* expanded GBM-CSCs for the development of novel oncolytic therapeutics it will be necessary to be able to control the tumour microenvironment *in vitro* within set limits. The use of a large-scale bioprocess with control capabilities, which is also able to effectively control oxygen levels in vitro, will aid in generating clinically-relevant numbers of GBM-CSCs.

6.2 Large-scale production of hGBM-derived CSC tissue in computer-controlled suspension bioreactors

6.2.1 Scale-up using suspension bioreactors

In order to generate a large-number of clinically-relevant CSCs, it is necessary to scale-up our current process to larger volume bioreactors. Large volume bioreactors have been

developed and used for the last 50 years for the culture of different organisms such as bacteria, yeast and mammalian cells for pharmaceutical applications (Eibl, 2009). These applications include protein production [i.e. production of tissue plasminogen activator (tPA) and recombinant erythropoietin (rEPO)] and viral vaccine production (i.e. Hepatitis A vaccine) from recombinant cells (Demain & Vaishnav, 2009). However, in comparison to the more robust cells currently used in industry, stem cells are fragile and are metabolically slower. Therefore, in order to scale-up production of stem/progenitor cells (or in our case CSC tissue), it is necessary to consider key variables which may have a more significant affect when moving from smaller volumes to larger volumes. Two of these key variables are (1) oxygen supply, and (2) hydrodynamic shear in the liquid medium.

The specific oxygen consumption rates of mammalian cells have been reported to be between $1.7x10^{-17}$ and $17x10^{-17}$ mol O_2/cell•s (Butler, 2004). In our laboratory, we have found that single cell suspensions of hNPCs and GBM-derived CSC tissue have specific oxygen consumption rates are $5.87x10^{-17}$ and $2.99x10^{-17}$ mol O_2/cell•s respectively (Baghbaderani et al., 2008). Typically for small bioreactors (less than 1.0 L) the oxygen demands of cells can be satisfied by gas diffusion from the headspace through the culture surface. However, this may not be adequate in larger bioreactors. As the working volume of the bioreactor increases, the surface area-to-volume (SAV) ratio (also referred to as the aspect ratio) decreases. If we were to consider that we have two bioreactors, one 125 mL and one 500 mL, the SAV ratio would decrease from 33.0 m^{-1} to 15.6 m^{-1}. Therefore, as we scale-up to larger volume bioreactors, the area available for oxygen transfer through the interfacial surface of the medium within the bioreactor, decreases. There are three parameters which could be modified in order to maintain adequate oxygen supply in order to meet the oxygen demands of the expanding cells (1) sparging oxygen into the culture, (2) increasing the agitation rate of the impeller, or (3) increasing the oxygen concentration in the headspace. Spargers have been implemented in bacterial fermentation, where high cell densities require high oxygen supply. However, since sparging is associated with the production of gas bubbles that can burst at the surface of the culture, it can cause cell damage which is not recommended for the cultivation of stem cells (Shuler & Kargi, 2002). Increasing the agitation rate is another option, since this would increase the dissolution of oxygen into the medium. However, as the agitation rate increases, the hydrodynamic shear increases which could be detrimental if it causes excessive cell damage. Increasing the oxygen concentration in the headspace is also a viable option for small bioreactors, however, this may not be sufficient to maintain adequate oxygen supply as the vessel volume increases. Thus far, we have evaluated the use of 500 mL bioreactors for the expansion of GBM-derived CSC tissue. From our work we have found that increasing the oxygen level in the headspace has been sufficient to maintain adequate oxygen supply within our bioreactor cultures.

Hydrodynamic shear is an important characteristic in the culture of cells. The Kolmogoroff theory of turbulent eddies (Cherry & Kwon, 1990) and the Nagata correlation (Nagata, 1975) can be used to determine the agitation rate to scale-up the cultures.

6.2.2 Process control

In order to have a reproducible supply of CSCs in a bioreactor culture process, it is necessary to control key culture process parameters within a strict limit. These parameters are typically temperature, dissolved oxygen concentration and pH. In order to control these

process variables (PV) we use standard process control techniques. Typically, the PV is measured by a sensor or probe that is connected to a computer control system (controller) [Figure 6]. The controller receives the measurement from the probe, called the control variable (CV), and compares it with a predetermined set point (SP) value (this difference is called the error or offset (ϵ)). If there is no difference between the CV and SP (ϵ=0), then the controller does not take any action. However, if there is a difference between the CV and the SP (ϵ≠0), then the controller initiates a corrective feedback in order to shift the process to the SP. The controller output is applied through a final control element (FCE) which manipulates the input values into the bioreactor in order to apply the corrective feedback necessitated by the controller. The manipulated input value is called the manipulated variable (MV). The method by which a controller determines the corrective feedback necessary is through the combination of proportional, integral and derivative control.

Proportional control (P-only) is the simplest control strategy. The controller output to the FCE is proportional to the error (ϵ) and is given by the equation:

$$MV = K_c\epsilon + bias \tag{3}$$

where K_c is the controller gain which is the ratio of the change in the controller output to the change in the error. The bias term is the output of the controller when the error is zero. There are a few shortcomings of using just a proportional control including its relatively slow response to a disturbance in a system, and often the system does not reach the predetermined SP.

Integral controllers (I-only) are used to minimize the error by continually changing the FCE. The governing equation for this controller is:

$$MV = \frac{1}{T_i}\int \epsilon dt + bias \tag{4}$$

where T_i is the integral time constant. However, the integral controller by itself has a response period that is grossly larger than the response period of the proportional controller. Typically, integral control is combined with proportional control (PI) which has a response period that is 1.5 times the response period of the P-only controller. The equation for a PI controller is:

$$MV = K_c\left(\epsilon + \frac{1}{T_i}\int \epsilon dt\right) \tag{5}$$

A PI controller provides better dynamic control than either a P-only or I-only controller. One disadvantage of a PI controller is that a sustaining error could cause the integral term to increase and saturate the controller output (known as reset windup) (Seborg, 2011).

Derivative (D-only) controllers anticipate the behaviour of a process by calculating the rate of the change of the error. The equation for derivative action is:

$$MV = T_d\frac{d\epsilon}{dt} \tag{6}$$

Where Td is the derivative time constant. Using derivative time decreases lags in the process response. In combination with the PI controller we obtain a PID controller which would have a response similar to P-only control. The overall process control equation is:

$$MV = K_c\left(\epsilon + \frac{1}{T_i}\int \epsilon dt + T_d\frac{d\epsilon}{dt}\right) \quad (7)$$

The growth and proliferation of hGBM-derived CSC tissue was therefore investigated in computer-controlled suspension bioreactors, using a PID controller.

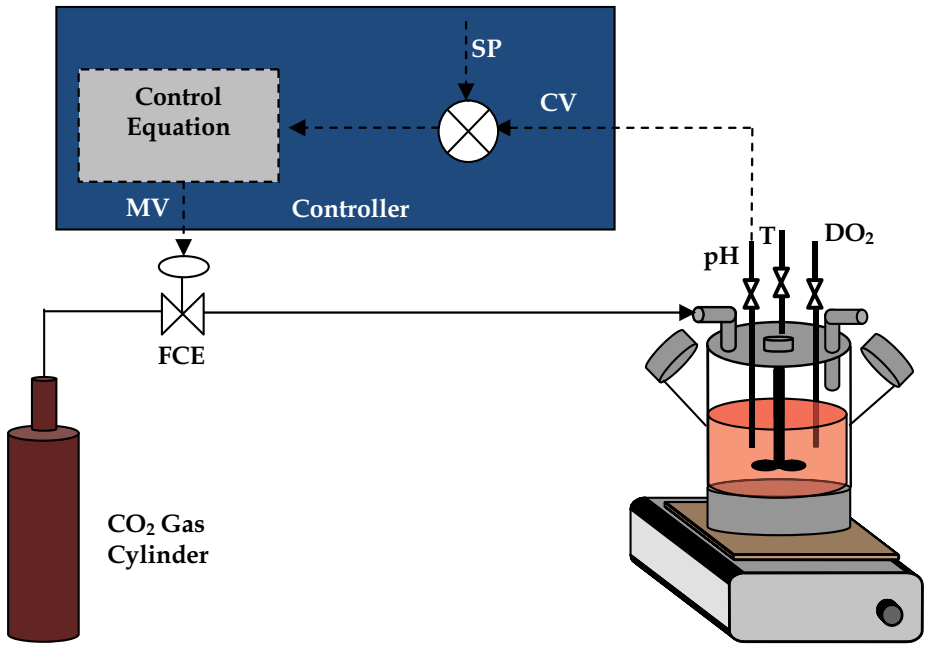

Fig. 6. A schematic diagram of a process control system for controlling pH, temperature (T) and dissolved oxygen (DO_2) within a suspension bioreactor system. Using pH control as an example: the pH probe measures the pH and transmits a signal carrying that pH value (CV) to the controller where it is compared with a pre-determined SP value. The deviation between the CV and SP is sent to the control equation, where if there is a deviation present, a corrective feedback is provided to the FCE to shift the culture PV to the SP. For example, if the controller recognizes that there is a pH increase in the medium, it will output a corrective feedback (MV) to the FCE, which in this case increases the CO_2 flow to the headspace, which in turn decreases the pH of the culture

6.2.3 Proliferation of hGBM-derived CSC tissue in computer-controlled suspension bioreactors

We inoculated two 500 mL computer-controlled suspension bioreactors at a density of $5.0x10^4$ cells/mL in PPRF-h2 medium operating in batch mode. Using the Kolmogoroff theory of turbulent eddies (Cherry & Kwon, 1990) and the Nagata correlation (Nagata, 1975) we determined that an agitation rate of 85 rpm would provide the same maximum shear as experienced by a 125 mL suspension bioreactor operating at 100 rpm. Our computer-controlled suspension bioreactor setup is illustrated in Figure 7. We controlled the dissolved oxygen concentration at 70% air saturation (15% atmospheric oxygen concentration), the pH at 7.4 and the temperature of the vessel at 37°C. Throughout the culture period we observed that the growth kinetics of hGBM-derived CSC tissue grown in the computer-controlled

suspension bioreactors were similar to hGBM-derived CSC tissue expanded in 125 mL suspension bioreactors operated in an incubator maintained at 5% CO_2 and 37°C. After 450 hours in culture, the hGBM-derived CSC tissue attained a maximum viable cell density of $1.12x10^6$ and $1.26x10^6$ cells/mL in the 125 mL and 500 mL bioreactors, respectively. Neurospheres generated in the 500 mL bioreactors had similar morphologies with the neurospheres expanded in the 125 mL bioreactors. However, we saw that the neurosphere size distribution in the 500 mL bioreactors had an observable shift towards larger neurosphere diameters and hypothesized that scaling-up our bioreactor process using maximum shear, would allow us to generate similar sized neurospheres in the 500 mL and 125 mL bioreactors. However, our observations here suggest that maximum shear may not be the only factor impacting neurosphere size within our bioreactors. Additionally, we found that oxygen supply through the headspace was sufficient in order to meet the oxygen demands of the cells. Further analysis of their differentiation characteristics and CD133 expression, showed that these cells retained similar phenotypic characteristics as neurospheres expanded in the 125 mL suspension bioreactors. The conclusion of this study was that a computer-controlled suspension bioreactor process would be amenable for the large-scale expansion of clinically-relevant numbers of GBM-derived CSC tissue. Through the use of a computer-controlled suspension bioreactor process, we can control key bioprocess parameters in order to generate high numbers of GBM-CSCs for the development of novel oncolytic therapeutics.

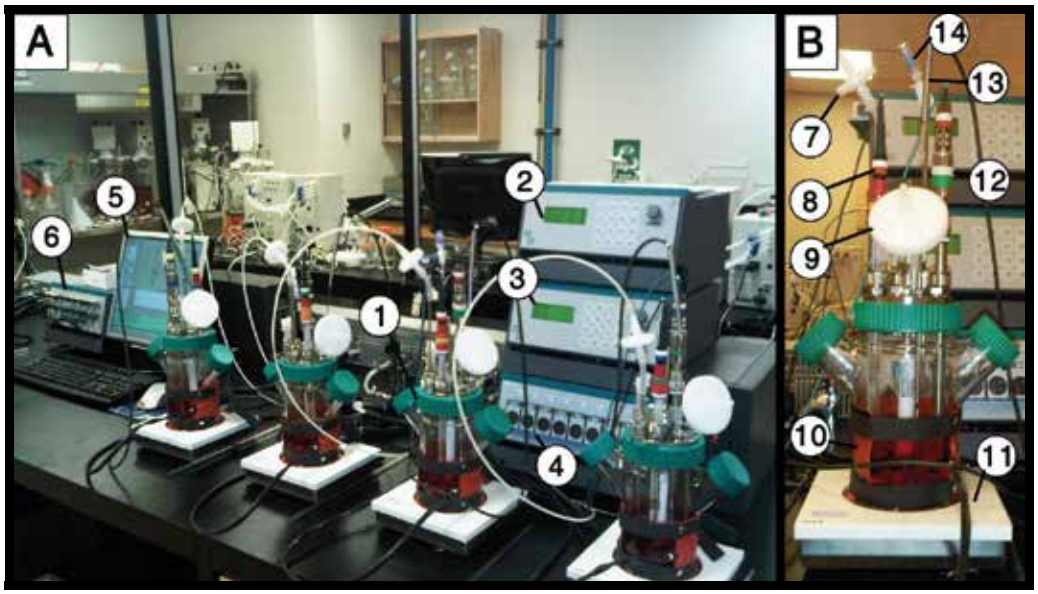

Fig. 7. Photographs of 500 mL DASGIP computer-controlled suspension bioreactors. (A) Experimental setup for the 500 mL suspension bioreactor system showing: (1) 500 mL suspension bioreactor, (2) temperature and agitation module TC4SC4, (3) gas mixing station MX 4/4, (4) multipump modules MP8, (5) DASGIP control and data acquisition computer, and (6) DASGIP sensor module PH8PO8. (B) A closer view of the 500 mL DASGIP suspension bioreactor, showing: (7) gas inlet to the bioreactor connected to O_2, N_2, CO_2 and air gas cylinders via gas mixing station MX 4/4, (8) pH probe, (9) gas outlet from bioreactor, (10) heating jacket, (11) stirring plate, (12) dissolved O_2 probe, (13) temperature probe and, (14) bioreactor sampling port

7. Conclusion and perspective

The highly aggressive brain cancer, hGBM, is thought to arise from a sparse population of cells within these tumours, with stem cell-like characteristics, called CSCs. Hence, the development of effective cancer therapies may rely on developing methods that specifically target these cells. However, the scarcity of CSCs *in vivo* represents a major impediment to such research, as there is an insufficient supply for basic biochemical and genetic analyses. It is therefore necessary to develop a bioprocess to expand CSCs *in vitro* in a controlled environment. Our objective was to produce a large amount of hGBM-derived brain CSC tissue in computer-controlled suspension bioreactors in order to facilitate the development of novel oncolytic therapeutics. In this chapter we focused on two areas of our research: - (1) the development of a culture medium to successfully grow the GBM-derived CSC tissue and, (2) the development of bioprocess conditions to grow GBM-derived CSC tissue in computer-controlled suspension culture. We employed successfully a serum-free medium (PPRF-h2), developed in our laboratory (Baghbaderani et al., 2010) for the expansion of human neural precursor cells to expand GBM-derived CSC tissue. Using this medium we were able to isolate and support the expansion of GBM-derived CSC tissue from primary tumours (Panchalingam et al., 2010). We also investigated a number of key bioengineering parameters on the expansion of GBM-derived CSC tissue within well-mixed suspension bioreactors. Through the refinement of these bioprocess parameters, we successfully expanded GBM-derived CSC tissue to large cell numbers in suspension bioreactors, while showing that this expanded GBM-derived tissue retained similar genomic characteristics to the initial cell population (Panchalingam et al., 2010). The bioreactor protocols we developed were applied to the expansion of hGBM-derived CSC tissue in computer-controlled suspension bioreactors, which we have shown are able to control our process within very strict limits. The use of a computer-controlled bioreactor process to expand hGBM-derived CSC tissue can play an important role in the development of novel drug treatments by ensuring a continuous homogenous supply of tissue for genomic analysis and high-throughput drug screening.

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9. References

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Cancer Stem Cells: The Role of the Environment and Methods to Identify Them

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1. Introduction

The study and investigation of cancer stem cells (CSCs) or tumour initiating cells (TICs) have received enormous attention over the last 15 years. CSCs are rare, quiescent and capable of self-renewing and maintaining tumour growth and heterogeneity. Better understanding of CSCs will lead to a new era of both basic and clinical cancer research, re-classification of human tumours and development of novel therapeutic strategies. Therefore, the biological properties of CSCs, the relevance of CSCs to cancer therapy, methodologies to identify them are essential in order to address real and efficacious therapeutic strategies to eradicate the cancer.

Primary tumours are responsible for 10% of cancer deaths. In most cases, the main cause of mortality and morbidity is the formation of metastases in sites distant from tissue in which the primary cancer is formed. The cancer cell detach from primary tumour and, through blood and/or lymphatic vessels, colonises new sites in which forms the secondary tumour. Accumulating evidences suggest that a subpopulation of tumour cells with distinct stem-like properties is responsible for tumour initiation, invasive growth and possibly dissemination to distant organ sites (Reya et al., 2001; Brabletz et al., 2005). These few cells can divide asymmetrically, producing an identical daughter cell and a more differentiated cell, which, during their subsequent divisions, generate the vast majority of tumour bulk (Caussinus & Gonzalez, 2005; Clevers, 2005). Many names have been used to identify this subpopulation, but the term "Cancer Stem Cells" (CSCs) has received wide acceptance.

CSCs hypothesis states that the cancer stem cell is a cell within a tumour possessing the capacity of self-renewal and to cause the heterogeneous lineages of cancer cells that comprise the whole tumour (Ailles & Weissman, 2007; Lobo et al., 2007). Experimentally, this population is identified by its ability to form new tumours through serial transplantations in immunodeficient non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice, re-establishing tumour heterogeneity (Sarry et al., 2011).

There are two basic topics that underline the hypothesis that CSCs may originate from normal tissue stem cells. First of all, CSCs share many features with normal stem cells, including self-renewal, differentiation, drug resistance and migration capacity. Secondly, the longevity of stem cells make them susceptible to accumulate genetic and epigenetic damages in such a way to make them good candidates for the emergence of the neoplastic transformation.

Existing therapies have enhanced the length of survival after diagnosis of cancer, but completely failed in terms of recovery. Cancer therapy failures may be due to inefficient effects of current therapy upon potentially quiescent CSCs, which remain vital and retain the capacity to regenerate the tumour (Dean et al., 2005). In most cases, current therapeutic strategies are developed to target the bulk of cancer and likely do not eradicate CSCs completely. CSCs are more resistant to therapies, due to survival advantage with increased anti-apoptotic activities and drug resistance due to increased levels of drug efflux pumps such as BCRP (breast cancer resistance protein) and MDR (multi-drug resistance) complexes (Steinbach & Legrand, 2007; Chuthapisith et al., 2010; Dean et al., 2005).

Therefore, the rarity of CSCs will require therapeutic strategies different from conventional ones. Specific recognition of CSCs from the tumour mass will be the first challenge (Bao et al., 2006; Woodward et al., 2007). The identification of CSCs-specific antigens may help develop specific targeting. Since the origins of CSCs vary from cancer to cancer, the development of therapeutic strategies targeting different CSCs populations will also be necessary. Future therapeutic strategies will need to integrate inhibition of these resistant mechanisms with CSCs killing components. Finally, multiple pathways/mechanisms will likely need to be targeted together for their effective elimination. Therefore, with further improvements in understanding of CSCs biology, we will be able to develop better diagnostic and therapeutic methodologies, with which to classify, treat and cure cancer.

Research on CSCs has its roots in the second half of the nineteenth century when, in 1858, Virchow (Virchow, 1858.) argued that all cells come from other cells, and all organisms are made up of cells providing scientific basis for cancer origin. With Virchow, the concept of cellular hierarchy, a column in the investigation of the cancer stem cells, was born. In 1875, after the birth of experimental embryology, Cohnheim (Cohnheim, 1875) hypothesized that stem cells 'misplaced' during embryonic development were the source of tumours that formed later in life. The first experimental evidence of the existence of normal hematopoietic stem cells was supplied by Till & McCulloch in 1961 (Till & McCulloch, 1961). They eradicated the blood system of recipient mice with whole-body ionizing radiation and then injected donor bone marrow cells into the tail vein of irradiated mice to assay how many cells were required to restore blood production. They detected colonies of blood cells in the spleens of the recipient mice containing all the different mature blood cells. The same results were obtained with transplantations of spleen colonies into secondary recipients. On the basis of these studies on the normal blood system hierarchy, the framework for investigation of cancer stem cell was set. Bruce and Van Der Gaag (Bruce & Van Der Gaag, 1963) demonstrated that only approximately 1% of transplanted malignant blood cells could form colonies in the spleen of mouse as well as Hamburger and Salmon (Hamburger & Salmon, 1977) showed that only a small subset of tumour epithelial cells could produce in vitro colonies. Since then, researchers speculated that these cancer cells were cancer stem cells. Later studies involving teratocarcinomas further showed that a single tumour cell can give rise to a new tumour and generate heterogeneous progeny, providing strong evidence for the clonal origin and self renewal of tumours (Makino, 1956; Bruce, 1963; Lewis, 1964). During the twentieth century, much attention was placed both on physical and chemical carcinogenesis and genetic mechanisms that underlie cancer development. In the 1980s, Fialkow (Fialkow, 1981, 1990.) demonstrated that a single progenitor-cell gives rise to clones that sequentially acquire additional mutations and generate a tumour both in Chronic Myelogenous Leukemia (CML) and Acute Lymphoblastic Leukemia (ALL). Although all these studies, the work that laid the foundations for all subsequent cancer stem cells

research was provided by John Dick in 1997 (Bonnet & Dick, 1997). Dick and co-workers showed that, in human acute myeloid leukaemia (AML), a rare malignant cell with ability to repopulate the entire original disease over serial transplantations, implying self-renewal and capacity to differentiate, was only found within the immature $CD34^+CD38^-$, and not $CD34^+CD38^+$, subpopulation (Bonnet & Dick, 1997; Lapidot et al., 1994). This work represented the foundation from which started the research on CSCs in both hematologic malignancies and solid tumours.
The first identification of CSCs in solid tumours was made by Al-Hajj in 2003 (Al-Hajj et al., 2003) who identified and isolated CSCs from breast cancer by using CD44 and CD24 markers.
Since, CSCs have been identified in a variety of solid tumours such as glioblastomas (Singh et al., 2003), melanoma (Fang et al., 2005), sarcoma (Tirino et al., 2008; Tirino et al., 2011), prostate (Collins et al., 2005), ovarian (Bapat et al., 2005), gastric (Takaishi et al., 2009) and lung cancers (Eramo et al., 2008; Tirino et al., 2009) as reassumed in Table 1.

Tumour type	CSCs phenotype	References
Breast	CD44+CD24-/low	Al-Hajj, 2003
Brain	CD133+	Singh, 2003
Melanoma	CD133+ CD20+	Fang, 2005
Sarcoma	CD133+	Tirino, 2008, 2011
Prostate	CD44+ $\alpha2\beta1$+ CD133+	Collins, 2005
Ovarian	Spheres	Bapat, 2005
Gastric	CD44+	Takaishi, 2009
Lung	CD133+	Eramo, 2007; Tirino, 2009

Table 1. CSCs identification in human solid cancer

2. Normal and cancer stem cells

Human development follows a predetermined program by which the zygote develops into a multicellular organism. The zygote gives rise to a totipotent ball of cells that further differentiates into the three germ layers: endoderm, ectoderm, and mesoderm (McClay, 1991) developing in turns into all tissues in the adult body. Tissues in which malignancies originate, such as the blood, brain, breast, skin, and gut, are organized as a cellular hierarchy with a small population of tissue-specific stem cells responsible for both development and maintenance/regeneration of tissues for the human lifetime (Cairns, 1981). A normal adult stem cell is defined as a somatic cell that can undergo extensive cell division and has the potential to give rise to both stem cells and cells that differentiate into specialized cells. Adult stem cells possess two unique characteristics: multipotency, which allows mature cells to compose specific organs or tissue, and self-renewal, which supplies an organ with an adequate number of cells to maintain the organ's function.
The first characteristic is the self-renewal, a special cell mitotic division, that enables a stem cell to produce another stem cell with essentially the same development and replication potential. The ability to self-renew enables expansion of the stem cell compartment in response to systemic or local signals, which trigger massive proliferation and maintenance of a tissue-specific undifferentiated pool of cells in the organ or tissue.

Differentiation is the second function of a stem cell and involves the production of daughter cells that become tissue-specific specialized cells. For example, the hierarchy in the blood system leads stem cells that first differentiate into transiently amplifying progenitors cells, then these cells rapidly proliferate for a short time and produce terminally differentiated cells, such as macrophages or basophiles.

Cancer stem cells have been defined as 'a cell within a tumour that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour' (Clarke & Fuller, 2006). These two definitive biological properties are what make the CSC the prime candidate for initiation of relapse, thereby becoming a crucial target for the development of novel therapies.

The CSC is commonly assumed to be developed from a normal tissue stem cell and, as such, thought to be the cell from which a malignancy originated. There is an ongoing debate over whether CSCs represent a mature tissue stem cell which has undergone malignant change or whether more differentiated cells re-initiate a 'stemness' programme as part of, or following, malignant transformation. Despite the multitude of regulatory systems that prevent abnormal proliferation during biologic normal processes, mutations that result in aberrant mitoses can occur. Most of the mutations leading to cancer regards the cell division, DNA damage, and aberrant signal transduction pathways. Stem cells may be preferential targets of initial oncogenic mutations because in most tissues in which cancer originates, they are the only long-lived cell populations and are therefore exposed to more genotoxic stresses than their shorter-lived, differentiated progeny (Pardal et al. 2003, Reya et al. 2001).

The cancer stem cell theory proposes that tumours have a cellular hierarchy that is a caricature of their normal tissue counterpart because they reflect the multipotency of the originally transformed cell.

The basis for this functional heterogeneity has been explained by two models. The stochastic model predicts that a malignancy is composed of a homogeneous population of cells, which generate their heterogeneity in response to particular combinations of endogenous and exogenous factors. Endogenously these would include gene dosage effects, transcriptional and translational control mechanisms, whereas exogenously cytokine concentrations, cell–cell interactions and particularly niche environment would all be important. Therefore, all tumour cells may give rise a tumour.

The CSCs model predicts that a malignancy is organised in a manner analogous to the normal tissue hierarchy with cancer/tissue stem cells able to produce identical daughter stem cells with self-renewal capacity, and committed progenitor daughter cells with limited, although potentially still significant, potential to divide. Therefore, the CSC model has a rare CSC at the apex, by which tumour heterogeneity originate as result of a random process of genetic changes and selective advantage. Although this, in the stochastic model, stemness exists as a functional phenotype, which could be shown by any member of the malignant population given the appropriate endogenous and exogenous factors. Most plausibly, having occupied a suitable niche, a cell now able to express its self-renewal programme and producing daughter cells which differentiate to populate the bulk malignancy, can become a CSC. The stochastic model does not yet predict whether stemness is found truly within each population, or whether cells first undergo a process of de-differentiation to a more tissue specific stem cell-like phenotype, reacquiring stemness. This plasticity within a cell lineage, between the CSC and non-CSC compartments, is known as bi-directional interconvertibility (Gupta et al., 2009). Another model that we consider is one in which cancer cells, stem cells included, are subject to a process known as clonal evolution. In clonal evolution, new clones

continuously develop, emerging with new genetic, and potentially epigenetic, changes. Environmental pressures result in constantly adapting cancer cell populations. These adaptations may change proliferation, metastatic potential or drug resistance, for example. It is also possible that evolution could generate novel clones with self-renewal potential, providing a rather more 'hard-wired', albeit evolving, route to the development of CSCs than does the process of interconvertibility described above as shown in primary and relapsed leukaemias by Mel Greaves (Greaves et al., 2010), albeit at the level of a limited number of known targets.

3. Cancer stem cells and niche

Stem cells are found in specific areas of an organ where a special microenvironment called the niche maintains stem cell functions. Stem cells and niche cells interact with each other via adhesion molecules and paracrine factors. They exchange molecular signals that maintain the unique characteristics of the stem cells. Therefore, stem cells, their progeny, and elements of their microenvironment make up an anatomical structure that coordinates normal homeostatic production of functional mature cells by cellular mechanisms that regulate the balance of self-renewal and differentiation. The microenvironment surrounding normal and cancer stem cells, which provides the stem cell niche, plays multiple roles including as a mechanical anchorage for the stem cells and in cross-talk communication mediated by direct contact and/or indirect extracellular factors.

For instance, Wnt ligands are produced and released from both stem cells and niche cells, BMP and Sonic hedgehog (Shh) are released from niche cells and epithelial cells respectively, and Notch signaling is transmembranously transmitted between neighboring. The microenvironment may also provide signaling via the cell receptor integrin as suggested by its expression.

The existence of a stem cell niche, or physiological microenvironment, has been studied for mammalian adult stem cells in the intestinal, neural, epidermal, and hematopoietic systems (Iwasaki & Suda, 2009; Shaker & Rubin, 2010).

Bone marrow (BM) hematopoietic stem cells (HSCs) are the best characterized stem cell population. Single HSCs are multipotent, highly self-renewing, and cycle with slow kinetics. Bone and marrow are intrinsically linked with HSCs, and their primitive progeny is located proximal to the endosteal surface of trabecular bone (Fig. 1).

Studies have shown that osteoblast (OB) cells are required for this localization. Genetically engineered increases in OB numbers lead to elevated HSC numbers without changes in committed progenitor populations. The Bmpr1a and activated parathyroid hormone–related protein receptor (PPR) studies provide mechanistic insights into OB-mediated HSC expansion. The Bmpr1a studies identified a specific subset of N-cadherin–expressing OBs that form an N-cadherin/β-catenin adherens complex with HSCs, perhaps mediating the attachment or adhesion of HSCs within their niche. N-cadherin is negatively regulated by c-Myc in differentiating HSCs, perhaps promoting displacement from the endosteum (Wilson, 2004). In the PPR studies, Notch signaling was implicated, because the Notch ligand Jagged 1 was highly expressed in OBs and Notch activated in HSCs. Also, Wnt protein was shown to promote HSC proliferation (Reya et al., 2003; Willert et al., 2003), and now, an additional study has shown that Notch and Wnt inputs are integrated by HSCs. Although this, the exact roles of Wnt and Notch signaling will require further analysis. The maintenance of HSC levels by these Wnt, Notch and Hedgehog signaling pathways could lie in controlling

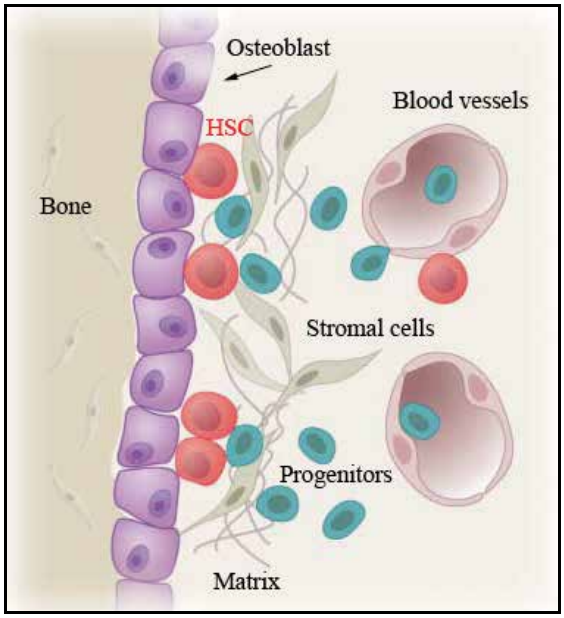

Fig. 1. Overview of Bone Marrow niche in which HSC are indicated. (Moore, Lemischka, 2006)

asymmetric cell division. Other mediators of HSC self-renewal have been identified; such as p21 (Cheng et al., 2000), p18 (Yuan et al., 2004) and bmi-1 (Park et al., 2003); but how these are controlled by extrinsic signals from the niche has not been determined. In addition, HSCs can be found in tissues that have no OBs (Taniguchi, et al., 1996). Thus, although BM-HSC niches are at least in part composed of OBs, other cell types may also provide this function. The contribution of other cellular elements, such as stromal cells or perivascular cells, is yet to be defined. It is has been shown that HSCs can be recruited to a ''vascular niche'' in the BM (Heissig et al., 2002). Such vascular structures could serve as components of extra-medullary niches. One intriguing study has demonstrated that HSCs express a calcium sensing receptor. Stem cells lacking this receptor fail to localize to the endosteal niche and do not function normally after transplantation (Adams et al., 2006). This study highlights the importance of the ionic mineral content of the bone itself and of the bone-derived matrix in the lodgment and retention of HSCs within the endosteal niche.

Another example of niche is the intestinal niche. The epithelial villus/crypt structure and its surrounding pericryptal fibroblasts and mesenchyme in the small intestine make up an anatomical unit that generates four cell lineages: absorptive enterocytes and the goblet, enteroendocrine, and Paneth cells of the secretory lineage. The crypt is a contiguous pocket of epithelial cells at the base of the villus. Intestinal stem cells (ISCs) and transit amplifying (TA) cells within the crypt regenerate the entire villus every 3 to 5 days (Potten & Loeffler, 1990) (Fig. 2).

Genetic marking shows that crypts are derived from individual or few ISCs and that each villus is the product of cells from several adjacent crypts (Gordon et al., 1992). There are four to six ISCs per crypt that are located in a ring about four cell diameters from the crypt bottom. Progeny of activated ISCs migrate upwards to become TA cells. When they reach the top of the crypt, TA cells stop proliferating, differentiate, and assume their appropriate positions within the villus structure. As such, proper cell-fate decisions are organized within

the microanatomy of the crypt structure. Asymmetric cell division mediated by oriented mitotic planes, together with defined migratory activities within the overall crypt structure, could produce the correct localization of distinct differentiated cell types. Although asymmetric cell division along the vertical crypt axis is an attractive mechanism, this process has yet to be rigorously demonstrated in the ISC system. Mesenchymal cells surround the crypt. It is likely that the mesenchymal signals that mediate different cell fates along the vertical crypt axis are spatially organized into distinct domains. The canonical Wnt pathway regulates ISCs. A Wnt gradient is predicted by the distribution of nuclear versus cytoplasmic β-catenin along the crypt axis (van de Wetering et al., 2002). A comprehensive study has now shown that Wnt signaling components are expressed by both crypt epithelial cells and surrounding mesenchymal cells, predicting an even broader role for this pathway in normal homeostasis as indicated by genetic studies (Gregorieff et al., 2005). There is also evidence that Wnt inhibitors such as Dkk3 may be expressed in a graded manner in this tissue, suggesting an intricate quantitative balance between positive and negative regulators of this pathway (Byun et al., 2005).

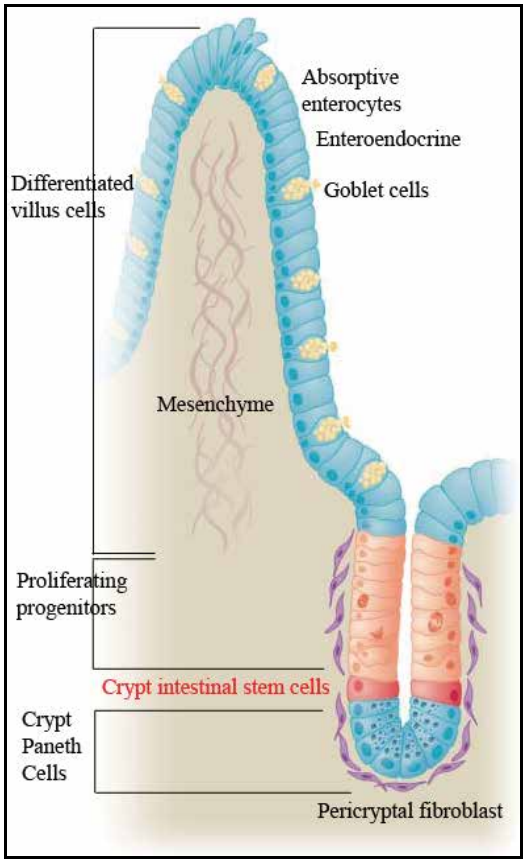

Fig. 2. Overview of intestinal niche in which the epithelial villus/crypt structure and its surrounding pericryptal fibroblasts and mesenchyme in the small intestine make up an anatomical unit that generates four cell lineages: absorptive enterocytes and the goblet, enteroendocrine, and Paneth cells of the secretory lineage. (Moore, Lemischka, 2006)

In light of these findings, it has been proposed that a "cancer stem cell niche" also exists and that interactions with this tumour niche may specify a self renewing population of tumour cells. The surrounding microenvironment (stromal fibroblasts, adipocytes, and endothelial cells, as well as the extracellular matrix) and the immune system are known to play important roles in cancer progression.

As already described above, the two cardinal characteristics of stem cells are the capacity to self-renew, in order to produce stem cells, and to differentiate in order to obtain the full repertoire of specialized cells that comprise the tissue in question.

Achieving a delicate balance between these two opposing processes is critical in the adult organism for maintaining proper tissue homeostasis and for repair and regeneration of tissues after injury. Excessive differentiation at the expense of self-renewal, for instance, can deplete the stem cell pool, whereas excessive self-renewal could lead to aberrant expansion and even tumorigenesis.

Niche cells provide a sheltering environment that sequesters stem cells from differentiation stimuli, apoptotic stimuli, and other stimuli that would challenge stem cell reserves. The niche also safeguards against excessive stem cell production that could lead to cancer. Stem cells must periodically activate to produce progenitor or transit amplifying (TA) cells that are committed to produce mature cell lineages. Thus, maintaining a balance of stem cell quiescence and activity is a hallmark of a functional niche. In fact, it is widely known that in general stem cells are in a quiescent state (G_0 phase in the cell cycle) and that this quiescence prevents the stem cells from entering into the cell cycle and undergoing differentiation. Different mechanisms ensure a proper balance between the production of stem cells and/or progenitor cells and differentiated cells. These could include Wnt, Notch, and Hedgehog signaling. Disregulation of these pathways can lead to tumour formation. Cancer and normal stem cells have much in common with regard to the maintenance system within their niches.

A representative example of cancer stem cells niche is observed in acute myeloid leukemia (AML) and its niche in BM. Dick and colleagues showed that anti-CD44 antibody treated non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice transplanted with AML cells exhibited a significantly lower rate of disease onset (Jin et al., 2006). Also, Van Etten and colleagues showed that there was impaired induction of chronic myeloid leukemia (CML)-like myeloproliferative disease among recipient mice that received transplanted *BCR–ABL1*-transduced CML progenitors from CD44-mutant donors (Krause et al., 2006). These results indicate that for both AML and CML, CD44 is essential for the homing and engraftment of the cancer stem cells to the niche. In other words, CD44-expressing leukemic stem cells adhere to the niche and bind to hyaluronic acid expressed by cells on the surface of sinusoidal endothelium or endosteum in BM; this binding is crucial for the niche's maintenance of the stem cells. Interestingly, this molecular mechanism resembles that of the interaction between normal HSC and the vascular niche described earlier.

Gilbertson and colleagues showed that brain tumour cells coexpressing Nestin and CD133, the fraction believed to contain the cancer stem cell, were found near the capillaries in the brain tumour (Calabrese et al., 2007). When these cells were cocultured, the cancer stem cells selectively adhered to the endothelial cells. This suggested that the endothelial cells secreted factors necessary to maintain the cancer stem cells and showed that cancer stem cells of brain tumours rely on endothelial cells, which form a vascular niche that maintains the capacity of the cancer stem cells for self-renewal, differentiation, and proliferation. In addition to its role in brain tumours, CD133 has been extensively studied in other kinds of

cancer, such as colon, prostate, and pancreatic cancers, and is now considered to be a tumour marker for those cancers (Table 1). Despite the growing interest in CD133, the functional role of CD133 itself remains unclear. CD133 is a cholesterol-binding pentaspan membrane glycoprotein and is associated with a membrane microdomain. The microdomain in the stem cells or progenitors has been proposed to be a carrier of important molecular factors necessary for the maintenance of stem cell properties. Therefore, it is hypothesized that the localized distribution of CD133 during cell division might reflect the localized distribution of the microdomains that determine the daughter cell's fate, that is, whether it remains as a stem cell or undergoes differentiation (Bauer et al., 2008).

Li and Neaves studied the dependence of stem cells on their niches and they hypothesized that the behaviors of cancer stem cells and normal stem cells are regulated by the niche to different degrees (Li & Neaves, 2006) The cancer stem cell is engendered by an intrinsic mutation that leads to its high proliferation. This highly proliferative state itself can alter the signaling balance between the niche and stem cells. Namely, the characteristics of the niche that function to maintain quiescence become relatively ineffective, and the characteristics of the niche that function to support proliferation and differentiation become more dominant. This model is supported by some clinical symptoms, one of which is the blast crisis of CML. It is important to note that many signaling pathways involved in the interaction between normal stem cells and their niches are also involved in the interactions between cancer stem cells and their niches, and can play a role as promoters of tumourigenesis and cancer proliferation. An identical set of proteins under slightly different conditions can deliver totally different results. Thus, the purpose of the niche is not only the cradling of existing cancer stem cells, but also the cradling of future incoming cancer stem cells. The niche is constantly sending passive signals of invitation to remote cancer stem cells. Matrix metalloproteinases (MMP) are well-known factors, not only for their contribution to the repair of inflammation and wounds, but also for their involvement in cancer invasion and metastasis. For example in lung cancer, vascular endothelial growth factor secreted by primary cancer cells induces specific MMP9 expression in lung endothelial cells and macrophages via vascular endothelial growth factor receptor (VEGFR) tyrosine kinase, resulting in the formation of the cancer stem cell niche. This means that the cancer cells can produce their own favorable microenvironment, the future cancer stem cell niche, from a distance by secreting factors that influence the protein composition at that site. Bone metastasis of prostate cancer has been shown to be supported by urokinase-type plasminogen activator (uPAR) or prostate specific antigen (PSA) secreted by prostate cancer cells through alteration of the growth factors in the bone microenvironment, thus enhancing the proliferation of the osteoblasts that serve as the cancer stem cell niche (Logothetis et al., 2005). Lung metastasis of breast cancer via secreted protein acidic and rich in cysteine osteonectin or MMP2 has also been found to be based on this mechanism (Minn et al., 2005). From both scientific and clinical viewpoints, the biology of the normal and cancer stem cell and their niche is expected to be one of the most promising fields of research to address an efficacy therapeutic strategy in order to cure and heal the cancer.

4. Signaling in normal and cancer stem cells niche

4.1 Wnt/β-catenin pathway

Wnt/β-catenin pathway is a conserved molecular system that plays a major role in development and homeostatic tissue self-renewal (Rattis et al., 2004). This pathway takes its

name from the wingless gene in Drosophila, homologue of human gene int-1 and was characterized for the first time by Rijsezijk et al., in 1987. The Wnt ligands activate a signaling pathway that induces changes in gene expression, physiology, cell adhesion, and polarity of stem cells. In mammals, the Wnt protein family comprises 19 highly conserved molecules. At least three pathways have been described, including the main one that interacts with two distinct families of cellular receptors: the Frizzled family of receptors (Fz) and the family of proteins related to the LDL receptor (LRP). In a physiological state, β-catenin level remains low due to constant turnover mostly by the destruction complex APC/Axin/GSK3b. Phosphorylation by this complex causes β-catenin to be degraded by the proteasome. When the Wnt binds to the cell surface receptor Frizzled and activates disheveled (Dsh), glycogen synthase kinase 3b (GSK3b) is dissociated from the destruction complex. As a result, free β-catenin accumulates and translocates into the nucleus. There, β-catenin binds to T cell factor (TCF) releasing it from a repressed state and initiates transcription of its target genes (Fig. 3).

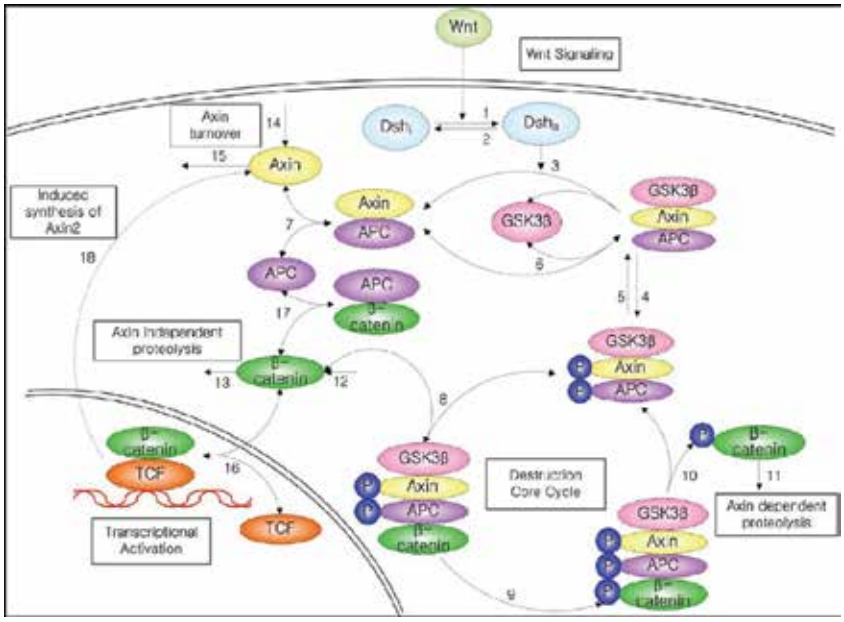

Fig. 3. Brief overview of Wnt/β-catenin pathway (Cho et al., 2006)

C-myc, cyclin D1, MMP7, and CD44 are some of the known target genes that may be relevant for a tumour. Among the factors that regulate the release of Wnt family proteins, there are also the BMPs. For example, intestinal tissue is produced by mesenchymal cells outside the crypts. A defect in this setting may cause an expansion of stem and progenitor cells that leads to a state-onset juvenile polyposis. Wnt proteins may also play a role through activation of II kinase calmodulin and protein kinase C, causing an increase of intracellular Ca2 + or Jun N-terminal kinase (JNK), under the control of cytoskeleton rearrangements of cell polarity.

Recently in the development of research on "cancer stem cells" a great interest in the Wnt pathway has taken mainly regarding the regulation mechanisms of self-renewal of stem cells, repair and tissue regeneration that are involved in cancer development when Wnt

signaling is activated in aberrant manner (Polakis, 2000; Reya & Clevers, 2005). In this context, recent experimental evidence together with encouraging clinical trials have focused attention on the role of molecular signaling pathway in tumours.
In summary, the organs in which Wnt proteins influence the process of stem cell self-renewal are the same organs that are studied as Wnt-dependent tumours. In fact, numerous studies have shown aberrant activation in many human tumours, such as colorectal, melanoma, head and neck and leukemia. This aberrant activation may be caused by mutations and/or deregulation of many different components of the Wnt signaling.

4.2 Notch pathway

Notch is known to promote the survival and proliferation of non neoplastic neural stem cells and to inhibit their differentiation.
Notch signaling mechanism consistis of four membrane receptors (called Notch1, 2, 3 and 4) and five ligands (eg Delta-like 1, Delta-like 2, Delta-like 4, Jagged1 and Jagged2). Notch receptors are eterodimeric proteins formed by NEC (extracellular subunit) and NTM (transmembrane subunit). Interaction of Notch receptors (Notch 1 to 4) with their ligands (Delta like 1, -3, -4, Jagged-1 and -2) leads to cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD) that migrates into the nucleus (Weinmaster, 1998). In the nucleus, NICD associates with a transcription factor, RBP-Jk (also known as CSL for *C*BF1/*S*u(H)/*L*ag-1) (Weinmaster, 1998; Artavanis-Tsakonas et al., 1999; Egan et al., 1998; Greenwald, 1998; Mumm & Kopan, 200) and activates transcription from the RBP-Jk DNA binding site (Fig.4). The NICD–RBP-Jk complex upregulates expression of primary target genes of Notch signaling, such as hairy and enhancer of split (HES)-1, -5, -7 and more recently isolated HES-related repressor protein (HERP)-1 to -3 in mammals (Davis & Turner, 2001; Iso et al., 2003). The HES and HERP families are basic helix–loop–helix-type transcriptional repressors and appear to act as Notch effectors by negatively regulating expression of downstream target genes (Chen et al., 1997; Ishibashi et al., 1995; Ohsako et al., 1994; Van Doren et al., 1994). Thus, many ligands, receptors, and effectors are involved in this pathway.

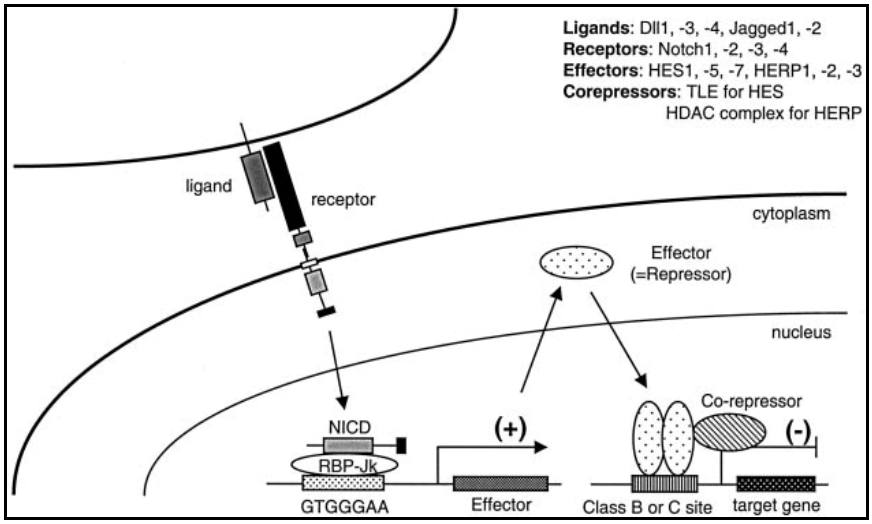

Fig. 4. Brief overview of Notch pathway (Iso et al., 2003)

Through this mechanism, the Notch signals provide an indication on how much of ancestors will remain undifferentiated or differentiate into different cell types. This type of signal is present in many different cell lines, including the nervous system and T lymphocytes (Hajdu et al., 2010).

4.3 Hedgehog pathway

Hedgehog (hh) signaling plays a role in many processes during embryonic development and remains active in the adult where it is involved in the maintenance of stem cell populations. Here, aberrant Hedgehog signaling in some cases can lead to certain forms of cancer. The hedgehog gene was originally identified in flies, where it is first required for patterning of the early embryo (Nusslein-Volhard & Wieschaus, 1980). In mammals, the Hh family consists of three different members, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) (Riddle et al., 1993; Roelink et al., 1994). Shh is the most broadly expressed member and is involved in the patterning and growth of a large variety of organs, including the brain, skin, lung, prostate, gastrointestinal tract, and skeletal system (Pasca di Magliano & Hebrok, 2003). Hedgehogs are secreted glycoproteins, which undergo posttranslational modifications, including autocatalytic cleavage and lipid modification, before binding to a transmembrane receptor in responding cells. Hh ligands act through the transmembrane proteins Patched1 (Ptch1) and Smoothened (Smo) to trigger an intracellular signal transduction pathway that results in the activation of the Gli zinc finger transcription factors (Fig. 1). The current model of ligand receptor signaling proposes that in the absence of Hh ligands, Ptch1 blocks the function of Smo. The binding of Hhs to Ptch1 releases this basal repression of Smo. As a consequence, Smo initiates an intracellular signaling cascade that is regulated by a multimolecular complex, leading to the action of the Gli proteins (Fig. 5).

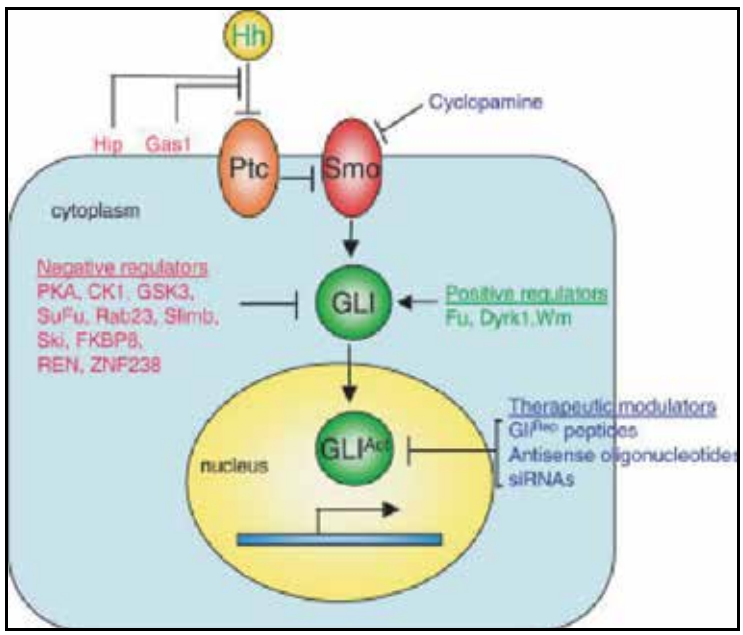

Fig. 5. Brief overview of Hedgehog pathway (Stecca et al., 2005)

5. Detection, isolation and characterization methodologies of cancer stem cells

5.1 Current methodologies

The CSCs can be identified and isolated by four main methodologies:

1. isolation of CSCs by flow cytometry according to CSC-specific cell surface markers (Al-Hajj et al., 2003; Singh et al., 2003; Fang et al., 2005; Tirino et al., 2008; Collins et al., 2005; Bapat et al., 2005; Takaishi et al., 2009; Eramo et al., 2008; Tirino et al., 2009; Dean et al., 2005);
2. detection of side population phenotype by Hoechst 33342 exclusion (Goodell et al., 1996; Song et al., 2010; Fukuda et al., 2009; Moserle et al., 2008);
3. ability to grow as floating spheres (Rybak et al., 2011; Zhong et al., 2010);
4. aldehyde dehydrogenase (ALDH) activity (Ma & Allan, 2010; Awad et al., 2010).

None of the methods above mentioned are exclusively used to isolate the CSCs, highlighting the imperative to delineate more specific markers or to use combinatorial markers and methodologies. Therefore, the "cancer stem cell" will be the cell that shows the following characteristics:

- expression of stemness markers;
- side population phenotype;
- capable of forming spheres;
- capable to form new tumours in mice.

5.2 Isolation and *in vitro* expansion of cells from tumour specimens.

5.2.1 Glioblastoma primary cell culture

Biopsy specimens are put in ice-cold Leibowitz-15 medium (L-15), washed in L-15 and mechanically dissociated using 2 scalpels. The dissociation into single cells is achieved by incubation in trypsin-EDTA solution and mechanical dissociation. Thereafter, trypsin-EDTA solution is blocked using 2 mg/mL BSA and washed in L-15 twice. Cells are cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, at 37 °C, 5% CO_2, in humidified atmosphere.

5.2.2 Breast primary cancer cell culture

Breast lesions, within 30 minutes of surgery, are immediately mechanically disaggregated and then enzymatically digested in a 1:1 solution of III Type collagenase/hyaluronidase. The digestive solution is incubated at 37°C for 4-18 hours on shaking bath. After filtration through a 30 µm pore filter, the cells are plated in DMEM or RPMI at 10% FBS, at 37 °C, 5% CO_2, in humidified atmosphere.

5.2.3 Lung primary cancer cell culture

Surgical specimens are washed several times and left overnight in DMEM–F12 medium supplemented with high doses of penicillin/streptomycin and amphotericin B to avoid contamination. Tissue dissociation is carried out by enzymatic digestion by 20 mg/ml collagenase II, for 2 h at 37°C (Eramo et al., 2008). Recovered cells (Fig. 6) are cultured in Bronchial Epithelial Cell Growth Medium or RPMI at 10% FBS, at 37 °C, 5% CO_2, in humidified atmosphere.

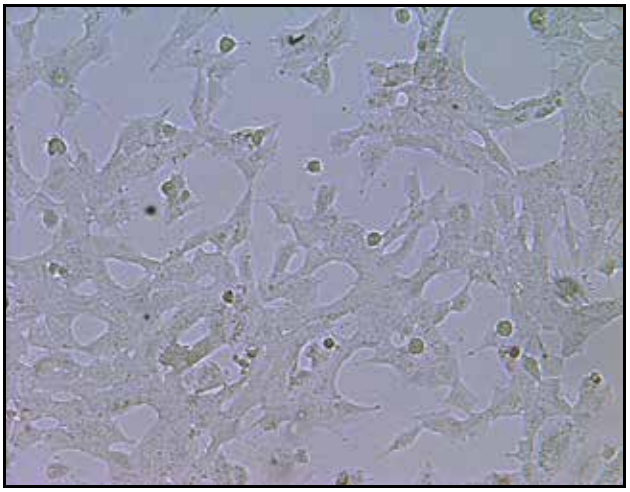

Fig. 6. Example of tumour lung cells obtained from lung biopsy (original magnification 100x)

5.2.4 Sarcoma primary cell culture

Tumours biopsies are dissected, minced and washed in PBS. After all visible clumps are removed, the cells are digested with 10mg/ml Collagenase IV or I (soft tissue or bone sarcoma, respectively), and 3mg/ml Dispase at 37 °C over night. Then cells are washed with PBS twice and filtered through a 70-µm filter to generate cell lines. Cells are cultured in DMEM at 10% FBS at 37 °C, 5% CO_2, in humidified atmosphere.

5.3 Markers expression and cell sorting by flow cytometry

Within 7 days of primary cultures, cells are detached with 0.5% BSA and 2 mM EDTA in PBS, counted and washed in 0.1% BSA in PBS. At least 200,000 cells are incubated with 1 µg/ml of fluorescent-labelled monoclonal antibodies or respective isotype controls at 4 °C for 30 min in the dark. After washing, the labelled cells are analysed by flow-cytometry using a cytometry cell sorter. The same procedure is also performed on spheres. The antibodies used are: mouse anti-human CD133/2 PE, mouse anti-human CD326 (EpCAM) FITC and PE, mouse anti-human cytokeratin (CK3-3E4) FITC, mouse anti-human CD24 PE conjugated, mouse anti-human CD29 PE-CyTM^5, mouse anti-human CD44 FITC, mouse anti-human CD90 FITC conjugated and mouse anti-human CD45 CY (Fig. 7). CD133 positive and negative fractions are sorted. The purity of sorted populations is routinely 90%. Aliquots of $CD133^+$ and $CD133^-$ sorted cells are evaluated for purity by flow cytometry. $CD133^+$ and $CD133^-$ sorted cell populations are cultured in standard medium, used for *in vivo* and *in vitro* experiments and spheres formation assay.

5.4 Side population assay

Cells are resuspended at 2.0×106 cells/ml in pre-warmed standard culture medium and divided into two portions. A portion is treated with 50 µM verapamil and the other is left untreated. Both portions are incubated in standard culture medium with 5 µg/ml Hoechst 33342 for 90 minutes at 37°C on shaking bath. Mix the cells well, and place in the 37°C water bath for 90 minutes exactly. Make sure the staining tubes are well submerged in the bath water to ensure that the temperature of the cells is maintained at 37°C. Tubes should be

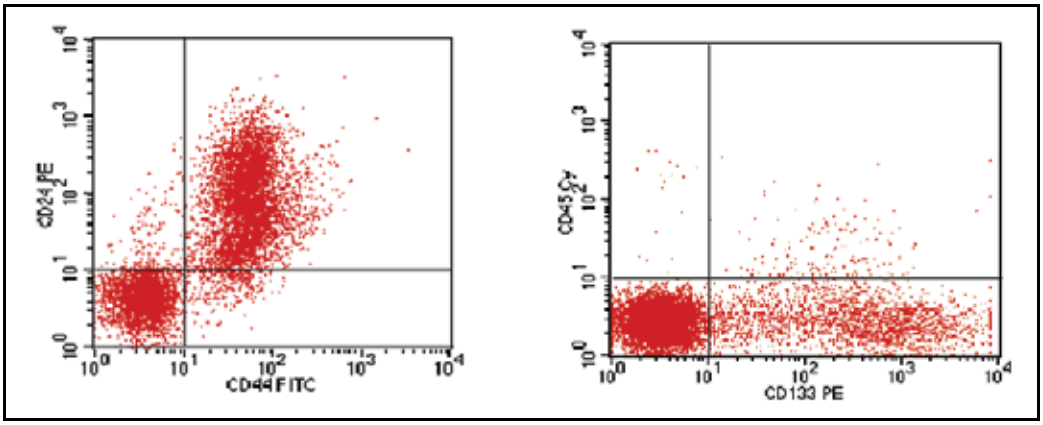

Fig. 7. Figure showing an example of cancer stem cells in breast cancer by expression of CD44 and CD24 and in lung cancer by expression of CD133

mixed several times during incubation. After 90 minutes, spin the cells down in the cold and re-suspend in cold PBS. All further proceedings should be carried out at 4°C to prohibit leakage of the Hoechst33342 dye. Add 2 µg/ml of 7-AAD or PI to the suspended cells and mix about 5 minutes before FACS analysis. This will allow for the discrimination of dead versus live cells as 7-AAD or PI permeates only cells that do not have an intact membrane. The Hoechst 33342 dye is excited at 350 nm ultraviolet and resultant fluorescence is measured at two wavelengths using a 424/44 BP and 675 LP filters for detection of Hoechst blue and red, respectively (Fig. 8). Side population (SP) positive and negative fractions are sorted. Aliquots of SP^+ and SP^- sorted cells are evaluated for purity by flow cytometry. SP^+ and SP^- sorted cell populations are cultured in standard medium, used for *in vivo* and *in vitro* experiments, analyzed for stemness markers and spheres formation assay.

5.5 ALDH activity

The ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) is used to isolate the population with a high ALDH enzymatic activity. Cells are suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 µmol/l per 1×10^6 cells) and incubated during 40 minutes at 37°C. As negative control, for each sample of cells an aliquot is treated with 50mmol/L diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. The sorting gates are established using as negative controls the cells stained with PI only. ALDH positive and negative fractions are sorted. Aliquots of $ALDH^+$ and $ALDH^-$ sorted cells are evaluated for purity by flow cytometry. $ALDH^+$ and $ALDH^-$ sorted cell populations are cultured in standard medium, used for *in vivo* and *in vitro* experiments, analyzed for stemness markers and spheres formation assay.

5.6 Spheres

5.6.1 Mammospheres

Single cells are plated at 1,000 cells/mL in ultra-low attachment plates (Corning) in serum-free DMEM-F12 supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 5 µg/mL insulin, and 0.4% BSA. Cells (Fig.8) grown in these conditions as non-adherent spherical clusters of cells (usually named "mammospheres") were enzymatically dissociated by incubation in a trypsin-EDTA

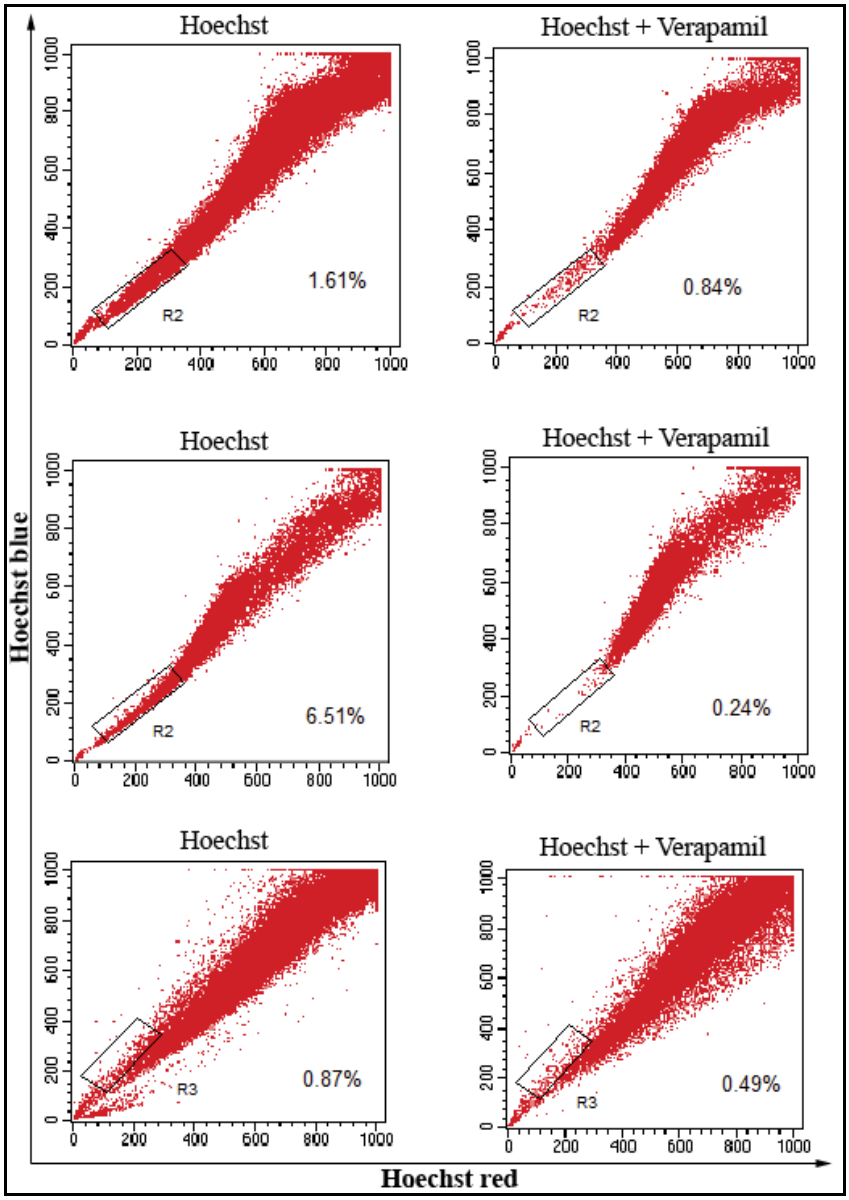

Fig. 8. Figure showing different samples in which side population is detected

solution or mechanically disaggregated every 3 days for 2 minutes at 37°C (Al-Hajj et al., 2003). Conversely, differentiation is induced by culturing mammosphere-derived cells for 8 days on collagen-coated dishes in DMEM-F12 supplemented with 10% FBS without growth factors.

5.6.2 Neurospheres

Tumour cells are resuspended in TSM consisting of defined serum-free neural stem cell medium, human recombinant EGF (20 ng/ml), bFGF (20 ng/ml), leukemia inhibitory factor

(10 ng/ml), Neuronal Survival Factor (NSF) (1x), and N-acetylcysteine (60 μg/ml). The cells are plated at a density of $3x10^6$ live cells/60-mm plate. Cells grown in these conditions as non-adherent spherical clusters of cells (usually named "spheres" or "neurospheres") are enzymatically dissociated by incubation in a trypsin-EDTA solution or mechanically disaggregated every 4 days for 2 minutes at 37°C (Singh et al., 2003). Conversely, differentiation is induced by culturing neurospheres-derived cells for 7 days on collagen-coated dishes in DMEM-F12 supplemented with 10% FBS without growth factors.

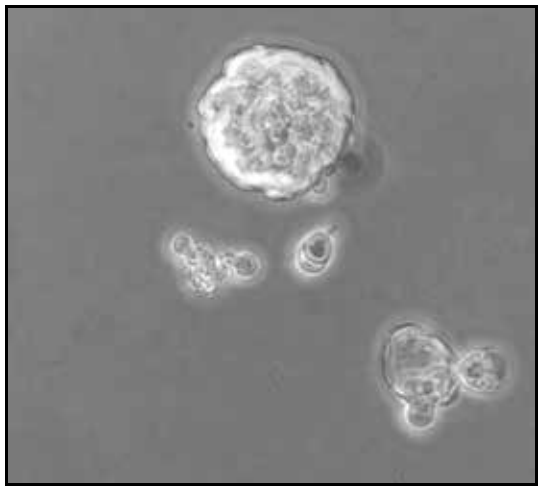

Fig. 8. Mammospheres (original magnification 40x)

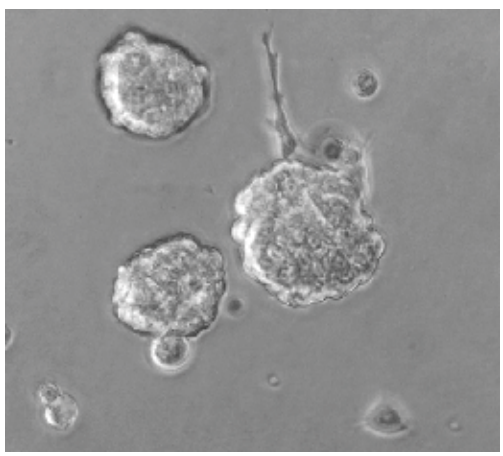

Fig. 9. Pneumospheres (original magnification 40x)

5.6.3 Pneumospheres

Tumour cells are cultured at clonal density in DMEM–F12 serum-free medium containing insulin (50μg/ml), apo-transferrin (100μg/ml), putrescine (10μg/ml), sodium selenite (0.03Mm), progesterone (2μM), glucose (0.6%), sodium bicarbonate (0.1%), BSA (0.4%), glutamine and antibiotics, and supplemented with 20μg/ml EGF and 10μg/ml bFGF. The

medium is replaced or supplemented with fresh growth factors twice a week until cells started to grow forming floating aggregates. Cultures are expanded by mechanical dissociation of spheres, followed by re-plating of both single cells and residual small aggregates in complete fresh medium. Cells (Fig. 9) grown in these conditions as non-adherent spherical clusters of cells (usually named "pneumospheres") were enzymatically dissociated by incubation in a trypsin-EDTA solution or mechanically disaggregated every 3 days for 2 minutes at 37°C (Eramo et al., 2007). Conversely, differentiation is induced by culturing pneumosphere-derived cells for 5 days on collagen-coated dishes in DMEM or RPMI supplemented with 10% FSB without growth factors. Another medium that are used to form pneumospheres is BEBM in ultralow attachment plates (Tirino et al., 2008).

5.6.4 Sarcospheres

Tumour Cells are plated at a density of 60,000 cells/well in 6-well ultra low attachment plates (Corning Inc., Corning, NY, USA) in DMEM/F12 cell medium, supplemented with 1% methylcellulose, progesterone (10 nM), putrescine (50 μM), sodium selenite (15 nM), transferrin (13 μg/ml), human recombinant insulin (10 μg/ml), human EGF (10 ng/ml) and human bFGF (10 ng/ml). Fresh aliquots of EGF and bFGF are added every day. After culture for 48–72 hours, spheres are visible at inverted phase-contrast microscope (Nikon TS 100, Nikon) (Fig. 4). Cells grown in these conditions as non-adherent spherical clusters of cells (usually named "spheres" or "sarcospheres") are enzymatically dissociated by incubation in a trypsin-EDTA solution every 3 days for 2 minutes at 37°C (Tirino et al., 2008). Conversely, differentiation is induced by culturing sarcospheres-derived cells for 2 days on collagen-coated dishes in DMEM supplemented with 10% FBS without growth factors.

5.7 Clonogenic assay

For clonogenic assays, spheres are mechanically disaggregated or detached with tripsyn-EDTA solution and the single cells obtained are plated in 96-well ultra low attachment plates for 20 days in sphere medium. The medium was changed twice a week to renew the growth factors. Singly dissociated primary sphere cells give rise to secondary spheres that, in turn, are able to form tertiary spheres. Undifferentiated spheres are passaged twenty times during the culture period.

5.8 Mesenchymal differentiating culture conditions for sarcospheres

To determine their differentiation potential, sarcospheres are cultured in osteogenic and adipogenic media supplemented with 10% FBS without EGF or bFGF. After one day of culture, spheres attached to the bottom of the flask and gradually migrated from the sarcospheres into adherent cells and after 15 days, they differentiated into mesenchymal lineages. Adipogenic medium: DMEM supplemented with 10% FBS, 1μM dexamethasone, 10μM recombinant human insulin, 200μM indomethacin, and 3-isobutyl-1-methyl-xantine (IBMX) for 15 days, changing the adipogenic medium twice a week. To detect adipocytes, immunocytochemistry for adiponectin (diluted 1:100 in PBS) was performed with a DAKO Cytomation En Vision+System-HRP kit (AEC), according to the manufacturer's instructions. Osteogenic medium: DMEM supplemented with 10% FBS, 0.1μM dexamethasone, 50μM ascorbate-2-phosphate, and 10mM β-glycerophosphate for 15 days. To detect osteoblasts, immunocytochemistry for osteocalcin (diluted 1:100 in PBS) was performed with the DAKO Cytomation En Vision+System-HRP kit.

5.9 Soft agar assay

One of the methods of analysing the transformed phenotype of the cells is the soft agar assay that measures anchorage-independent growth, which is an indicator for assessing cell transformation. In order to assess the anchorage-independent growth properties of spheres versus adherent cells or $CD133^+$ cells versus $CD133^-$ cells, soft agar assay is performed as following. Cells are detached with Tripsyn-EDTA solution (adherent cells) or mechanically disaggregated (spheres) for 5 minutes, counted and 500, 1000 and 5000 cells per well in 24-well were plated, in triplicate. The test is performed using 0.8% and 0.3% agar in IMDM as the base and top layers, respectively. Spheres and adherent cells are plated and incubated for 21 days at 37 °C in a humidified atmosphere at 5% CO_2 and 50 µl of standard medium are added twice a week. At the end of the incubation period, colonies (Fig. 10) is stained with nitrobluetetraziolium (NBT) at a concentration of 50 mg/100 ml in PBS and counted using an inverted microscope. The colony efficiency is calculated as proportion of colonies per total number of seeded cells. The data are analyzed by Image Pro Plus software.

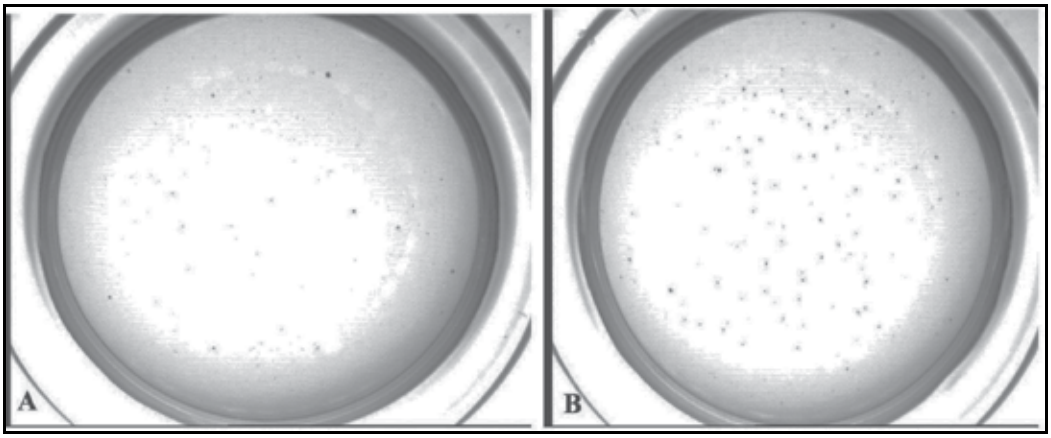

Fig. 10. Figure showing colonies derived from adherent cells (A) and spheres (B). Spheres formed colonies with major efficiency than adherent cells

5.10 *In vivo* tumourigenicity evaluation

In order to evaluate the tumourigenicity of spheres versus adherent cells or $CD133^+$ cells versus $CD133^-$ cells, *in vivo* experiments are performed using Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice. Cells are detached with Tripsyn-EDTA (adherent cells) or mechanically disaggregate (for spheres) for 5 minutes and counted. Cells are diluted in PBS, mixed with matrigel and injected subcutaneously in six-week-old female NOD/SCID mice at following serial dilutions: 1 and 5 x 10^2; 1 and 5 x 10^3; 1 and 5x10^4; 1 and 5 x 10^5; 1x10^6 cells. Mice are monitored every 3 days for the appearance of subcutaneous tumours. After 60-80 days, mice are sacrificed and the tumour tissue collected, in part fixed in buffered formalin and in part minced to re-obtain the cell line. Tumour volume is calculated by the formula (length x width^2)/2. Haematoxylin and eosin staining are performed to analyse tumour histology (Fig. 11). The injection experiments are in triplicate. Regarding to the possibility to regenerate tumours in mice, several publication have challenged the frequent assertion that CSCs are necessarily a rare phenomenon, by showing that assay conditions can have a significant effect on the engraftment of transplanted

malignancies. Limitations on the ability of recipient microenvironmental/niche factors to successfully provide the survival and growth signals required to support engraftment are compounded by damage to cells during isolation and preparation, the effect of residual recipient immunity and, in haematological malignancies, a lack of homing factors to allow leukaemic stem cells to engraft a suitable bone marrow niche environment. The development of mouse strains more heavily immune-suppressed than the SCID and NOD/SCID mice used in early AML studies has been a major step forward. NOD/SCID mice with additional knock out of the IL2-R γ chain (NSG and NOG mice) lack all B, T and NK cells and have deficiencies in macrophage and complement function and are the current gold standard species to regenerate tumour in *in vivo* experiments.

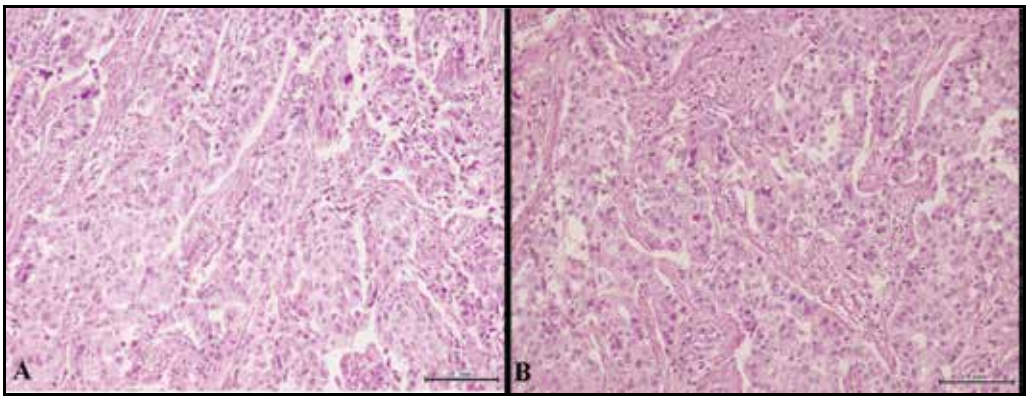

Fig. 11. Figure showing hematoxylin and eosin staining in human tumour (A) and tumour derived from xenograft (B)

5.11 Cryopreservation of tumour cells

For freezing, tumour cells or spheres are harvested by trypsin-EDTA solution for 2 minutes at 37°C and centrifuged at 800g. The pellet is washed once with fresh growth medium and cells are re-suspended in the standard medium containing 10% DMSO (freezing medium) to yield a final concentration of 1.5 x10^6cell/ml. Cells are transferred into 2-ml cryogenic vials and cells are gradually cooled at a rate of 1°C/min and stored in liquid nitrogen. For thawing, vials are quickly thawed by immersion in a 37°C water bath and cells are gently re-suspended in 12 ml of growth medium and rinsed twice with the same medium prior to re-plating under standard growth conditions.

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Modulation of Multidrug Resistance on the Same Single Cancer Cell in a Microfluidic Chip: Intended for Cancer Stem Cell Research

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1. Introduction

A small population of cancer stem cells has been identified in a range of haematopoietic and solid tumours. These cells retain features of normal stem cells including self-renewal, pluripotency and altered gene expression, and might represent the cell of origin of these tumours (Dean et al., 2005)(Fallica et al., 2011). The possible eradication of cancer stem cells might offer revolutionary advances in the treatment of cancer. However, cancer stem cells are rare and are hard to destroy due to the occurrence of high level of multidrug resistance (MDR) proteins.

Tumor cells carrying the MDR phenotype are often associated with the over-expression of drug efflux pumps, among which the membrane-bound energy-dependent P-glycoprotein (Pgp) is one of the important classes (Marthinet et al., 2000)(Persidis, 1999)(Locke et al., 2003). The Pgp efflux pump, which belongs to the superfamily of ATP binding cassette (ABC) transporters (i.e. ABCB1) (Higgins, 2007) and is encoded by the MDR1 class of genes (Gros et al., 1986), actively transports drugs out of the cancer cells. This has caused the intracellular drug concentration to be lower than the drug's efficacy threshold within cancer cells (Marthinet et al., 2000), leading to the failure of many forms of chemotherapy (Higgins, 2007). Gaining a better insight into the mechanisms of cancer stem cell resistance to chemotherapy might lead to new therapeutic drug targets and better anti-cancer treatment strategies (Dean et al., 2005)(Shervington and Lu, 2008)(Dean, 2009)(Donnenberg and Donnenberg, 2005).

To improve the chemotherapy sensitivity, Pgp inhibitors or MDR modulators have been employed, with their effects on MDR reversal studied (Ren and Wei, 2004)(Wang et al., 2000)(Efferth et al., 2002)(Medeiros et al., 2007)(Meaden et al., 2002). For instance, Ren *et al.* studied the efflux of doxorubicin in human carcinoma cells (Ren and Wei, 2004). But the assay conducted by a fluorescence plate reader is not amendable to the study of rare cells such as cancer stem cells. Wang *et al.* developed a method to quantitatively assess Pgp inhibitors by flow cytometry (Wang et al., 2000). While this method is widely used to study MDR modulation, it does not provide the information of an individual single cell, such as its time-dependent drug transport kinetics. In addition, flow cytometry requires a large

number (i.e. 100,000) of cells in the starting population to achieve reliable results (Wang et al., 2005). This is a challenging requirement especially when only a limited amount of rare cells is available.

Since 2000, the microfluidic technique has been widely used for cell biology applications (Li and Li, 2010)(Salieb-Beugelaar et al., 2010). This technique has several advantages for conducting cellular assays: (1) the liquid channels or physical microstructures are compatible with the micrometer-sized biological cells, and these features make single-cell manipulation, or single-cell capture most applicable; (2) the microfluidic devices can be used to analyze a small amount of cells, and this aspect is advantageous in bioanalytical applications because biological samples are often limited in quantity; (3) this technique has a low reagent consumption, and this reduces the assay costs; and (4) the microfluidic technique can integrate multiple steps on a single microdevice, including cell sample introduction, fluid control, single-cell capture, cell lysis, reagent mixing, and analyte detection.

In cellular applications, single-cell analysis is preferred to the traditional bulk cellular analysis because the former can study cellular heterogeneity and provide information on cell-to-cell variations (Sims and Allbritton, 2007)(Li and Li, 2010)(Di Carlo and Lee, 2006). Additionally, physicochemical modelling of biological processes also demands data to be obtained from a single cell (El-Ali et al., 2006). To date, the single-cell applications using the microfluidic devices include intracellular signalling (Li and Li, 2005)(Wheeler et al., 2003)(Li et al., 2004), pathogen identification(Zeng et al., 2010), myocyte contraction (Li and Li, 2005)(Li et al., 2007)(Cheng et al., 2006)(Klauke et al., 2003), drug discovery (Li et al., 2009)(Li et al., 2007), patch-clamp recording (Chen and Folch, 2006), multidrug resistance (Li et al., 2008), cell nanosurgery(Jeffries et al., 2007), electroporation (Ryttsen et al., 2000), genetic analysis (Liu et al., 2010)(Hong et al., 2004), protein analysis (Schumann et al., 2008), forensic analysis (Liu et al., 2010), cell culture (Peng and Li, 2004), photobleaching of cellular fluorescence (Peng and Li, 2005), and so on.

Recently, we have developed a microfluidic single-cell analysis approach for the study of multidrug resistance modulation by real-time monitoring of drug efflux in MDR cancer cells (Li et al., 2008). In order to distinguish drug effects of MDR modulators on cancer cells, control experiments on untreated cells are needed, but cell heterogeneity in multidrug resistance can obscure any positive drug response. To address this issue, we have introduced and tested the concept of same-single-cell analysis (SASCA) in which the same cell is used as its control in order to compare the effect of MDR modulators on drug efflux (Li et al., 2008). In addition to measuring drug efflux, we have also developed a new microfluidic approach to study MDR modulation effects of drug candidates by monitoring drug accumulation.

2. Methodology

This section will briefly introduce the design of the microfluidic chip, followed by the procedures for single-cell selection and retention, and the same-single-cell approaches for drug efflux and drug accumulation measurements. Finally, the procedures for conventional cell assays such as flow cytometry and cytotoxicity tests are listed.

2.1 Microdevice design and fabrication

The layout of the glass microfluidic chip is shown in Figure 1a. The chip consists of 3 channels, 3 reservoirs and 1 chamber containing a cell retention structure. Reservoir 1 is

used to introduce cells; reservoir 2 serves as the waste outlet, and reservoir 3 is used for reagent delivery. The glass chip was fabricated by Canadian Microelectronic Corporation (CMC, Protolyne® Chip) by the standard microfabrication procedure, with a channel depth of 20 μm (Manz et al., 1992). An image of the microchip filled with a red food dye is shown in Figure 1b. Figure 1c illustrates the 3-dimensional view of the cell retention structure, with the retained single cell treated with a flow of drug solution.

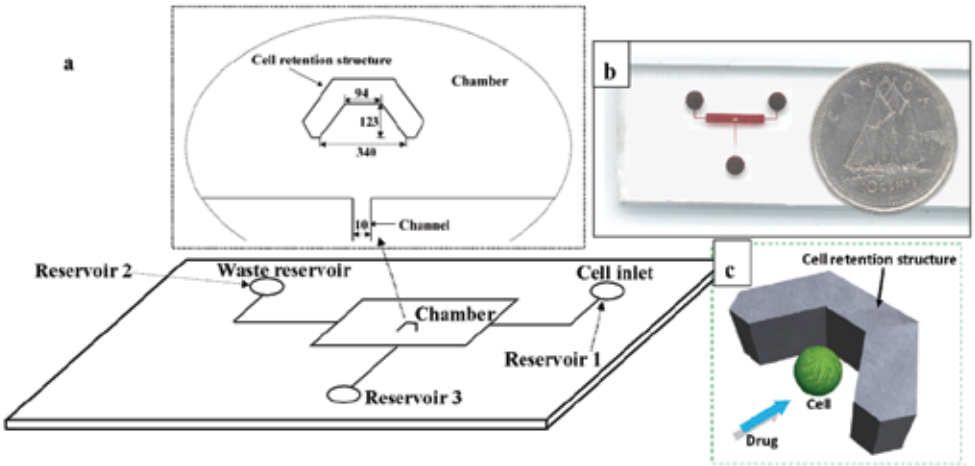

Fig. 1. Layout of the microfluidic chip. (a) The schematics of the microfluidic chip consisting of 3 solution reservoirs and a cell retention structure, with the dimensions (in μm) shown in the inset. (b) An image of the microchip filled with a red food dye. A Canadian dime (10-cent coin) was placed on the chip for size comparison. (c) A schematic diagram of the 3-dimensional illustration of the cell retention structure. Reprinted with permission from American Chemical Society and Royal Society of Chemistry (Li et al., 2008; Li et al., 2011)

2.2 Cell culture

The human wild-type (WT) T-cell leukemic cell line CCRF-CEM (CEM/WT, drug-sensitive) was obtained from ATCC. This cell line and its multidrug-resistant vinblastine-selected subline (CEM/VLB0.05, drug resistant) were maintained in α-MEM medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin, as previously reported (Li et al., 2008).

2.3 Single-cell selection and retention

After a cell suspension (~0.1 x 10^6 cells/mL) was introduced into reservoir 1, the cells flowed unidirectionally (from the right to the left) into the microchip. By adjusting the liquid levels of reservoirs 1 and 2, a desired cell was adjusted to flow near the entrance of the cell retention structure. Then, a liquid flow at the central reagent channel was induced to direct the cell into the retention structure. The cell was allowed to settle further for ~ 15 min (900 s) before the fluorescence measurement was started. Figure 2 shows the image of a single CEM/VLB cell retained in the cell retention structure. The post-retained cell remained alive, which was confirmed using a live stain (i.e. fluorescein diacetate), as shown in the inset of Figure 2. This approach has been widely used to selectively retain single round-shaped

cancer cells and rod-shaped cardiomocytes (Li and Li, 2005; Li and Li, 2006; Li et al., 2007; Li et al., 2008; Li et al., 2009; Li et al., 2011)

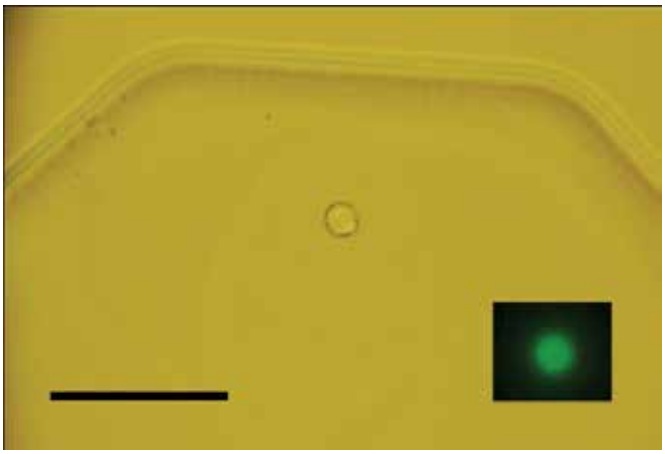

Fig. 2. An optical image of a retained live single CEM/VLB cell, which had been stained by fluorescein diacetate (25 μM) as shown in the inset. The scale bar is 50 μm. Reprinted with permission from American Chemical Society (Li et al., 2008)

2.4 On-chip drug efflux study on single cells

As previously described, an optical detection system was employed for simultaneous bright-field observation and fluorescence measurement (Li et al., 2009). We measured the uptake of the drug daunorubicin (DNR) because it is a substrate of the MDR1 transporter (Murthy and Shah, 2007) (Fu and Roufogalis, 2007), and we used the fluorescent method since DNR is inherently fluorescent (i.e., λ_{em}=590 nm, λ_{ex}=470 nm). The chip was shuttled back and forth across the detection aperture window so that the signals for the cell or its vacant region in the cell medium were obtained in turn. When the cell was inside the detection aperture, the cellular fluorescence was measured; whereas the background signal was measured when the cell was outside the detection window.

We have conducted experiments using two methods: different-single-cell analysis (DISCA) and same-single-cell analysis (SASCA). In the DISCA method, after one CEM cell was selected and retained in the cell retention structure, the cell media in all the reservoirs were removed, and then reservoir 3 was filled with DNR (35 μM) and left to flow in the microchannel for ~1000 s. There was virtually instantaneous replacement of the cell medium around the cell by the DNR solution because the space in front of the cell retention structure was small. Fluorescent measurement was initiated to observe DNR accumulation in the cell (e.g. Cell 1), see Figure 3a. After the accumulation stage, the solutions in all reservoirs were replaced by the cell medium (i.e. no DNR) and it was left to flow for ~1800 s. At the same time, the cellular fluorescent intensity during drug efflux in the cell medium was measured.

This procedure was repeated on a second retained cell (e.g. Cell 2). Briefly, it was treated with 35 μM DNR for ~1000 s, and drug efflux was conducted in cell medium containing a MDR inhibitor candidate compound (i.e. VER, IQ or ART) for ~1800 s. The two sets of fluorescent intensity curves are depicted in the schematics shown in Figure 3a. Comparison of the 2 drug efflux curves (Ed1 vs Ed2) were made subsequently, see Figure 3a inset.

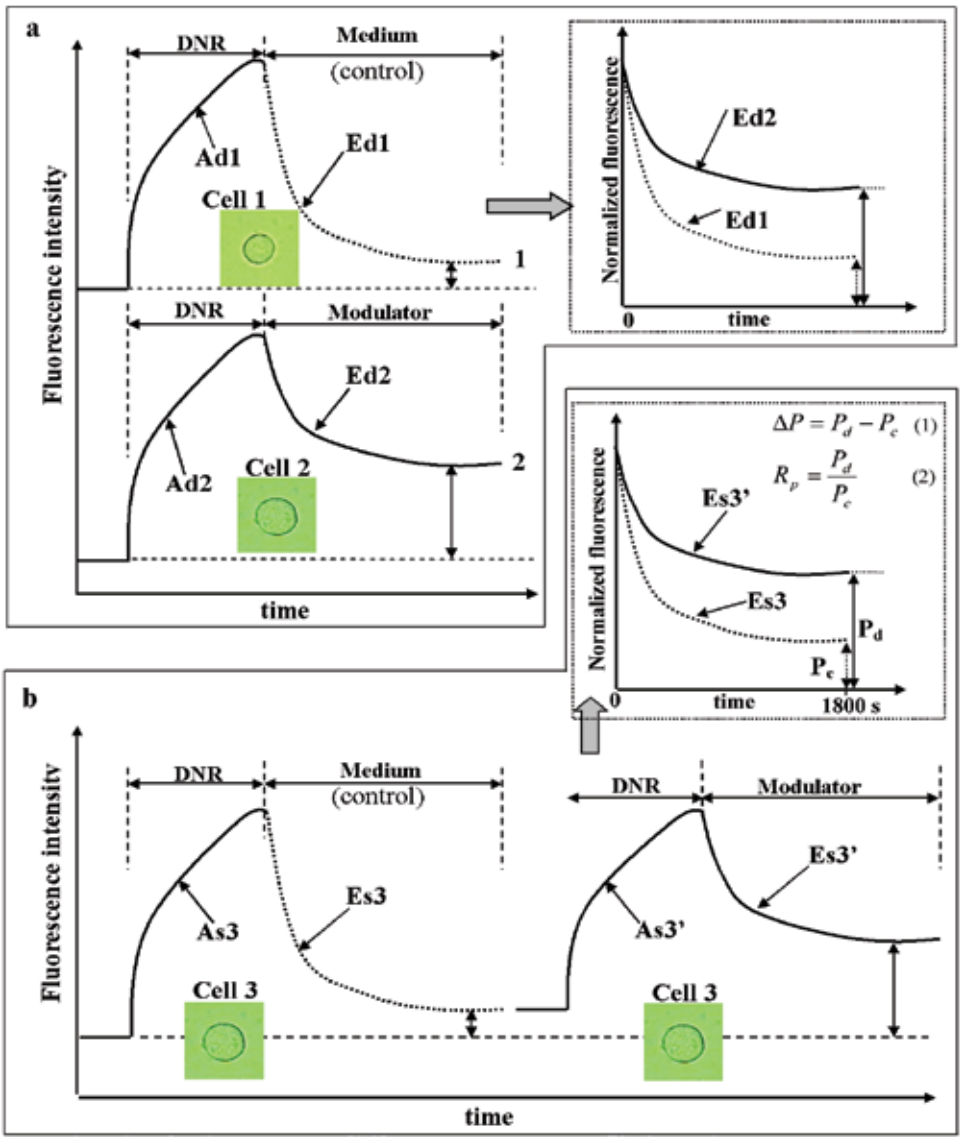

Fig. 3. The schematic illustration of DISCA and SASCA for single-cell MDR efflux study. (a) In DISCA, two different cycles of accumulation and efflux occur on two different cells, namely cell 1 (Ad1 and Ed1), and cell 2 (Ad2 and Ed2). MDR modulation is evaluated by comparing the normalized fluorescent intensity of Ed2 and that of Ed1 (different cell control) shown in the inset. (b) In SASCA, one and the same cell was used in the 2 cycles of drug accumulation (As3, As3′) and efflux (Es3, Es3′). MDR modulation is evaluated by comparing the normalized fluorescent intensity of Es3′ with that of Es3 (same cell control) shown in the inset. P_d and P_c are the DNR retention percentages in the MDR modulator solution, and in the medium alone (control) at efflux time of 1800 s, respectively. These percentages are determined by dividing the cell fluorescent intensity at efflux time of 1800 s, with the maximum fluorescent intensity before the efflux stage. Reprinted with permission from American Chemical Society (Li et al., 2008)

In the same-single-cell analysis (SASCA) method, only one retained cell (e.g. Cell 3) was used. It was first treated with 35 μM DNR for ~1000 s in the drug accumulation stage. Thereafter, drug efflux was observed in cell medium alone for ~1800 s (control experiment). Then, the same cell was treated a second time with 35 μM DNR for ~1000 s, and a second drug efflux was observed in cell medium containing a MDR inhibitor candidate compound (i.e. VER, IQ or ART) for ~1800 s. The fluorescent intensity curves are depicted in the schematics shown in Figure 3b. Comparison of the 2 efflux curves (Es3 vs Es3′) are shown in Figure 3b inset. The time needed to complete this procedure was ~115 min, as described in our previous report (Li et al., 2008).
After all drug treatments and cell measurements, trypan blue was used to treat the cell in order to evaluate the cell viability.

2.5 On-chip drug accumulation study on single cells

In SASCA, only one retained cell (e.g., cell 3) was used. The cell was first treated with DNR (8.8 μM) in the absence of MDR modulators for ~1400s to test drug accumulation (control experiment). Thereafter, the same cell was treated with a DNR solution in the presence of a MDR modulator for ~700 s. Since we expect that if the modulator has a positive drug effect, there is greater drug accumulation, as displayed by the instant change of the slope depicted in Figure 9b.

2.6 Flow cytometry

Flow cytometry was performed using a fluorescence-activated cell sorter (FACS) to confirm the microfluidic single-cell analysis results, utilizing a previously reported procedure (Wang et al., 2000). Briefly, an aliquot (750 μL) of the cells (300,000 cells/mL) in the cell culture medium was transferred to a plastic tube containing 750 μL of incubation medium with DNR (35 μM). Drug accumulation was conducted at 23 °C for 30 min in the dark. After centrifugation (200 g for 5 min) and removal of the supernatant, the cells were re-suspended in the cell medium alone (i.e. without DNR) and incubated at 23 °C for an additional 30 min (the efflux phase). After removing supernatant, cold HBSS was then added to each tube to quench the drug efflux. The cell suspension was transferred to a FACS tube, and was stored on ice (for less than 15 min) before analysis. Fluorescent intensity (excitation at 488 nm, emission at 570 nm) was measured and displayed as single-parameter histograms, based on the acquisition of data from 10,000 cells. The procedure was repeated for the efflux study of the cells in the presence of a MDR modulator compound (e.g. VER, IQ and ART). The time needed to complete this procedure was ~149 min. as described previously (Li et al., 2008).

2.7 MTT cytotoxicity assay

The sensitivities of CEM/WT cells and CEM/VLB cells to the cytotoxic drug DNR were determined by the 96-well microtiter plate assay (Jow et al., 2004; Li et al., 2009). To summarize, after 1 day of cell seeding in each well, 100 μL of test compounds at various concentrations were separately introduced in each well. After the cells were further incubated for 3 days, 40 μL of MTT (5 mg/mL) was added to each well and incubated at 37 °C for 3 h. Finally, after removing the supernatant and adding DMSO, a microplate reader was used to measure the absorbance of each well at 570 nm. The medium without test compounds and DMSO alone were used as the negative control and the positive control, respectively. All tests were carried out in triplicate.

3. Results and discussion

3.1 Multidrug resistance and drug sensitivity

We first investigated the different drug responses of the drug-resistant cells (i.e. VLB cells) and the wild-type (WT) cells using the MTT cytotoxicity assay. Since VLB cells, but not WT cells, have over-expressed Pgp pumps, this discrepancy leads to the difference in the drug sensitivities of these two types of CEM cells toward the drug DNR. This difference was confirmed by the results of cytotoxicity assay (see Figure 4a). It is estimated that the IC_{50} values of DNR (half maximal inhibitory concentration of a drug) for CEM/WT and CEM/VLB0.05 are ~80 nM and 15 μM, respectively. Thus, the CEM/VLB cells are ~180 fold more resistant to DNR, as compared to the CEM/WT cells. This higher drug resistance of the MDR cancer cells is a primary cause of chemotherapy failure, when patients develop multidrug resistance.

3.2 Different-single-cell analysis (DISCA) for drug efflux study in a microchip

The purpose of this drug efflux study was to test a MDR modulator compound (e.g. verapamil or VER) for a possible MDR reversal effect. To achieve this, DNR efflux was conducted on a CEM/VLB cell (test cell) in the presence of the modulator compound and then on another cell (control cell) in the absence of the compound. Since two cells are measured, this analysis is called different-single-cell analysis (DISCA). As described in Figure 3a inset, the two efflux curves (Ed1 vs Ed2) obtained from the two different cells (i.e. Cell 1 and Cell 2) were compared.

In the results shown in Figure 5a, we observe the typical drug efflux curves of different single drug-resistant cells (CEM/VLB) in various DNR-free solutions such as VER, IQ, and the cell medium alone. It was observed that the DNR efflux was initially fast (i.e. 0-500 s). Afterwards, the efflux rate slowed down and the cellular fluorescence did not change significantly. In addition, less DNR was retained (i.e. 0.2-0.4 or 20-40%) when the efflux was conducted in the medium (the efflux-in-medium curve) as compared to the efflux conducted in VER (the efflux-in-VER curve). This observation is consistent with the notion that VER interferes with the drug efflux process by binding to the Pgp efflux pump, thus resulting in more DNR retention (Akiyama et al., 1988; Wang et al., 2000). We also studied the MDR effect of another compound, IQ, an ingredient of the traditional Chinese herb licorice (Sung and Li, 2004; Cai and Li, 2007). It has been reported that IQ has an anti-tumor effect on human gastric cancer (Ma et al., 2001) and leukemia (Li et al., 2009), prostate cancer (Kanazawa et al., 2003) and hepatoma (Hsu et al., 2005). However, no obvious inhibition effect of IQ on DNR efflux was observed, as the efflux-in-IQ curves were similar to the efflux-in-medium curves (see Figure 5a).

Figure 5a shows the typical curves of DNR efflux among results obtained from multiple individual cells. The typical curves represent the cluster of the curves for most cells, as opposed to some outliers, see Figure 5b-d. Even in the same cluster, some cellular variations were observed. For instance, Figure 5b shows the variations in the cluster of the drug-resistant cell line (CEM/VLB) and that of the CEM wild-type cell line (CEM/wt). Although it can be comfortably inferred that there is a lower drug retention in the CEM/VLB cells than in the CEM/WT cells, which was attributed to the overexpressed Pgp pumps in the CEM/VLB cells (Higgins, 2007), there are still many variations observed in both clusters. It can be observed that some curves of CEM/VLB cells are even close to those of the CEM/WT cells, indicating some CEM/VLB cells have more drug retention, which is likely due to less Pgp pump activities.

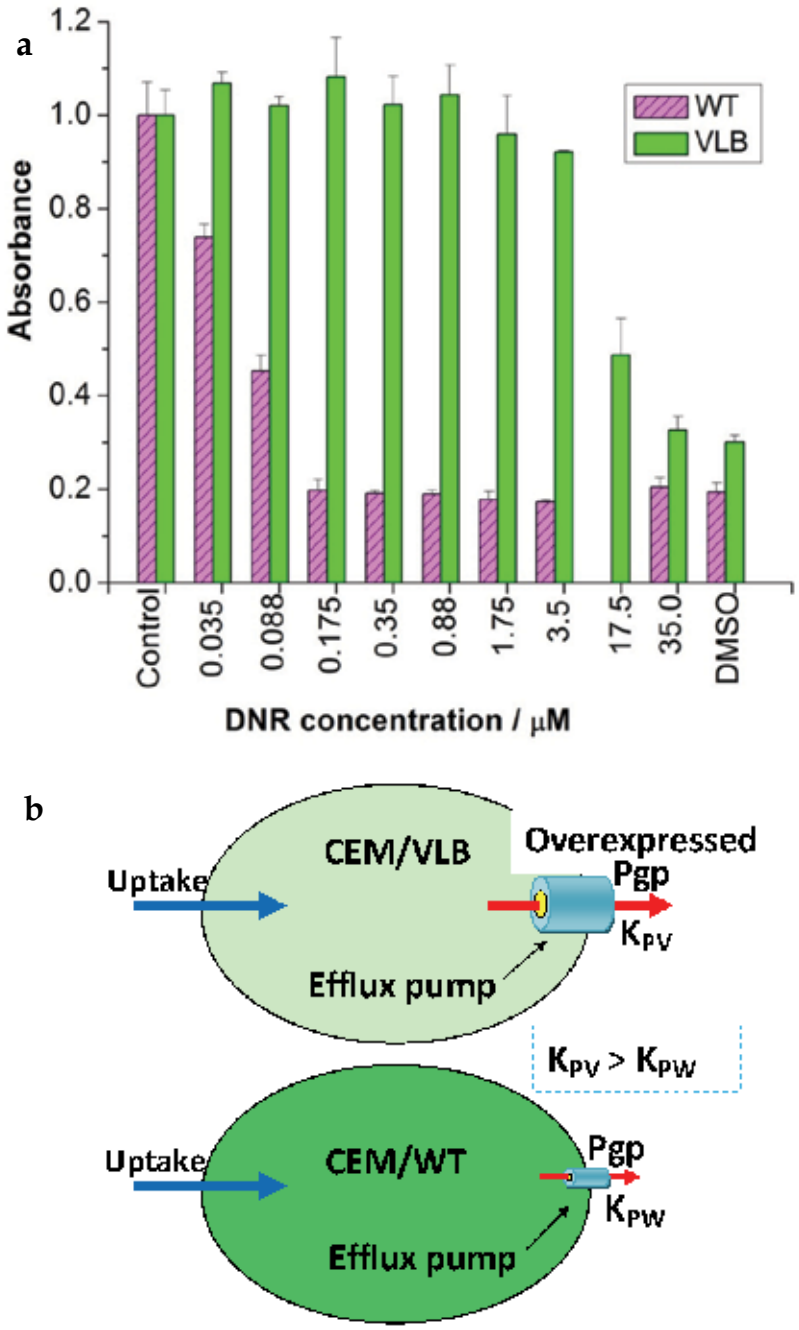

Fig. 4. (a) Cytotoxicity assay to show the different drug sensitivities of WT and VLB cells toward DNR. (b) A drug accumulation model to describe the higher drug sensitivities (or lower drug resistance) of the WT cells, as compared to the VLB cells. K_{PV} and K_{PW} are the drug efflux rates in VLB and WT cells, respectively. Reprinted with permission from Royal Society of Chemistry(Li et al., 2011)

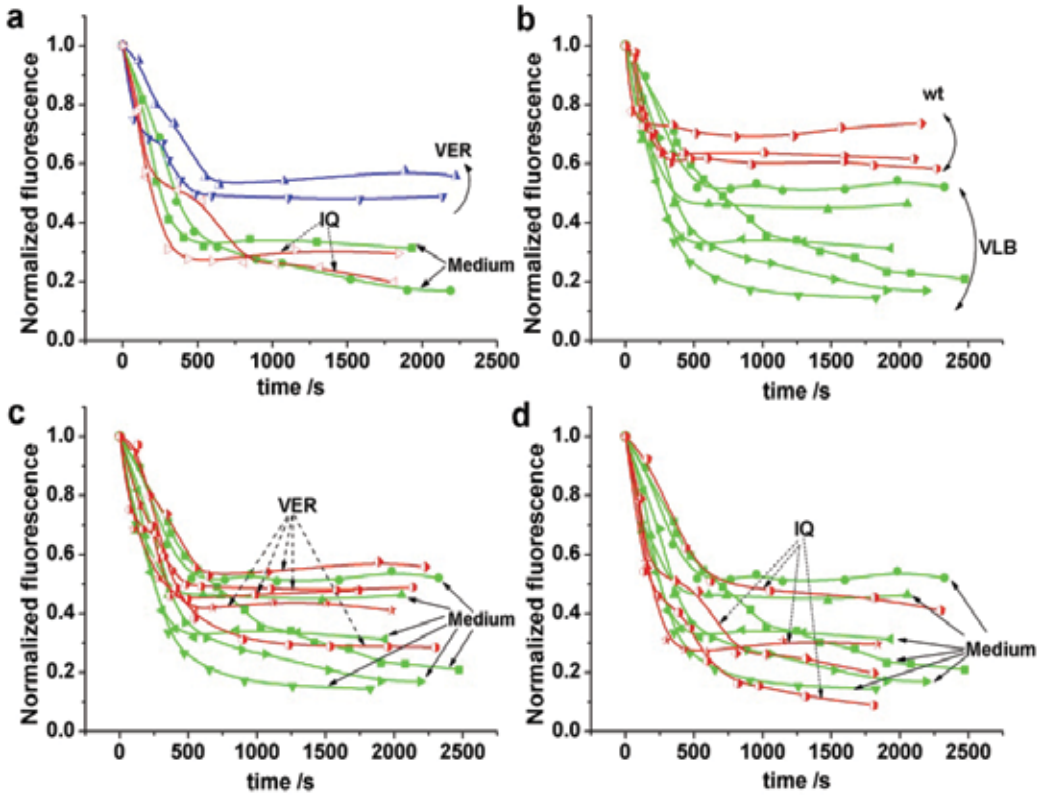

Fig. 5. Modulation of DNR efflux by VER and IQ studied by DISCA in a microchip. (a) The typical curves of DNR efflux of CEM/VLB cells in the medium (green, solid symbols), in 50 μM VER (blue, half-filled symbols) and 100 μM IQ (red, hollow symbols). (b) Comparison of DNR efflux in medium between CEM/WT (red, half-filled symbols) and CEM/VLB (green, solid symbols) cells. (c) The effect of VER (red, half-filled symbols in panel) on the DNR efflux in CEM/VLB cells. (d) The effect of IQ (red, half-filled symbols in panel) on the DNR efflux in CEM/VLB cells. DNR, 35 μM. IQ is isoliquiritigenin; VER is verapamil; ART is sodium artesunate. Reprinted with permission from American Chemical Society (Li et al., 2008)

In the cases of drug efflux in VER and in IQ, the MDR reversal effects from these compounds cannot be concluded due to the enormous cell variations in the efflux-in-VER cluster, and in the efflux-in-IQ cluster, as shown in Figure 5c and 5d, respectively. Although it is well known that verapamil (VER) could inhibit the MDR efflux and result in more drug retention (Akiyama et al., 1988; Wang et al., 2000), this positive effect of MDR reversal cannot be verified in Figure 5c as some efflux-in-VER curves are mixed with the efflux-in-medium curves. Since the effect of IQ on MDR reversal is unknown, it is difficult to draw any conclusion when we examine Figure 5d. Under this situation, we selected the typical curves in each of the 3 cases (shown in Figure 5a) as obtained from many single-cell experiments, and tentatively concluded that there is a MDR reversal effect by VER, but not by IQ.
More single-cell measurements could help the data interpretation and this is one of the reasons why conventional FACS requires a large number of cells to achieve reliable results (Wang et al., 2005). Nevertheless, it may not be possible to conduct many measurements in

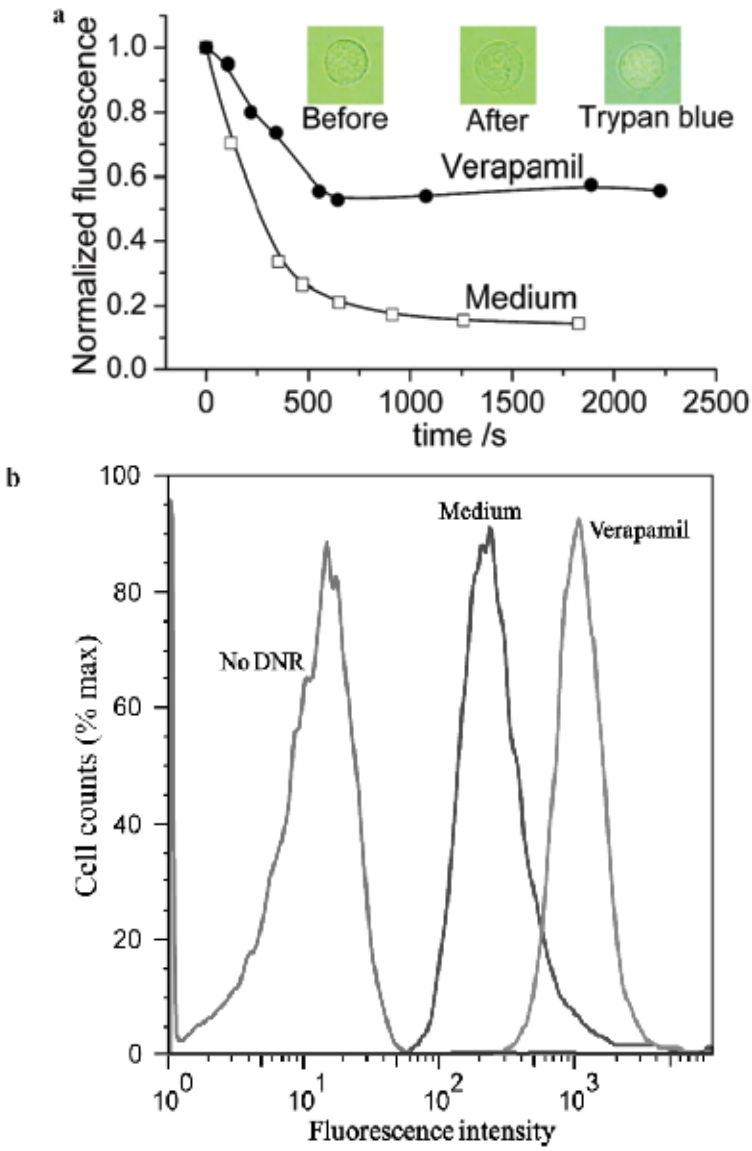

Fig. 6. Modulation of 50 μM verapamil (VER) on DNR efflux of CEM/VLB cells studied by SASCA and confirmed by flow cytometry. (a) SASCA method used to study the effect of MDR reversal by VER on one and the same CEM/VLB cell. The fluorescence intensities of DNR efflux in medium and in VER have been normalized for easy comparison. The insets show the cell morphologies before and after the experiment, and after trypan blue treatment. (b) Flow cytometry study of the modulation of DNR efflux in CEM/VLB cells by VER. The histograms represent the normalized percentage of cell counts plotted against the fluorescent intensity expressed as log relative fluorescence. The histograms depict, from left to right, the cells without DNR accumulation; the cells with DNR efflux in medium alone; and the cells with DNR efflux in VER. DNR, 35 μM. Reprinted with permission from American Chemical Society (Li et al., 2008)

the case of cancer stem cells because of the limited number of cells available. It is because one drawback of using DISCA for the MDR efflux study is the inevitable variations in cellular properties, such as the MDR activities. In a worse case scenario: if the control cell has a lower drug efflux ability (e.g. the highest efflux-in-medium curve in Figure 5c), and the test cell has a greater drug efflux ability (e.g. the lowest efflux-in-VER curve in Figure 5c), the MDR reversal effect of the candidate compound will not be obvious, leading to a false negative conclusion.

3.3 Same-single-cell analysis (SASCA) for drug efflux study in a microfluidic chip

In order to minimize cellular variations, we conduct the drug efflux study in which the same cell is employed as the control cell as well as the test cell. This analysis is thus called same-single-cell analysis (SASCA). This demands the single cell to be retained long enough during the course of the drug efflux study, and is achievable by the cell retention ability of the microfluidic chip. The schematic diagram in Figure 3b shows the concept of the SASCA, in which 2 cycles of DNR accumulation and efflux steps are conducted on the same single cell (e.g Cell 3). As depicted in the inset of Figure 3b, the 2 efflux curves (Es3 vs Es3′) obtained from one and the same cell are compared.

Since the well known effect of MDR reversal of verapamil (VER) cannot be verified by DISCA as shown in Figure 5c, we conduct SASCA to verify this effect. Figure 6a shows that there is indeed a greater DNR retention when the cell efflux was conducted on the same cell in the presence of VER and so it is clear that this well known modulator has reversed the DNR efflux process. More SASCA data confirm the same positive result of MDR reversal by VER (see Table 1).

Conventional flow cytometry was also performed to confirm the effect of VER. From Figure 6b, it is found that the fluorescent intensity (plotted as the x-axis), which indicates the intracellular DNR retention, is greater when the drug efflux study is conducted in VER than in the cell medium alone, corroborating the microfluidic results. In the flow cytometry experiment, during the drug accumulation and efflux procedures (a total of 60 min.) and before data collection, no cellular information is collected. But, in SASCA, the full profile of the drug transport in the same single cell is recorded even during drug accumulation and efflux. In addition, this approach has provided information about the cell morphology, which can be seen in the cell images in Figure 6a inset, showing little cell shape changes, and non-staining by trypan blue. This data suggested that the cell was still viable, indicating not only that the drug-resistant cell was not killed by DNR due to substantial drug efflux, but also that the microfluidic method was robust enough for long-term cell measurement. The cell images could also be useful in documentation and data interpretation of the MDR reversal response. Furthermore, SASCA can comfortably measure one cell selected from a small cell population of ~100, in contrast to flow cytometry that requires ~100,000 cells to achieve high yield in data collection. A full comparison between flow cytometry and SASCA, in terms of experimental time, information content, throughput and automation, has been previously reported (Li et al., 2008).

In a similar manner, the effect of IQ on DNR efflux was studied. It is clear in Figure 7a that IQ did not result in a greater DNR retention, and so IQ was ruled out as a MDR modulator candidate. Again, the use of the same cell as both the control and test cells in SASCA rules out the variations among different cells, and assists in a conclusive data interpretation. More experiments on IQ (see Table 1) were conducted, and the same observation (no MDR reversal effect by IQ) was achieved. The microchip data are also consistent with the histograms

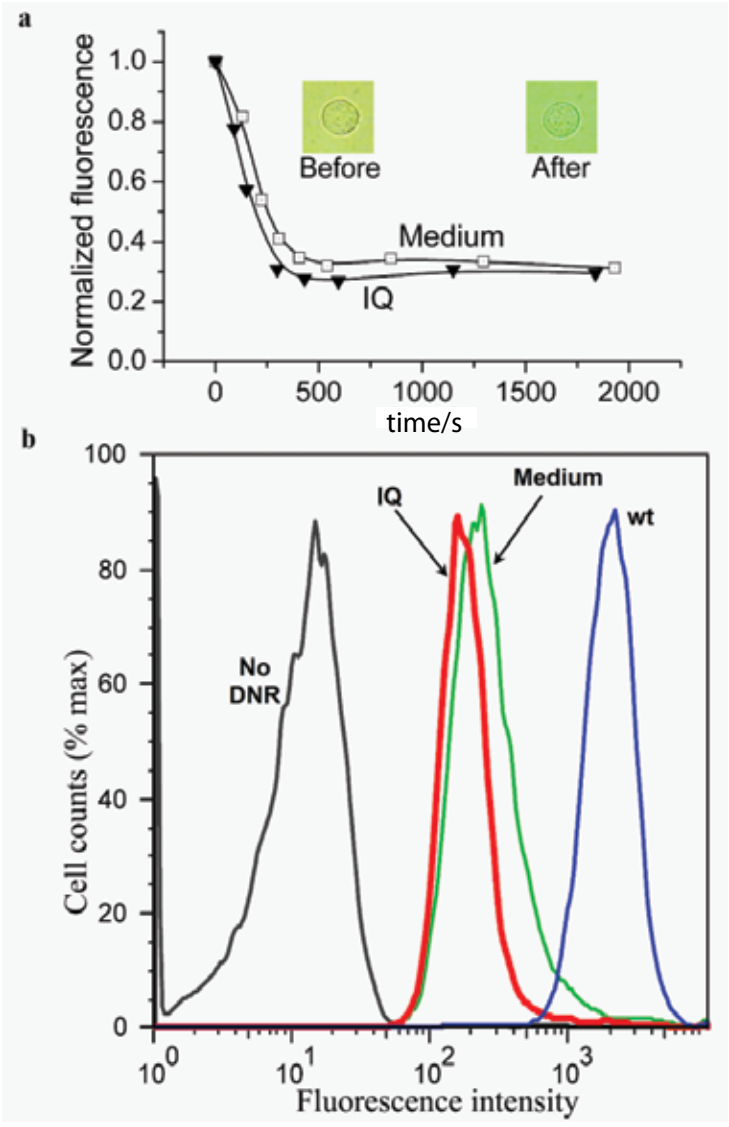

Fig. 7. Effect of IQ on DNR efflux of CEM/VLB cells studied by SASCA and confirmed by flow cytometry. (a) Effect of 100 μM IQ on MDR reversal studied on one and the same CEM/VLB cell. Other conditions are the same as Figure 6a. (b) Flow cytometry study of DNR efflux in CEM cells as treated by IQ. The histograms represent, from left to right, the CEM/VLB cells without DNR accumulation; the CEM/VLB cells with DNR efflux in IQ (100 μM); the CEM/VLB cells with efflux in medium alone; and the CEM/WT cells with DNR efflux in medium alone. Others conditions for flow cytometry are the same as Figure 6b. Reprinted with permission from American Chemical Society (Li et al., 2008)

obtained by conventional flow cytometry (Figure 7b), which shows the CEM/VLB cells have a similar DNR retention in IQ as in medium alone, while DNR retention in CEM/WT cells is greater than that of the CEM/VLB cells. Similar to the VER experiment, the flow cytometry

data did not give more information than SASCA regarding the conclusion of the IQ effect, although the histograms provide valuable information about cell distribution, which may be important in applications such as the cell-cycle study.

	Verapamil (VER)			Isoliquiritigenin (IQ)			Artesunate (ART)			
	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Cell 9	Cell 10
P_d (%)	42.2	28.8	55.7	29.6	45.3	8.8	19.3	35.7	68.8	66.1
P_c (%)	31.7	17.2	14.4	31.7	40.8	14.4	14.9	10.8	47.6	38.0
ΔP (%)	10.5	11.6	41.3	-2.1	4.5	-5.6	4.4	24.9	21.2	28.1
Average ΔP (%)		21±17			-1.1±5.1			19.7±10.6		
$R_p = P_d / P_c$	1.33	1.67	3.87	0.93	1.11	0.61	1.30	3.31	1.45	1.74
Average R_p		2.3 ±1.4			0.88 ±0.25			1.95 ±0.92		
G_p		3.83±0.86			0.72±0.21			1.39		

Table 1. DNR efflux in CEM/VLB cells as modulated by IQ, VER and ART using SASCA. See eqs (1-3) for the definitions of P_d, P_c, ΔP, R_p, and G_p. Reprinted with permission from American Chemical Society (Li et al., 2008)

To quantitatively evaluate the MDR modulations or MDR reversal effects of various drug candidates, we define 2 parameters, ΔP and R_p, as follows:

$$\Delta P = P_d - P_c \quad (1)$$

$$R_p = P_d / P_c \quad (2)$$

where P_d and P_c are the DNR retention percentages in the MDR modulator solution and in the medium alone (control), respectively, see Figure 3b inset.
In flow cytometry, the fluorescence geometric mean ratio, G_p, is used, as defined in equation 3 (Medeiros et al., 2007) (Wang et al., 2000).

$$G_p = G_d / G_c \quad (3)$$

where G_d and G_c are the fluorescence geometric means in the MDR modulator solution and in the medium (control), respectively.
ΔP gives the DNR retention percentages that the drug candidate can reverse, i.e. the larger the ΔP value, the greater is the drug's MDR reversal. On the other hand, a negative ΔP value means that the drug candidate potentiates the drug efflux, resulting in less drug retention. R_p is defined for comparison with the data obtained from the flow cytometry method (i.e. G_p). The higher is the R_p value above 1, the greater is the MDR inhibition effect; R_p =1 means that the drug candidate does not have any MDR modulation effect. If R_p is less than 1, it means the drug candidate potentiates the drug efflux.
Several experiments of 50 μM VER by SASCA show that the highest values of ΔP and R_p are 41.3 and 3.87, respectively (Table 1), indicating that Cell 3 would have the greatest MDR reversal effect. This demonstrates the capability of SASCA to evaluate the different cellular abilities in response to drug efflux modulation. To compare with the flow cytometry data, the averaged values were used, and the average ΔP and R_p for VER (50 μM) are 21±17% and 2.3±1.4 (n=3), respectively (Table 1). The values of ΔP and R_p for IQ (100 μM) are similarly compared, and they are -1.1±5.1% and 0.88 ±0.25 (n=3), respectively. The R_p values (2.3±1.4

and 0.88±0.25) for VER and IQ are similar to the G_p values (3.83±0.86 and 0.72±0.21) obtained from flow cytometry data.

The difference in R_p and G_p values may result from the differences between these two measurement methods, and the amount of tested cells, but this does not affect the conclusion of MDR modulation in both cases. Although R_p is defined for comparison with the results obtained from the flow cytometry method (e.g. G_p), we believe ΔP should be used in evaluating the drug candidate's MDR reversal effect in SASCA since the P_d and P_c values have already been normalized into the percentages. Moreover, R_p may be substantially affected by the denominator (P_c), especially when the value of P_c is very small.

The MDR modulation effect of another herbal ingredient, sodium artesunate (ART) was also studied. ART, which was derived from 'Qinghao', was first discovered as an anti-malarial drug (Dai and Chen, 1999), and subsequently found to have anti-cancer property (Wang et al., 2002). Figure 8a shows that the DNR efflux is reversed in the presence of ART, as compared with the efflux in the cell medium (medium 1, before ART). In order to confirm this finding, a second efflux step in the medium (medium 2) was conducted after the MDR reversal by ART. It was found that the efflux-in-medium curve in the second case was consistent with that in the first, thus confirming the finding that the MDR modulation effect of ART was genuine. More same-single-cell experiments also confirmed the effect of ART (300 μg/mL) on MDR efflux, as shown by the mean values of ΔP and R_p, which are 19.7±10.6% and 1.95 ±0.92, respectively (see Table 1). It has been reported that ART (60 μg/mL) significantly increased DNR accumulation in the MRP1-expressing cells, but not in the MDR1-expressing CEM cell line (Efferth et al., 2002). We discovered that when ART was more concentrated than 200 μg/mL, it produced a beneficial MDR inhibition effect, consistent with the flow cytometry data (Figure 8b). No inhibition effect on MDR efflux was observed in lower concentrations of ART (100 μg/mL).

3.4 Simpler, faster, and more reliable method of microfluidic same-single-cell analysis

The study of multidrug resistance based on drug efflux is fairly complicated because it involves multiple cycles of drug uptake and drug efflux, and each assay is time-consuming. In addition, the time period between the two drug efflux processes (i.e. test and control or $t_{e1} + t_r + t_{a2}$,), as shown in Figure 9a, might cause slight differences in the cellular health status or Pgp activities. Although multiple controls can be adapted to confirm cell status as we reported previously (Li et al., 2008), it complicates the assay process. To overcome these problems and work toward an 'identical' control, we are reporting a new microfluidic SASCA approach for the study of MDR modulation by monitoring drug accumulation in single cells. Based on the same concept of SASCA, this new approach is simpler, faster and more reliable for MDR study by investigating drug accumulation, instead of drug efflux.

As illustrated in the Figure 9b inset, during the drug accumulation stage in the presence of DNR, the drug uptake process and the drug efflux process by Pgp pumps occur simultaneously. In contrast to WT cells, more DNR is pumped out of MDR cells, leading to low drug accumulation in these cells. But when the MDR cells' Pgp pumps are inhibited by a MDR modulator compound (e.g., VER), less DNR is pumped out, leading to a higher intracellular drug accumulation (i.e., the MDR reversal effect). Therefore, we can choose to measure DNR accumulation in order to determine whether a compound has the MDR reversal effect, instead of the time-consuming measurement of the DNR efflux process (Li et al., 2008). We call this approach SASCA-A to differentiate it from the previous approach of same-single-cell analysis, termed as SASCA (Li et al., 2008).

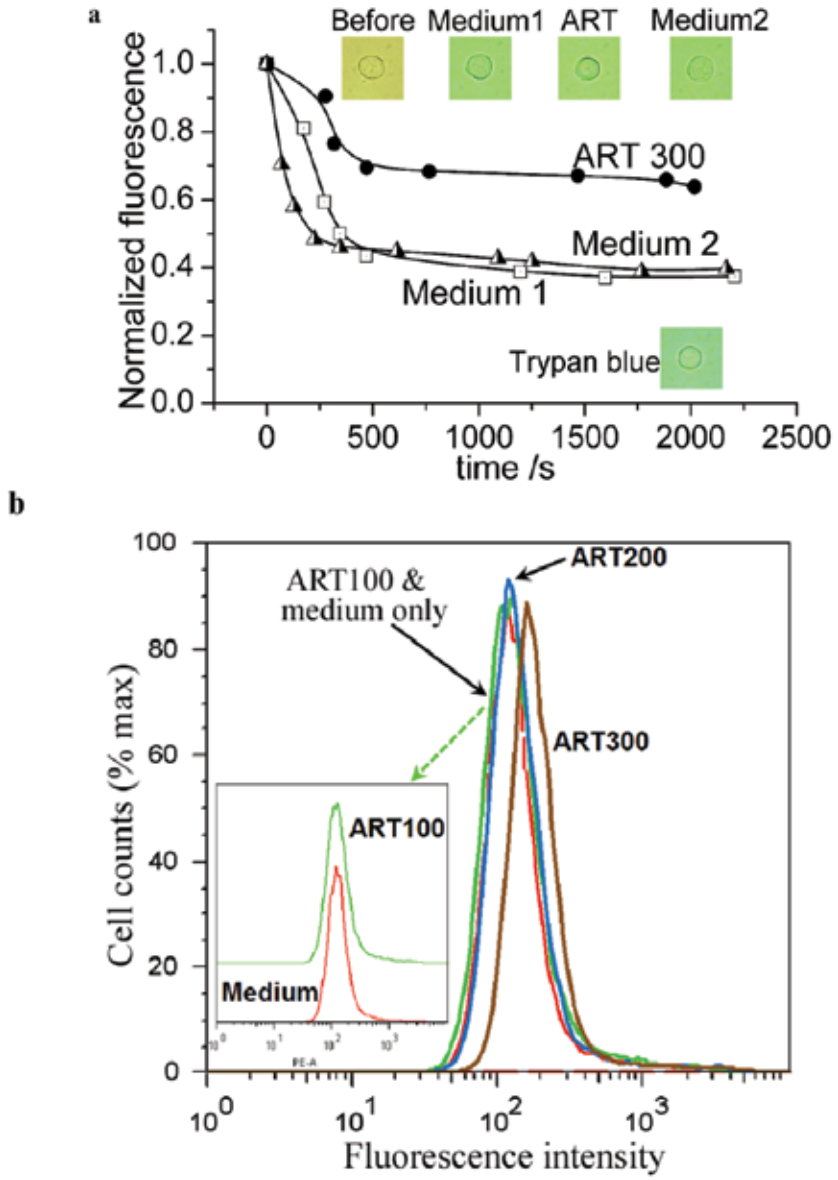

Fig. 8. Modulation of ART on DNR efflux of CEM/VLB cells as studied by SASCA (a) and confirmed by flow cytometry (b). (a) Effect of MDR reversal by ART (300 μg/mL) studied by SASCA on one and the same CEM/VLB cell in a microfluidic chip. A second efflux in medium (Medium 2) confirmed that the effect of 300 μg/mL ART (ART300) was genuine after the first efflux in medium (Medium 1). Both efflux steps in medium alone were used as control experiments. Other conditions are the same as Figure 6a. In (b), flow cytometry revealed the effect of artesunate (100, 200, 300 μg/mL) on DNR efflux in CEM/VLB cells. The histograms from the medium alone and 100 μg/mL ART are offset vertically and shown in the inset to provide greater detail. Others conditions for flow cytometry are the same as Figure 6b. Reprinted with permission from American Chemical Society (Li et al., 2008)

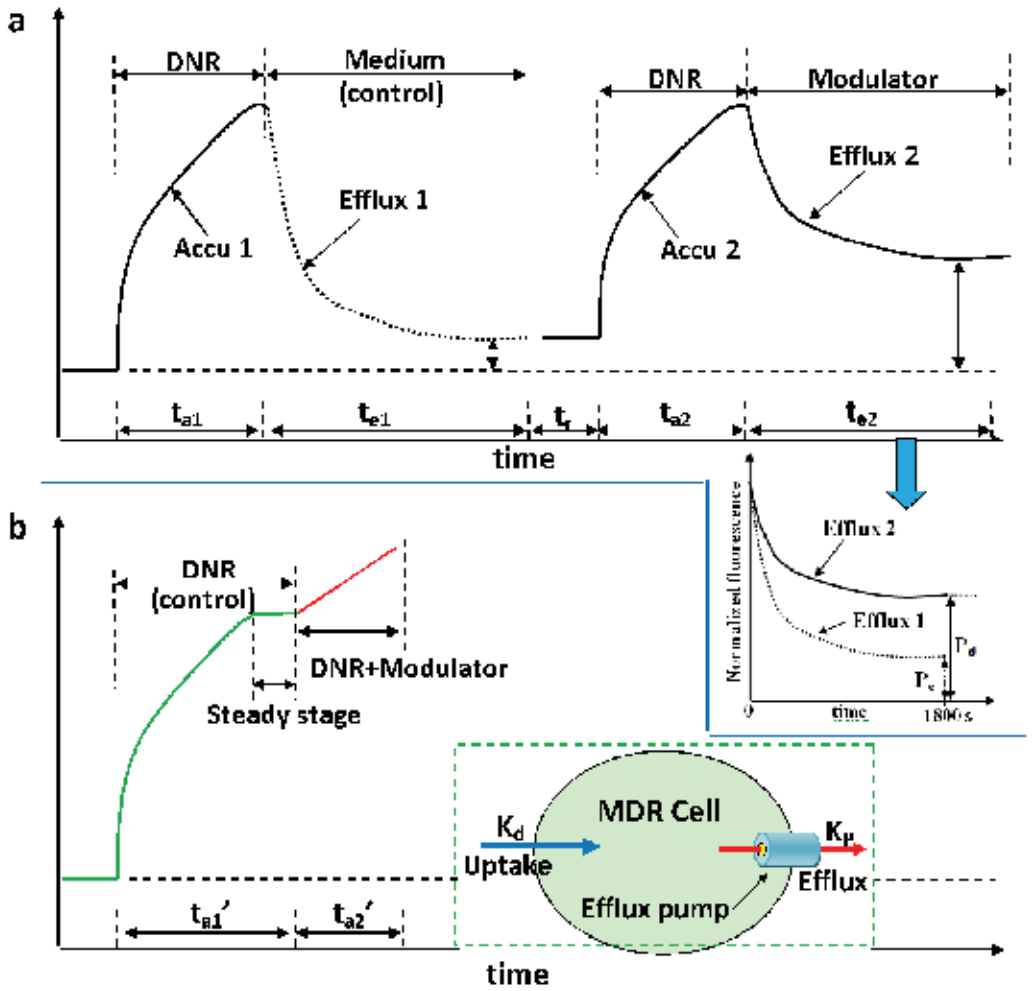

Fig. 9. The schematic illustration of two SASCA approaches for single-cell multidrug resistance study (a) by a microfluidic method measuring drug efflux process and (b) by a new approach via investigating drug accumulation. The inset depicts a mathematical model to describe the kinetics of drug accumulation, with K_d and K_p representing the drug uptake rate and drug efflux rate, respectively. Reprinted with permission from Royal Society of Chemistry (Li et al., 2011)

In the SASCA-A experiment, we used a lower concentration of DNR (e.g., 8.8 μM) to minimize cytotoxic effect on cells. It was found that the accumulation was initially fast, then it reached a relatively 'steady' state (after 470 s as seen in Curve 3 in Figure 10). This showed a balance of the drug uptake and efflux processes, as previously observed (Ren and Wei, 2004)(Wang et al., 2000). It was only when the DNR concentration was low enough and the accumulation was relatively slow that the 'steady' accumulation stage was obvious. During this 'steady' state, if an MDR inhibitor-containing DNR solution is applied (see Figure 9b and 10), the MDR modulation will tip the balance at this 'steady' state, and the drug accumulation rate will increase instantly. This is shown by the abrupt slope change in the

curve (see Figure 9b and 10). This provides us a simple and fast means to monitor MDR modulation using this SASCA-A method, without a long waiting period which is inevitable in the previous SASCA method (Li et al., 2008).

Figure 10 shows an obvious slope transition in the drug accumulation curve when a VER–containing DNR solution was applied to the cell at 1370 s (Curve 3). The slope transition indicates a MDR reversal effect in which there is a faster DNR accumulation as compared to the 'steady' state before VER was added. Thus, the MDR reversal effect of VER can easily be determined by the new approach of microfluidic SASCA-A. However, when an IQ-containing DNR solution was added, no obvious slope transition was observed (see Curve 2). This was similar to the negative control when only DNR (without MDR inhibitors) was applied (at 1562 s in Curve 1), which indicated that IQ did not have MDR reversal effect on CEM cells. More SASCA data confirmed the different reversal effects from VER and IQ, as listed in Table 2. The SASCA-A method can readily conclude the effects of not only VER but also IQ, directly on the same single cell, by minimizing the cellular variations among different single cells. The effects of VER and IQ were further confirmed by the conventional technique of flow cytometry (data not shown).

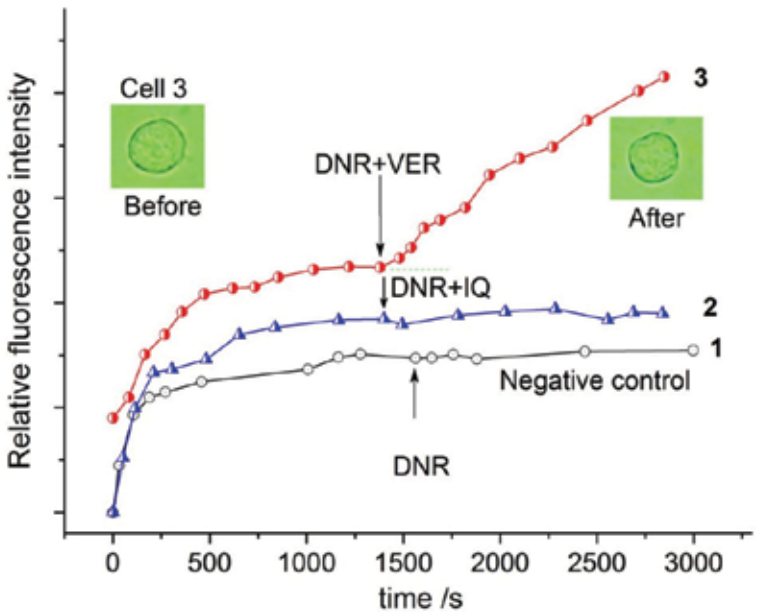

Fig. 10. MDR modulations from VER and IQ in CEM/VLB cells by the new approach of the same-single-cell analysis of drug accumulation (SASCA-A). DNR solutions (8.8 μM) in the presence of VER (50 μM) and IQ (100 μM) were applied to cells at 1370 s (Curve 3) and at 1402 s (Curve 2), respectively. In Curve 1, 8.8 μM DNR in the absence of MDR modulators was added at 1562 s as a negative control. The relative fluorescence intensities have been offset for clarity. The inset shows the images of the cells before and after the VER experiment. Reprinted with permission from Royal Society of Chemistry (Li et al., 2011)

In a similar manner, this microfluidic SASCA-A approach was applied to study the effect of PSC 833 on MDR modulation. PSC 833 (Valspodar, a non-immunosuppressive cyclosporin D derivative) is a less toxic Pgp inhibitor (Thomas and Coley, 2003). As shown in Figure 11,

a slope transition was observed as soon as 3 μM PSC 833 in a DNR solution was applied to the cell at 1458 s (see Curve 2), indicating a faster drug accumulation due to the inhibition of Pgp pumps by PSC 833. More same-single-cell experiments also confirmed the MDR reversal effect of PSC 833 on drug accumulation in MDR cancer cells. This is consistent with previous reports that PSC 833 can inhibit Pgp pumps and improve the chemotherapy response (Warmann et al., 2002). As a control, when the same PSC 833 solution was applied to CEM/WT cells (at 1368 s on Curve 3), no obvious slope transition occurred and the drug accumulation in the cells still increased linearly with the same slope as before. This implied

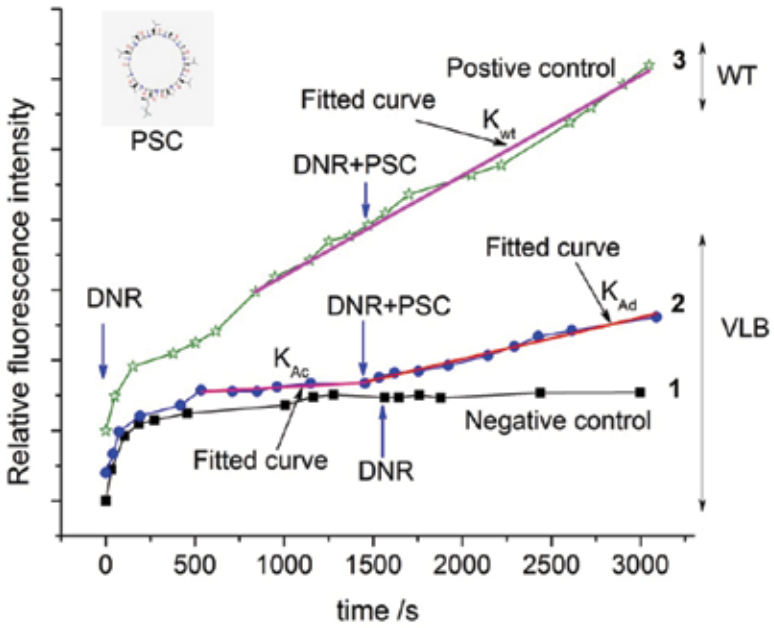

Fig. 11. MDR modulations from PSC 833 in CEM cells by the microfluidic approach of SASCA-A. DNR solutions (8.8 μM) in the presence and absence of 3.0 μM PSC 833 were applied to CEM/VLB cells at 1458 s in Curve 2 and at 1562 s in Curve 1, respectively. In Curve 3, a DNR solution (8.8 μM) in the presence of 3.0 μM PSC 833 was applied to a CEM/WT cell at 1465 s. The relative fluorescence intensities have been offset for easy comparison. The different segments of Curve 2 and Curve 3 have been linearly fitted and shown as straight lines along the curves. K_{Ac} and K_{Ad} are the slopes of fitted lines before and after applying drug modulators in CEM/VLB cells, and K_{wt} is the slope of the fitted line in CEM/WT cells. Reprinted with permission from Royal Society of Chemistry (Li et al., 2011)

that the MDR modulator did not have any reversal effect on the drug accumulation in CEM/WT cells, as were similarly observed in previous reports (Hu et al., 1990). During the drug accumulation in WT cells as seen in Curve 3, no obvious 'steady' state was observed, and yet the accumulation rate was fairly constant after 838 s. As time went on, there was an increasingly greater deviation in the amount of drug accumulation between WT cells (Curve 3) and drug-resistant cells (Curve 1). Accordingly, the SASCA-A approach cannot only readily identify MDR modulators, but can also clearly distinguish CEM sensitive cells from MDR cells. This method might be used to distinguish MDR cells or cancer stem cells from a population of cells.

To quantitatively evaluate the MDR modulations or MDR reversal effects of various drug candidates using the SASCA-A method, two parameters have been defined, namely, the ratio of drug accumulation rates (denoted as R_A), and the MDR reversal percentage ($\Delta R_A\%$), as follows:

$$R_A = \frac{K_{AD}}{K_{AC}} \tag{4}$$

$$\Delta R_A\% = \frac{K_{AD} - K_{AC}}{K_{wt}} \tag{5}$$

where K_{Ad} and K_{Ac} are the slopes of the linearly fitted lines on the drug accumulation curves of the CEM/VLB cells in the presence of drug plus MDR modulators (modulators + DNR) and in the absence of MDR modulators (DNR only), respectively; K_{wt} is the slope of the fitted line on the curve of the CEM/WT cell.

In this way, R_A indicates how much a MDR modulator affects the drug accumulation in a MDR cell. Therefore, the greater R_A is, the stronger the reversal effect of MDR modulators will be. Since R_A does not reveal how close the MDR reversal of the VLB cell is to the drug accumulations in the WT cell, we have also defined $\Delta R_A\%$. Based on these two equations, the values of R_A and $\Delta R_A\%$ were calculated and listed in Table 2. Both average values of R_A and $\Delta R_A\%$ confirm the positive MDR reversal effects by VER and PSC 833, but not by IQ. The average values of $\Delta R_A\%$ of VER, IQ, and PSC 833 are 57.4, -0.1, and 29.5, respectively, showing that 50 μM VER has a greater MDR reversal effect as compared to 3 μM PSC 833. From Table 2, we also found that different cells responded to MDR modulators differently. The values of $\Delta R_A\%$ of VER on cell 1, 2, and 3 are 67.0%, 52.7%, and 52.3%, respectively. Cell 1 shows the highest response on the MDR reversal effect from VER among the cells tested in this work. This kind of cellular heterogeneity in MDR modulation was also observed in previous reports (Li et al., 2008).

	verapamil (VER)			isoliquiritigenin (IQ)			PSC 833 (PSC)		
	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 7	Cell 8	Cell 9
R_A	7.2	9.1	27.5	0.9	0.9	1.1	4.2	16.5	4.5
Average R_A		14.6±11.0			1.0±0.1			8.4±7.0	
K_{wt}					0.0081±0.0005				
$\Delta R_A\%$	67.0	52.7	52.3	-0.7	-0.6	1.0	27.9	33.8	26.9
Average $\Delta R_A\%$		57.4±8.4			-0.1±1.0			29.5±3.7	

Table 2. DNR accumulation in CEM/VLB cells as modulated by VER (50 μM), IQ (100 μM), and PSC833 (3 μM) using SASCA-A. For notations, see Figure 11 and eqn (4-5). Reprinted with permission from Royal Society of Chemistry (Li et al., 2011)

3.5 Dynamics of drug accumulation in MDR cells

We believe the understanding of drug accumulation dynamics in MDR cells will benefit the design and exploitation of novel MDR inhibitors. Therefore, we have also developed a mathematical model to describe the process of drug accumulation. As shown in the Figure 9b inset, the drug accumulation rate is determined by the drug uptake process as well as the drug efflux process by Pgp pumps. Assuming that the drug uptake is by passive diffusion

and the drug efflux is controlled by an enzymatic process based on the Michaelis-Menten equation (Peng and Li, 2004, Agarwal et al., 2007), we developed the following equation to describe the drug accumulation dynamics (See the reference (Li et al., 2011) for the derivation).

$$\frac{dc}{dt} = \frac{A}{V} \times \left(\underbrace{D \times \frac{c_{out} - c_{in}}{x}}_{(a)} - \underbrace{\frac{v_{\max} \times c_{in}}{K_m + c_{in}}}_{(b)} \right) \tag{6}$$

where C is the concentration of DNR; t is the time; C_{out} and C_{in} are the extracellular and intracellular DNR concentrations, respectively; D is the diffusion coefficient; A is the cell surface area; V is the cell volume; x is the cell membrane thickness; and v_{max} and K_m are the maximum rate and the Michaelis-Menten constant, respectively, in the Michaelis-Menten equation for the drug efflux process.

From this equation, we find the initial drug accumulation is dominated by the drug uptake process when there is a large gradient (C_{out}-C_{in})/x between extracellular and intracellular DNR concentrations. Due to this large gradient, the initial drug accumulation rate is very fast, as shown by Curve 1 in Figure 11 (before 100 s). When the gradient across the cell membrane becomes smaller, the drug uptake becomes slower (see part (a) of eqn (6)). The drug efflux process begins to play a significant role in the dynamics of the drug accumulation process (see part (b) in eqn (6)). When the drug uptake rate is close to the drug efflux rate, that is, part (a) is similar to part (b) in eqn (6), it will reach a 'steady' state, as shown by the part of curve 1 (Figure 11) after 450 s. However, when a MDR modulator is applied, it will bind to Pgp, and inhibit the drug efflux pump, leading to a decrease of the maximum efflux rate (v_{max}) shown in eqn (6). Accordingly, the drug uptake process again dominates, as shown by the upward slope transition in the drug accumulation (see Curve 2 in Figure 11). Therefore, the data obtained from the SASCA-A method can help us to understand the drug accumulation in multidrug resistance. Further work about the simulation of the various processes involved in multidrug resistance is underway. A note of caution: the cellular kinetic response can be misinterpreted by averaging the data obtained from bulk analysis (Di Carlo and Lee, 2006).

4. Conclusion

The same-single-cell analysis conducted in a microfluidic chip has demonstrated the advantages in identifying MDR modulators, and in quantifying the MDR reversal effect of drug candidates during the drug efflux stage based on the defined parameter of ΔP. It has been demonstrated that SASCA is superior to DISCA by ruling out the difference in MDR activities among different cells and presenting a conclusive result about the effect of MDR modulators by using the same cell as both the test and the control cell.

SASCA is also compared with the conventional flow cytometry method in the study of MDR modulator candidates. The time needed to conduct SASCA is shorter than flow cytometry, and the microfluidic operation will become less tedious after automation in cell manipulation and fluorescent data collection. Moreover, the microfluidic SASCA method can provide time-dependent drug transport kinetics and cell morphological information, and only a small amount of cells are needed to confirm the findings. Therefore, this technique may have significant potential for investigating drug resistance in minor cell subpopulations (e.g. cancer stem cells) that may be the key determinant of clinical response to chemotherapy.

In addition, this microchip-based method, SASCA, is envisioned for clinical use as a companion diagnostics method, e.g. to check the MDR profile and drug responses so as to identify personalized drugs before patient treatment starts. This is an important step in determining the drug efficacy in individual patients. This approach will be even more robust if multiple parallel channels/structures are used to improve throughput (Di Carlo et al., 2006)(Faley et al., 2008). Moreover, the time-dependent data from SASCA can help us to understand the kinetics and mechanism of drug accumulation or efflux in MDR cancer cells.
Compared to the SASCA approach for the MDR modulation study in the drug efflux stage (Li et al., 2008), the new method—microfluidic SASCA-A has four significant advantages. (1) It is simpler; as illustrated in Figure 9, it does not need multiple drug accumulation and drug efflux stages as used in the previous SASCA method. (2) It is faster; a typical SASCA-A experiment could be finished within 2200 s ($t_{a1}'+t_{a2}'$ in Figure 9b). That is about one fourth of the time used in the previous SASCA experiments ($t_{a1}+t_{e1}+t_r+t_{a2}+t_{e2}$). As a more efficient assay method, this improves the throughput of MDR assays. (3) SASCA-A provides more 'identical' and reliable controls. Here, the previous SASCA has two limitations. First, the previous SASCA needs a longer measurement time. Long term exposure to cytotoxic drugs is detrimental to cells because the cell status or health conditions may change over time. To make sure the cell still have similar conditions, a second control experiment could be conducted, as we reported previously in testing ART (Li et al., 2008). Nevertheless, this dual control experiment makes the assay more complicated, and consumes more time. Secondly, there is a time interval of ~ 3700 s ($t_{e1}+t_r+t_{a1}$ in Figure 9a) between the two efflux stages in the previous SASCA approach; that is, the two efflux stages start at different life time points of a cell. Even though these points refer to the same cell, they are still not true identical controls. In terms of Pgp, which has been the most studied drug efflux protein (Perez-Tomas, 2006) (Gillet et al., 2007)(Davey et al., 1996), research shows that after the drug molecules bind to the transmembrane domains of the protein, the ATP-binding domains are activated and the drug molecules are then transported out of the cell by a major conformational change of Pgp via an enzymatic process (Perez-Tomas, 2006)(Higgins, 2007). During this time interval of ~ 1 h, any change in the enzymatic process might cause changes in the drug efflux abilities of Pgp pumps.
Considering these two limitations from the previous SASCA method, SASCA-A takes less time and it compares the time points just before MDR modulator tests. Therefore, SASCA-A has a more 'identical' and reliable control than SASCA. (4) SASCA-A may be more accurate in representing the patient's cells during cancer chemotherapy in which the cancer patient is exposed to a continuous drug infusion for a defined period of time.
In conclusion, it is found that it is less time-consuming to evaluate MDR reversal effects of drug candidates by studying the drug accumulation stage than the efflux stage using SASCA. Having said that, in the investigation of MDR dynamics, it might be less complex to study the drug efflux stage than to study the drug accumulation stage. It is because in the former case, only the drug efflux process is involved, but in the latter case, both drug uptake and efflux processes should be accounted for. Therefore, the combination of studying both stages will likely provide insight for the overall mechanism of multidrug resistance, especially when associated with cancer stem cells.

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Part 7

A Biologist's View

28

Evolution of Cancer Stem Cells

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1. Introduction

Theodosius Dobzhansky (1973) proclaimed, "Nothing in biology makes sense except in the light of evolution." But how can evolution make sense of something as manifestly maladaptive as metastatic tumors, leukemia, and lymphomas? How does evolution explain cancers invading and destroying vital tissues? How are "[u]ncontrolled cell proliferation" (Sherr, 1996) and "[g]enetic lesions that disable key regulators" (Sherr, 2000) reconciled with evolution?

Possibly cancers appeared unbidden in vertebrates without having evolved! This possibility cannot be dismissed out of hand, since animals on invertebrate branches of the metazoan tree, Lophotrochozoa, Ecdysozoa, bilaterians, coelomates, and deuterostomes develop non-malignant growths spontaneously *but not cancers* (Sutherland, 1969; Matz, 1969).

On the other hand, *induced* malignancies in *Drosophila* (Gateff & Schneiderman, 1969), "[a]lthough not naturally occurring" (Gonzalez, 2007), and aberrant patterns of cell death and changes in specification in *Caenorhabditis elegans* suggest that cryptic cancers exist in invertebrates. Moreover, widely distributed molecular homologues (i.e., genomic equivalents) in metazoans point to fundamentally "conserved" or "canonical, core pathways" common to human cancers and invertebrate tissues (Potts & Cameron, 2011). For example, "ancestral forms of myc and max [onco]genes … [appear in] the early diploblastic cnidarian *Hydra*" (Hartl et al., 2010), and a portion of an acute myelogenous leukemia gene (AML1) has 67% identity over 387 base pairs with 69% amino acid identity with the *Drosophila* segmentation gene *runt* (Erickson et al., 1992). In addition, cell death is induced by genotoxic stress in *Drosophila* as it is in cancers (Jin et al., 2000).

Other molecular evidence also supports the notion of cancer's evolution. For instance, evolutionary creativity, competition, and selection are suggested by redundancy of the human p53 cancer suppressor gene known as the "guardian of the genome" (Levine & Oren, 2009). Moreover, the planarian homologue of human p53 "functions in stem cell proliferation control and self-renewal" (Pearson & Alvarado, 2010); "ancestral forms" of p53 "mediate … multiple stress responses in the soma" of *C. elegans*; and "a primordial p53 ancestor gene which appeared early in phylogenesis" is found in the squid, *Loligo forbesi* (Schmale & Bamberger, 1997).

More direct evidence for cancers in invertebrates has emerged from efforts to evaluate effects of pollutants on animals. For example, a transmissible sarcoma that breaks out epizootically in Maryland soft-shell clams, *Mya arenaria*, would seem to be infectious but may also be synergistically promoted by contamination with the pesticide chlordane (Farley

et al., 1991). Herbicide contamination is also correlated with outbreaks of gonadal neoplasms (seminomas and dysgerminomas) and catastrophic declines of reproduction in softshells (Gardner et al., 1991a) and in hard shell clams (*Mercenaria spp.*) (van Beneden, 1994). Similarly, the eastern oyster, *Crassostrea virginica*, develops neoplasm at multiple sites when exposed to suspensions of Black Rock Harbor sediments known to contain "genotoxic carcinogens, co-carcinogens, and tumor promoters." And winter flounders fed on the blue mussel, *Mytilius edulis,* raised on contaminated sediments develop renal and pancreatic neoplasm "demonstrating trophic transfer … up the food chain" (Gardner et al., 1991b). The carcinogens in polluted effluvia, such as polynuclear aromatic hydrocarbons, chlorinated hydrocarbons, pesticides, and/or metals sequestered by aquatic bivalves induce liver neoplasm in teleosts and in human beings (Stegeman & Lech, 1991).

Animals, larvae, and embryos also play tricks on cancers that seem rooted in an evolutionary past. For example, an aqueous extract from the common clam (*Mercernaria mercenaria*) promotes regression in viral induced tumors in hamsters and melanomas in mice (Li et al., 1972). The soft coral, *Sarcophyton glaucum,* produces an anti-tumor agent effective against the development of chemically induced mouse skin and rat colon carcinoma (Narisawa et al., 1989). And receptors for the snail hemagglutinin HP present "on leukaemic lymphocytes … in combination with conventional surface marker analysis provides a new important tool for monitoring patients with CLL [chronic lymphocytic leukemia]" (Hellström et al., 1976).

Another argument in favor of cancers' evolution relies on reminiscences of recapitulation, namely that presumptive ancestral types of animals and younger human beings foster fewer malignancies than adult human beings. For instance, in *Drosophila,* "metastases nearly always occur in transplanted [adult] hosts rather than in the larva in which the primary tumours first arose" (Gonzalez, 2007). In human beings, the "overall incidence of cancer in persons under 15 years of age is one-thirtieth that of the population as a whole … Indeed, most pediatric cancers consist of leukemias, lymphomas, and sarcomas … In contrast, more than 80% of adult cancers in the United States are carcinomas … and 8% are hematopoietic with a higher preponderance of myeloid leukemia than is observed in children … Carcinomas are rare in persons under age 30, rising exponentially in incidence thereafter" (Sherr, 1996).

Rather than never having had cancers, invertebrates and human young seem to have evolved successful strategies of cancer suppression, at least before anthropogenic pollution lowered the bar to tumorigenicity. Conceivably, in human beings, inclusive fitness (the sum of advantages that project a living thing's progeny into the next generation) pushed cancers generally and carcinomas and myelomas in particular into adult years beyond the reproductive prime.

On balance, evidence of cancers having evolved is abundant and robust. If Dobzhansky is right, therefore, the light of evolution will yet illuminate the biology of cancers (Shostak, 1981, 2007-8; Zimmer, 2007).

2. Studying cancers' evolution

In order to avoid ambiguity, the sense in which the "evolution of cancer" is used here must be distinguished from the sense in which the "evolution of cancer" is typically used in the oncology literature. Oncologists typically equate "cancer's evolution" with "tissue independent [gene-expression] signature[s] associated with metastasis" (Ramaswamy et al.,

2003; van 't Veer et al., 2002) or mutational patterns (aka spectra) appearing throughout a cancer's development from a single cell. The "cancer genome … [is said to leave] an archaeological record bearing the imprint of [mutagenic and DNA repair] processes" (Stratton, 2011).

This developmental/genetics' sense of "cancer's evolution" is *not* the sense in which the term is used here. Here, "cancers' evolution" refers to cancers' proterozoic origins and subsequent history of adaptations leading to contemporary malignancies.

Phylogenomic analysis would be the method of choice for studying cancers' evolution in the sense intended here, and, no doubt, such an analysis will be feasible when "[o]ver the next 5 to 7 years … tens of thousands of cancer genomes will be sequenced … an essentially complete set of cancer genes … revealed … [and] the complete catalog of somatic mutations provided by the sequence of the cancer genome" (Stratton, 2011). Today, however, cancer's phylogenomics are inaccessible. Rather, spotty spectra of mutations all but obliterate the trail of cancers' genomic phylogeny. Instead of genomic coherence, clonal diversity in cancers is found in copy number DNA profiling (Notta et al., 2011) and multiplexing fluorescence in situ hybridization (Anderson et al., 2011). The results of single-nucleus sequencing in two ductal human breast cancers and paired liver carcinomas show that "metastatic cells arise late in tumour development" and that tumors grow by "punctuated clonal evolution" with few persistent intermediates (Navin et al., 2011). Making matters worse, rather than translocations at oncogenic sites producing cancers (Bohr et al., 1987; Croce, 2008), single catastrophic events lead to massive chromosomal rearrangements, and chaotic chromosomal architecture (Stephens et al., 2011; Berger et al., 2011; Tubio & Estivill, 2011). Furthermore, outside the cellular mainstream, "cancer can be initiated in cells … [with] long-term reconstituting ability … [and] self-renewal capacity" (Visvader, 2011); metastases may be formed where malignant niches recruit cells from local or circulating sources (König et al., 2005); and tumors may arise from long dormant cancer initiating cells (CICs) "with a metastatic potential … [to] disseminate … even at a premalignant stage" (Ansieau et al., 2008). Ultimately, "it is unclear how best to assess the effects of new genetic lesions on … growth, differentiation, tumorigenicity and functionality" (Pera, 2011).

Cancers' evolution is thus pursued here the old-fashioned way, by following Charles Darwin's lead and asking, "[without supposing] that the modifications were all simultaneous … [how would d]ifferent kinds of modification … serve the same general purpose" (Darwin, 1958 [1872])? In the case of cancer, the notion of a "general purpose" is epitomized by cancers' stem cells invading normal tissues and destroying their cells while metastasizing, and, growing elsewhere in the organism to the same effect. Darwin's question becomes, therefore, what "kinds of modification" would produce cancers' stem cells?

Two distinctly different possible answers stand out: (1) Cancers' stem cells arose from normal self-renewing cells which added invasiveness, destructiveness, and metastasis to their repertoire of cell behaviors; (2) Cancers' stem cells and normal tissues' stem cells arose through competition within cell populations in response to evolutionary pressures and adaptive advantages.

3. Is the stem cell the root of cancers' evolution?

Did a rudimentary stem cell provide the ancestral branch or common root of cancers' stem cells? The cancer stem cell theory encapsulates this idea by proposing that normal tissues

and cancers converge on stem cells. The problem is to find common ground among the many cells identified as both normal and cancer stem cells.

In general, stem cells fall into three or four categories: adult stem cells, separated into organ stem cells (OSC aka somatic stem cells) and hematopoietic stem cells (HSC), germ stem cells (GSC), and embryonic stem cells (ESC). Each of these has its malignant complement: cancer stem cells (CSCs) in solid tumors complement OSCs in solid organs; malignant HSCs (malHSCs) in leukemia, lymphoma, and related cancers represent the malignant counterpart of HSCs of normal blood and lymph; malignant GSCs (malGSCs) in testicular and ovarian cancer are the malignant counterparts of oogonia and spermatogonia; malignant embryonic stem cells (malESCs), thought to be present in small cell cancers and other malignancies, resemble (hypothetical) retained or reproduced post-embryonic ESCs.

The list is easily expanded by adding other cells called stem cells (see below), but the list is also contracted by squeezing one or another so-called stem cell into the above categories. For example, mesenchymal stem cells (MSCs) resemble HSCs or marrow stem cells (also MSCs) in several ways including differentiating as skeletal muscle, fat, cartilage, or bone (Young et al., 2004). Even GSCs are easily absorbed in the HSC category, inasmuch as both types of stem cells are derived embryonically from wandering, infiltrating, and colonizing cells (see Shostak, 1991), and both are especially plastic in the range of cells ultimately differentiating from their stock.

The most problematic stem cells are the ESCs. Whether they exist in adults at all is uncertain, although OSCs and HSCs are sometimes said to be virtual ESCs. This claim would seem vastly exaggerated, since neither OSCs nor HSCs possess ESCs' prime virtue of differentiating into cells of all three germ layers. Rather, ESCs are subsumed by germ layers in early development and disappear entirely in the parenchyma and stroma of adult organs during morphogenesis. OSCs and HSCs then emerge fresh in adult tissues.

Some similarities between the behavior of embryonic and cancer cells suggested that cancers originated from leftover or restored embryonic cells, but, historically, the alternative idea that stem cells produced metastases took precedence. This idea is traced to Rudolf Virchow. Even if he didn't use the term, he clearly attributed metastasis to unique proliferative cells as distinguished from differentiated cells. He wrote, "the transference … disposes different parts to a reproduction of a mass of the same nature as the one which originally existed," although, later, he added that he "must confess" that he can do no more than "allow it to be possible that the diffusion by means of vessels may depend upon a dissemination of cells from the tumours themselves" (Virchow, 1971 [1863]).

Evidence of stem cells as a source of cancer was indecisive until it became overwhelming: injected single murine embryonal carcinoma cells (ECCs) produced teratocarcinomas (Kleinsmith & Pierce, 1964); cells of a non-T cell line produced human acute lymphoblastic leukemia (ALL) (Kamel-Reid et al., 1989); "primitive hematopoietic cells" as opposed to "committed progenitors" produced human acute myelogenous leukaemia (AML) (Lapidot et al., 1994; Bonnet & Dick, 1997); small "$CD44^{+}CD24^{-/low}$ $Lineage^{-}$ [cell] populations" uniquely formed breast cancers (Al-Hajj et al., 2003), and as few as one hundred CD133 positive cells from human brain cancers recreated "classical histopathological features of [the patient's original] tumour type" in immuno-compromised mice (Singh et al., 2004). The idea of "a small subpopulation of leukemic stem cells that possess extensive proliferative capacity and the potential for self-renewal" was quickly generalized to a cancer stem cell theory according to which cancers were stem cell-supported and metastases were stem cell-dependent (see Lobo et al., 2007).

Cancer stem cell theory's great attraction was the explanation it offered for two of malignancy's great enigmas, namely, recurrence and the enhanced resistance to chemo- and radiation therapy displayed by the returning cancer (O'Brien et al., 2007–8; Gilbert & Ross, 2009; Ropolo et al., 2009). The explanation was seductively simple: Chemo- and radiotherapies targeted the abundant, rapidly dividing non-stem cancer cells, while rare stem cells dividing at low rates escaped the effects of treatment and regenerated the cancer. Moreover, since selection for a predisposition to resistance was also in play, the recurrent cancers had enhanced resistance to similar therapies. The prognostic and therapeutic implications were unmistakable: the fewer stem cells, the more promising the prognosis; eradicating a cancer depended on eliminating all stem cells.
Cancer stem cell theory soon launched a virtual cancer stem cell industry. Its business was to define, find, and isolate cancer stem cells for the purpose of destroying them.

3.1 Defining stem cells

Stem cells' principal attribute is self-renewal, the ability to maintain or expand a specific population of stem cells through cell division while also producing cells that give rise to a tissue's or a cancer's bulk (characteristic) cells. Self-renewal takes place in either a maintenance or expanding mode. In normal adult tissues at homeostasis, OSCs, HSCs, GSCs, and possibly ESCs undergo maintenance self-renewal by dividing asymmetrically thereby giving rise to different sibling cells (Chartier et al., 2010). Generally, one cell replaces the stem cell and one enters a "transit amplifying" (TA) pathway of division, terminal differentiation, and disposal. CSCs, malHSCs, malGSCs, and malESCs also perform asymmetric division, producing stem and bulk tumor cells, (Norton, 2007-8; Powell et al., 2010; Quyn et al., 2010), but following a premalignant transition and in growing cancers some stem cells also undergo expanding self-renewal by symmetric division (Tomasetti & Levy, 2010) thereby giving rise to identical self-renewing sibling cells, enlarging the cancer stem cell population and contributing to tumorigenesis.
The difference in the mode of self-renewal places cancer and normal stem cells on a sliding scale rather than separate stem cell branches. Some other differences between normal and cancer cells are also differences of degree rather than kind. For example, ECCs produce benign cells and normal tissues within teratocarcinomas (Pierce, 1974) and differentiate into normal mammary epithelium in epithelial-free mammary fat pads of athymic (aka nude) mice when mixed with mouse mammary epithelial cells (Bussard et al., 2010a).
But defining stem cells by self-renewal may still not homogenize them. According to the "gold standard assay" (Clarke et al., 2006), putative stem cells renew themselves while giving rise to tumors following transplantation in vivo and to tumor-like nodules following serial passage in vitro. The assay breaks down, however, for identifying stem cells that resist transplantation and nodule formation.
A different assay identifies stem cells without recourse to transplantation or passage. This assay relies on the retention of label by cells in long-term pulse-chase experiments and the premise that stem cells divide rarely. For example, putative smooth muscle stem cells of the uterine myometrium are labeled in perpetuity by a pulse with the DNA nucleotide-mimic 5-bromo-2–deoxyuridine (Szotek et al., 2007). These "label-retaining cells" (LRCs) are also found in the endometrial epithelium and stroma (Chan & Gargett, 2006), intestinal absorptive and gland epithelium, mucous epithelium of the tongue (Fellous et al., 2009), mammary epithelium (Booth et al., 2008), neurons (Das et al., 2003), satellite reserve skeletal

muscle cells (Shinin et al., 2006; Conboy, Karasov, & Rando, 2007; Kuang et al. 2009), and in cancers of the breast (Trosko, 2006; Bussard et al., 2010b) and intestine (Barker et al., 2008). LRCs are also found in yeast (Klar, 1987), bacteria, plants, fungi, the round worm, *Caenorhabditis elegans,* the fruit fly, *Drosophila,* and elsewhere (Tajbakhsh et al., 2009).

A sluggish division rate might represent an anti-mutation adaptation since delaying cell division provides an opportunity for correcting replication errors and performing DNA repair. Thus, in cancers' stem cells, the post- and pre-mitotic gaps (see below) function as checkpoints for DNA damage and damage response signaling networks (Bao et al., 2006; Kuntz & O'Connell, 2009). Lengthening these gaps and suspending progress through the cycle, therefore, would aid in repairing damaged DNA (Wang et al., 2009). On the other hand, cells with damage too severe to be adequately repaired are dispensed without replicating their errors.

But if the LRC divided repeatedly after acquiring the labeled DNA precursor, the cell might have remained labeled because it retained labeled "immortal strands" of DNA while casting off unlabeled DNA strands replicated during the chase phase of the experiment (Cairns, 2006). The retention of "immortal strands" of DNA would also seem an anti-mutation adaptation, since it would help keep stem-cell DNA pristine by reducing opportunities for errant base substitution during replication (Cairns, 2006; Seaberg & van der Kooy, 2003; but see Sotiropoulou et al., 2008). "Immortal strand" retention may "apply to only a subset of stem cell lineages" (Neumüller & Knoblich, 2009), and epigenetic changes, such as an increase of methylation, may accumulate in "immortal strands" thereby compromising the efficacy of this "anti-mutation" adaptation (Genereux, 2009). But asymmetric division is a decidedly regulated process in some stem cells where it occurs, for example, in the GSCs of male *Drosophila* where the older "centriole is always in the centrosome that is … retained by the stem cell" (Gonzales, 2007). Hence, retaining "immortal strands" is not a mere coincidence and is presumably adapted to some function such as mutation prevention.

3.2 Finding and isolating stem cells

Putative stem cells are found by in situ hybridization with antibodies for specific antigens. For example, antigens for *Lgr5* gene products label LRCs in intestinal glands and hair follicles (Bussard et al., 2010b). Some markers are associated predominantly with malignant stem cells. For example, human breast cancer cells are $CD44^{+}CD24^{-/low}$ (Al-Hajj et al., 2003), leukemic stem cells (LSCs) are CD34 positive CD38 negative (Bonnet & Dick, 1997), and colon cancer (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) and human glioma cells are CD133 positive (Singh et al., 2004). Other markers change with malignant progression. For example, the "$CD133^{+}$, epithelial-specific antigen-positive … population is increased in primary non-small cell lung cancer (NSCLC) compared with normal lung tissue and has higher tumorigenic potential in SCID mice and expression of genes involved in stemness, adhesion, motility, and drug efflux than the $CD133^{-}$ counterpart" (Bertolini et al., 2009). But problems arise over the antigen detected, the antibody used, the specificity of the antigen/antibody complex (see Lobo et al., 2007; Rao et al., 2010), and how closely tied the antigen is to a self-renewal signal pathway (Barker & Clevers, 2007).

Happily, some techniques accommodate multiple criteria allowing for "cross checking." Conspicuously, cytometric cell sorting allows researchers to combine multiple criteria for stem cells while providing living cells for further experimentation. With the help of fluorescence-activated cell sorting (FACS; Watt, 1998; Osborne, 2010), researchers can isolate presumptive OSCs, HSCs, CSCs, malHSCs, and putative malESCs in a "side population"

(SP) of cells able to reduce their load of supravitally absorbed dye (i.e., they exhibit Hoechst 33342 or Rhodamine 123 "effluxing"). Much like chemotherapeutic reagents, incorporated Hoechst 33342 and Rhodamine 123 are pumped out of (i.e., "effluxed" from) presumptive stem cells via the action of transporters (i.e., members of the ABC transmembrane protein family such as the ABCG2 transporter pump in mice) said to be uniquely over expressed in stem cells and embedded in their boundary lamella. Thus, presumptive stem cells have been isolated in SP fractions of cells from a host of normal organs, tissues, and cell populations: bone and dental tissues, cardiovascular tissue, endometrium (lining the uterus), endothelia (lining blood vessels), epidermis, gastrointestinal epithelium, mammary gland, neural tissue, pituitary and thyroid glands, and elsewhere (Welm et al., 2003; see Telford, 2010).

And some SP cells originating from cancers also pass the "gold standard assay" and form tumor-like nodules in minimum, low adhesion medium, while they produce histologically recognizable tumors in histo-compatible mouse strains such as immuno-incompetent nude mice, immuno-compromised non-obese diabetic (NOD), severe combined immunodeficient (SCID) mice, combined NOD/SCID mice, and more severely genetically compromised NOD/SCID mice. These SP cells also carry stem cell-relevant antigens and cell markers, for example, antigens associated with high plasticity (Sox2 and Oct4, but see Lengner et al., 2007), embryonic activities (stage-specific embryonic antigens [SSEA], Nanog, Sox4, Isl-1, and Pax6; see Konala et al., 2010), and specific histotypic markers (pituitary specific factor [Prop1]) alone and in combination (Garcia-Lavandeira et al., 2009).

A problem arises, however, about the size of a transplantable stem cell population identified operationally in the SP fraction. When does size exceed reasonable expectations for "a small subpopulation" conforming to traditional expectations for stem cells? Consider, for example, a "tumorigenic subpopulation with [melanoma] stem cell properties enriched in a $CD20^+$ [SP] fraction [that] produces tumor-like non-adherent spheroids in culture with the plasticity of neural crest stem cells and a capacity for self-renewal" (Fang et al., 2005). A small percentage (<0.1%) of these cells are transplantable in NOD/SCID mice, but as much as 20% of "melanoma tumor stem cells" (MTSCs) positive for neural growth factor receptor CD271 (Boiko et al., 2010) give rise to tumors in more highly immune-compromised mice (i.e., NOD/SCID mice lacking the interleukin-2 gamma receptor, i.e., natural-killer cell activity; Quintana et al., 2008). This high percentage of melanoma cells able to transfer the tumor to these mice "suggests that either virtually every melanoma cell is a CSC because it can induce de novo tumors in xenograft assays irrespective of any known stem cell marker, or that melanoma is not hierarchically organized into subpopulations of tumorigenic and nontumorigenic cells and the CSC model does not apply" (Roesch et al., 2010).

3.3 Normal and malignant stem cells: Comparisons and contrasts

Stem cells sit on top of differentiation pyramids of cells (Reya et al. 2001). Hence, inevitable similarities appear in normal and malignant stem cells. "Indeed, in several tissues, normal stem cells and cancer stem cells (CSCs) have been identified using the same set of markers" (Dey & Rangaragan, 2010). For example, paired antigens are found in lung parenchyma and malignant adenocarcinoma of the lung (Kim et al., 2005) and in pancreatic acinar and pancreatic cancer cells (Hermann et al., 2007).

Some similarities are readily attributed to routine functions performed by normal and malignant cells. For example, cells of both types undergo mitotic cycling, periodically going through mitosis (M [chromosomal events prior to and accompanying cell division]) followed by a post-mitotic gap (G_1), a period of DNA synthesis (S), and a pre-mitotic gap

(G_2). And some similarities may be superficial (i.e., analogies instead of homologies). Conspicuously, "self-renewal" in stem cells may be a consequence of "transformation" or immortalization (Shay et al., 2001). Immortalized normal cells even become tumorigenic when introduced in immuno-compromised mice. For example, human B-lymphoblastoid cell lines immortalized by the Epstein-Barr virus become cancer-like in several ways: expressing telomerase (the ribonucleoprotein that elongates telomeres), exhibiting aneuploidy (an abnormal number of chromosomes), sustaining mutations in the cancer suppressing p53 gene, and failing to undergo apoptosis (Sugimoto et al., 2004). And immortalization is effected by a variety of devices that may be irrelevant to oncogenesis or over-determined: fusion with cancer cells, treatment with carcinogens, transfection with particular oncogenes such as myc, activation of normal cellular proto-oncogenes, transformation with Epstein-Barr virus, retrovirus-mediated oncogene transduction, human T-cell leukemia virus type 1 (HTLV-1) and simian virus 40 large T-antigen oncogene, human papillomavirus, etc.

On the other hand, some difference may be rationalized with the help of reasonable argument. For example, the difference between symmetric and asymmetric division may be reconciled if division in stem cells is facultative rather than constitutive and if the same cells that contribute to homeostasis via asymmetric division can support growth via symmetric division (Morrison & Kimble, 2006). In male rats, for example, differences in the mode of division depend on conditions. Large cells with outer membranes rippling with amoeba-like pseudopods (as opposed to cells with a smooth outline) are committed GSCs (aka gonocytes) that perform both asymmetric and symmetric division. Although male GSCs maintain a steady state population as spermatogonia in adults, the cells proliferate symmetrically and generate spermatogenic colonies when transplanted to infertile testes (Orwig et al., 2002). Thus, at least some stem cells would seem able to divide both asymmetrically and symmetrically.

Greater difficulty is encountered rationalizing differences in label-retaining cells (LRCs), namely, their presence among OSCs and CSCs versus their absence in HSCs and malHSCs (but see Wilson & Trumpp, 2006). Caveats aside, if HSCs and malHSCs are not LRCs, they cannot differentially segregate new and "immortal strands" of DNA during asymmetric division (Kiel et al., 2007). And other differences cannot be ignored. For example, while OSCs and (most) CSCs are confined to niches, HSCs and malHSCs circulate in peripheral blood (and umbilical blood, in the case of the fetus and newborn). Furthermore, unlike products of OSCs and CSCs, products of HSCs and malHSCs, and their dormant memory cells (see below) may regain self-renewal.

HSCs also exhibit far greater potential than OSCs and give rise to clones of hematopoietic proliferative precursors or progenitors (HPPs) with greater competences than transit-amplifying cells (TACs) produced by OSCs. In vivo, bone marrow derived HSCs known as stromal cells have a reputation for extraordinary "transmutation" to nerve and other non-hematopoietic cells, even if, in vitro, their range of transformations narrows to osteoblasts, chondrocytes, adipocytes, and possibly myoblasts (Prockop, 1997). Consequently, HSCs were once thought to be available for extensive "reprogramming" and multilineage differentiation compared to other stem cells. Prior to 2006 when induced pluri-potential stem cells (iPSCs) came along, HSCs were supposed to be the great hope of regenerative medicine (Trounson, 2009).

Reprogrammability is not open ended, however, and early hopes for HSCs' did not pan out despite their vast multi-potentiality. HSCs failed to exhibit pluripotency (the ability to

differentiate into tissues formed by all germ layers) when injected into blastocysts (Geiger et al., 1998) and failed to differentiate as cardiac myocytes when injected into damaged hearts (Murry et al., 2004). Some claims for HSCs' multipotency may have been exaggerated as a consequence of fusion with differentiated cells (Terada et al., 2002; Ying et al., 2002; Wagers & Weissman, 2004). The ability to fuse may be an interesting characteristic of HSCs and HPPs, but it is not especially promising as a method in regenerative medicine. Ultimately, instead of progress toward applications in regenerative medicine, "confusion looks set to continue" (Check, 2007).

In addition, significant differences abound among post-stem cell (non-self renewing) products in normal tissues and cancers. TACs and HPPs both divide symmetrically producing clones of bulk cells committed to determined pathways of terminal differentiation and disposal, but HPPs have vastly greater competences for differentiation than TACs. The products of CSCs and malHSCs also differ in their plasticity, with malHSCs sometimes called "primitive HSCs" because of the greater range of malignant phenotypes available to them.

Typically, the malignant phenotype "progresses" from dividing and invading cells destroying tissue locally to metastasizing cells repeating these processes at new sites. In the process, CSCs produce cancer transit amplifying cells (CTACs). The CTACs of less malignant cancers, such as teratocarcinomas, undergo terminal differentiation in any of a variety of directions. More generally, the "difference between cancer and normal tissue renewal is that in normal tissue renewal, the number of cells that are proliferating is essentially equal to the number of cells terminally differentiating (undergoing apoptosis), whereas in cancer the number of cells that are proliferating ([cancer] transit-amplifying cells) is greater than the number of cells that are entering terminal differentiation, because of maturation arrest of the cancer cells in the transit amplifying population" (Sell, 2008). In more malignant carcinomas, CSCs or CTACs pass through an epithelial-to-mesenchymal transition (EMT), become motile, and all the more malignant and metastatic (Prindull & Zipori, 2004). Likewise, malHSCs produce malignant HPPs (malHPPs) that not only display the malignant phenotype but are recruited to metastastatic sites from circulation. MalHPPs have also been accused of re-acquiring self-renewal with its consequent resistance to radiation and chemotherapy (Lapidot et al., 1994).

Disposal also takes place through different mechanisms in the products of different stem cells. In cellular apoptosis or caspase-dependent cell fragmentation, cell fragments known as apoptotic bodies are ingested and digested by neighboring cells (known as entosis) leaving healthy tissue behind. In tissue disposal or caspase-independent programmed cell death, aka autophagy, cytokines attract leukocytes and immune cells inducing an inflamed response and mass destruction. Other cellular disposal methods include phagocytosis by macrophages in localized centers (e.g., spleen, thymus) of effete cells marked by components of the complement and/or immune system, and the shedding of mature cells at topographically external surfaces.

Unlike normally produced TACs and HPPs, AMLs produce massive numbers of malHPPs that die before differentiating (Bonnet and Dick, 1997). In contrast, CTACs may have prolonged lifetimes as a consequence of delayed programmed cell death. "When baseline levels of autophagy are compared with many cancer cells and noncancerous cells from the same tissue, decreased autophagy is observed in many cancer cells … [C]ells within the center of the tumor, deprived of an adequate blood supply have upregulated autophagic flux to allow for survival in the hypoxic and low nutrient microenvironment … Many cancer

therapies considered over the last couple of years have been thus paradoxically aimed at either inducing or reducing levels of autophagy" (Demaria et al., 2010).
In sum, the closer one looks the harder it seems to harmonize stem cells. Even *bona fide* stem cells do not fall comfortably into a single category. Stem cells cannot be present in small and large numbers, divide infrequently and frequently, be both long-lived and short-lived and both capable of retaining "immortal strands" of DNA and not. Oncologists, like other scientists suffer from the tendency to lump phenomena together and to over-generalize, but lumping cells together under the "stem" umbrella does not illuminate the mysteries of cancer. Thus, the possibility of tracing cancers' stem cells' origins to a rudimentary stem cell must be abandoned and the search begin again elsewhere.

4. Are cell populations the roots of cancers' evolution?

Did cancers' stem cells evolve through mutual competition and selection in cell populations? The problem answering this question is that little is known about cell populations and virtually nothing about their evolution.
Cell populations are groups of cells sharing developmental and morphological characteristics. Cell populations are the constituents of tissues (i.e., epithelia, connective, blood and lymphatic, muscle, and nerve tissue), of parenchyma (i.e., major, conspicuous or characteristic cell type), and stroma (i.e., supporting the parenchyma) of organs (Baker, 1988; Hughes, 1989; Harris, 1999). Initially, "cell populations constituting multicellular organisms … [were] roughly classified, based on their kinetics, into three main groups," static, transit, and stem (Lajtha, 1979). This classification required amendment, since "transit" cells were derived from stem cells and did not, therefore, constitute a unique class, and other cell populations were not static, transit, or stem (e.g., the endothelium of vessels).
Table 1 is a new taxonomy for animal cell populations at homeostasis based on three dichotomous descending divisions: (1) Classes of attached or epithelial-like cell populations versus unattached or amoeba-like cell populations, (2) subclasses of steady state versus static cell populations, and (3) subsets of stem versus non-stem cell populations. Both stem and non-stem populations are found in three of the four subclasses, the exception being the attached, static state subclass containing only stem-like (reserve) cell populations. In addition, the subset of unattached, static, non-stem cell populations is partitioned into cell populations with stress-induced and developmentally produced dormancies.

4.1 Classes, subclasses and subsets of cell populations

Attached or epithelial-like cells are mounted on an extracellular membrane (e.g., the basal lamella of the epidermis) and share intimate contacts with each other in the form of intercellular and gap junctions or synaptic junctions. Nuclei are typically enclosed in a cytoplasm limited by a plasmalemma, but cells may also fuse in syncytia containing multiple (nondividing) nuclei. Cells in attached populations have limited plasticity or range of differentiation. Mono-potent cells differentiate into only one type of cell, and oligo-potent cells differentiate into a few related types of cells.
In contrast, unattached or amoeba-like cells are embedded or suspended in extracellular material and do not have intimate contacts with each other. Amoeba-like cells may have intercellular bridges (sex cells; see Shostak 1991) or be fused in plasmodia (*Physarum*) with mitotically active nuclei (as distinct from syncytia). Unattached amoeba-like cells also tend to be oligo-potent or multi-potent, having competence to differentiate into more than one cell type epitomized by germ cells.

Steady state cell populations produce as many cells by cell division as they lose through terminal differentiation and cell disposal. In contrast, static state cell populations do not produce new cells and lose cells primarily as a result of wear-and-tear, trauma, and aging.
Stem cell-supported populations are hierarchal containing different types of dividing cells, some of which (i.e., stem cells) are self-renewing and also give rise to clones of terminally differentiating cells. The populations may cycle at a constant rate, and be homogeneous, or they may cycle at different rates, move out of phase, and be heterogeneous.
In contrast, non-stem cell populations are non-hierarchal containing uniformly dormant cells or more or less identical cells that are both dividing and differentiated. Cells divide symmetrically in or out of phase. They are non-hierarchal, since they are more or less uniformly differentiated, although differentiation may proceed stochastically, regressively, or progressively across spatial and physiological gradients.

4.2 Specific categories of somatic cell populations

All adult somatic cell populations fall into eight categories (Table 1): (1) cache cells (CCs), (2) organ stem cells (OSCs), (3) reserve cells, (4) neoblasts, (5) stressed cells, (6) quiescent cells, (7) hematopoietic stem cells (HSCs), and (8) mesenchyme. Cache cells and neoblasts are primitive cells in the attached (epithelial) and unattached (amoeboid) categories, respectively. Other normal cells in these classes represent derived cells including germ cells placed in the HSC category. Neoplasm occurs and cancers develop in all but two of the categories, namely 4 and 5.
The origin of germ stem cells (GSCs) from amoeboid, neoblasts, and interstitial cells in invertebrates, and conspicuously from wandering cells in vertebrates relegates GSCs to the unattached cell line and places them in the HSC category. The amoeboid spermatozoon of nematodes makes the case plainly, and, like vertebrates' HSCs, embryonic GSCs invade and colonize ectopic sites (germinal ridges).
Because embryonic stem cells (ESCs) are not recognized in adult tissue, they do not appear in Table 1. Neoplasm typically attributed to malignant malESCs, however, is cited in categories 2, 7, and 8.

4.2.1 Cache cells (CC) and cancer cache cells (CCCs)

The parenchyma of glandular organs (e.g., liver) is typically comprised of CCs. The cells "appear mitotically equivalent" (Rhim et al., 1994) and uniformly differentiated (but see Alison, 1998; König et al., 2005). The population is non-hierarchal and steady state. Cells are mono-potent, committed to their specific cell type. Their state of differentiation may change stochastically or gradually. In the liver, for example, CC differentiation regresses as cells move centripetally on septa.
Cells are called "cache cells" because they constitute a "horde" of similar cells that can exceed normal rates of proliferation during regeneration the way a computer's "cache memory" promptly retrieves data (Shostak, 2006). Previously, parenchymal cells, such as hepatocytes were dubbed "expanding" cells (Leblond, 1972), because nearly all of them undergo cell division during regeneration (e.g., induced by partial hepatectomy), and the population's size expands virtually exponentially (Bucher & Swaffield, 1973). But at homeostasis the size of CC populations does not change, and the notion of expansion is inappropriate.

Attached, epithelial-like, mono- oligo-potent:

Steady state:

Non-stem, symmetrical division, differentiated, non-hierarchal:

(1) Cache cells (CCs) and cancer cache cells (CCCs)

CCs: superficially uniformly differentiated, mono-potent; CCCs: hepatoma carcinoma, angiosarcoma, (lymphangiosarcoma, or hemangiosarcoma), Kaposi sarcoma

Stem, asymmetrical division, hierarchal populations:

(2) Organ stem cells (OSCs), cancer stem cells (CSCs), and malignant embryonic stem cells (malESCs)

OSCs: self-renewing, homogeneous, produce TACs: symmetrically dividing, clonally committed, terminally differentiating, limited potency; CSCs: expanding, metastatic, produce CTACs: adenocarcinomas, non-small cell lung cancer (NSCLC); malESCs: heterogeneous tumors differentiate in embryo-like patterns (melanoma, glioblastoma)

Static state:

Stem-like, induced asymmetrical division, hierarchal:

(3) Reserve and reserve cell-derived cancer cells

Undifferentiated, arrested, retain ability to divide and differentiate, mono-potent; malignancies: rhabdomyosarcomas

Unattached, amoeba-like, oligo- multi-potent:

Steady state:

Non-Stem, symmetrical division, non-hierarchal:

(4) Neoblasts

Undifferentiated, cell division regulated by nutrition, multi-potent

Static state:

Non-Stem, stress (starvation) induced mitotic arrest, retain ability to divide, non-hierarchal:

(5) Stressed (regeneration or stockpile) cells

Undifferentiated, stress induced mitotic arrest, may resume mitosis when stress is lifted (i.e., animals fed)

Non-Stem, developmentally induced mitotic arrest, retain ability to divide, non-hierarchal:

(6) Quiescent cells and their derived cancer cells

Differentiated, developmentally induced mitotic arrest (e.g., Hayflick limit); may be irretrievably arrested and nil-potent (*C. elegans*) or resume division conditionally and oligo-potent (vertebrates); malignancies: fibrosarcoma, synovialsarcoma

Steady state

Stem, asymmetrical division, hierarchal:

(7) Hematopoietic stem cells (HSCs), malignant HSCs (malHSCs); malignant ESCs (malESCs), germ stem cells (GSCs) and malignant GSC (malGSCs)

HSCs: heterogeneous, produce hematopoietic proliferative precursor (HPPs) and memory cells, multi-potent; malHSCs (aka cancer initiating cells [CICs]), expanding metastatic malignant (malHPPs), leukemia, lymphomas; malESCs: small cell lung carcinoma; malGSCs: testicular and ovarian cancers

Static state

Stem-like, retain ability to divide, non-hierarchal:

(8) Mesenchyme (aka mesenchymal stem cells) and mesenchyme derived cancers (also malESCs)

Unifferentiated (fibroblast-like), arrested, oligo- multi-potent, malignancies: chondrosarcomas, osteosarcomas, malignant fibrous histiocytoma, and liposarcoma

Table 1. Classification of cell populations

The regeneration of CC populations would seem dependent on multiple controls. Regeneration in the liver, for example, tapers off when a normal mass is approximated irrespective of morphology, but a liver with its regenerative capacity exhausted by severe or chronic liver disease may yet regenerate as a function of proliferation by small stem-like oval cells in the intrahepatic bile ductules and (possibly) through the recruitment of extra-hepatic stem cells from bone marrow (König et al., 2005).

The mesothelium of the plural, pericardial, and peritoneal cavities, and the endothelium of vessels belong in the CC category, although endothelium is sometimes said to harbor stem cells (Potten et al., 1979). Endothelium may also consist of "mixed" CC and OSC-supported populations, and in glioblastoma, the presence of the same genomic alterations in a high percentage of endothelial cells and glioblastoma cells suggests that malignant neural cells transform into endothelium (Ricci-Vitiani et al., 2010; Wang et al., 2010) without cell fusion (Wurmser et al., 2004). The pancreatic parenchyma may also consist of "mixed" cell populations. Pancreatic islet cells divide symmetrically and thus qualify as CCs, although β pancreatic islet cells do not replace cells lost in type 1 diabetics (Dor et al., 2004). Pancreatic acini, on the other hand, harbor "multi-potent" stem cells with "a limited capacity for self renewal" (Weir & Bonner-Weir, 2004; Seaberg et al., 2004; Sangiorgi & Capecci, 2009; but see Brennand et al., 2007; Ku, 2008).

Polyploidy (i.e., abnormal multiples of the chromosome number) and binuclearity (i.e., the presence of two nuclei in a cell) are widespread among CCs. These conditions do not represent adaptations to streamlining regeneration, since smaller mononuclear diploid cells provide most new cells during regeneration (Sigal et al., 1999). Polyploidy and binuclearity may represent accommodations to increasing metabolic demands, since cells with these traits accumulate with age, chronic stress, and oxidative injury (Goria et al., 2001). But nuclei of binuclear cells may also be evidence of degenerate change. When two nuclei fuse and divide symmetrically, they produce tetraploid cells (Guidotti et al., 2003), and "ploidy reversal" or "reductive mitoses" occurring despite bipolar spindles results in chromosomal imbalance and aneuploidy (Duncan et al., 2010) conducive of cancer (Ganem et al., 2009).

CC populations may spawn symmetrically dividing cancer cache cells (CCCs). Endothelial CCCs, for example, are probably the source of angiosarcomas (lymphangiosarcoma, or hemangiosarcoma), and the spindle cells of Kaposi sarcoma may also be CCCs. Angiosarcomas and glioblastomas would seem to be composed of CCCs, but CCCs may become CSCs in "mixed" tumors consisting of CCC-like differentiated cells and undifferentiated CSC-like cells (e.g., polycythemia [myeloproliferative neoplasms]; Jepson, 1969).

Malignant hepatoma cells of hepatocellular carcinomas are archetypal CCCs. They divide symmetrically, rapidly and are sensitive to chemo and radiation therapy. Irradiated cells may be arrested at the G_2/mitosis checkpoint if the DNA damage caused by radiation exceeds a threshold of two chromatid breaks or "a few" double-strand breaks (Ishikawa et al., 2010). Surprisingly, rat malignant hepatoma cells are oncogenic or not depending on their site of introduction and age of a host. Possibly, instead of a homogeneous CCC population, a heterogeneous population includes subsets able or not to establish themselves in different circumstances (McCullough et al., 1998).

4.2.2 Organ stem cells (OSCs) and cancer stem cells (CSCs)

OSCs exhibit self-renewal by asymmetric division. They are the classic label-retaining cells (LRC) thought to divide infrequently and frequently retain "immortal DNA" strands. In

contrast the TACs produced by OSCs divide rapidly and symmetrically producing clones of cells, typically with limited potency.

OCSs occupy distinct niches where they undergo self-renewal (Li & Xie, 2005). Some niches are conspicuous such as the corneal limbus basal layer (Sun et al., 2010), the bulge of hair follicles (Clayton et al., 2007; Hsu et al., 2011), and the ends of intestinal glands between enteroendocrineocytes (Potten & Loeffler, 1990; Barker & Clevers, 2007). But some niches, such as the subventricular zone of the cerebral cortex and spinal cord (Lois & Alvarez-Buylla, 1993; Weiss et al., 1996; Merkle et al., 2004; Maric et al., 2007; Doetsch et al., 2009) are only identified loosely as areas of asymmetric division (Lajtha, 1979; Tumbar et al., 2004) and might not truly qualify as niches, since the "simple location of stem cells is not sufficient to define a niche. The niche must have both anatomic and functional dimensions, specifically enabling stem cells to reproduce or self-renew" (Scadden, 2006).

In the mammalian epidermis, self-renewal is constrained by the differential expression of β-1 integrins and binding to the extracellular matrix (Lavker & Sun, 2000). The niche determines if TACs form hair follicles, hair, and sebaceous glands (Hsu et al., 2011) or if blocks of cells moving outward through the epidermis toward the surface synthesize a variety of keratins and finally differentiate as disposable squames (Blanpain & Fuchs, 2006). Epidermal cells occupying other niches produce fingernails, toenails, claws, and hooves. In the small intestine, basal glandular niches (Barker & Clevers, 2007; Fellous et al., 2009) produce TACs that divide and differentiate. Absorptive, dome (M), and goblet cells (Lelouard et al., 2001) move outward and are disposed of en masse at the intestinal surface. Parietal and chief cells, enteroendocrinocytes, and exocrinocytes stay in the gland until they are disposed of individually.

Some astrocyte stem cells in the central nervous system (CNS) exhibit moderate oligopotency, since the products of their division differentiate as disposable neurons and glial cells (Quian et al., 2000; Doetsch, 2003; Walton et al., 2006). The CNS is derived from neuralectoderm, and, hence, from epithelium, but neuro/glioblasts produced by astrocyte stem cells are motile and amoeba-like, and the ependymal home of astrocytes (Weiss et al., 1996) lacks a basal lamina and therefore does not qualify as an epithelium. Neuro/glioblasts, thus have taken on amoeboid characteristics after de-epithelializing.

The relationship of OSCs to CSCs is ambiguous. Some solid tumors supported by CSCs share antigens with OSCs, and a stem subset among otherwise non-tumorigenic cells may express tumorigenicity (Bonnet & Dick, 1997; Al-Hajj et al., 2002; Hermann et al., 2007). CSCs do not necessarily arise in the same niches as those occupied by OSCs. For example, basal cell carcinoma arises in inter-follicular epidermis rather than the hair follicle's bulge (Youssef et al., 2010). On the other hand, malignant stem cells, such as those of non-small cell lung cancer (NSCLC), an adenocarcinoma, may be derived in situ from bronchioalveolar OSCs following malignant transformation, and the CSCs of breast and colon cancers share affinities with OSCs.

4.2.3 Reserve cells and cancers derived from reserve cells

Reserve cells are undifferentiated dormant cells within a differentiated (typically, but not exclusively static) parenchyma derived from attached cells. Reserve cells include astrocytes (Rice et al., 2003; Martens et al., 2002) and pancreatic acinar cells (Sangiorgi & Capecci, 2009), but satellite cells (also known as quiescent myoblasts) in skeletal muscle are archetypal (Hawke & Garry, 2001). The satellite/skeletal muscle framework suggests that satellite cells are mammalian skeletal muscles stem cells held in mitotic abeyance.

Satellite cells reside within or beneath the external lamina of muscle fibers (in the sublaminal space or zone between the lamina and the sarcolemma of the muscle fiber) and are distributed evenly along the length of muscle fibers (with the exception of the neuromuscular junction). The sites occupied by satellite cells constitute a diffuse niche adapted to permit regeneration over the length of muscle fibers. During skeletal muscle regeneration, satellite cells become self-renewing, albeit briefly (Schultz, 1996) via asymmetric division. The stem cells exhibit differential "immortal DNA" strand retention, and the precursors of muscle fuse with sarcomeres and differentiate as skeletal muscle (Tajbakhsh et al., 2009).

Reserve cells seem to have left the division cycle in the G_1 post-mitotic gap. Following trauma, the proportions of satellite cells in S and G_2 increase rather than drop-off demonstrating that cells have moved through the cycle (or that other cells have undergone apoptosis disproportionately; Relaix et al, 2006).

Reserve cells are frequent suspects in the cancer lineup. Rhabdomyoblasts, or embryonic and fetal skeletal muscle cells appear in benign rhabdomyoma, in malignant rhabdosarcoma, embryonic, alveolar, and adult rhabdomyosarcomas. The precise etiology of rhabdomyoblasts is uncertain, but satellite cells may be their precursors (Merlino & Helman, 1999; Mercer et al., 2006).

4.2.4 Neoblasts

"Neoblast" is the generic term for dividing amoeboid cells in many well-fed, sponges, cnidarians, flatworms, and other protostomes. Neoblasts exhibit multi-potentiality during steady-state homeostasis, during regeneration, and somatic asexual reproduction, differentiating into a wide range of cells in the animal's body. Hence, neoblasts are also called "stem cells" in the sense that they "branch" out and differentiate into a variety of non-dividing cells, although they do not fulfill the additional stem-cell criterion of occupying a niche and representing a small slowly dividing part of a proliferative population. A distinguishing characteristic of neoblasts in flatworms and elsewhere is that cell division is down regulated by stress such as that brought on by starvation (Newmark & Alvarado, 2000; Reddien & Alvarado, 2004).

No malignant growths are attributed to neoblasts. Cell division in neoblasts seems to be held in check by homologues of the human p53 cancer suppressor gene which "functions in [planarian] stem cell proliferation control and self-renewal" (Pearson & Alvarado, 2010).

4.2.5 Stressed cells

Stressed cells (aka regeneration or stockpile cells) in invertebrates are derived from neoblasts and similar cells after entering mitotic arrest typically induced by starvation (Hong et al., 1998). In flatworms, the rate of cell division in neoblasts declines to a "basic level" as a result of starvation (Nimeth et al., 2004). These stressed cells are arrested at the G_2 stage, presumably as an adaptation for a rapid return to mitosis. G_2 arrested cells disappear following the resumption of feeding. The resulting highly plastic neoblasts then resume differentiating along multiple paths. In other animals, stressed cells may abandon the division cycle in G_1. When arrest is persistent, these cells are identified as G_0 or G_1/G_0 cells. No malignancies are attributed directly to stressed cells, although malignant cells may be "stressed."

In vertebrates, mitotically arrested cancer cells suffering from energy deficiencies due to a carbohydrate deficit might be considered stressed cells. Cancers acquire their energy largely

by glycolysis. Indeed, cancers' demand for glucose, known as cancers' "sweet tooth," and the enhancement of glycolysis, known as the Warburg effect after Otto Warburg who discovered it in 1924, are dose dependent and correlated with the aggessivity of the malignancy in vivo (Elstrom et al., 2004). The Warburg effect leads to the excess production of lactate that induces several oncogenes, causes an acid environment protecting cancer cells from the immune system, and allows pyruvate to scavenge endemic hyperoxides. At the same time, reduced cofactors remove free radicals and relieve high oxidative stress created meeting demands of rapid cell division (Kim et al., 2009). The Warburg effect also explains why tumors light up in positron emission tomography (PET) with a glucose radioisotope (Garber, 2004) and suggests that cancers might be selectively starved with low carbohydrate, high fat or insulin-induced hypoglycemia/lactate supplemented therapeutic diets.

Stress in mammals also triggers immuno-suppression that can be tumorigenic rather than therapeutic. For example, indirect deleterious effects of stress promote tumor development in rodents and human beings. Tumorigenesis under stress seems to result from immune suppression of natural killer cell activity (Ben-Eliyahu et al., 1999). For example, oxidative stress in myeloid cells makes them capable of inhibiting T-cell proliferation. The presence of oxidatively stressed cells "in a premetastatic niche … [may] help incoming tumor cells [i.e., CSCs] survive by inducing local immune suppression via inhibition of effector immune cells and by helping to evade immune system control, thus promoting metastasis growth" (Kusmartsev et al., 2008).

4.2.6 Quiescent cells and cancers derived from quiescent cells

Quiescent cells become mitotically dormant in the course of development (rather than as a consequence of stress). They are widespread in invertebrate adults. For example, in *C. elegans,* after adding cells throughout four larval stages, the hermaphrodite adult winds up with 959 quiescent somatic nuclei (1031 in males) arrested in G_0/G_1 (van den Heuvel, 2005).

In vertebrates, quiescent cells are represented conspicuously by fibroblasts (aka fibrocytes). Arrested in G_1, fibroblasts comprise numerous non-hierarchal, static state cell populations forming the bulk of stroma in organs including loose and dense, regular and irregular connective tissues. Osteocytes, chondrocytes, and possibly cardiac myocytes are also quiescent cells (Grounds et al., 2002).

Remarkably, although fibroblasts are not ordinarily dividing, they support division in other cells. An underlying layer of irradiated, non-multiplying "feeder" fibroblasts in vitro sustains cell division in other cells (e.g., embryonic stem cells, epithelial, and cancer cells). "Feeder" fibroblasts are employed to "condition" tissue culture media thereby promoting cell division and aiding the establishment and upkeep of fragile cell lines (Puck et al., 1956).

Fibroblasts can be provoked into division. They divide in the vicinity of wounds, in the uterine stroma during pregnancy, and in the breast during lactation. Dividing fibroblasts tend to remain fibroblasts although fibroblasts may be oligo-potent and differentiate into fat cells. And perichondral and periosteal fibroblasts also differentiate into cartilage and bone cells.

Freshly explanted fibroblasts in tissue culture perform a large but limited number of divisions (e.g., 50–70) after which the cells enter a period of "mitotic quiescence" that may last months but is eventually followed by cell death. Known as the Hayflick limit after Leonard Hayflick who discovered it in the early 1960s, the number of divisions performed by freshly explanted fibroblasts in vitro is inversely proportional to the age of the organism from which the fibroblasts were taken (Hayflick & Moorhead, 1961; Kill & Shall, 1990).

Possibly, telomeric shortening is the "replicometer" determining cells' Hayflick limit and mortality (Hayflick, 2000). The alleged "immortality" of transformed and cancer cells in vitro may be due to the over expression of telomerase and consequent maintenance of telomeres (Chan & Blackburn, 2002; Hackett & Creider, 2002; Shay et al., 2001).
Fibroblasts produce benign leiomyomas (aka fibroids), malignant fibrosarcoma, and synovialsarcoma. Fibroblasts are not otherwise prone to malignancy (but see mesenchyme below).

4.2.7 Hematopoietic stem cells (HSCs), malignant HSCs (malHSCs), germ stem cells (GSCs), and malignant GSC (malGSCs); malESCs (see 2 and 8)

HSCs are the root stem cells of blood and lymphatic cells and all their derivatives both in circulation and sequestered in connective tissue. HSCs also sprout branches virtually everywhere: osteoclasts in bone, microglia in the central nervous system, dendritic cells in epithelia, and macrophages (e.g., histiocytes and dust cells) in lungs and elsewhere.
Human HSCs are typically rhodamine 123 low and CD34 positive. HSCs, like their malignant counterpart, malHSCs, or "primitive HSCs" are typically heterogeneous with respect to rates of self-renewal and differentiation (Uchida et al., 1996; Hope et al., 2004). Cells range from rarely dividing stem-like cells to those on the verge of committed HPPs (Osawa et al., 1996).
HSCs are highly multi-potent and HPPs widely competent, differentiating across a wide range of cell types. Even after reserving the title HSCs "for cells already committed to a hematopoietic phenotype" (Herzog et al., 2003), HSCs include common myeloid progenitors (CMPs), similar to cells in *Drosophila* that provide endothelial cells lining vessels in addition to blood cells (Owusu-Ansah & Banerjee, 2009), highly plastic bone marrow-derived stem cells (BMDSCs or BMSCs), and marrow stromal cells (MSCs aka mesenchymal stem cells) that produce clones differentiating into fat, cartilage, and bone (see Kode & Tanavde, 2010). HPPs include multi-potent adult progenitor cells (MAPCs), and prolific myelogenous blast cells that give rise to the multitude of circulating and fixed blood and lymphatic cells. And while the small lymphocyte seems genuinely non-dividing (Bekkum et al., 1971), medium and large proliferative lymphocytes in lymphopoietic organs, germinal zones, and nodules remain in contention for dividing T or B lymphocytes as well as cells playing a host of roles in immunity.
HPPs can also become dormant "memory cells" (members of the B lymphocyte domain) that resume proliferation in response to unique antigens and growth factors (Ohta et al., 1998). Memory cells are way stations responsible for the secondary antibody response characteristic of acquired immunity and may function as "first responders" to new antigens.
The plasticity of HSCs and malHSCs suggests that they are accessible to extensive reprogramming and expansion of potential in the process of forming clones. Reprogramming, if that is what it is, may also occur in malHPPs. For example, BMDSCs pass through a "metaplasia/dysplasia/carcinoma progression" into adenocarcinoma of the stomachs of C57BL/6 mice chronically infected with *Helicobacter pylori* (Houghton et al., 2004). Moreover, BMDSCs form stromal myofibroblasts in esophageal adenocarcinoma including epithelial tumor cells and endothelial cells (possibly) following fusion with host cells (Hutchinson et al., 2010).
The production of malHSCs may also be determined by conditions rather than an inherent commitment to this particular fate. For example, "primitive" HSCs that become the malHSCs of leukemia/lymphoma stem cells (LSCs) over express the cancer inducing *bcl-2*

oncogene in the presence of serum containing the KIT ligand (also known as the steel factor cytokine or stem cell factor [SCF]) and undergo stimulated cell division at the onset of malignant differentiation (Domenet al., 1998; Domen & Weissman, 2000).

MalHSCs or LSCs also seem to be members of a heterogeneous population of cells differing in rates of self-renewal and degrees of commitment (e.g., in AML; Hope et al., 2004). LSCs seem to be common, since "more than 10% of cells in many mouse leukemia and lymphomas are transplantable" (Adams et al., 2007). In fact, AML cells in mice are easily transplanted to nonirradiated histocompatible (congenic) recipient mice (Kelly et al., 2007; Adams & Strasser, 2008) leaving the impression that bulk AML cells rather than stem cells as such are capable of propagating the malignancy.

The germ line fits the HSC mold. The adult male germ line beyond dormant spermatogonia is easily placed in this category of stem cells. Even dormant spermatogonia (Clermont, 1962) can be placed in the stem-cell category allowing that they mimic reserve cells. And the adult female germ line of mammals, once thought to be static, is now conceded to be stem. GSCs in the ovarian surface epithelium (OSE or germinal epithelium) produce primary follicles in vitro and in vivo while in contact with underlying connective tissue (Johnson et al., 2004; Bukovsky et al., 2005).

Germ line niches such as the basal compartment of seminiferous tubules (Lin, 1998; De Rooij & Grootegoed, 1998) may exert "extrinsic" influences on asymmetric divisions, but "intrinsic" cellular influences also affect the geometry of chromosomal delivery and the "unequal distribution of key regulators" (Kim & Hirth, 2009). In the *Drosophila* ovary, the position of oogonia near the end of terminal filaments seems to depend on the expression of the *piwi* gene that suppresses GSC differentiation while promoting self-renewal (Lin, 1997). Further down the filament, the oriented asymmetrical division of GSCs creates the cystoblast or germ-line cyst that gives rise to "assembly line organization, with each egg chamber representing a differentiated stem cell product whose position along the ovariole corresponds to its birth order" (Cox et al., 1998).

Remarkably, in *Drosophila*, asymmetric division in mutant GSCs takes place in the absence of the centrosome. "[C]entrosoms are not required for the proper orientation of the spindle relative to the … niche in female GSCs," but centriole orientation is essential for embryogenesis (Stevens et al., 2007), and spindle mis-orientation consequent to mutations may contribute to tumorigenesis. The activities of "tumour suppressors, *lgl, dig* and *scrib,* in controlling the asymmetric segregation of cell-fate determinants in larval neuroblast … [suggest] that impaired cell-fate determination itself could cause tumour growth" (Gonzalez, 2007).

MalGSCs are the presumptive cause of malignant testicular and ovarian cancers (Lin, 1997). Evidence linking malGSCs to GSCs is weak, but the nuclei of spermatogonia bear "cancer/testis" antigens (e.g., Brdt, SSX, NY-ESO-1, members of the melanoma antigen and SPANX families; MacLean & Wilkinson, 2005). (For malESCs see 4.2.2 and 4.2.8.)

4.2.8 Mesenchyme (aka mesenchymal stem cells [MSCs]) and cancers derived from mesenchyme

Mesenchyme is defined classically as the highly hydrated connective tissue of embryos (Shostak, 1991), but the drier adult connective tissue of bone, skeletal muscle, dermis, and heart are often said to contain mesenchyme (see Kode & Tanavde, 2010). The appellation "mesenchyme" is also attached to pericytes, contractile cells sharing the basal lamella with endothelium in capillaries and small venules. In addition, MSCs are frequently equated with

HSC-derivatives, marrow stromal cells (also MSCs), BMDSCs, and MAPCs. In effect, "mesenchyme" in adults is a synonym for a subset of generally quiescent fibroblasts readily mobilized for mitosis by growth factors. Mesenchymal cells are not known to be self-renewing and are not confined to a recognized niche, but they may otherwise resemble reserve fibroblasts. Alternatively, mesenchyme may be compared to a normally slowly dividing CC-like population but especially active in regeneration.

Mesenchyme's relationship to embryonic connective tissue must not be taken too literally or dismissed too lightly. Wnt genes link malignant mesenchyme to embryonic signal pathways. In malignant fibrous histiocytoma (aka high-grade undifferentiated pleomorphic sarcoma) expressing the DKK1 gene, the gene's protein, Dkk1, is an inhibitor of the Wnt developmental program. Inhibiting Wnt2 signaling in human MSCs or their progenitor cell products transforms them into malignant fibrous histiocytoma-like tumor cells following injection into immuno-compromised mice. Amazingly, reestablishing Wnt signaling in malignant fibrous histiocytoma returns the cells to their normal connective tissue status (Matushansky et al., 2007). Regrettably, Dkk1 does not perform the same trick in carcinomas.

Mesenchyme may also be a source of malESCs responsible for malignancies of soft tissue, in particular, following the malignant transformation of perivascular "mesenchymal" cells (Iwasake et al., 1987). Malignant chondrosarcomas and osteosarcomas may also have mesenchymal etiologies as may malignant fibrous histiocytoma and liposarcoma.

5. Evolution of normal and cancer cell populations

"Chance and necessity" (Monod, 1971) are the motors that drive evolution over the rocky road of Darwinian competition and selection. Multicellular animals have been on that road a long time and chance and necessity have had ample opportunity to work their magic on the tissues and cancers of animals. Epithelial cell populations would have the most ancient roots if attached cells evolved from biofilms and biomats (recently reassigned to the pre-Phanerozoic; Arp et al., 2001; Bengston et al., 2009). Newly discovered fossils of epithelial-like organisms clock in at 2.1 billion years before the present (El Albani et al., 2010). Amoeboid cells have ancient roots too if not quite as ancient as epithelia. Acritarchs associated with marine algae suggest that unicellular eukaryotes were around somewhere between the late Paleoproterozoic and Early Mesoproterozoic epochs, 1.6–1.3 billion years before the present (Knoll et al., 2006).

Competition between these life forms inevitably drove them into conflict, and a form of conflict resolution known as "escape toward" would have driven epithelia and amoeba into symbiotic relationships. Presumably, somewhere, some time, or, more likely, in many places and many times symbiotic relationships were attempted and an occasional one proved successful. Evolution's creative powers were then unleashed especially when "Life got big" (Narbonne, 2011) in the wake of fluctuating levels of free oxygen in the post-glacial early Ediacara (Yuan et al., 2011).

Today, the placozoan, *Trichoplax adhaerens* (Grell & Ruthmann, 1991) may be the last surviving purely epithelial metazoan, while vast numbers of amoeboid organisms testify to the continued viability of the amoeboid way of life. Competition and selection in epithelial/amoeba symbiotic organisms, however, proved more innovative and inventive, and led to the enormous diversity of tissues and organs found across the multicellular animal kingdom.

5.1 Evolution of cell populations and tumors with epithelial lineages

The evolution of epithelial-derived cell populations turns out to track increasingly sophisticated controls over cell division during the production of increasingly complex tissues. Solid tumors compete with epithelia and their derivatives largely by defeating controls over cell division while accommodating to tissue complexity.

The origins of an epidermis can be found in freshwater sponges (Demospongiae, Haplosclerida). Surface pinacocytes form an epithelium exhibiting close intercellular junctions that resist permeability and the diffusion of small-molecules while offering high transepithelial electrical resistance and a transepithelial potential. Pinacocytes retain these properties during regeneration and asexual reproduction and are not transformed into other types of cells (Adams et al., 2010).

In Hydra (Cnidaria, Hydrozoa), the epithelial epidermis and gastrodermis are composed of cache-like cell populations. In the epidermis, potency is limited to surface epithelium, battery cells, and possibly nerve and gland cells in the foot, while in the gastrodermis, potency is limited to digestive cells and possibly some digestive gland cells. The rate of cell division in these epithelia is a function of the availability of food (Shostak, 1979, 1982). Sustenance levels of feeding support the production of cells in sufficient quantities for maintenance (homeostasis) and regeneration. Feeding above sustenance levels supports growth, and further feeding supports asexual reproduction as well (Campbell, 1967a, 1967b; Shostak, 1968, 1974). Restraints on the growth of epithelia appear in some anthozoans (e.g., anemones), however, where body size and asexual reproduction are constrained (Shick & Hoffmann, 1980).

Regulation of growth increases in Platyhelminthese and Aschelminthes. Flatworms have a quiescent cellular epidermis (Rieger et al., 1991), and in adult round worms, with the exception of smaller species that retain a small number of cells, the subcuticular "epidermis" is a syncytium with quiescent nuclei plus a row of quiescent lateral line seam cells (Wright, 1991). The mere presence of multiple nuclei within a unified cytoplasm is not the explanation for mitotic dormancy, since nuclei in plasmodia such as those of insect eggs and the true slime mold, *Physarum*, divide abundantly. Rather, the absence of mitosis in syncytia would seem an adaptation for inhibiting growth.

Growth is also constrained internally as an accommodation to an unyielding integument or exoskeleton in animals where complexity militates against removing excess cells via asexual reproduction. The regulation of growth within the organism would also seem to have been a prerequisite for the evolution of complex internal organs (Extavour et al., 2005), and curbs on cell division seem to have ratcheted up with the complexity of parenchymal differentiation. In contemporary vertebrates, cells that have left their niches in embryos, such as sensory and motor neurons and skeletal muscle do not divide at all. Mitosis seems to have been curbed entirely in the course of evolution of highly differentiated cell populations where growth would be disruptive.

Other tissues adopted the steady state to meet size constraints without sacrificing the flexibility inherent in cellular replacement. CC populations epitomize steady-state cell populations, losing and gaining cells at the same rate in dynamic equilibrium. OSC populations then branched off CC populations when cell division was further restricted in a self-renewing population separated from the bulk of dividing TACs (Stanger et al., 2007). In OSC-supported populations, asymmetric cell division is confined to cells that remain in their niche following division. Reserve (satellite) cells evolved from OSCs by the further restriction of cell division to the point of arrest in G_1 "until needed."

Epithelial-derived tissues seem to have invested heavily over the course of their evolution in preventing oncogenic mutations. Thus, "the G_2/M checkpoint is invariably activated in cancer cells in response to DNA damage" (Wang et al., 2009). In G_2 arrested cells, entry to mitosis is blocked when Cdc25 phosphatases fail to remove the inhibitory phosphorylation of (inactivated) complexes of mitotic CDK, Cdc2 (aka Cdk1) and B-type cyclins. Moreover, the chief regulator of the G_1/S checkpoint is the tumor-suppressor p53 gene whose products also prevent the expression of NANOG and other embryonic stem cell factors associated with malignancy (Zbinden et al., 2010). The widespread retention of the "immortal strand" of DNA by OSCs and satellite cells would also seem an anti-mutation adaptation. The presence of label-retaining cells (LRCs) in breast and intestinal cancers (Trosko, 2006; Bussard et al., 2010b; Barker et al., 2008) suggests that these tumors' CSCs are derived from OSCs.

On the other hand, the cells of solid tumors seem to have devised mechanisms for competing successfully with the cells of normal solid organs. CCCs override the rules governing steady state dynamics in CC populations, and CSCs may have branched off OSCs by violating the terms of stem-cell regulation. "Mixed" cancers containing stem and non-stem cells (e.g., pancreatic cancer and myeloproliferative neoplasm) suggest that CCCs may also step-up to CSCs with increased malignancy. Thus, CCCs are equipped with two deadly weapons, the step-up to CSC and the epithelial-to-mesenchymal transitions (EMT) (Prindull & Zipori, 2004). With these weapons, tumor cell populations not only undermine the restraints imposed by cell-to-cell communication, but they escape the limits imposed by asymmetric division. Malignant cells increase in number, break out of their niche, and overpower normal defenses (Powell et al., 2010; Quyn et al., 2010).

Some solid tumors seem to begin as pure accidents. For example, cancers develop following "chromosome missegregation" of "lagging chromosomes" in damaged aneuploid hepatocytes (Ganem et al., 2009). And other epithelial cancers may be initiated, promoted, or progress through the accumulation of breaks, translocations, and errors of replication that prevent tumor suppressor genes from completing DNA repair, create aberrant products in signaling pathways, or permit the notorious EMT (see Ansieau et al., 2008). Genetic and epigenetic changes in some solid tumors suspend normal terminal differentiation and disposal, turning rapidly dividing TACs into malignant CACS. These malignancies are hotly pursued under the rubric of targeted therapy (Gilbert & Ross, 2009). For example, the Wnt/β-catenin, Hedgehog, and Notch signal transduction pathways of cell division are also pathways of differentiation and offer especially vulnerable points for therapeutic attack (Taipale & Beachy, 2001). In addition, these pathways are associated with tumor suppressors, such as PTEN (Stambolic et al., 1998) suggesting still other opportunities for therapeutic intervention.

5.2 Evolution of cell populations, leukemia, lymphomas, germ, and soft tissue cancers with amoeboid lineages

Amoeba-like cells are the obvious choice for ancestors of neoblasts, for unattached cells, and for freely moving cells including germ-line cells in multicellular animals. Contemporary amoebas even behave much like neoblasts and like scavenger blood cells in today's multicellular animals. "Interestingly … environmental cues such as temperature, starvation, and high population are potent inducers of autophagy in yeast, *Dictyostelium* and mammals … [as well as] dauer formation in *C. elegans*" (see below; Meléndez & Levine, 2009). Presumably, stress provokes ancient mechanisms in these cells' adaptive repertoire including the suspension of cell division.

Many amoebas cease dividing following starvation but resume cell division after turning to cannibalism. Similarly, large amoeboid cells or archeocytes in sponges (Porifera) acquire reserves by cannibalizing adjacent trophocytes in response to seasonal adversity and produce an encapsulated gemmule. When growth conditions return, the gemmule "hatches." Cells stream through the capsule's micropyle and commence cell division and morphogenesis.

Amoebas also exhibit multi-potentiality. For example, amoebas of the cellular slime mold (aka social amoeba), *Dictyostellium discoideum,* attracted by cyclic adenosine monophosphate (cAMP) to its source, congregate and differentiate into distinctively contrasting cells of slug and fruiting body (see Bonner, 1988; Margulis et al., 1990). Likewise, in freshwater sponges, multi-potential amoebocytes emerging from reduction bodies differentiate as choanocytes as well as various types of amoebocytes (Bisbee et al., 1989). In general, sponge amoeboid cells contribute to growth, maintenance, asexual reproduction, and regeneration by generating a variety of cells: fiber cells or desmocytes, muscle or myocytes, spongin-producing sponbioblasts, food-containing trophocytes, pigmented chromocytes, large archaeocytes, gland, and germ cells (Hanson, 1977).

In Cnidaria, amoeboid cells or interstitial cells produce as many as seven types of cnidocysts (average 3 per species; Shostak & Kolluri, 1995) as well as sensory and motor neurons, several types of gland cells (Hwang et al., 2007), and germ cells (Littlefield, 1985, 1991). Amoeboid cells also fill regression bodies in response to adversity, undergo multi-potent differentiation during regeneration (Shostak, 2005), and participate in asexual reproduction through budding, regenerative fragmentation, strobilation, and fission (Shostak, 1993).

In well-fed flatworms, multi-potential neoblasts proliferate and differentiate (Newmark & Alvarado, 2000). By replacing effete cells, neoblasts maintain specialized organs, the epidermis enclosing the animal, the gastrodermis lining its gut, and the "fixed" parenchymal cells between these epithelial layers. Neoblasts also aid in remodeling the animal during regeneration and reconstituting it during asexual reproduction (Pellettieri et al., 2010). In starving animals, neoblasts become dormant stressed cells but return to the neoblast status upon the resumption of feeding.

Likewise, larvae of the celebrated round worm, *C. elegans,* respond to stress by "conditional cell cycle arrest" (Hong et al., 1998). Thus stressed newly hatched, L1 larvae cease developing and enter the dauer diapause. Stressed cells remain in mitotic suspension indefinitely, prolonging the life of the larva (hence *dauer*), but, when conditions permit, the cells return to mitotic cycling, and development resumes (Meléndez & Levine, 2009) along determined lines of differentiation (Sulston et al., 1983).

Amoeba-like cells left a long evolutionary line of descendants in vertebrates from connective tissue to germ with blood and lymph cells prominently in the middle. HSCs and malHSCs are enormously plastic and spawn a variety of blood, lymphatic, and connective tissue cell types, normal and malignant. Their version of "stemness" has unique features. HSCs and malHSCs appear outside their niche in circulation. Recruitment or self-seeding is also characteristic of these stem cell. Thus HSCs repopulate organs (e.g., bone marrow, lymph nodes, and thymus) depleted by disease or radiation, while the arrival of circulating malHSCs (aka cancer initiating cells [CICs]) at sites of metastasis and the further recruitment of circulating malHSCs or malHPPs would seem at least partially responsible for the growth of leukemia/lymphomas (Zon, 2008). Recruitment might also be a point of attack for intervention. Leukemia/lymphomas might be kept from growth and brought back to the steady state by recreating the "environmental guidance" that prevents recruitment (McCulloch, 1983).

Fibroblasts of connective tissue seem to have adopted quiescence as a way of restraining growth, although cell division may still be an option as it is in so-called mesenchyme. Benign growths of fibroblasts do not compare with malignant sarcomas presumably of mesenchymal origin.

Both male and female germ lines clearly evolved from amoeboid cells as witness the extensive intercellular bridges present in pre-germ cells (see Shostak, 1991), while the "pseudopods" on the outer lamellae of male gonocytes would seem perfect reminiscences of amoeba. On the other hand, the epithelial-like zona pellucida (i.e., an extracellular membrane) surrounding mature mammalian eggs would seem a harbinger of epithelialization of the future blastocyst.

5.3 Evolution of cell populations with malignant embryonic cell lineages

Because the rates of cell division in some tumors, leukemia, and lymphomas actually approach exponential growth (see Shibata & Kern, 2007-8), cancers are sometimes said to represent the release of arrested embryonic cells (Sell, 2008) or a transformation of adult cells to an embryonic state (Weinberg, 1996). But high rates of cell division are also found in normal adult OSCs. Mouse intestinal OSCs, for example, divide once a day (Barker & Clevers, 2007). The appearance of an abundance of dividing cells in cancers (Norton, 2007-8; Tomasetti & Levy, 2010) may also be exaggerated as a consequence of stem cell recruitment (Zon, 2008).

Attributing cancers to anything resembling ESCs is all the more difficult, since normally, there are no ESCs in adults. ESCs that appear briefly in the mammalian blastocyst's inner cell mass and embryonic plate exist afterwards only in tissue culture or briefly following re-introduction into blastocysts.

Normally, in amniotic vertebrates, and conspicuously in placental mammals, the first wave of embryonic cells is diverted from embryogenesis toward establishing maternal contact. As the blastocyt implants in the uterus, massive numbers of small cells become motile. Strictly and irretrievably determined, these cells migrate beneath the chorion, fill out chorionic villi, and form the rudiments of a maternal/embryonic exchange system. Gradually, other embryonic plate cells, no longer ESCs, accumulate and fall under local and global commands directing them into germ layers, endo-, ecto-, and mesoderm. Subsequently, endoderm folds into the foregut; endo-mesoderm vesicles converge into the heart-forming region; dermo-myotomes and the neural crest de-epithelialize, and motile cells are released; gonocytes occupy the germinal ridges, and hemocytoblasts colonize liver and bone marrow (see Shostak, 1991). The local and global forces controlling these activities are so powerful that they can even bring small numbers of cancer cells, such as those of teratocarcinomas, into line and direct them toward normal pathways of differentiation in all germ layers (Minsk & Illmensi, 1976).

The possibility that latent ESCs continue in adults seems remote in light of their virtual absence in germ layers and differentiating tissues, but ESCs are sometimes thought to be represented by OSCs, HSCs, and GSCs, and these latent ESCs are even said to be the sources of malESCs. Thus, the notorious EMT is thought to be reminiscent of de-epithelialization in embryonic tissue releasing motile invasive cells. This possibility would seem especially apt for melanomas, malignant schwannomas (neurolemmacytomas) and malignant peripheral nerve sheath tumors (neurofibrosarccomas or triton tumors), all bearing putative ESC markers while resembling retarded embryonic cells differentiating along neural crest lines.

Other malignant cells said to be malESCs are small cells (typically smaller than a red blood cell but larger than a platelet; see Konala et al., 2010), as well as very small embryonic-like stem cells (VSEL-SCs) or small embryonic-like stem cells (SELSCs) expressing "early stem cell markers" such as CXCR4 and CD4, and "signature ESC genes" such as NANOG, a member of the HEDGEHOG-GLI signaling cascade, CD133 (Zbinden et al., 2010), Oct-4, and SSEA-4 (see Zuba-Surma et al., 2010; Sharma & Krishan, 2010). Like embryonic cells, the small malignant cells are multi-potent, differentiating into a variety of tumors from "pediatric sarcomas (e.g., rhabdomyosarcoma, neuroblastoma, Ewing-sarcoma Wilm's tumor) … [to adult] malignancies (e.g., stomach cancer)" (see Kucia et al., 2007). The most aggressive of these are probably small cell lung carcinoma (SCLC aka oat-cell carcinoma) and small-cell carcinomas appearing, if rarely, in the prostate and cervix (Mooi, 2001).

A difference in the number of mutations in two cancers (i.e., in their "mutational burden") provides the best evidence, if only suggestive, for a unique type of cancer cell, albeit not necessarily a malESC. The mutational burden for small-cell lung cancer (Pleasance et al., 2010) is only about half that of a non-small cell lung cancer (Lee et al., 2010). The difference does not seem to be due to mechanisms of mutation or efforts the cells make to correct errors in their DNA, since the frequencies of predominant changes in DNA, such as transversion of $G{\bullet}C \rightarrow T{\bullet}A$, are similar in both tumors, as are genomic rearrangements and gene translocations. Furthermore, mutation rates in the transcribed strands of DNA are lower than in the non-transcribed strands in both cancers. The different mutational burdens, therefore, would seem due to the small cell lung cancer's cells having accumulated mutations over a shorter period of time than the non-small cell lung cancer's cells. Conceivably, ES-like small cells residing in a dormant state would not accumulate as many mutations as non-small adult cells dividing regularly.

6. Conclusions

The present search for the ancestral branch and root of cancers' stem cells began by testing the merits of opposing hypotheses: A rudimentary stem cell is the ancestor of cancers' stem cells; the stem cells of different cancers evolved in different ancestral cell populations. The first hypothesis proposes that "self-renewal" unifies stem cells, while the second hypothesis proposes that cancers' different stem cells are unrelated.

Unexpectedly, the first hypothesis founders on irreconcilable differences among stem cells. Above all, OSCs and CSCs turn out to be label-retaining cells (LRCs), while HSCs and malHSCs are not (or not demonstrably). Thus, OSCs and CSCs preserve "immortal strands" of DNA and/or divide sluggishly, while HSCs and malHSCs do not preserve "immortal strands" and/or divide comparatively rapidly. What is more, while asymmetrical division occurs in both normal stem cells supporting steady-state populations and reserve cells supporting static cell populations, CSCs and malHSCs have added symmetric division to their modes of cell division (or have fallen back on embryonic habits) while exhibiting the malignant phenotype. In addition, HSCs and malHSCs are vastly more plastic than OSCs and CSCs, and the fate of clones and the disposal of products of terminal differentiation are also different. Thus, "stemness" is different in OSCs and CSCs, on one hand, and HSCs and malHSCs on the other, and stem cells cannot be brought under the umbrella of a unifying concept. The notion of a rudimentary stem cell giving rise to all stem cells must, therefore, be abandoned as without foundation.

Which leaves the possibility that the stem cells of different cancers arose through competition in cell populations. The similarities of CSCs to OSCs in their stem, steady state, attached cubbyhole, and of malHSCs to HSCs in their stem, steady state, unattached cubbyhole fit expectations, but the presence of malignancies in six of the eight categories of cell populations, including non-stem cells (Table 1), suggests that cancer/normal competition went well beyond stem populations. In each of these six categories, the cancer and normal cells have more in common with each other than they have with cells in other categories suggesting that each of these cancer and normal cell pairs arose in a common cell-population ancestor and adopted their normal and malignant phenotypes by competition.

Genomic evidence suggests, moreover, that the evolution of cancers in cell populations is ongoing (Notta et al., 2011; Anderson et al., 2011). For example, competition seems to have trimmed differences between lymphoblastic leukemia and breast cancer cells. Their "transcriptomes" (all the RNA produced in a cell population) or gene expression profiles (demonstrated through laser capture micro-dissection and DNA microarrays) display "extensive similarities" from initiation through progression (Ma et al., 2003) and from original masses to remote metastases (Weigelt et al., 2003). Furthermore, evolution is at work among genetically distinct lymphoblastic leukemia cells. These branch out into multi-clonal cancers, and, in lymphoblastic leukemia, the competitive regenerative capacity of cells growing in immuno-compromised mice (and the prognosis for patients from whom the cells were derived) changes with the tumors' genetic profile.

Of course, the old-fashioned Darwinian methodology employed here cannot say definitively if competition within cell populations gave rise to normal and malignant stem cells, but the evolutionary scenario sketched out here provides a model for future testing. According to this scenario, the evolution of animal cell populations began in symbiotes of epithelial and amoeboid cells in the pre-Phanerozoic. Initially, cell growth was indeterminate, subject only to the availability of resources. Excess cells were simply relegated to propagules of asexual reproduction. But restraints on cell division evolved in response to limitations imposed by animal size. Cellular quiescence or dormancy evolved in animals of small size and brief lifespan and in sequestered tissues, while the steady state evolved in long-lived, large animals and in tissues meeting size constraints while producing new cells in response to stress and contingency. A limiting scaffold determined the number of cells permitted in the steady state population while cell division was permitted to fill gaps.

More subtle controls were required to accommodate turnover in steady state cell populations sustaining cell loss in the process of meeting normal functional demands. Stem cells evolved when niches replaced the scaffold supporting steady state cell populations, and asymmetric division permitted the retention of one out of every two cells produced by division. Epithelial-derived stem cell populations placed a higher priority on controlling cell division than amoeboid-derived stem cells, it would seem, because attached cells are under greater pressure to conform to size limitations than freely moving cells. Thus, steady state CC populations evolved into stem-cell populations when cell division in self-renewing OSCs was constrained by the requirement to divide sluggishly while retaining the "immortal strand" of DNA. Cell division in "primitive" HSCs was not as greatly restrained in evolving self-renewing HSCs presumably due to the ease of disposing of excess cells.

Ultimately, cell populations produced the animals' tissues and organs. CC and OSC populations became organismal surface layers, the substance (parenchyma) of organs,

nerve, smooth muscle, and skeletal muscle equipped with reserve cells. Motile amoeba-like cells became amoeboid archeocytes, interstitial cells, neoblasts, stressed cells, the quiescent cells of connective tissues, and (probably) cardiac myocytes. HSCs' precursors also gave rise to mesenchyme and germ cells, and hemocytoblasts evolved in animals with mesothelial-lined cavities (Hartenstein, 2006). Epithelial and amoeboid characteristics also mixed, for example, as eggs epithelialized by oriented spindles, and amoeboid cells emerged from germ layers by de-epithelialization.

Likewise, cancers evolved through similar competition and selection in six of the eight categories of cell populations. And like their normal counterparts, cancers also mixed epithelial and amoeboid characteristics. For example, metastatic sites collect free cancer-initiating cells (CICs) and produce carcinomas, while the epithelial-to-mesenchymal transition (EMT) creates metastatic, invasive, and destructive amoeba-like cells from carcinomas.

As always, evolution is a push and pull process. Inclusive fitness has deployed successful strategies for neutralizing cancers in most animals. For example, small animals that discharge excess cells in reproductive propagules are not troubled by cancers, and other small animals having turned off growth in adult soma are preadapted to "cancer free" life. This option is not available for large animals, such as human beings, obliged to maintain cell replacement in steady state cell populations at homeostasis, but large animals are not bereft of alternative defenses against cancers. For example, inclusive fitness, it would seem, pushed most human cancers into the time of life beyond the prime reproductive years, and we are also well equipped with massive systemic defenses, such as the immuno-surveillance system. Of course, cancers evolved countermeasures such as recruiting stromal barriers to macrophages, and evolution exapted some cancers with a buffer of slowly dividing stem cells providing stubborn resistance to the best efforts at chemo- and radiotherapy.

Ultimately, competing evolutionary forces may resolve conflict and reach a detente. Thus, some (ancient?) malignant and normal cell populations would seem to have reached equilibrium (e.g., adenomas derived from CCCs) and many cancers are all but unknown except when induced by radiation, carcinogens, etc. On the other hand, highly malignant cancers (e.g., melanomas and small cell lung cancers) are far from equilibrium and may be newly evolving or easily provoked by conditions of contemporary life.

In sum, Dobzhansky has been vindicated, and the light of evolution brightens the outlook for making sense of cancer. Most importantly, researchers equipped with an evolutionary perspective may now be able to devise effective strategies for preventing cancers, detecting them early, and bringing those cancers that cannot be prevented into equilibrium with normal tissues.

Obviously, cancer's should not be given a competitive edge through exposure to anthropogenic carcinogens such as those in cigarette smoke, air pollutants, the polynuclear aromatic hydrocarbons, chlorinated hydrocarbons, pesticides, and/or metals in effluvia and food. Researchers should seek clues for prevention in the prophylactic devices deployed against cancers by most animals and our own young. Researchers hoping to detect, monitor, and track cancers should also take a hard look at perturbations in the biometrics of normal tissues competing with neoplasm rather than relying solely on the detection of cancer's markers. Finally, by correcting cancers' equation of state for competition and selection, cancers' evolution in the past should be plotted and steps taken to thwart the flow of cancers' evolution in the future.

7. References

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Acronyms and Abbreviations

2D: two-dimensional
3D: three dimensional
5-FU: 5-fluorocytosine
5α-R: 5α-reductase;
AAs: anaplastic astrocytomas
ABC transporters: Adenosine triphosphate-binding cassette transporters
ACTH: adrenocorticotropic hormone;
ADC: adenocarcinoma
ADP: adenosine diphosphate
AKT: subfamily of the serine/threonine protein kinases
ALCAM: activated leukocyte cell adhesion molecule
ALDH: aldehyde dehydrogenase
ALL: acute lymphoblastic leukemia
AML: acute myelogenous leukemia
Ang1: angiopoietin-1
antagomiR: anti-miRNA oligonucleotide
APC: antigen presenting cell
APC: adenomatous poliposys coli
AR: androgen receptor
ARACNe: reconstruction of accurate cellular networks
ASC: adult stem cells (see OSC)
ASCL1: achaete-scute complex homolog 1
ATM: ataxia-telangiectasia-mutated
ATM/ATR: ataxia telangiectasia mutated/AT Rad3-related
ATO: arsenic trioxide
ATRA: all-trans-retinoic acid
BAA: BODIPY-aminoacetate (fluorescent substrate)
BBB: blood-brain barrier
BCC: basal cell carcinoma
BCR: B cell receptor
BCRP; breast cancer resistance protein
BDNF: brain-derived neurotrophic factor
bFGF: basic fibroblast growth factor
bHLH: basic helix-loop-helix
BLI: bioluminescence imaging
BM: bone marrow
Bmi-1: a polycomb protein
BMP: bone morphogenetic protein
BMSC: bone marrow-derived stem cells
BM-MSC: bone marrow-derived mesenchymal stem cells
BrdU: 5′- bromo-2′-deoxyuridine

BTSC: brain tumour stem cell
CII: Mitochondrial complex II
CAF: cancer (carcinoma)-associated fibroblasts
cAMP: cyclic adenosine monophosphate
CAR: coxsackie-adenovirus receptor
CBC: clonotypic B cell
CBTRUS: Central Brain Tumor Registry of the United States
CCC: cancer cache cell
CCL5: chemokine (C-C motif) ligand 5
CD-AT-MSC: cytosine deaminase expressing AT-MSCs
CD: co-diffusing (surface markers)
CD::UPRT: cytosine deaminase::uracil phosphoribosyltransferase (a suicide gene)
Cdc: cyclin dependent kinase
CDK: cyclin-dependent kinase
cDNA: copy deoxyribonucleic acid
CEA: carcinoembryonic antigen
CGH: comparative genomic hybridization
CGNP: cerebellar granule neuron precursors
CIC: cancer initiating cell (aka TIC, CSC)
CK: cytokeratin
CK1: casein kinase 1
CLP: common lymphoid progenitor
CML: chronic myeloid leukemia
CMP: common myeloid progenitor
CNS: central nervous system
CoA: nuclear co-activator proteins
CoR: nuclear co-repressor proteins
CRC: colorectal cancer
CRT: chemo- radio-therapy
CSC: cancer stem cell (aka cancer-maintaining cell, CIC, TIC)
CSCLC: CSC-like cells
CSD: cold-shock domain
CTAC: cancer transit amplifying cell
CTL: cytotoxic T lymphocyte
DC: dendritic cell
DCIS: ductal carcinoma in situ
DDR: DNA damage response
DHT: 5a- dihydrotestosterone
DIF: differentiation-inducing factors
DISCA: different-single-cell analysis
DKK: Dikkopf (a secreted Wnt antagonist)
DLL4: delta like ligand 4
DMAPT: dimethyl-amino-parthenolide
DMEM: Dulbecco's modified Eagle's medium
DNA: deoxyribonucleic acid
DNR: daunorubicin
DSB: double-strand break

DSH (DVL, Dvl, also DSH/DVL): Disheveled phosphoprotein
EBRT: external beam radiation
EC: endothelial cell
ECC: embryonal carcinoma cells
ECM: extracellular matrix
EGCG: epigallocatechin-3-gallate
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ELP: early lymphocyte progenitor
E/M-MP: membrane E-cadherin^{low}/cytoplasmic E-cadherin^{high}/ CD133^{high}
EMMPRIN: extracellular matrix metalloproteinase-inducer
EMT: epithelial to mesenchymal transformation
eNOS: endothelial nitric oxide synthase
EpCAM: Epithelial cell adhesion molecule
EPC: endothelial progenitor cells
ER: estrogen receptor
ESC: embryonic stem cell
ESCC: embryonic stem cells cell cycle
ET: endothelium
ETS factor: epithelium-specific transcription factor
FABP2: fatty acid binding protein 2
FACS: fluorescence-activated cell sorting
FFPE: formalin-fixed paraffin embedded (specimen)
FGF: fibroblast growth factor
FLT: fetal liver tyrosine kinase
FLT3: FMS-like tyrosine kinase 3 (
FRP (also sFRP): Frizzled related proteins (a secreted Wnt antagonist)
FSC: forward scatter cell
G_0: post-mitotic dormancy
G_1: post-mitotic gap
G_2: pre-mitotic gap
GAG: glycosaminoglycan
GBM: glioblastoma multiforme
GC: germinal centre
GCNF: germ cell nuclear factor
G-CSF: granulocyte-colony stimulating factor
GFAP: glial fibrillary acidic protein
GFP: green fluorescent protein
GIN: genomic instability
GKS: Gamma Knife surgery
Gli: glioblastoma (family of transcription factors)
GPCR: G-protein coupled receptor
GPI: glycosylphosphatidylinositol
GRNs: gene regulatory networks
GSC: germ-line stem cell (aka gonocyte)
GSC: glioma stem cell
GSEA: gene set enrichment analysis

GSI: gamma secretase inhibitor
GSK: glycogen synthase kinase
HA: hyaluronan
HAT: histone acetyltransferase
HDAC: histone deacetylase
HDAC histone deacetylase inhibitors
HER2-targeting agent: Herceptin (trastuzumab)
HERP: HES-related repressor protein
Hes3; Hairy and Enhancer of Split 3
HGF: hepatocyte growth factor
Hh: Hedgehog
HHM humoral hypocalcaemia of malignancy
HHMI: Howard Hughes Medical Institute
HIF: hypoxia-inducible factor
HMEC: human mammary epithelial cell
HMLE: human mammary epithelial cell
HMLEN: *HER2/neu*-infected HMLE
HMLER *H-Ras*^{V12}-infected HMLE
HNSCC: head and neck squamous cell carcinoma
HPC: hematopoietic progenitor cell
HPPC: hematopoietic proliferative precursor cell
HPV: human papillomavirus
HSC: hematopoietic stem cell
Hsp: heat shock protein
HSV: herpes simplex virus
ICP: immortal cell progenitor
IDO: indoleamine-2,3-dioxygenase
IFN: interferon
IGF: insulin-like growth factor
IGFBP: insulin-like growth factor binding protein
Ig: immunoglobulin
IgH: Ig heavy chain
IGS: invasiveness gene signature
IHC: immunohistochemistry
IL: interleukin
IL-3Rα: interleukin-3 receptor alpha
INP: intermediate neural progenitor
iPSC: induced-pluripotency stem cell
ISC: intestinal stem cell
IWP: inhibitors of Wnt production and secretion
IWR: inhibitors of Wnt response
JNK: Jun N-terminal kinase (or Wnt/calcium pathway)
KLK: kallikerin-related peptidase
LBX: Ladybird homeobox
LCC: large cell carcinoma
LCM: laser capture microdissection
Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5

LH: luteinizing hormone
LH-RH: luteinizing hormone releasing hormone;
LIC: leukemia initiating cell
LIF: leukemia inhibitory factor
LL: Lewis lung (carcinoma)
LMPP: lymphoid-primed multipotent progenitor
LRC: label-retaining cell
LRH: liver receptor homolog
LRP: lung resistance-related protein
LRP: low density lipoprotein receptor related protein
LSC: leukemic stem cell
Lupeol: Lup-20(29)-en-3β-ol
M: mitosis (ordinarily referring to chromosomal events accompanying cell division)
mAb: monoclonal antibody
Mac: macrophage antigen
malESC: malignant embryonic stem cell
malHSC: malignant hematopoietic stem cell
MAPC: multipotent adult progenitor cell
MAPK: mitogen-activated protein kinase
MBD: methylated CpG binding domain
MDR: multi-drug resistance
(MEK)-MAPK: kinase-mitogen-activated protein kinase kinase
MELK: maternal embryonic leucine zipper kinase
MET: mesenchymal- epithelial transition
MET-PET: methionine positron emission tomography
MGMT: O^6-methylguanine-DNAmethyltransferase
MGUS: monoclonal gammopathy of undetermined significance
MHC: major histocompatibility complex
MIM: mitochondrial inner membrane
miRNA: micro-ribonucleic acid
MitoVES: mitochondrially targeted vitamin E succinate
MM: multiple myeloma
MMP: matrix metalloproteinase
MP: main population (as opposed to side population [SP])
MPO: myeloperoxidase
MPP: multipotent progenitor
MR: magnetic resonance
MRI: Magnetic resonance imaging
mRNA: messenger RNA
MRP: multiple resistance-associated proteins
MSC: marrow stromal cell or mesenchymal stem cell
MSC: mesenchymal stromal/stem cells
mTOR: mammalian target of rapamycin
MV: mosaic vessels
NRD: negative regulatory domain
NF-κB: Nuclear factor of kB

NHL: non-Hodgkin lymphoma
NICD: Notch intracellular domain
NK: natural killer cell
NKT: natural killer T-cell
NLRCs: nonlabel retaining cells
NLS: nuclear localization sequence
NMSC: normal mammary stem cells
NO: nitric oxide
NOD/SCID: non-obese diabetic/severe combined immunodeficiency
NPC: neural precursor cell
NSAID: nonsteroidal anti-inflammatory drugs
NSC: neural stem cell
NSCLC: non-small cell lung cancer (carcinoma)
oncomiR: oncogenic miRNA
OB: osteoblast
Oct4: Octamer 4
OPC: oligodendrocyte precursor cells
OS: overall survival
OSC: organ stem cell (aka somatic/adult stem cell)
OSCC: oral squamous cell carcinoma
OSM: Oncostatin M
OSMR: OSM receptor
PAP: prostate acid phosphotase
PAR: protease activated receptor
PCR: polymerase chain reaction
PD: population doublings
PDEF: prostate-derived epithelium-specific transcription factor
PDGF: platelet-derived growth factor
PFS: progression-free survival
PGBC: post-germinal center B cell
Pgp: P-glycoprotein
PI: propidium iodide
PI3K: phosphoinositide 3-kinase also phosphatidylinositol-3-kinase
PIG: placental growth factor
PINS: Partner of Inscuteable (a cortical cell polarity determinant)
PLCγ: phospholipase Cγ
PLK: fetal liver tyrosine kinase
PMA: phorbol 12-myristate 13-acetate
PPARγ: Peroxisome proliferator-activated receptor gamma
PPR: parathyroid hormone–related protein receptor
primiRNA: primary-miRNA
PSA: prostate specific antigen
PSC: prostate stem cell
Ptch1: Patched1
PTEN: Phosphatase and tensin homolog
PTH: parathyroid hormone

PTHrP: parathyroid hormone-related protein
PTL: parthenolide
QOL: quality of life
R: radioresistant
RA: Retinoic acid
RAR: Retinoic acid receptor
RARE: retinoic acid response element
Rb: retinoblastoma protein
Rho: Rhodamine123
Rbpsuh or Rbp: Recombining binding protein suppressor of hairless
RDGN: retinal determination gene network
RGC: radial glial cell
Rlx: relaxin
RNA: ribonucleic acid
RNAi: RNA interference
ROS: reactive oxygen species
R-Smad: receptor-related Smad protein
RT: radiation therapy
RTK: receptor tyrosine kinase
RT-PCR: real-time reverse transcription- polymerase chain reaction
S: period of DNA synthesis or replication in the cell cycle
SAA: serum amyloid A
SASCA: same-single-cell analysis
SC: stem cell
SCF: stem cell factor
SCID: severe combined immunodeficiency
SCLC: small cell lung carcinoma
SCP: senescent cell progenitor
SCT: stem cell transplantation
SDF: stromal derived factor
SF: steroidogenic factor
SFU: sphere forming unit
SGZ: subgranular zone
SHh (also Shh): Sonic Hedgehog
siRNA: small interfering RNA
SMO: smoothened
SP: side population (as opposed to main population [MP])
SPEM: spasmolytic polypeptide-expressing metaplasia
SQC: squamous cell carcinoma
SSC: side scatter cell
SSEA: Stage Specific Embryonic Antigen
STAT: Signal transducer and activator of transcription (an oncogene)
S-TRAIL: secretable TRAIL
SUMO: small ubiquitin-related modifier
SVZ: subventricular zone
T: testosterone
TAC: transition amplifying cell

TACE: tumor necrosis factor alpha converting enzyme
TAF: tumor-associated fibroblast
T-ALL: T-acute leukemia and lymphoma
TAM: tumor associated macrophage
TCF/LEF: T cell factor/ lymphoid enhancer factor
TCSF: tumor cell-derived collagenase stimulatory factor
TF: transferrin protein
TF: tissue factor
TFR: transferrin protein receptor
TG: transglutaminase
TGF-β: transforming growth factor beta
TIC: tumor initiator cell (aka CIC, CSC, tumor initiating cell)
TIM3: T-cell Ig Mucin-3
TMZ: temozolomide
TNC: tenascin-C
TNF: tumor necrosis factor
TOS: tocopheryl succinate
TPP+: positively charged triphenylphosphonium
TRAIL: TNF- related apoptosis-inducing ligand
TRBP: transactivating response RNA binding protein
TRG: tumor regression grading
Trk: tyrosine kinase
TSA: trichostatin
TSC: tumor stem cells
TSmiR: tumor suppressor miRNA
TSC: tumor stem cell (aka CSC)
TTF: Thyroid transcription factor
TVPC: Tumor vasculogenic stem/progenitor cell
UCLA: University of California, Los Angeles
uPAR: urokinase-type plasminogen activator
UTR: untraslated region (of mRNA)
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
VER: verapamil
VSEL-SC: very small embryonic-like stem cell
VM: vasculogenic mimicry
YB-1: Y-box binding protein-1

Edited by Stanley Shostak

Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancers' stem cells' evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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