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Breast Cancer

Focusing Tumor Microenvironment, Stem cells and Metastasis

Edited by Mehmet Gunduz and Esra Gunduz

BREAST CANCER – FOCUSING TUMOR MICROENVIRONMENT, STEM CELLS AND METASTASIS

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Meet the editors

Dr. Mehmet Gunduz graduated from the Faculty of Medicine of Hacettepe University in Ankara, Turkey in 1990. From 1995 until 2009 he studied and worked in Japan, both at Wakayama Medical University and Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences at various academic levels (from PhD candidate to Assistant Professor). During this period, he led a discovery and identification of roles of several cancer-related genes such as ING1 and 3. He has also been a visiting scientist in MD Anderson Cancer Center University of Texas, Houston, USA. Since March 2011, he is holding the position of a Professor at the Departments of Otolaryngology and Medical Genetics in School of Medicine Fatih University in Ankara. He has over 150 publications in internationally indexed journals, and over 160 presentations at various levels in national and international meetings. He also served as a reviewer for a number of journals.

Dr. Esra Gunduz is Associate Professor of Medical Genetics at Fatih University Medical School, Ankara, Turkey. She completed her PhD program in 2005 at Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Japan. She was awarded by the Japan Society for the Promotion of Science (JSPS) as a post-doctoral fellow in 2006. In 2009 she was appointed as Assistant Professor at Fatih University, and in 2010 she was promoted to Associate Professor at the same faculty. Her research focuses mainly on tumor suppressor genes. In her lab she leads various ongoing projects for her research teams. She contributed to the literature with forty articles in peer reviewed journals and over sixty presentations both in national and international meetings.

Contents

Preface

Cancer is the leading cause of death in most countries and continues to increase mainly because of the aging and growth of the world population as well as habitation of cancer-causing behaviors such as smoking and alcohol. Based on statistics of the GLOBOCAN 2008, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 (Siegel et al. Ca Cancer J Clin 61:212-236, 2011). Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Thus cancer researches, especially breast cancer, are important to overcome both economical and physiological burden. The current book on breast cancer aims at providing information about recent clinical and basic researches in the field. The book includes chapters written by well-known authors, who are worldwide experts in their research areas. The current book covers topics such as characteristics of breast cancer cells, molecular tumor classification methods, in vitro cancer models, breast cancer and microenvironment, breast cancer stem cells, gene regulation in breast cancer, and mechanism of breast cancer cell interaction, invasion as well as metastasis. We hope that the book will serve as a good guide for the scientists, researchers and educators in the field.

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Part 1

Breast Cancer Cell Lines, Tumor Classification, *In Vitro* Cancer Models

1

Breast Cancer Cell Line Development and Authentication

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1. Introduction

Inarguably, the development of cell culture and the ability to grow human cells *in vitro* has revolutionized medicine and scientific research. In the nearly sixty years since the first successful culture of immortalized human tumor cells in the lab in 1952, new fields of research have emerged and new scientific industries have been launched. Without cell lines, medicine would not be as advanced as it is today. Modern techniques that allow for manipulation of cell have allowed for a more complete understanding of the of fundamental basics of cellular and molecular biology and the biological system as a whole.

Different types of cell lines exist. Lines are maintained as continuous cultures, are established as primary cultures for transient studies, are created as explants of tumor or tissue samples, or cultivated from a single individual cell. Cell lines, especially cancer cell lines, are ubiquitous and are used for everything. By using cell lines, our understanding of cells and genes, how they function or malfunction, and how they interact with other cells has increased the pace of discovery and fundamentally changed how science is conducted. Cell lines have been established as a model of specific disease types. Individual cell lines have been derived from specific disease states and therefore possess specific characteristics of that disease state. Therefore, they are exceptionally useful to gain insight into normal physiology and how that physiology changes with onset of disease. Novel treatments and therapeutic strategies are investigated in cell lines in order to gain a fundamental detailed understanding of how a cell will react. Initial protocols are developed and tested in cell lines prior to use in animal models or testing in humans. This has enormous implications in discovery and reducing unintended side effects.

The first breast cancer cell line was established in 1958. Today, lines modeling the varied types of breast cancer help to develop targeted therapy and to provide a molecular signature of gene expression. Cell lines of estrogen/progesterone receptor (ER/PR) positive, ER/PR negative, triple negative (ER/PR/Her2), normal mammary epithelium, metastatic disease, and more are so widely used that it is nearly impossible to identify a recent discovery that hasn't used cell line models at some point during development.

Unfortunately, significant shortcomings of the use of cell lines exist. Cell lines are a model system. They do not always predict the outcome in humans and therefore, do not replace use of whole organisms. They are grown and tested in isolation, therefore the influence of neighboring cells or organs is non-existent in cell culture systems. Over time, cells can differentiate resulting in a change in phenotype from the original culture. Cell lines can

become contaminated by infectious agents such as mycoplasma or even by other cell lines. Such contamination may not be readily detectable and can result in dramatically different results leading to false or irreproducible data. Some of these issues can be addressed to thwart the waste of reagents, money, and time. This includes testing and authenticating cell lines while they are actively grown and in use in the lab. Companies exist that can test for mycoplasma infection or DNA fingerprinting of cell lines to authenticate a particular cell line. Other shortcomings are merely inherent to this model system and must simply be identified and addressed.

2. A brief history of cell culture

Since the first successful establishment of a human cancer cell line in 1952, cell lines have been the backbone of cancer research. They have provided the understanding of systems at the molecular and cellular levels. Cell lines are used in the vast majority of research labs to understand the fundamentals of basic mechanisms as well as the translation to clinical settings.

Modern tissue culture techniques were made possible through the contributions of many scientists across the world whose attempts to understand physiology and to establish a source of tissue to study lead to fundamental changes in our understanding of biology and medicine. Among the contributions include those of Sydney Ringer at the University College London, who determined the ion concentrations necessary to maintain cellular life and cell contractility, and ultimately created Ringers Solution. Through his seminal work in the 1880s, Ringer described the concentrations of calcium, potassium and sodium required to maintain contraction of a frog heart and began the steps towards modern day cell culture (Miller, 2004; Ringer, 1882, 1883). In 1885, Wilhelm Roux at the Institute of Embryology in Germany cultured chicken embryonic tissue in saline for several days. This was followed by the work of Ross Harrison at the Johns Hopkins University in 1907, who was the first to successfully grow nerve fibers in vitro from frog embryonic tissues. While this was the outgrowth of embryonic tissue, these tissue cultures were successfully maintained *ex vivo* for 1 - 3 weeks (Skloot, 2010)(Ryan, 2007b). In 1912, Alex Carrel at the Rockefeller Institute for Medical Research successfully cultured the first mammalian tissue, chicken heart fragments. He claimed to maintain beating chicken heart fragments in culture for over 34 years and outliving him by one year (Ryan, 2007a). Although controversy as to whether these cultures were authentic or supplemented with fresh chicken hearts still remains (Skloot, 2010). This controversy may have slowed progress towards the establishment of cell lines in culture to some degree, it did not prevent work to create a source of material and model systems to allow for testing *in vitro*.

It would be another 40 years before the establishment of the first continuously growing human cell line, however steady advances towards that goal were ongoing. Carrel, working with Charles Lindbergh, worked to create novel culturing techniques that included use of pyrex glass. This glass could be heated and sterilized to reduce, or preferably eliminate, bacterial contamination. This led to the creation of the D flasks in the 1930s which improved cell culturing conditions by reducing contamination (Ryan, 2007c).

Tissue culture took another leap forward in 1948 when Katherine Sanford at Johns Hopkins was the first to culture single mammalian cells on glass plates in solution to produce the first continuous cell line (Earle et al., 1943; Sanford et al., 1948). Prior to this, tissues were attached to coverslips, inverted and grown in droplets of blood or plasma.

Her work set the stage for modern practices of growing cells in media on plates or flasks (Sanford et al., 1948).

2.1 Establishment of the HeLa cell line and cell line production

Indoubtedly, the most important factor to change biomedical research and our understanding of disease at the cellular and molecular levels was the establishment of the first continuously growing human cell line, the HeLa cell (Gey et al., 1952). In 1952, Henrietta Lacks was a patient with adenocarcinoma of the cervix treated at the Johns Hopkins Hospital. A portion of her tumor was used in the laboratory of George Gey at Johns Hopkins University and the revolution of modern biomedical research began. These cells were grown in roller flasks in specialized medium containing serum developed by Evans and Earle et al. and continued to proliferate (Evans et al., 1951). Almost 60 years later, these cells are still proliferating in laboratories across the globe and used to increase our understanding of cellular mechanisms from cell signaling, to the implications of weighlessness/zero gravity on cellular aging, and everything in between. The implications of establishing this cell line have been tremendous and is still ongoing. HeLa cells have not stopped growing and neither has the vast amount of knowledge gleened from them.

In 1953, Gey demonstrated that HeLa cells could be infected with the polio virus and therefore were a useful tool for testing the efficacy of the polio vaccine that was under development. This set the stage for the mass production of cell lines for distribution and use worldwide. The National Science Foundation established the first production lab at the Tuskegee Institute in 1953 that would provide HeLa cells to scientists involved in the development of the polio vaccine (Brown and Henderson, 1983). The goal was to ship at least 10,000 cultures per week. At the peak of production, 20,000 cultures were shipped per week and a total of 600,000 cultures were shipped in the two years the lab was in existence (Brown and Henderson, 1983). This, along with the Lewis Coriell's development of the laminar flow hood to reduce contamination of cell cultures and methods to freeze and recover cell lines (Coriell et al., 1958; McGarrity and Coriell, 1973, 1974)(Coriell and McGarrity, 1968; Greene et al., 1964; McAllister and Coriell, 1956; Silver et al., 1964), led to the establishment of cell repositories to house and distribute cells. It also led to the development of tumor specific cancer cell lines that created models of different types of human cancer and to an explosion of understanding of how cells work without the influence or perturbation of other cells. These models were also an ideal system to test novel therapeutics and treatment strategies without use of whole animals or humans.

2.2 Culturing cells

The terms tissue culture and cell culture are used interchangeably, but in reality they are two distinct entities. While both methods are derived from specific cells isolated from the whole organism, the cultures established are quite different and used for different endpoints (Freshney, 2010a).

Tissue, or primary, cultures are established from isolated tissue or organ fragment, most commonly from tumor slices (McAteer and Davis, 2002). These primary cultures can be used either for immediate experimentation to determine how primary cells operate or to establish a continuous cell line. Generally, primary cultures are established through placing an organ explant into culture media and allowing for outgrowth of cells or by digesting the tissue fragment using enzymatic or mechanical digestion. By definition, these cultures are

transient. Primary culture refers to the period of time the primary tissue/organ fragment is kept in culture *in vitro* prior to the first passage or subculturing of cells, at which time they are referred to as a cell culture. This could range from days to a few weeks at most (MacDonald, 2002).

Cell lines are primary cultures that have been subcultured or passaged and can be clonal, terminal or immortalized cells (McAteer and Davis, 2002). Clonal cell cultures are created by selecting a single cell that will proliferate to establish a single population. Terminal cell lines are able to grow in culture for a few generations before senescence occurs and the cell line can no longer survive in culture media. Immortalized cell lines are able to grow in culture forever. These immortalized cell lines can occur naturally, such as HeLa cells, or through transformation events, such as Epstein-Barr Virus transformation. All types of *in vitro* cell cultures are used in breast cancer research.

3. The establishment of human breast cancer cell lines

The first human breast cancer cell line, BT-20, was established by Lasfargues and Ozzello in 1958 from an explant culture of a tumor slice from a 74 year old caucasian woman (Lasfargues and Ozzello, 1958). These cells are estrogen receptor alpha (ER) negative, progesterone receptor (PR) negative, Tumor Necrosis Factor alpha (TNF-α) positive, and epidermal growth factor receptor (EGFR) positive (Borras et al., 1997). While BT-20 is the oldest established breast cancer cell line, it is not the most commonly used line. By far, the most widely used breast cancer cell line worldwide is the MCF-7 cell line (Table 1 and Figure 1)(Burdall et al., 2003). Established in 1973 by Soule and colleagues at the Michigan Cancer Foundation, from where it derives its name, MCF-7 cells were isolated from the plural effusion of a 69 year old woman with metastatic disease (Soule et al., 1973). Since its establishment, MCF7 has become the model of ER positive breast cancer (Lacroix and Laclercq, 2004). Establishment of other cell lines has followed, including ones from other breast cancer types such as BRCA mutant, triple negative, HER2 overexpressing, and those derived from normal mammary epithelial cells such as MCF-10A cells (Soule et al., 1990) (Table 2).

Cell line use in labs is ubiquitous and continues to increase. From 2000 - 2010, the publication of manuscripts using the 10 most commonly used cell lines has almost tripled (2.8% increase) (Figure 2). Clearly demonstrating that the importance of, need for, and use of breast cancer cell lines will not diminish in the near future. Evaluation of the existing lines indicates that most breast cancer cell lines in use are derived from metastatic cancer and not other breast cancer phenotypes (Borras et al., 1997). Indeed, the overall success rate of establishing a cell line is only 10%. Most of the cell lines that exist today have been derived from pleural effusion instead of from primary tumors and are primarily ER - lines (Table 2 and reviewed in (Lacroix and Laclercq, 2004). This is surprising since ER - breast cancer is detected in only 20 - 30% of all primary tumors, whereas ER + tumors are detected 55-60% of the time (Ali and Coombes, 2000; McGuire et al., 1978). The reason for this discrepancy remains unknown, however it has been postulated that this could be because ER - cells are easier to establish in culture than ER + or that as cells are grown in culture, the epithelial like phenotype is lost while more mesenchymal traits are retained, therefore cells in culture appear to undergo a endothelial to mesenchymal transition (EMT) *in vitro* which is associated with the ER - phenotype (Lacroix and Laclercq, 2004). This suggests that culture systems are a model of metastatic disease that can grow in isolation and not a model the

wide heterogeneity of disease that is detected clinically. Although current cell lines are derived form only a subset of primary cancers, overall these lines are a reliable model to study the fundamental questions concerning cell growth, death, and the basic biology of breast cancer. Indeed, many advances in breast cancer biology have been made using cell culture systems and should not be dismissed because of these concerns.

Cell line	No of publications 1/1/2000 to 12/31/2010	origin
BT-20	79	breast
MCF7	11813	pleural effusion
MDA-MB-231	3489	pleural effusion
MDA-MB-435 *	719	pleural effusion
MDA-MB-468	486	pleural effusion
SkBr3	372	pleural effusion
T47D	1168	pleural effusion
ZR75.1	96	ascites
BT474	251	pleural effusion
MCF-10A	451	subcutaneous mastectomy
* not a breast cancer cell line		

Table 1. List of commonly used cell lines, the number of citations and their origin

3.1 Breast cancer cell lines as models of primary tumors

Using breast cancer cell lines clearly hold advantages over use of animal or human models. Beyond the ethical implications of animal or human use, the advantages to using cell lines include the ease of obtaining cell lines (can be purchased from commercial sources), the ease of harvesting large numbers of cells (can be grown in culture for long periods of time to accumulate the necessary concentration), and the ability to test an individual cell type without confounding parameters such as other cell types or local microenvironment (to date, no two cell lines can grown simultaneously in culture for extended periods). Conversely, much debate has circulated concerning the applicability of the data derived from isolated cell lines to the predicted outcomes in humans. One area that this debate has been most contentious has been regarding the importance of the immune system in cancer development. Clearly, the microenvironment and infiltrating immune cells contribute to development and progression of disease, therefore individual cells grown in isolation will lack the influence of other neighboring cells (Voskoglou-Nomikos et al., 2003). Genetic, epigenetic and cytotoxicity studies that focus on outcomes in breast cells clearly benefit from use of cell culture systems. The fundamental understanding of the underlying genetic or molecular pathways involved in breast cell growth and its response to cytotoxic agents are best understood in isolated cell culture systems (Voskoglou-Nomikos et al., 2003).

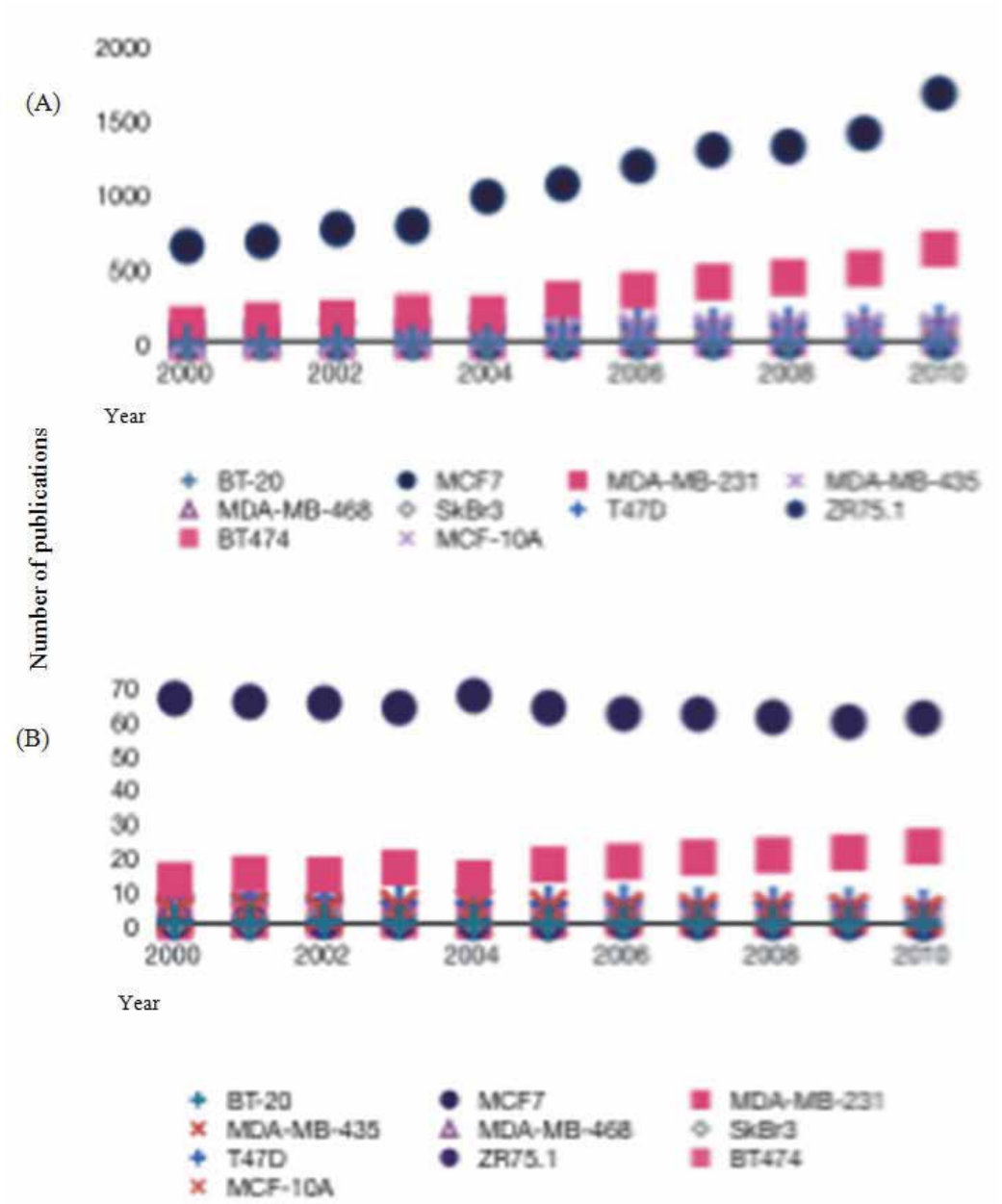

Fig. 1. The total number of publications per breast cancer cell line from 2000 through 2010. The most commonly used cell line is the ER+ MCF7 cell line, followed by ER - MDA-MB-231 cell lines. Many other cell lines are in use, however the number of publications using these models is quite small. A. Total number of publications using breast cancer cell lines. B. Each breast cancer cell line as a percentage of the total breast cancer cell lines used per year.

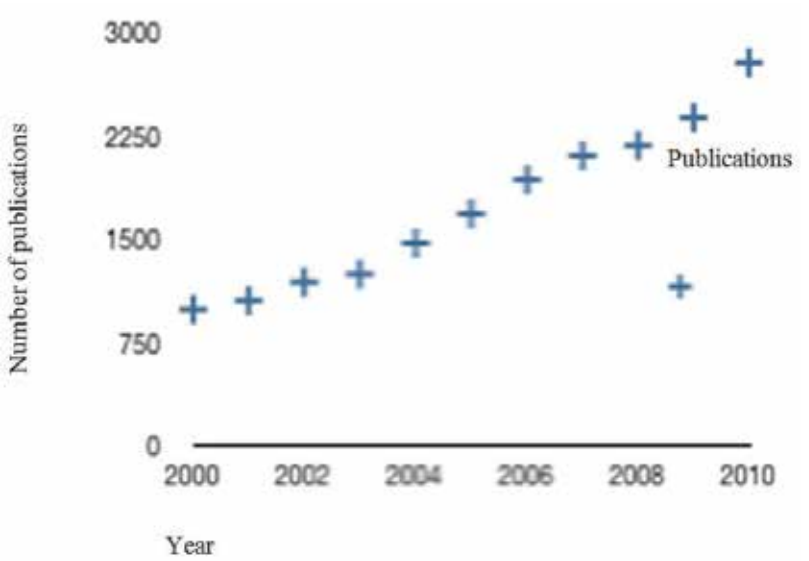

Fig. 2. The total number of publications using breast cancer cell lines from 2000 through 2010. Use of breast cancer cell lines has steadily been rising since 2000.

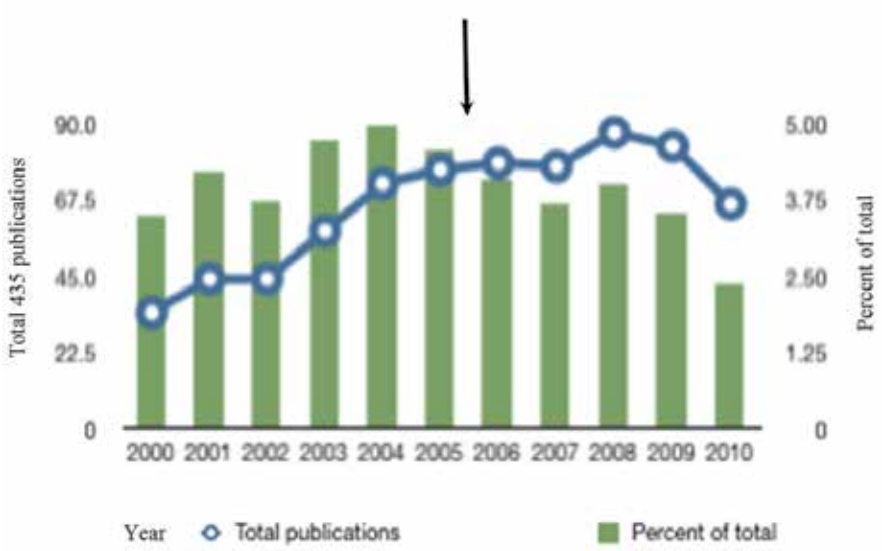

Fig. 3. Number and percent of papers published using MDA-MB-435 cells from 2000 - 2010. The tumor type that gave rise to MDA-MB-435 cells has been controversial since 2000. In 2004, STR profiling confirmed that MDA-MB-435 was not a breast cell line but rather has been contaminated with the M4 melanoma cell line. There has been a subsequent drop in the use and publication of these cells. Shown is the total number of papers published using MDA-MB-435 cells (green bars) and the percent of the total number of publications use MDA-MB-435 cells (blue circles). Arrow denotes when MDA-MB-435 were identified as M14 melanoma cells.

cell line	year established	origin	ER/PR status
BT-20	1958	primary tissue	-/?
SK-Br-3	1970	pleural effusion	+/+
SW13	1971	?	?
MDA-MB-134-VI	1973	pleural effusion	+/-
MDA-MB-157	1973	pleural effusion	?
MDA-MB-175-VII	1973	pleural effusion	?
MDA-MB-231	1973	pleural effusion	-/-
MDA-MB-361	1973	brain metastasis	?
MDA-MB-330	1973	pleural effusion	?
MDA-MB-415	1973	pleural effusion	?
MDA-MB-436	1973	pleural effusion	?
MDA-MB-453	1973	pleural effusion	-/-
MDA-MB-468	1973	pleural effusion	-/-
MDA-MB-157	1974	pleural effusion	?
MCF7	1974	primary tissue	+/+
CAMA-1	1975	pleural effusion	?
SW527	1977	?	?
Hs578Bst	1977	non-tumorigenic breast tissue	-/-
Hs578T	1977	primary tissue	-/-
ZR-75-1	1978	ascites	+/+
ZR-75-30	1978	ascites	?
BT483	1978	primary tissue	?
DU4475	1979	primary tissue	?
T47D	1979	pleural effusion	+/+
MCF10A	1984	non-tumorigenic breast tissue	-/-
MCF10F	1984	non-tumorigenic breast tissue	-/-
MCF10-2A	1984	non-tumorigenic breast tissue	-/-
184A1	1985	normal mammoplasty (transformed)	?
184B5	1985	normal mammoplasty (transformed)	?
UACC-812	1986	primary tissue	-/-
UACC-893	1987	primary tissue	-/-
HCC38	1992	primary tissue	-/-
HCC70	1992	primary tissue	-/-
HCC202	1992	primary tissue	-/-
HCC1008	1994	lymph node	-/-
HCC1143	1994	primary tissue	-/-
HCC1187	1994	primary tissue	?/-

cell line	year established	origin	ER/PR status
HCC1395	1994	primary tissue	-/-
HCC1419	1994	primary tissue	-/-
HCC1428	1995	pleural effusion	?
HCC1500	1995	primary tissue	+/+
HCC1569	1995	primary tissue	-/-
HCC1806	1995	primary tissue	-/-
HCC1937	1995	primary tissue	-/-
HCC1954	1995	primary tissue	+/+
HCC2157	1995	primary tissue	-/+
HCC2158	1996	primary tissue	-/?
HCC1599	1998	primary tissue	-/-
AU565	1998	pleural effusion	?

Table 2. Commercially available cell lines, their establishment date, and hormonal receptor status

Debate has also centered on whether cell lines grown in culture maintain the same genotypic/phenotypic changes that are detected in the primary tissues from which they are derived. Characterization of breast cancer cell lines has been ongoing since their establishment in 1958. In general, breast cancer cell lines are representative models of the primary breast tumors they are derived from (Kao et al., 2009). Initial characterization including karyotyping and comparative genomic hybridization (CGH) demonstrate that, when created and propagated in culture, cell lines maintain the same mutations and chromosomal abnormalities as their primary tumor samples (Lacroix and Laclercq, 2004). While new mutations and chromosomal instability develop in cultured cell lines, overall the genotype remains generally consistent between primary cells and cell lines (Lacroix and Laclercq, 2004). Due to differences in the *in vitro* environment, lack of surrounding naturally occurring microenvironment, and selection pressures, differentiation in culture can occur (Kao et al., 2009; Lacroix and Laclercq, 2004; Voskoglou-Nomikos et al., 2003). Because cancer cells are inherently unstable, differences between same cell line grown in different labs under different environments, even if the growth conditions are the same, are evident (Lacroix and Laclercq, 2004; Osborne et al., 1987). This impacts experimentation as data derived from one lab may not be reproducible in another lab, even is using the same cell line. Caution must be taken when relying on one or two cell lines to draw conclusions.

Use of more modern molecular techniques to characterize cell lines has revealed that while differences between primary cells and cell lines do exist. These techniques do confirm, however, that cell lines maintain the molecular distinction found the primary tumors. Gene expression changes detected in primary tumors are not dramatically different to those found in culture systems, even when cultures are grown directly on plastic in 2D cultures or in reconstituted 3D cultures (Vargo-Gogola and Rosen, 2007). Direct comparison of primary tissue to cultured cells revealed "close similarities" between molecular profiles (Dairkee et al., 2004). Indeed, even epigenetic changes found in primary cancers are similarly detected

in cell lines (Lacroix and Laclercq, 2004). This suggests that cell lines are an appropriate model of primary disease and, depending on the research focus, cell lines will faithfully reflect the processes of primary tissues.

Since cell lines generally remain faithful in terms of the molecular and genetic profiles of the primary tumor from which they are derived, it is critical to consider the correct model system. While ER/PR status of primary tumors leans predominantly toward ER+ expression (55-60%), most breast cancer cell lines have been derived from ER - tumors or pleural effusions (McGuire et al., 1978)(Table 2). Therefore it is of utmost importance to select the proper model to answer the experimental question. A detailed analysis of the applicability of cell lines to accurately model primary breast tumors revealed that overall breast cancer cell lines as a whole do model primary tumors, however on an individual basis, one specific cell line does not accurately mirror a primary breast tumor, even with the same gene expression profile. Since variability in cell lines exist, it is generally thought that to more accurately predict outcomes in primary tissue, a panel of breast cancer cell lines rather than just 1 or 2 individual lines should be tested. Using panels more accurately reflects primary breast tumors and will help translate findings from *in vitro* studies to *in vivo* therapeutic options (Dairkee et al., 2004).

Microarray analysis clearly defined primary breast tumors and breast cancer cell lines at the genetic level. Perou and others have conducted detailed studies using microarray platforms and determined a molecular signature of gene expression changes found in primary breast cancer tumors (Alizadeh et al., 2001; Perou et al., 1999b; Perou et al., 2000b; Ross et al., 2000; Sorlie et al., 2001). These signatures are used to understand the molecular basis of breast cancer and to define different subtypes of cancer that occur naturally in humans. It was also developed as a diagnostic tool to detect breast cancer tumors earlier and to facilitate proper treatment based on a gene signature. Based on these studies, 5 molecular signatures and types of primary breast tumors have been identified. These are luminal A, luminal B, basal-like, HER2+, and normal-like profiles (Perou et al., 1999a; Perou et al., 2000a; Ross et al., 2000; Sorlie et al., 2001). Prior to establishment of these molecular signatures, diagnosis was determined by receptor expression status, i.e. ER/PR/HER2, and treatment regimes assigned accordingly. Using this molecular approach, luminal A and luminal B tend to also be ER + expressing tumors, basal-like encompasses ER - tumors, HER2+ incorporate those HER2+ expressing tumors, and normal-like have similar expression patterns to non-cancerous cells (Perou et al., 1999a; Perou et al., 2000a; Ross et al., 2000; Sorlie et al., 2001). Such molecular characterization will lead to providing more personalized therapy to patients. Efficacy of drugs in different subtypes will be easily determined and accurately assigned to patients expressing a similar molecular profile. While such personalized medicine may be still in the future, some current breast cancer treatment options that exist today are based on the molecular profile of the tumor. For example, tumors expressing the estrogen receptor are treated with selective estrogen receptor modulator (SERM) or other similar anti-estrogen compound whereas tumors lacking ER do not receive the same therapy. Similarly, HER2+ tumors are susceptible to trastuzumab because of HER2 expression. In the future as molecular characterization improves and new chemotherapeutics are developed, more personalized options will be available.

Do cell lines reflect the molecular signature of primary tumors? In a direct comparison of the molecular profiles from cell lines and primary tumors, Kao et. al. found that instead of the 5 breast cancer subtypes identified in primary breast tumors, cell lines can be divided into three main groups, luminal, basal A, or basal B phenotypes (Kao et al., 2009). Luminal cells

contained all ER + cell lines, both Basal A and B consisted of all ER - cell lines. HER2+ cell lines were grouped into the luminal. Basal A contained the HCC cells and BRCA1 mutant cells, whereas basal B genotype contained non-tumorigenic lines including MCF10A cells (Kao et al., 2009). This highlights that breast cancer cell lines are a model of disease.

Cell lines are merely a model of breast disease that aim to provide clinical predictability of outcomes in humans. To directly test the applicability of breast cancer cell lines, xenograft cancer models, and mouse breast cancer models to clinical outcome, Voskoglou-Nomikos et. al. compared outcomes *in vitro* to those in xenograft models, to mouse models and phase II clinical trails (Voskoglou-Nomikos et al., 2003). In these comparisons, a general correlation between relative risk (predictive value of a drug in cell line) and the phase II human trial (tumor/control ratio) existed for *in vitro* cell lines. A general predictive value when using xenograft models to predict outcome to chemotherapy was detected, however this was dependent on the drug tested and the grade/type of tumor analyzed (Voskoglou-Nomikos et al., 2003). Overall, Vaskoglou-Nomikos et. al. concluded that cell lines and xenograft models were good predictors of clinical phase II trial outcomes, but are reliable predictors only when testing cytotoxic drugs and when using the correct model system. These models generally were not predictive of human outcomes when testing non-cytotoxic drugs (Voskoglou-Nomikos et al., 2003). Taken together, these studies emphasize the critical need to establish more breast cancer cell lines that model the heterogeneity of breast cancer and to employ many *in vitro* and xenograft model systems using multiple cell lines per experiment to reliably predict clinical outcome.

4. Contamination

Overt contamination of cell lines, such as bacterial, fungal or yeast infections, is readily detectable merely by altered appearance of the culture and can be rectified without impacting the quality or reproducibility of the data. Less overt contamination, such as mycoplasma and cell line cross-contamination, can occur undetected and can seriously jeopardize experimental findings. While it is well recognized that periodic testing for mycoplasma is a necessary requirement when using cell lines, cross-contamination with other cell lines is less recognized as a problem and therefore and cell authentication practices are not routine.

Cell line cross-contamination is most evident in the case of MDA-MB-435 cells. When Ross et. al. published the molecular profiles of breast cancer cell lines in 2000, the MDA-MB-435 cell line consistently fell outside the range of profiles of the other breast cancer cell lines and clustered with melanoma cell lines (Ross et al., 2000). This sparked great debate about the authenticity of the this line. Derived in 1976 from the pleural effusion of a 31 year old patient with metastatic adenocarcinoma of the breast, initial debate suggested that this was still a breast cancer cell line, but had been derived from a patient who may have also had undiagnosed melanoma (Cailleau et al., 1978). Data indicating that MDA-MB-435 cells expressed a mixture of both melanoma and epithelial markers fueled this debate, however the overwhelming belief was the these were indeed breast cancer cells (Chambers, 2009; Sellappan et al., 2004)(Figures 2 and 3). Indeed, early characterization of the cell line indicated that they were highly metastatic and secrete milk proteins, findings consistent with those of breast cancer cells (Howlett et al., 1994; Price, 1996; Price et al., 1990; Price and Zhang, 1990; Sellappan et al., 2004; Suzuki et al., 2006; Welch, 1997). Confusingly, MDA-MB-435 cells also expressed the melanocyte markers tyrosinase, melan A and S100 (Ellison et al.,

2002; Sellappan et al., 2004). Because of such conflicting results, these data just propagated the debate instead of satisfactorily squelching it as intended. MDA-MB-435 cells were still used and published as a breast cancer cell line (Figure 3).

Finally in 2007, DNA fingerprinting, or short tandem repeat (STR) analysis, in conjunction with SNP analysis, cytogenetic analysis, and comparative genomic hybridization using the earliest stocks of MDA-MB-435 cells revealed that these cells were identical to the M14 human melanoma cells and were melanoma rather than breast cancer cells (Garraway et al., 2005)(Rae et al., 2007). Rae et. al., who conducted the analysis, concluded that at some point early in passage, MDA-MB-435 cells were contaminated with M14 melanoma cells which took over the colony, leading to the establishment of a M14 melanoma cell line rather than a breast cancer line (Rae et al., 2007). This change was never detected. Stocks were unknowingly mislabeled, marked as MDA-MB-435 cells and distributed. Still, after the molecular characterization was published, debate as to whether MDA-MB-435 were really M14 melanoma cells or if M14 were really MDA-MB-435 breast cell still existed (Chambers, 2009). Ultimately, it was determined that MDA-MB-435 cells were really M14, based on the original 1974 publication that initially characterized the morphology, growth and tumorigenicity of MDA-MB-435 cells. In the original paper, MDA-MB-435 cells were reportedly non-tumorigenic in nude mice. After the initial creation in 1974, the MDA-MB-435 cells were not extensively used for testing until the 1990s when Price et. al. used these cells. At this time, MDA-MB-435 cells were characterized as a tumorigenic cell line (Cailleau et al., 1978; Price, 1996; Price et al., 1990; Price and Zhang, 1990).

While impossible to reconstruct that actually happened, this indirect evidence suggests that the MDA-MB-435 cells were contaminated with M14 melanoma cells and the original breast cancer cells died off. Subsequent frozen stocks were of the contaminating M14 cell lines, although they were labeled as MDA-MB-435 cells. No one was aware of this misidentification. Therefore, M14 cells were masquerading as MDA-MB-435 cells and used as a model of breast cancer until 2007. A total of 1803 PubMed indexed articles using MDA-MB-435 cells were published over that period (Figure 3). Since 2007, however, the number of publications using MDA-MB-435 cells has diminished, indicating that it is generally accepted that these cells are clearly not breast cancer cells and therefore should not be used as such.

4.1 Authentication

Cell line cross-contamination is hardly a new problem in tissue culture studies, although it still remains largely ignored. When HeLa were the only human cell line and few scientists studied them, cross-contamination was not a concern(Buehring et al., 2004; Skloot, 2010). Now, it is estimated that 20 - 30% of all cell lines are inadvertently contaminated (Alston-Roberts et al., 2010; Buehring et al., 2004; Gartler, 1968; Rojas and Steinsapir, 1983). Gartler et. al, was the first to highlight the problem in 1967 at the Second Decennial Review Conference on Cell, Tissue and Organ culture (Gartler, 1968). He was the first to demonstrate that many cultures from many labs were contaminated with other cell lines, primarily by HeLa cells. This meant that a significant amount of research was incorrectly interpreted because it was conducted in a different cell line and therefore the data were false. His findings were largely ignored. Over the years, others, including MacLeod, Freshney, Nardone, Alston-R, Buehring and Capes-Davis, have also documented contamination with HeLa and other cell lines, including cross-species

contamination, however this issue has rarely been adequately addressed (Alston-Roberts et al., 2010; Bartallon et al., 2010; Buehring et al., 2004; Capes-Davis et al., 2010; Freshney, 2008; MacLeod et al., 2008; MacLeod et al., 1999; SDO et al., 2010). Recent efforts have again been made to increase awareness of this problem and many calls for action have been published (Buehring et al., 2004; Capes-Davis et al., 2010; Freshney, 2008, 2010b; Lichter et al., 2010; MacLeod et al., 2008; MacLeod et al., 1999; SDO et al., 2010). A group of concerned scientists gathered and created the ATCC Standard Development Organization (ATCC SDO) to develop standards for cell authentication and with maintaining databases of STR profiles.

Eliminating contamination has an easy solution. Cell line authentication using a standardized technique, Short Tandem Repeat Analysis (STR), can provide an unique DNA fingerprint of the cell line (Azari et al., 2007; Bartallon et al., 2010; Masters et al., 2001; Nims et al., 2010; Parson et al., 2005). STR is inexpensive, standardized, and provides proven methodology to produce cell line identities that is reproducible between labs. An aliquot of DNA can be analyzed and compared with known STR profiles to authenticate the cell line. STR profiles for the most commonly used cell lines are freely available and STR services are available at many universities or companies. According to the standards developed by the ATCC-SDO, cells in active use should be authenticated by STR every 2 months (SDO et al., 2010). The ATCC-SDO also recommends that such documentation of authenticity be provided with grant applications and with manuscript submission. Many funding agencies and journals agree with this idea and suggest that scientists provide such documentation prior to acceptance of a manuscript, however at this time, this is merely a recommendation.

5. Future directions

Use of breast cancer cell lines as models of breast disease will not diminish in the near future. These cell lines are an excellent resource to test novel hypotheses and to gain greater understanding about how cells work and how breast cancer can be treated. On the whole, the established cell lines are a good model for disease, however additional cell lines should be created. The addition on new lines, especially those derived from various forms of breast cancer will only strengthen the data gleaned from them. Likewise, cell authentication should become a routine part of experimental procedures. By periodically ensuring the cell lines being tested are truly the correct lines will eliminate the generation and publication of false data. Authentication will save money and potentially careers if done of a routine basis.

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2

In Vitro Breast Cancer Models as Useful Tools in Therapeutics?

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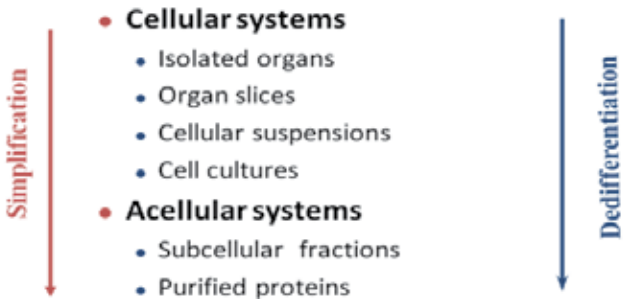
1. Introduction

The increased use of animals in fundamental and applied research due to the remarkable drug development in the 20th century has been an important matter of concern for people at large, but also for the scientific community. This led Russel and Burch to examine the decisions which could meliorate this situation, and they proposed, in 1959, the principle of the 3Rs (Reduce, Refine, and Replace) nowadays largely admitted as an ethical and incontrovertible principle (Russell & Bursch 1959). Alternatives to animal experiments (Scheme 1) then knew a fantastic boom with the permanent objective of a high scientific quality in order to prevent, treat and cure human illness.

Reaching the equilibrium between *in vitro* and *in vivo* models, observing the 3Rs rules, is very difficult. Effectively, *in vitro* systems allow an excellent control of all parameters of the experiments, and then, good quantifications. More the models are simple, more they are easy to handle, but more they also are dedifferentiated and keep away from the *in vivo* situation.



Scheme 1. *In vitro* systems as alternatives to the use of animals.

Within the framework of this book, the question becomes now: how the 3Rs could be the best way to phase out animal experiments when considering breast cancer? We try to bring some response elements in this chapter, emphasising the *in vitro* models the most useful and the most frequently used. But we also show that no model is perfect and sufficient by itself, and that pure *in vitro* models also need assistance of *in vivo* ones.

2. Models for investigation on breast cancer

2.1 Established breast cancer cell lines

2.1.1 The different cell lines and their main properties

Significant amounts of data on breast cancer have been collected over the past 40 years, thanks to the use of established cell lines. The first breast cancer cell lines (BCCL) have been established in the sixties-seventies and very few new cell lines have been developed since. Only a hundred of BCCL are currently available and three of them have been extensively studied and represent now nearly 80% of the 35 000 publications mentioning breast cancer cell lines (Lacroix & Leclercq 2004).

Most of the cell lines were created from cells derived from metastasis or from pleural effusion. Pleural effusions contain large amounts of well isolated tumour cells and few contaminating cells such as fibroblasts, thus making their recovery and growing easier than those of cells directly derived from primary tumours or metastasis. Moreover, metastatic cells are highly dedifferentiated cells, which allow their cultivation more successfully than the primary tumour cells.

The three more used BCCL (MCF-7, MDA-MB-231 and T47D) are issued from pleural effusion of an invasive ductal carcinoma (Soule *et al.* 1973; Cailleau *et al.* 1974; Keydar *et al.* 1979), and they mainly differ by their oestrogen receptor (ER) and progesterone receptor (PgR) status: MCF-7 and T47D are ER+ PgR+ while MDA-MB-231 is ER- PgR-. Among these three cell lines, MCF-7 was the most often used during the last ten years: it has been cited in 53% of all the scientific papers mentioning BCCL, while MDA-MB-231 and T47D were respectively cited in about 18% and 7% of these articles (calculation made on the basis of a Medline-based survey in March 2011).

The use of these lines has many technical advantages.

- The complete control of environmental conditions and standardised culture conditions ensures the reproducibility of results between experiments and laboratories.
- Maintaining cells in culture is much less costly than working on animal models. Besides the fact that some animal models are expensive by themselves, the care of animals and the staff necessary to a good work in an animal house are the main drain of resources. Conversely, the medium and the staff time required to growth cells are cheaper, thus allowing the widespread use of BCCL.
- Cryopreservation enables long-term conservation of the same strain and can theoretically permit the use of these cell lines indefinitely.

These advantages have allowed to gather essential data for the study of breast cancer in the last 40 years, making these cell lines reference models in the field with the establishment of a complete genetic and proteinic profile.

2.1.2 The main drawbacks of these models

- Stability/instability

In practice, these strains, although cryopreserved, undergo dedifferentiation resulting from multiple subcultures, and leading to the lost of special characteristics. Moreover, differences in the culturing practices (medium composition, time between subcultures, subculturing technique, etc.), can explain some divergences observed for a same strain in different laboratories.

- Simplicity

The relevance of cellular models is controversial since their over-simplicity implies difficulties in extrapolating results from the cell line to the tumour in humans and thus raises the question of their representativeness.

Indeed, cell lines are homogeneous, theoretically consisting only of a single cell type (pure and clonal) due to the way they are established:

- The dislocation of tumours is followed by isolation of cells, in order to obtain the most stable culture during subcultures.
- The culture conditions eliminate some types of cells present in the original tumour, unable to grow on a synthetic surface, or whose rate of development is much lower than the one of the surviving cells.
- Cells in culture do not undergo the influence of nervous and hormonal regulatory systems active *in vivo.*

These particularities reduce the similarity with the primary tumour.

- Limited representativeness

The hundred of available cell lines do not cover all of the tumour features found in patients. Furthermore, the proportions of some characteristics are sometimes reversed, such as the ER and PgR status which is very different in cell lines, compared to that found in the patient population (Lacroix & Leclercq 2004). These dissimilarities can be explained by the fact that most lines are derived from pleural effusion and metastases containing cells which are already different from the original tumour and thus, more or less representative of this tumour. Indeed, the ER/PgR status sometimes differs between the metastasis and the original tumour from the same patient. Based on these observations, several teams have worked on the development of cell lines derived from a primary tumour (Amadori *et al.* 1993; Gazdar *et al.* 1998; Shen *et al.* 2009), which are much more representative of the *in vivo* cancerous tissues that lines derived from metastases, but which suffer from the same problems related to their relative homogeneity and instability in a long term use. Moreover, the establishment of cell lines from primary tumours remains a difficult achievement, failures mainly being the result of contamination by the stroma surrounding the tumour.

- Confusion with some cell lines

Besides these previous drawbacks, many criticisms have been made against BCCL because some of them have been proven not being from breast cancer origin. Indeed, some lines were contaminated by other cell types during their first years of use, then spread to other laboratories, and used on a large scale without further verifications of their true origin. Several cell lines were denounced as false, whereas it was not the case (Fogh *et al.* 1977; Nelson-Rees & Flandermeyer 1977). These contaminations have been subjects of controversial for a long time. However, studies have shown with certitude that two cell lines were not from their supposed origin.

The MCF-7-ADRr cell line was developed in 1986 by Batist. It is derived from the lineage of human mammary adenocarcinoma MCF-7 and was rendered resistant to adriamycin treatment after exposition to increased concentrations of this drug. The obtained resistant cell line was also resistant to other agents such as actinomycin D, vinblastine and vincristine. However in 1998, the lineage between MCF-7 and MCF-7-ADRr became controversial, as shown by DNA fingerprinting studies and genetic comparison, so that the true origin of the cell line was undetermined and the cell line was renamed NCI/ADR-RES. Liscovitch and Ravid, in 2007, have collected data showing that NCI/ADR-RES were carcinoma ovarian

cells (Liscovitch & Ravid 2007), and experiments of Affymetrix SNP array analysis at the Sanger Institute (Cancer Genome Project) and of karyotyping, helped to put in evidence an indisputable resemblance of NCI/ADR-RES with the OVCAR-8 human ovarian carcinoma cell line. The most likely scenario is that the stock of MCF-7 cells from the National Cancer Institute used in 1986 for the development of the lineage, was contaminated with OVCAR-8 cells before the first generation of MCF-7-ADR-r. OVCAR-8 cells are naturally resistant to adriamycin, and the *in vitro* selection probably eliminated the MCF-7 cells and allowed the survival of OVCAR-8 cells (Liscovitch & Ravid 2007). It can be noted that MCF-7-ADRr are no longer distributed by the international cell bank ATCC.

The second misidentification concerns the MDA-MB-435 cell line established by Cailleau and colleagues in 1978. This cell line has been controversial in 2000, further to the results of DNA microarray analysis which suggested that these cells might be of melanocyte origin (Ross & Perou 2001). Some other results, obtained by microsatellite comparison analysis, karyotyping and comparative genomic hybridisation experiments (Rae *et al.* 2007), confirmed that MDA-MB-435 cells are in fact M14 melanoma cells.

However, these two cell lines, MCF-7-ADRr and MDA-MB-235, are still used as breast cancer cell lines for some studies and are used for publications in international journals, while it has been proven that they are not from breast cancer origin (Lacroix 2008). The verification of the origin of a cell line is essential, and a way of ensuring that the cell lines are really from a well-defined origin is to make a short tandem repeat (STR) profiling. This method is used to confirm the identity of a cell line by comparison to a known profile and a periodic re-authentication of cell lines is advisable. Moreover, banks of cell lines such as ATCC guarantee the exact origin of their cells. Several authors suggested to prove the authenticity of the cell lines used for each publications (Burdall *et al.* 2003; Lacroix 2008).

2.1.3 Non cancerous immortalised cells as controls

It should be noticed that the study of mammary tumours also involves the use of non cancerous cells which were immortalised. These cell lines were derived from healthy breast tissue, but only few models, obtained by different methods, are available.

- The immortalisation could be the consequence of a particular composition of the growth medium. This is the case for the non-tumourigenic epithelial cell lines MCF-10A (adherent cells) and MCF-10F (floating cells) which were established from the same sample in the nineties (Soule *et al.* 1990). These cell lines were produced by a long-term culture in a special medium containing a low concentration of Ca^{2+} and no serum addition, which resulted in the apparition of immortalised cells with normal features of mammalian epithelial cells.
- Two other cell lines were derived from a mammoplastic surgery. These cells named MCF-12A and MCF-12F became spontaneously immortal after unexpected exposition to high temperatures (45°C during 72 hours, Pauley *et al.* 1991).
- Another cell line, hTERT-HME1 was obtained from the HME1 cells (Human Mammary Epithelial) which were immortalised by infection with the retrovirus pBabepuro+hTER. The immortality feature results from the exogenous expression of the telomerase gene coming from the viral infection (Van der Haegen & Shay 1993; Gollahon & Shay 1996).

- Under chemical pressure, normal cells in culture can also be immortalised. This is the case for some cell lines as 184A1 and 184B5 which were obtained by exposition to benzo[a]pyrène, a chemical carcinogen, leading to clonal events which are the origin of these immortal cell lines (Stampfer 1989).

The use of these "non cancerous" cell lines is important to give a comparison point to results obtained with cancerous cell lines. However, there are drawbacks and controversy to their use, the major one concerning the way they were obtained. Indeed, if they are still non-tumourigenic, they suffer of genetic modifications which lead them to become immortal. They are looking like normal cells, but they are not.

2.1.4 Breast cell lines and metabolism of therapeutic drugs

- Drug metabolism

The metabolic equipment of a cell can explain its sensitivity/resistance to drugs. Indeed, any xenobiotic molecule (therapeutic drugs included) undergoes the same metabolic fate in the cells. Briefly, enzymes of Phase I (essentially cytochromes P450 (CYP) dependent enzymes) ensure a bioactivation of the molecules while enzymes of Phase II conjugate the metabolites issued from Phase I to endogenous molecules (glucuronic acid, glutathione, sulfates…) in order to make them more water-soluble and to facilitate their elimination. Finally, transporters of Phase III are responsible for exporting these last products out of the cells. Each human organ is equipped with these enzymes, but their expression pattern differs quantitatively and qualitatively. The liver is the most efficient organ in metabolising processes, even if we know that some enzymes are more specifically expressed in non hepatic tissues.

When considering the usefulness of breast cell lines as *in vitro* tools to predict sensitivity or resistance to a molecule, it is easy to perform, in first line, simple cytotoxicity tests. However, in order to explain the reasons of these cells behavior, or to predict the metabolism of a new compound, the knowledge of the metabolic equipment of the cells is necessary. As it is impossible, and not very interesting, to decline the results of the literature concerning breast cell lines and assays with the numerous chemical molecules which have been, precisely or not precisely, tested, we chose two examples of therapeutic drugs, used in breast cancer, that need to be bioactivated by CYP before exerting their deleterious effects in the cells: oxazaphosphorines and ellipticine.

- Metabolism of oxazaphosphorines

The oxazaphosphorines generally used in pharmacology (*i.e.* cyclophosphamide (CPA), ifosfamide (IFO), and trofosfamide) represent an important group of chemotherapeutic agents. However, their use is limited by severe toxic side effects. New oxazaphosphorines derivatives have been developed in order to improve selectivity and to reduce toxicity but they won't be studied here, due to their bioactivation process which is different from that of previous molecules (Zhang *et al.* 2005).

Both CPA and IFO, the most widely used as alkylating agents, are prodrugs whose metabolism involves different cytochromes P450 (CYPs) catalysing 4-hydroxylations leading to acrolein and nitrogen mustards capable of reacting with DNA molecules leading to cell apoptosis and/or necrosis. Another pathway consists in an N-dealkylation whose last product is the toxic chloroacetaldehyde (Figure 1) (Rooseboom *et al.* 2004; Zhang *et al.* 2005). All these metabolites are highly reactive metabolites responsible for urotoxicity, neurotoxicity and nephrotoxicity. As all the mechanisms underlying these toxicities are not

elucidated, Mesna (Sodium 2-mercaptoethanesulfonate) is often used to limit these side effects (Giraud *et al.* 2010).

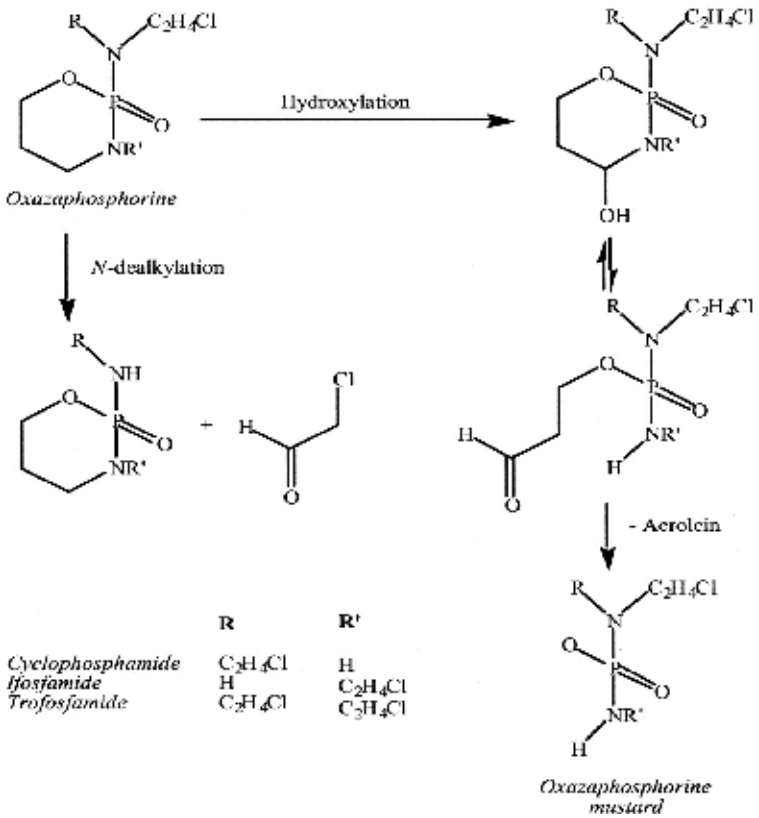


Fig. 1. **First phase of metabolism of the oxazaphosphorines by CYPs**: hydroxylation leads to oxazaphosphorine mustards, and N-dealkylation results in chloracetaldehyde formation. From Rooseboom *et al.* 2004 with permission from ASPET.

As already mentioned, several CYPs are involved in these drug metabolism: CYP2B6 (Wang & Tompkins 2008; Mo *et al.* 2009; Bray *et al.* 2010), CYP3A4 (Kivisto *et al.* 1995), but also CYP2A6 (Di *et al.* 2009), CYP2C9, CYP2C19, CYP3A5 (Bray *et al.* 2010) and probably others. Figure 2 below, extracted from Wang & Tompkins 2008, shows the expression of the different human hepatic CYP and their contribution to metabolize clinically-used drugs. No analog study was performed in breast tissue, and *a fortiori* in breast cancer cell lines. However, the literature reports the presence of CYP3A4 (the CYP enzyme the most involved in drug metabolism) in MCF-7, T47D and MDA-MB-231 (Nagaoka *et al.* 2006; Chen *et al.* 2009; Mitra *et al.* 2011), of CYP2B6 in MCF-7 and T47D (Lo *et al.* 2010) whereas this information is not available for MDA-MB-231. While CYP2D6 and splicing variants similar to those found in breast cancer tissues were shown expressed in MCF-7 (Huang *et al.* 1997), no information about this CYP, to our knowledge, was related for T47D and MDA-MB-231.

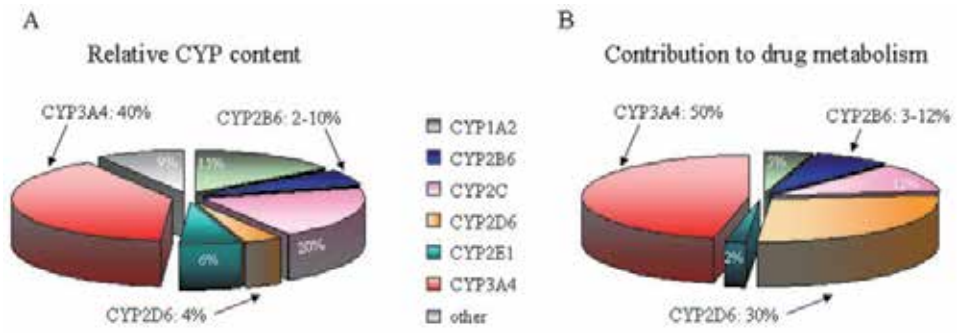


Fig. 2. Hepatic CYP expression (A) and their contribution to metabolism of clinically-used drugs (B). From Wang & Tompkins 2008, permission granted by Bentham Science Publishers Ltd.

- Metabolism of ellipticine

Another example is given by ellipticine. This alkaloid compound found in several plants (Ochrosia, Aspidoserma subincanum, Bleekeria vitiensis) is a topoisomerase poison often used in ovarian and breast cancer treatment. It is also a prodrug whose efficiency depends on CYP activation. 13-hydroxy- and 12-hydroxy-ellipticine, responsible for the formation of DNA adducts, are generated by CYP1A1/2, CYP3A4 and CYP2C9.

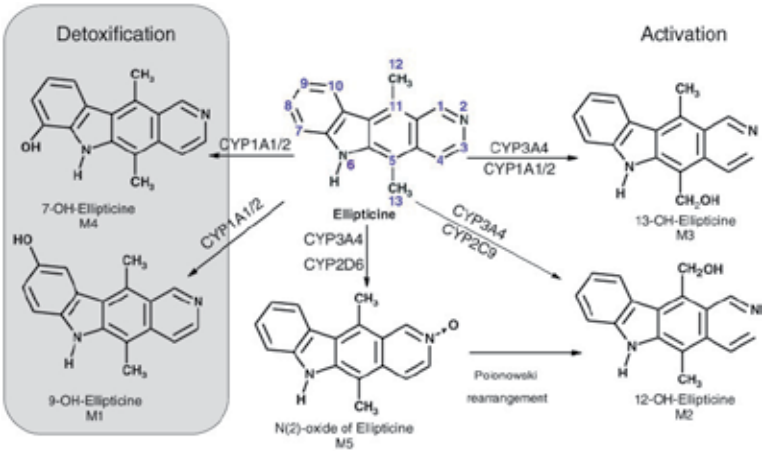


Fig. 3. Main pathways of ellipticine metabolism. Reprinted from Stiborova *et al.* 2011, ©2011, with permission from Elsevier.

Members of the CYP1 family are usually expressed in extrahepatic tissues and it is not strange to find CYP1A1 in MCF-7 (Androutsopoulos *et al.* 2009; Stiborova *et al.* 2011), in MDA-MB-231 and T47D (Macpherson & Matthews 2010). We already mentioned the presence of CYP3A4 in the three cell lines, but no precise information is available for CYP2C9.

This slight overview shows that the three main breast cancer cell lines are able to give interesting information about drugs that have to be bioactivated before exerting their deleterious effects in cancer cells. However, we must keep in mind that polymorphic variants of the genes coding these enzymes, or splicing variants, may influence the

pharmacology of any drugs. Very few information about that are available in patients, but no study was performed in breast cancer cells.
BCCL have been created to study tumour development and related mechanisms and to test molecules potentially active. They are inevitable models for many studies. However, their extensive use in all areas of research on breast cancer remains sometimes controversial due to the over simplicity of the model, the instability of the strain, the existence of "false cell lines" and the failures of representativeness of the tumour. Thus, it clearly appears that these models are not sufficient to answer all the questions on breast cancer, and it is essential to turn to complementary models. Consequently, new models were introduced in the late 70s. They were used to a lesser extent than cell lines for a long time, but they tend to be more used now.

2.2 Improving representativeness of the model: Direct culture of tumour fragment

There are several methods to circumvent the problem of representativeness of BCCL, e.g. the direct culture of tumour fragments. The first attempts in this direction were made in the late 60s from tumours of $1mm^3$ volume (Matoska & Stricker 1967). However, these cultures were proven difficult due to the high thickness of the samples, preventing the diffusion of nutrients and oxygen to the center of the sample, and thus, avoiding a long-term cultivation *in vitro*. This method has been modified over time, and with the use of microtome, problems associated with diffusion of nutrients have been resolved. The samples are now constituted of extremely thin slices of about 150 to 200 µM thick (Nissen *et al.* 1983).
This type of model was used to study the different inter-tumoural cell interactions and also to test the sensitivity to drugs (Milani *et al.* 2010). The slice tumour model associated with the development of microscopic analysis methods, such as the triple-fluorescence viability assay developed by Van Der Kuip, allowed the study of the cytotoxic effect of Taxol on this breast cancer model (Van Der Kuip *et al.* 2006).
Another example of drug study is the evaluation of the action of cytokines and cytotoxic drugs on animal (MMTV-Neu mice) breast cancer slices, especially the monitoring of apoptosis increase and DNA damage after treatment with interferon-gamma or doxorubicin (Parajuli & Doppler 2009).
The last noticeable example is the use of a tropism-modified oncolytic adenovirus, and a wild-type adenovirus on these slices to treat breast cancer. The results showed that the modified oncolytic adenovirus can infect and replicate in breast cancer tissue slices, suggesting the great potential of this model for evaluating the potential of oncolytic adenovirus constructs (Pennington *et al.* 2010).
This list is not exhaustive and the literature shows that a lot of results were obtained by the slice culturing method, more particularly on the study of drugs effects like tamoxifen or paclitaxel (Conde *et al.* 2008; Sonnenberg *et al.* 2008; Rajendran *et al.* 2011).
Although used since the late 70's, the slice technique evolved over time and was adapted to technological innovations. We may especially underline the use of silicon sensor chips wearing electrodes and sensors as a carrier of culture slice. The samples are deposited on the chip and data concerning the tumour-slice are analysed continuously during its cultivation and during its contact with drugs; measurements are made in real-time by the readout of ionic-sensitive field effect transistors and an oxygen electrode. This model was used to study the effects of Taxol on 200 slices of breast cancer, which revealed a dose-dependent decrease

of the metabolic activity showed by the measurement of a decrease in the acidification of the medium (Mestres *et al.* 2006).

This technique has advantages and drawbacks. The direct culture of tumour fragment has the major advantage of preserving tissue architecture and all the cell populations constituting the human tumour. This method is thus a valuable technique which permits to take into account the whole tumour environment *in vivo*, allowing the investigation of the role of 3-dimensional structures and stromal interactions in tumour. It also allows to study the response of a particular tumour type to environmental stimulations, drugs, and cytokines under well-defined and reproducible conditions.

However, the culture of tumour samples presents limitations that do not allow its widespread use. Obtaining tumour samples is submitted to ethical constraints relative to the use of patient samples for research. In addition, it must be performed under ideal conditions. Thus, the samples have to be prepared very quickly after their excision, which means that the research laboratory should have particular facilities to have a direct access to fresh tissues. Moreover, the samples excised by the surgeon are becoming smaller and smaller, due to early diagnoses, and the major part of the samples is kept for diagnosis. Then, if some sample is still available for research, priority is given to research on biomarkers of the tumour in order to give personalised therapies, and, only after, it is disposable for fundamental research. Additionally to the availability restrictions, the same sample cannot be used for many tests because of the limitations of growth of this tissue *in vitro*. Repetition of assays and comparative measurements are thus more difficult with this model.

The use of samples from animal models with mammary tumour partially resolves the problem of availability of samples, but it also raises questions on the representativeness of the samples with human breast tumours. High improvements for providing human tissues of good quality will be brought by the emergence of biobanks.

2.3 Circumventing the lack of diversity: Co-culturing of cell lines

The co-culturing represents another way to circumvent the lack of cell diversity found in cell lines and to allow understanding of the tumoural proliferation mechanisms and inter-cellular interactions within a tumour. It is an indispensable tool to elucidate the regulation of the tumour by epithelial and stromal components surrounding it.

This model can be used by different ways: co-culturing of two cell types with a direct contact or co-culturing with a separating porous membrane between both cell types. The first method implicates to be able to differentiate the two cell types by microscopy. For that the use of fluorescent markers is a valuable tool (see Figure 4 for an example of co-culture of MDA-MB-231 with hASCs (adipose stem cells) respectively stained by the lipophilic tracers DiI (dialkylindocarbocyanines) and DiO (dialkyloxacarbocyanines), Pinilla *et al.* 2009).

The second method allows a relative isolation of the two cell types, the porosity of the membrane separating them allowing the exchange of substances. The two techniques give complementary information on the behavior of cells studied, especially the crucial role of the inter-cellular communication (Cappelletti *et al*. 1991).

In example, we could cite the co-culture of MDA-MB-231 and MCF-7, which has highlighted the importance of the heterogeneity of tumours for their growth and the role of oestrogen receptors. In this study, the co-culture of MCF-7 and MDA-MB-231 (respectively ER+ and

ER-) in a membrane separation system, was characterised by an increase of the MCF-7 cells growth rate in comparison with monocultures. This suggests that complex interactions between heterogenous cells population in tumour could explain the variability in tumour progression between different patients and the failure in response to endocrine treatment for some patients with ER+ tumours.

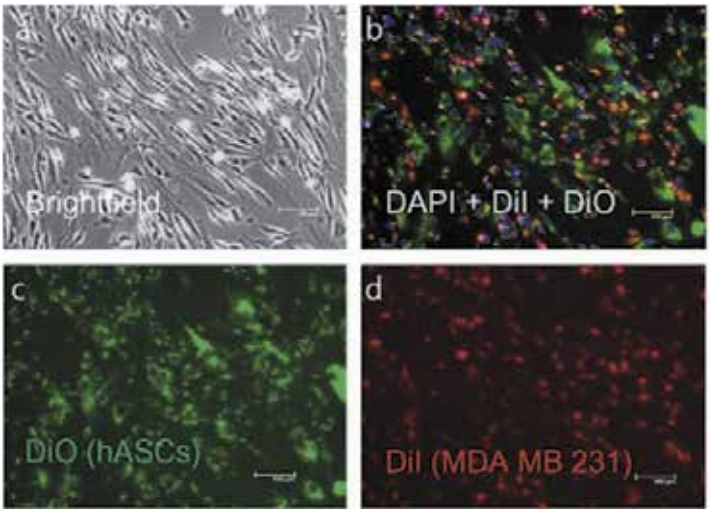


Fig. 4. Human stem cells derived from adipose tissue (hASCs) and breast cancer cells (MDA-MB-231) cultured in a monolayer co-culture system. (a) Direct microscopic observation of the co-culture of MDA-MB-231 and hASCs cells. (b) Overlay of DiO (hASCs), DiI (MDA-MB-231) and DAPI (nucleus) stainings. (c) DiO staining of hASCs derived stem cells (green). (d) DiI staining of MDA-MB-231 breast cancer cells (red). Reprinted from Pinilla *et al.* 2009, ©2009, with permission from Elsevier.

Another example concerns the direct co-culturing of MCF-10A, a non-cancerous breast cell line, with the cancerous one MCF-7. An exposure to hormonal treatment with 17β-estradiol was able to inhibit the proliferation of MCF-7 cells in this co-culture, whereas this phenomenon was not observed in a monoculture of MCF-7. This highlighted the complex interactions between ER+ MCF-7 and ER- MCF-10A cells which may reflect physiologically relevant mechanisms of the paracrine regulation of cell proliferation (Spink *et al*. 2006).

The co-culture of MCF-7 with fibroblasts derived from normal biopsies or from cancer biopsies also allowed to highlight the crucial role of fibroblasts in breast tumours. The results of two studies, one in direct co-culturing (Samoszuk *et al*. 2005) the second in membrane separated system (Dong-Le Bourhis *et al.* 1997), showed that MCF-7 growth rate was inhibited by fibroblasts issued from non cancerous tissues, but not by fibroblasts issued from tumourous tissues or serum-activated fibroblasts which enhanced MCF-7 growth rate. This suggests that fibroblasts could release some tumour growth inhibiting or activating factors.

The role of tumour-associated macrophages in the proliferation of tumour cells was also studied by co-culturing macrophages with MCF-7 cells in a membrane separated system. This co-culture lead to a significant increase of MCF-7 invasiveness *in vitro* (Hagemann *et al.* 2004).

These repeatable techniques have permitted to highlight the regulation of mammary tumours by the surrounding stroma and the complex interactions between the cell subtypes of the tumour.

2.4 A model with a tumour-like structure and cell diversity: 3-D culture

Another particular model allows cells to grow in 3-dimensions, generally with a matrix support (Yuhas *et al.* 1978). This type of culture permits an *in vitro* depiction of tumour tissue more accurate than classical 2-dimensional cultures in monolayers, as this last model does not correctly imitate the architecture and cellular gradients of oxygen and nutrients that are found in poorly vascularised regions of the tumour.

Only few cell lines are spontaneously able to establish spheroid architectures under certain culture conditions, but most of the systems require the use of synthetic or non-synthetic matrix. Systems are most often made of agar matrices or collagen support (Kim *et al.* 2004b). The Figure 5 show the growth of a MCF-7 spheroid growth in a hydrogel agarose matrix system (Fritsch *et al.* 2010).

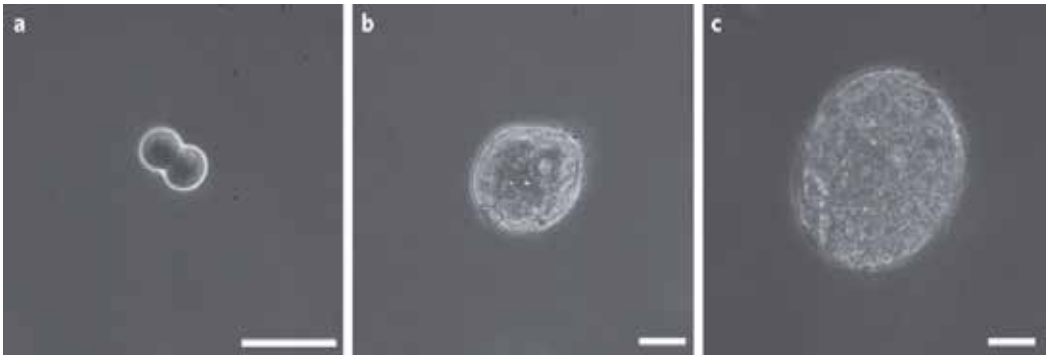


Fig. 5. Growth of a MCF-7 tumour spheroid in agarose hydrogel. The pictures represent the spheroid at 2 days old (a), 11 days old (b) and 27 days old (c) (the scale bar represent 50 µm). Reprinted by permission from Macmillan Publishers Ltd: Nature Physics, Fritsch *et al.* 2010, ©2010. http://www.nature.com/nphys.

Co-culturing of multiple cell types on these 3-dimensional systems is often used to study the relationship between cells, while simulating the tumour architecture with the most fidelity. These systems generally implicate the cultivation of tumour cells with other cell types like stromal, endothelial, fibroblasts and immune-competent cells. Moreover, this type of model, structurally like-looking the tumour, can be used quite indefinitely because it relies on the use of immortalised cells lines. This allows circumventing the problem of the lack of samples which is the major drawback of the tumour fragment culturing.

More advanced systems have been derived from this principle; one can cite the microfluidic-based 3-dimensional culturing (Bauer *et al.* 2010) that allows to grow multicellular tumour spheroid on a microchannel support, in order to analyze complex and heterotypic cellular interactions between breast cancer cells and fibroblast from the surrounding stroma. It has many advantages compared to the standard 3D culture: the culture volume and the number of needed cells are smaller than in standard support, the molecules are only distributed by diffusion mechanisms and the model is adapted to high throughput screenings.

2.5 Xenografts: An intermediary model between cell lines and *in vivo* models

We previously saw that some *in vitro* models tend to provide essential information on the inter-cellular interactions, by taking more or less into account the 3-dimensional structure of the tumour, but none of them benefit from the nervous and hormonal regulations found in the living organism.

There are particular models which can do perfectly the junction between *in vitro* and *in vivo* models, the xenografts. They are obtained by injecting cancer cells, usually derived from established cell lines, into a living organism. They are called xenografts because the injected cells are of human origin but are introduced into an animal organism, usually an immunodeficient rodent. The injection can be orthotopic (in breast gland) or heterotopic (localised in another part of the body, usually subcutaneously).

The xenograft model has the advantage of using cells from human tumour cell lines for which a significant amount of data was collected *in vitro*, and to study their behavior *in vivo*. There are several models available for research on breast cancer, principally using immunodeficient mice. The model nude is by far the most commonly used (Kim *et al.* 2004a). It is characterised by an absence of a functional thymus and active T cells (Kindred 1971). The second common model is the SCID mouse (severe combined immunodeficiency). These mice have a deficit in VDJ recombinases that allow the binding of specific and non-specific parts of immunoglobulin and T cell receptor (Bosma & Carroll 1991). See Figure 6 illustrating the two common models of mice used for breast cancer xenografts: nude and SCID.

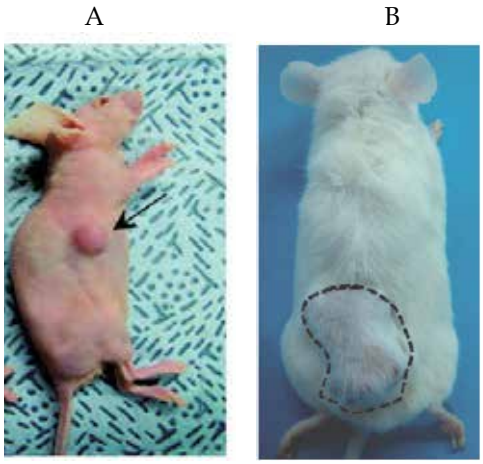


Fig. 6. **Nude (A) and SCID (B) mice models** xenografted respectively with MCF-7 and MDA-MB-231 breast cancer cell lines. (A) was taken from Nizamutdinova *et al*. 2008, by permission of Oxford University Press, and (B) was taken from Wang et al. 2010, with permission from ASBMB journals.

The injected breast cancer cells mostly come from established cell lines like MCF-7, MDA-MB-231, T-47-D or ZR-75-1. The first experiments of cell transplantations were made in the 80s, and opened onto success in the establishment of malignant tumours in nude mice (Ozzello & Sordat 1980; Kim *et al.* 2004a). Since then, a lot of models have been developed for investigation of new treatments, therapeutic targets and establishment of new cancer detection method by medical imaging.

This technique is widely used to test the effect of new antitumourous compounds or therapeutic methods, for example to test new virotherapies. Thus, a benign virus

Coxsackievirus 21 (CVA21) was intravenously injected in SCID mice xenografted with MDA-MB-231 breast cancer cells. CVA21 virus targets the receptors ICAM-1 and DAF that are overexpressed in breast cancer cells. In this experiment a rapid lysis focused on cancer cells was observed in all mice, making this virus a good candidate for use in systemic therapy (Skelding *et al.* 2009). See Figure 7 illustrating the effect of the virus on xenografted mice, visualised by bioluminescent analysis.

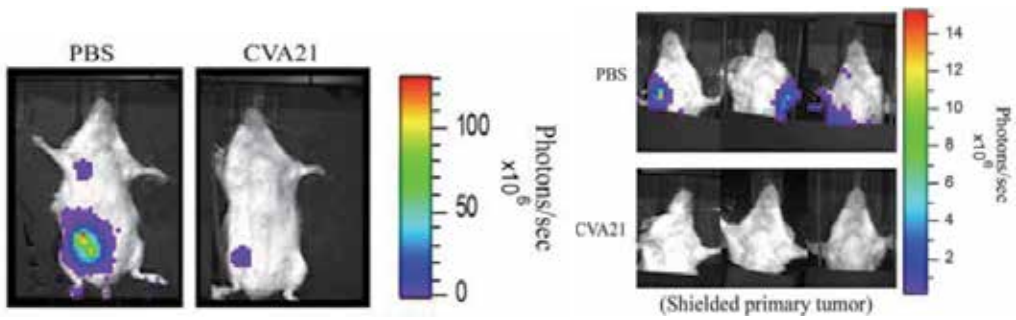


Fig. 7. **Observation of the oncolytic activity of CVA21 virus in SCID mouse xenografted with MDA-MB-231-luc**. The breast cancer cells were xenografted into the mammary fat pad, mice were then treated with PBS or CVA21. Metastases were detected 3 weeks post-cell injection. The mice on the pictures are representative for bioluminescent observation at day 42 post treatment. From Skelding *et al.* 2009, with kind permission from Springer Science and Business Media B.V.

In the investigation of new treatments, the vitamin D3 receptors constitute good targets as they are present in over 80% of mammary tumours and they are negative growth regulator of both oestrogen-dependent and independent breast cancer cells *in vitro*. In a study published in 1998 it was shown that EB1089, a vitamin D3 analog, was able to highly reduce the growth of tumour in nude mice xenografted with MCF-7 cells (tumours were 4-fold smaller than those in untreated mice). This reduction was resulting from an enhancement of apoptosis and reducing proliferation of tumour epithelial cells, suggesting the great potential of vitamin D3 analogs such as EB1089 against human breast cancer (VanWeelden *et al.* 1998).

This model can also be used to explore new potential targets for anticancer therapies. A good example is the targeting of receptor ERβ. In an experiment, standard T47D ERα+ ERβ- and modified T47D ERα+ ERβ+ (T47D stably transfected with a plasmid allowing the expression of the receptor ERβ), were xenografted in SCID mice. 17β-estradiol was then injected into mice. The treatment triggered an acceleration of tumour growth in mice xenografted with the native T47D strain, and conversely a regression of tumours T47D ERβ+. These results emphasize the antagonistic role of ERβ receptors that appear to play an antitumourigenic role, and offered prospects for the development of ER-selective inhibitors. (Hartman *et al.* 2006).

The targets cited above are non exhaustive. Many other therapeutic targets are tested with xenografts models, as it is the case of the VEGF pathway implicated in tumour angiogenesis (Le *et al.* 2008), or of cell cycle regulating proteins such as CDK kinases (Fry *et al.* 2004).

The use of established cell lines for producing xenografts raises several questions about their relevance. The murine model presents considerable differences with the human body, concerning the biochemical and physiological regulation. Moreover, the stroma that will grow surround the tumour will be of murine origin and it will result in a chimeric tumour

which biology may significantly differ from human one (Kim *et al.* 2004a). Furthermore, in humans, the immune system plays an important role in the fight against tumour, whereas in xenografts models the immune system is totally absent.
The xenograft model has some limitations but is the most accomplished of all models because it takes into account the complexity of the organism.
Besides the xenografts, there are also murine models which can develop tumours spontaneously or under the influence of inducing compounds (Russo & Russo 1996). Although the achievement of these models is easy, their use is largely debated because of their relevance to the clinical situation. Indeed, murine breast cancers are most often caused by viral infections and are not hormone dependent, whereas a considerable proportion of human cancers are oestrogen dependent. To date there is no evidence suggesting a viral induction of breast cancer in humans. The biology of spontaneous rodent tumours differs from the human ones. The size, the oncogenic targets or the degree of maturation and differentiation of cells differ between the two species, making them hardly comparable.

3. Conclusion

In this chapter, we described the main models used in breast cancer research in order to obtain results of high scientific quality. In summary, we can say that BCCL models allow repeatable experiments with simple material and methods. They are inevitable models for basic studies and mechanistic explorations, but their use is still controversial owing to their approximate representativeness of breast tumours in human and to the existence of misidentified cell lines.
Cultures of cancerous tissues preserve the tumour architecture and the cell diversity of a tumour but this model suffers of limited reproducibility and cannot be easily maintained for a long time. Co-culture systems offer an alternative with reproducible long term culture systems, and offer the possibility to study the relations between different types of cells in tumour, but this model suffers from the same controversies as BCCL as it mainly relies on their use.
3-dimensional systems allow the mimicking of the tumour architecture and microenvironment, but very few cell lines are able to form spheroids under specific conditions.
Considering the advantages and drawbacks of these models, the xenografts appear to be good alternative models as they enable to take into account the tumour structure, its microenvironment, the role of the metabolism and they preserve the cell diversity of the tumour. But as other models, they also have drawbacks principally due to the metabolic and physiological differences existing between human and rodents, and to the fact that the role of the immune system against tumour is not taken into account with the immunodeficient rodent models used for xenografts.
Application of the 3Rs principle leaded to the development of all these models, but we showed that none of them is sufficient by itself and able to perfectly mimic breast cancer in human. However it clearly appears that all these models are essential to accumulate data and information to fight breast cancer.

4. Acknowledgements

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3

Insulin-Like-Growth Factor-Binding-Protein 7: An Antagonist to Breast Cancer

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1. Introduction

1.1 The insulin-like growth factor (IGF) system

The insulin-like growth factor (IGF) system has been shown to have an integral role in normal growth and development, and in the pathophysiology of various cancers. The IGF system is comprised of a series of circulating ligands (IGF-1, IGF-2), transmembrane receptor tyrosine kinases (IGF-1R, IGF-2R, and the insulin receptor (IR), high affinity ligand-binding proteins (IGFBP1-6), IGFBP proteases, and several low affinity IGFBP-related proteins (IGFBP-rp1 to 10) that work in unison to regulate cell growth [1].

There are two key circulating ligands, IGF-1 and IGF-2, which share approximately 50% structural homology with insulin[2]. IGF-1 is produced primarily in the liver in response to circulating levels of growth hormone(GH) [3]. IGF-1 and IGF-2 are highly homologous small peptide hormones of approximately 7 kDa molecular mass, which are important mitogens that affect cell growth and metabolism [2]. IGFs interact with specific cell surface receptors, designated type I and type 2 IGF receptors, and can also interact with insulin receptor (IR).

The type I IGF receptor (IGF-1R) is a transmembrane heterotetramer consisting of 2 extracellular alpha subunits and two intracellular beta subunits linked by disulfide bonds (fig 1). The intracellular component of IGF-1R has intrinsic tyrosine kinase activity that requires ligand binding for activation [4]. The IGF-1R and the IR share approximately 60% homology which allows them to form hybrid receptors [5]. As a result of this homology, IGF-1R can be activated not only by IGF-1 but also IGF-2 and insulin, although the affinity of IGF-1R for IGF-2 and insulin is approximately 10 fold and 1000 fold lower than for IGF-1, respectively [6]. The type 2 IGF receptor (IGF-2R), which is identical to the cation-independent mannose-6-phosphate receptor, binds IGF-2 with 500 fold increased affinity over IGF-1[7]. IGF-2R does not bind insulin. Most of the biological activity of IGF-2 is thought to be mediated through binding IGF-1R[7]. IGF-2 is known to function primarily as a scavenger receptor, regulating circulating IGF-II levels through internalization and degradation [7].

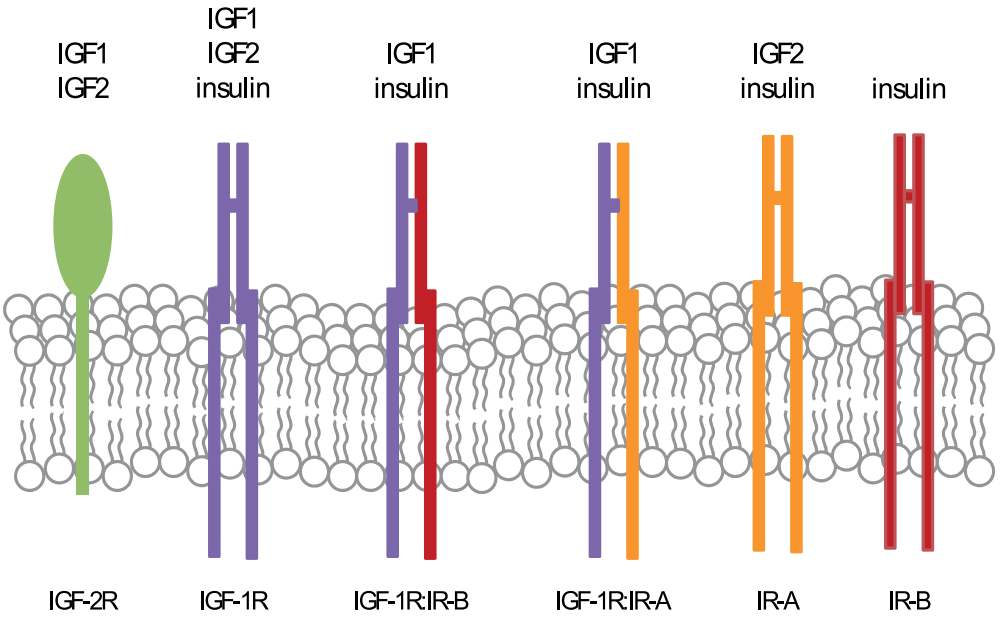

Fig. 1. Cell surface receptors for IGFs and insulin. Illustration of the different transmembrane receptors and ligands of the IGF system. Purple represents the alpha and beta subunit of IGF-1R; red represents the alpha and beta subunit of the IR-B; orange represents the alpha and beta subunit of the IR-A ; green represents the IGF-2R. The potential ligand(s) is shown above the respective receptor.

Two distinct insulin receptor isoforms have been identified and are known to hybridize with IGF-1R. The insulin receptor isoform A (IR-A), the IR fetal isoform, is generated by alternative splicing through the deletion of exon 11 of the insulin receptor gene whereas the insulin receptor isoform B (IR-B) retains exon 11 [8]. IR-A is the predominant isoform expressed in fetal tissues and cancers with ubiquitous expression, whereas IR-B appears in postnatal life within insulin-target tissues, such as muscle, adipose tissue and kidney [9,10,11]. Data obtained from murine 32D hemopoietic cells demonstrated that IR-A preferentially induces mitogenic and anti-apoptotic signals, whereas IR-B predominantly induces cell differentiation signals [12]. IR-A, but not IR-B, binds IGF-II with high affinity and operates as a second physiological receptor for this growth factor [13]. The two IR isoform half receptors (composed of one alpha and one beta subunit) can heterodimerize, resulting in the formation of either homologous IR-A/IR-A or IR-B/IR-B receptors as well as the hybrid IR-A/IR-B insulin receptors [14](fig 1). Heterodimers can also form between IGF-1R and IR, resulting in the hybrid IGF-1R/IR-A and hybrid IGF-1R/IR-B. Hybrid IGF-1R/IR receptors are believed to mostly bind IGF-1, although they can also bind insulin but with a much lower affinity [15]. The IGF system is also regulated by a group of at least six high affinity ligand-binding proteins, the insulin-like binding proteins (IGFBPs), as well as low affinity ligand-binding proteins (IGFBP-rp1 to 10).

2. The IGFBP superfamily

Unlike insulin, IGFs circulate in biological fluids complexed to a family of structurally related binding proteins, called IGF-binding proteins (IGFBPs). The IGFBP superfamily can be subdivided into two groups: the high affinity IGFBPs (IGFBP1 to 6) and the low-affinity IGFBPs (IGFBP7 to 10, and IGFBP-rP5 to 10). [16].

High affinity binding proteins (IGFBPs)

There are, to date, six well characterized mammalian IGFBPs, designated IGFBP-1 through -6. IGFBPs are capable of binding IGF-1 and IGF-2 with higher affinity than their interactions with the IGF-1R, but do not bind to insulin. Some IGFBPs compete for activity of IGFs at the receptor level and antagonize IGF signaling, while others (eg. IGFBP2 and IGFBP5) appear to amplify IGF signaling [17]. Therefore, IGFBPs function not only as carriers of IGFs, thereby prolonging the half-life of the IGFs, but also act as modulators of IGF availability and activity[18]. Apart from their ability to inhibit or enhance IGF actions, all the IGFBPs have been reported to exert distinct biological actions such as cell proliferation, differentiation, migration, angiogenesis and apoptosis through an IGF/IGF-1R-independent manner [19,20,21,22,23].

All six IGFBPs share approximately 35% sequence identity with each other. The primary structures of mammalian IGFBPs appear to contain three distinct domains of roughly similar sizes: the conserved N-terminal domain, the highly variable midregion, and the conserved C-terminal domain. Within their N-terminal domain, all IGFBPs share a common conserved cysteine-rich domain termed IGFBP motif (GCGCCXXC) (fig 2). The IGFBP motif is encoded by a single exon, has overall similar topology and is only present in vertebrates [19]. Ten to 12 of the 16-20 cysteines found in the prepeptides are located within this domain. In IGFBP1-5 these 12 cysteines are fully conserved, whereas 10 of the 12 cysteines are invariant in IGFBP6 [19]. The midregion is believed to act structurally as a hinge between the N and C terminal domains. Posttranslational modifications (glycosylation, phosphorylation) of the IGFBPs has been found only in the midregion so far. The C-termini of IGFBPs, like the N-terminal domain, are highly conserved, and contain the remaining 6 of the total 16-20 cysteines. The primary sequence of all members of the IGFBP family surrounding the last 5 cysteines is strikingly similar (~40%), implying that the tertiary structure of the C-terminal domain should be almost identical. Interestingly, the amino acid sequences embracing these last 5 cysteines share 37% similarity with the thyroglobulin-type-1 domain, a structural motif occasionally employed as an inhibitor of proteases [19,24]. It has been hypothesized that the N and C-terminal domains are capable of acting independently of each other based on the fact that the cysteines within each of the conserved regions are even numbered, and that proteolytic cleavage products of IGFBPs contain either the C or N-terminal regions. Indeed, disulphide linkages have been shown to form typically within each conserved domain, rather than between domains[25,26]. All the IGFBPs are encoded by 4 exons, except IGFBP3 which has an extra exon, exon 5, that is not translated. The striking observation is the correlation between these IGFBP exons and the three protein domains of IGFBPs. The N-terminal domain is encoded within exon 1 in all of the IGFBPs, as is the 5′ untranslated region and a few amino acids of the midregion. Exon 2 encodes the nonconserved midregion. Both exons 3 and 4 encode for the conserved C-terminal domain. The containment of the N-terminal domain within one exon, combined with the ability to bind IGFs, supports the concept of an IGFBP superfamily [27,19].

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IGFBP-1       MSEV-----PVAR-VWLVLL-LLTVQVGVT------AG-----AP--WQ-----------CA-PC---SAEKL-ALC-PP-------VSA-------SCS-EVTR--SAGCGCCPM--CALPLGAACG 72
IGFBP-2       MLPRVGCPALPLPPPPLLPLLPLLLLLLGAS-----GGGGGARAEVLFR----------CP-PC---TPERL-AACGPFRVAPPAAVAAVAGGARMPCA-ELVR--EPGCGCCSV--CARLEGEACG 102
IGFBP-3       MQRA-------RPTLWAAALTLLVLLRGPPVAR--AGASSGGLG-PVVR----------CE-PC---DARAL-AQCAPP-------PAV--------CA-ELVR--EPGCGCCLT--CALSEGQPCG 82
IGFBP-4       ML----------PLCLVAAL-LLA--AGP--------GPS--LGDEAIH----------CP-PC---SEEKL-ARCRP--------PVG--------CE-ELVR--EPGCGCCAT--CALGLGMPCG 68
IGFBP-5       M------------VLLTAVLLLAAYAGP--------AQS--LG-SFVH----------CE-PC---DEKAL-SMCPPS-------PLG--------C--ELVK--EPGCGCCMT--CALAEGQSCG 68
IGFBP-6       MT---------PHRLLPPLLLLLALLLAAS------PG-----G-ALAR----------CP-GC---GQGVQ-AGC-PG---------G--------CV-EEEDGGSPAEGCAEAEGCLRREGQECG 72

IGFBP-7       M------ERPSLRALLLGAAGLLLLLL--P----LSSSSSS---D----------T----CG-PC---EP----ASCPPL------PPLG--------CLLGETR---DACGCCPM--CARGEGEPCG 72
IGFBP-8       M-TAASMG-PVR---VAFV-VLLALC----------------SRPAVGQN----------CSGPC--RCPDEPAPRCPA----------G--------VS--LVL---DGCGCCRV--CAKQLGELCT 69
IGFBP-9       MQSVQSTSFCLRKQCLCLTFLLLHLL----------------GQVAATQR----------CPPQCPGRCPATP-PTCAP----------G--------VR--AVL---DGCSCCLV--CARQRGESCS 76
IGFBP-10      M-SSR-IA---R--ALALVVTLLHL-----------------TRLALST----------CPAAC--HCPLEA-PKCAP----------G--------VG--LVR---DGCGCCKV--CAKQLNEDCS 65
IGFBP-rP5     MQIP-----------RAALLPLLLLLLAAPASAQ------LSRAGRSAPLAAG-------CPDRC---EP----ARCPPQ--------PEH--------CEGGRAR---DACGCCEV--CGAPEGAACG 77
IGFBP-rP6     MKSV----------LLLTTLLVPAHLVAAW------------SNNYAVD----------CPQHC---DS----SECKSS--------PR--------CK-RTVL---DDCGCCRV--CAAGRGETCY 66
IGFBP-rP7     MRGTPKTH------LLAFS--LLCLL----------------SKVR-TQL----------CPTPC--TCPWPP-PRCPL----------G--------VP--LVL---DGCGCCRV--CARRLGEPCD 65
IGFBP-rP8     MRWFLPWT-LAAV-TAAAASTVLATALSPAPTTMDFTPAPLEDTSSRPQF----------CKWPC--ECPPSP-PRCPL----------G--------VS--LIT---DGCECCKM--CAQQLGDNCT 88
IGFBP-rP9     MQGLLFSTLLLAG-LAQFCCRVQGTG--PLDTTPEGRPGEVSDAPQRKQF----------CHWPC--KCPQQK-PRCPP----------G--------VS--LVR---DGCGCCKI--CAKQPGEICN 87
IGFBP-rP10    MLPP-----PRPAA-ALALPVLLLLLVVLTPP--PTGARPSPGPDYLRRGWMRLLAEGEGCA-PC---RP----EECA----------APRG-------CLAGRVR---DACGCCWE--CANLEGQLCD 91


IGFBP-1       ---VA-TA--RCAR--GLSCRALPGEQQP-LHALTRGQ---G-ACVQES -----DAS---AP-HAAEAGSPESPESTEITEEEL-LDNFHLMA----------------P 143
IGFBP-2       ---VY-TP--RCGQ--GLRCYPHPGSELP-LQALVMGE---G-TCEKRR -----DAEYGASPEQVADNGDDHS-EG-GLVENHV-DSTMNMLGGGGSAG-RK------P 183
IGFBP-3       ---IY-TE--RCGS--GLRCQPSPDEARP-LQALLDGR---G-LCVNAS AVSRLRAYLLPAPPAPGNASESEEDRSAGSVESPS-VSSTHRVSD--PK--FH------P 167
IGFBP-4       ---VY-TP--RCGS--GLRCYPPRGVEKP-LHTLMHGQ---G-VCMELA EI---EAIQESLQ--PSDKDEGDHPNNS------------F--S---P----C------S 132
IGFBP-5       ---VY-TE--RCAQ--GLRCLPRQDEEKP-LHALLHGR---G-VCLNE- -----KSYREQVK-IERDSREHEEPTTSEMAEE------TY--S---PKI-FR------P 139
IGFBP-6       ---VY-TP--NCAP--GLQCHPPKDDEAP-LRALLLGR---G-RCLPAR -----------AP-AVAEENPKESKPQAGTAR--------------------------P 129

IGFBP-7       GGGAG-RG--YCAP--GMECVKSRKRRKGKAGAAAGGPGVSG-VCVCKS ----------RYP--VCGSDGTTYPSGCQLRAASQ-----RAES--------R------G 144
IGFBP-8       ----E-RD--PCDPHKGLFCDFGS-PANRKIGVCT-AK--DGAPCIFGG ---------TVYR--SGESFQSSCKYQCTCLDGAVGCMPLCSMDVRLPSP-DC------P 149
IGFBP-9       ----D-LE--PCDESSGLYCDRSADPSN-QTGICT-AV--EGDNCVFDG ---------VIYR--SGEKFQPSCKFQCTCRDGQIGCVPRCQLDVLLPEP-NC------P 156
IGFBP-10      ----K-TQ--PCDHTKGLECNFGA-SSTALKGICR-AQS-EGRPCEYNS ---------RIYQ--NGESFQPNCKHQCTCIDGAVGCIPLCPQELSLPNL-GC------P 146
IGFBP-rP5     ---LQ-EG--PCGE--GLQCVVPFGVPAS-ATVRRRAQ--AG-LCVCAS ----------SEP--VCGSDANTYANLCQLRAASRRSERLHRPPVIVLQRGACGQGQEDP 162
IGFBP-rP6     ---------MKCGP--GLRCQPSNGEDP--FGEEF------G-ICKD-- -----------CP---YGTFGMDCRETCNCQSG---------------------------- 120
IGFBP-rP7     ----Q-LH--VCDASQGLVCQPGAGPGG-RGALCLLAE--DDSSCEVNG ---------RLYR--EGETFQPHCSIRCRCEDGGFTCVPLCSEDVRLPSW-DC------P 146
IGFBP-rP8     ----E-AA--ICDPHRGLYCDYSGDRPRYAIGVCAQVV---GVGCVLDG ---------VRYN--NGQSFQPNCKYNCTCIDGAVGCTPLCLR-VRPPRL-WC------P 168
IGFBP-rP9     ----E-AD--LCDPHKGLYCDYSVDRPRYETGVCAYLV---AVGCEFNQ ---------VHYH--NGQVFQPNPLFSCLCVSGAIGCTPLFI-----PKL-AG------S 163
IGFBP-rP10    LDPSAHFYG-HCGE--QLEC---R-LDTG--GDLSRGE-VPEPLCACRS ----------QSP--LCGSDGHTYSQICRLQEAA------RA----------R------P 156
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Fig. 2. Partial amino acid sequence alignment of human IGFBP-1 to 10, and IGFBP-rP5-rP10. The consensus IGFBP motif which relates all of these sequences as a family is boxed. Consensus cysteine residues are shown in red. The matriptase consensus site sequence for cleavage is indicated in blue. Alignment was performed using the Clustalw2 sequence alignment program (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/msa/clustalw2/). Small gaps were introduced to optimize alignment. Nomenclature for the IGFBP7-IGFBP15, IGFBP-rPs: IGFBP7, IGFBP-rP1;mac25/TAF/PSF1; IGFBP8, IGFBP-rP2, CTGF; IGFBP-rP3,NovH; IGFBP-rP4, Cyr61; IGFBP-rP5, L56/HtrA; IGFBP-rP6, ESM-1; IGFBP-rP7, WISP-2/CTGF-L; IGFBP-rP8, WISP-1; IGFBP-rP9, WISP-3; IGFBP-rp10, Bono1.

Low affinity binding proteins (IGFBP-rPs)

Upon comparison of the IGFBP N-terminus in other cysteine-rich proteins, another group of proteins that were structurally related to the IGFBP family were identified, IGFBP-related proteins (IGFBP-rPs). Based on sequence alignment, the N-terminal domains of the IGFBP-rPs have significant similarities to the IGFBPs (40-57%) within their N-terminal domains, conserving all of the 12 cysteines within the N-terminal domain, including the consensus IGFBP motif. Past the N-terminus, the similarities decrease significantly to less than 15%. Unlike the IGFBPs, the IGFBP-rPs do not contain the thyroglobulin-type 1 domain at the C-terminus [28]. Their low affinity for IGFs together with their conserved structural homology to the IGFBP family suggested that these IGFBPs may have unique biological properties independent of their capacity to bind IGF. The first protein proven to be functionally related to the IGFBPs was IGFBP-rP1(IGFBP7)[29,30]. A group of highly related, cysteine-rich proteins were subsequently identified as part of the IGFBP-like family, termed the CCN family of proteins, including connective tissue growth factor (CTGF)[16], *nov* (nephroblastoma overexpressing) oncogene [31],*cyr61* [32*]*, and three genes (WISP-1, WISP-2, and WISP-3) that are upregulated in Wnt-1-transformed cells and are aberrantly expressed in human colon tumors [33]. HtrA (IGFBP-rP5) refers to a family of serine proteases who's main functions are protein quality control, and have been implicated in

tumour suppression and in the control of proliferation, migration and neurodegeneration (reviewed in [134]). IGFBP-rP10 (Bono1), the most recently identified member of the IGFBP family, with the highest homology to IGFBP7 at the amino acid level (42.2%), has been shown to be involved in the proliferation of osteoblasts during bone formation and bone regeneration [135]. This chapter will preferentially focus on IGFBP7.

IGFBP7 overview

The gene for human IGFBP7 is localized to chromosome 4q12-13 [34]. The mouse homolog shares 87.5% nucleotide identity and 94.4% similarity with human IGFBP7 [35]. IGFBP7 amino acid sequence has an overall 40-45% similarity and 20-25% identity to IGFBPs. The protein is produced as a precursor of 282 amino acids, which is processed to a mature 27 kD protein of 256 amino acids with one N-glycosylation site resulting in a secreted mature protein of 33 kD [16,30,27]. Structurally, the region of similarity of IGFBP7 to IGFBPs is confined to the N-terminal domain, encompassing the common IGFBP motif in a region containing 11 out of the 12 conserved cysteines [36](fig 2). Another domain found within the

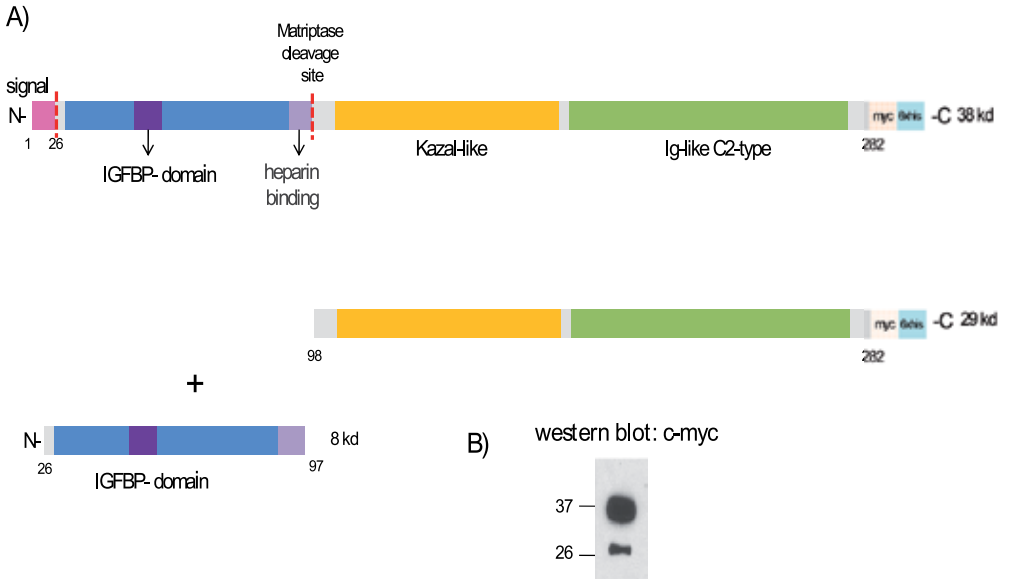

Fig. 3. Processing of recombinant IGFBP7 protein. A)Full length IGFBP7 protein is shown beginning with the signal sequence in red, which is cleaved off upon secretion from the cell. The N terminal contains the consensus IGFBP domain (dark purple), and the heparin binding domain (light purple). Kazal-like motif is shown in yellow and the Ig-like C2 domain is indicated in green. As a result of overexpression through the pSec-Tag2B plasmid, the protein is tagged in our system with *myc* and *his* at the C terminal, as shown in light pink and blue, respectively. Matriptase cleavage site is C terminal to the heparin binding domain between amino acid 97 and 98. Cleavage results in the production of 2 fragments, the N terminal portion (8 kd) and the C terminal 29 kd fragment. B) Western blotting of conditioned medium from MDA-MB-468 overproducing breast cancer cell line with anti-myc antibodies produces 2 bands, corresponding to the predominant large 38 kd protein, and the minor 29 kd cleaved protein.

N-terminus is a heparin sulfate binding site, consisting of 20 amino acid residues including 7 basic amino acids, which allows weak cell adhesion by interacting with cell surface-associated heparin sulfate proteoglycans [37](fig 3). Immediately adjacent to the N terminal domain is a stretch of 30-45 amino acid residues that has 30% similarity to the Kazal family of serine proteinase inhibitors, including the human pancreatic secretory trypsin inhibitor [38]. This domain, known as a KI domain, is also found in follistatin, leading to the hypothesis that IGFBP7 was a follistatin-like protein [35]. IGFBP7 can be proteolytically cleaved to a two-chain form by the type II membrane-bound serine proteinase, matriptase [39](fig 3). Cleavage occurs between K(Lys)97 and A(Ala)98, resulting in a 26 kD protein comprised of the C-terminal domain, and an 8 kD peptide corresponding to the N-terminal domain [40](fig 2,3). Cleavage results in almost a complete loss of both insulin/IGF-1 binding activity, while increasing cell adhesion activity [40].

IGFBP7-interacting proteins

Four groups independently identified the human IGFBP7 protein. One of these groups cloned the mac25 cDNA from normal leptomeningial and mammary epithelial cells, with expression of IGFBP7 decreased in the corresponding tumor cells [36,34]. The protein was shown to be able to bind IGFs, albeit with much lower affinity than IGFBPs [30]. During that same period, two other proteins were purified and characterized that were subsequently shown to be identical to the protein encoded by *mac*25. First, tumor adhesion factor (TAF) was isolated from the conditioned media of a human bladder carcinoma cell line, and promoted cell adhesion activity [41]. Second, prostacyclin-stimulating factor (PSF) was isolated from the conditioned media of human dipoid fibroblasts [42]. It was so termed due to its ability to stimulate prostacyclin production in endothelial cells, but not in patients with diabetes mellitus [43,44]. Finally, T1A12 was identified by subtractive cDNA cloning using RNAs from a normal breast epithelial cell line Hs578Bst and the breast cancer cell line Hs587T [45].

The ability of IGFBP7 to bind both IGF-1 and IGF-2, albeit with lower affinity than IGFBPs, led to its renaming as IGFBP7 [30]. However, IGFBP7 is unique amongst its family members in that it can bind insulin with high affinity, whereas IGFBPs 1-6 can only bind insulin with low affinity. This ability of IGFBP7 is due to the exposure of the insulin binding site at the amino terminal region due to lack of conserved cysteine residues in the C-terminal end, which are important for IGF binding by IGFBPs [46,47]. IGFBP7 can compete with insulin receptors for binding of insulin, thus preventing insulin-stimulated autophosphorylation of the insulin receptor β subunit[47]. IGFBP7 also contains a 'follistatin module' in its protein sequence, and has been shown to bind activin, a member of the TGF-β superfamily of growth factors [48]. Activin and its receptors are associated with growth modulation in glandular organs. Specifically, when activin signaling is disrupted or lost in normal mammary cells, malignant progression is potentiated, as demonstrated by the global decrease in the abundance of activin and its receptors in high grade breast cancer [49].

Another binding partner is type IV collagen. IGFBP7 co-localizes with type IV collagen in the vascular basement membrane [29]. IGFBP7 also can bind to cell surface-associated heparin sulfate proteoglycans, specifically, syndecan-1[40]. IGFBP7 has also been shown to bind certain CC chemokines, specifically, RANTES, SLC, and the CXC chemokine, IP-10 [50].

Expression

IGFBP7 is found in some biological fluids, such as serum, urine, CSF and amniotic fluid [51]. In normal human adult sera, the median IGFBP7 was 21.0 μg/liter. IGFBP7 is expressed in a

variety of normal tissues including heart, spleen, ovary, small intestine and colon [52]. Immunohistochemistry performed on normal human tissues showed a ubiquitous intense staining of peripheral nerves, smooth muscle cells, including those from blood vessel walls, gut, bladder, breast and prostate. Cilia from the respiratory system, epididymis, and fallopian tube also demonstrated intense positive staining. Most endothelial cells were seen to be positive, whereas fat cells, plasma cells and lymphocytes were negative. Specific IGFBP7 expression was limited to certain cell types in the kidney, adrenal gland and skeletal muscle [52]. IGFBP7 has also been shown to play a role in endometrial physiology. IGFBP7 expression is increased in the receptive versus prereceptive endometrium, and rises sharply again in late luteal phase. The protein was localized at the apical part of the luminal and glandular epithelium, as well as in stromal and endothelial cells [53]. Strong expression of IGFBP7 has also been seen in high endothelial vessels (HEV)[50].

Oncogene induced senescence

Normal cells have a limited proliferative lifespan, after which they enter a state of irreversible growth arrest. This process, originally observed by Hayflick and Moorhead and called replicative senescence, is believed to result in human cells from telomere shortening as a consequence of cell division [54,55]. This was thought to be a failsafe mechanism preventing the expansion of aged cells[56]. Almost three decades ago, it was observed that normal cells are refractory to oncogene transformation [57]. Ectopic expression of the oncogene H-RAS^{G12V} in normal fibroblasts induced senescence that was later shown to be telomere-independent, representing another type of senescence triggered by oncogenes, called oncogene-induced senescence (OIS)[58,59]. OIS, together with oncogene-induced apoptosis, has been suggested to act as a true barrier to cancer, once cellular damage is inefficiently repaired[56,60]. OIS can be triggered by activated oncogenes like BRAF^{E600} or RAS^{V12} or by the loss of tumor suppressor proteins, like PTEN or NF1[61,62,63]. OIS is often characterized by the upregulation of the CDK inhibitors p15^{INK4B},p16^{INK4A}, and p21^{CIP1}, as well as by an increase in senescence-associated β-galactosidase (SA-β-Gal) activity [64,65]. Acute inactivation of certain genes, such as Rb or p53, can reverse OIS [66,67,68]. A typical example of OIS occurs in melanocytic nevi, which are benign skin lesions that rarely progress to melanoma [69,70]. Nevi are growth arrested and display classical hallmarks of senescence, including expression of SA-β-Gal, and the cell cycle inhibitor, p16^{INK4A} [62,71,72]. Activating BRAF mutations account for up to 82% of melanocytic nevi [73]. Senescent cells secrete a broad spectrum of factors, primarily involved in IGF and TGF-β signaling, ECM remodeling and inflammation [74,75,76,77,78]. Together, these secreted factors are referred to as the Senescence-Messaging Secretome (SMS) or the Senescence-Associated Secretory Phenotype (SASP) [79,78]. IGFBP7 has been identified as one of these factors responsible for the establishment and/or maintenance of OIS [34,75].

3. IGFBP7 as tumor suppressor in various cancers

IGFBP7 has been shown to be a tumor suppressor in a variety of solid cancers (summarized in Table 1). Its expression is lost upon progression to more aggressive cancer types. Loss of expression is associated with poor prognoses. Reexpression or exposure of cancer cell lines to IGFBP7 results in either senescence or apoptosis, and when these IGFBP7-expressing cell lines are xenografted in mice, tumor growth is inhibited.

Breast cancer

IGFBP7 has been shown to be a tumor suppressor in breast cancer. IGFBP7 was identified as one of the genes overexpressed in senescent human mammary epithelial cells (HMEC) (10 fold higher than quiescent cells of the same origin), and which was upregulated in normal mammary epithelial cells by all-*trans*-retinoic acid [34,80]. We cloned the gene for IGFBP7 by subtractive hybridization from the Hs568T breast cancer cell line and found IGFBP7 to be downregulated in primary breast cancer tissues. In normal breast tissue, IGFBP7 protein expression is concentrated in the cytoplasm of luminal epithelial cells, in ducts and acini of normal and benign primary breast tissues as well as other luminal, normal human cellular structures, suggesting an important role for IGFBP7 in the maintenance of normal breast and tissue architecture in general [45].

Cancer type		Down-regulated	Up - regulated	IGFBP7 Introduction	Effect	Reference
Breast	MCF-7			Overexpressed	G0-G1 arrest Senescence	[86]
	MDA-MB-468	pERK1/2		Overexpressed	↓ Tumour-genicity	[85
	Xenograft-MDA-MB-468			Overexpressed	↓Growth and migration	[85]
	MDA-MB-231		pp38 p53, p21	Exogenous Protein	↓Cell growth ↑Senescence ↑Apoptosis	Manuscript submitted
Colorectal	SW620, COLO205, HT29			5-Aza-dc	↓Cell migration/invasion	[93]
	RKO, CW2	E-cadherin B-catenin pRB	p53	Overexpressed	G1 arrest Senescence	[95]
	DLD-1				↓Anchorage independent growth ↑Cell adhesion	[98]
	Xenograft-DLD-1			Overexpressed	↓ Tumour-genicity	[98]
	Xenograft-HT29, SW620			Exogenous protein	↓ Tumour-genicity	[89]
Hepatocellular	PLC/PRF/5	SMARCB1 BNIP3L p27	pERK1/2 cyclin D1 cyclin E	shRNA targeting IGFBP7 mRNA	IFNα resistance ↑ Cell growth ↓Apoptosis	[106,117]
Melanoma	Nevi	pERK1/2	RKIP	Exogenous protein	Senescence	[75]
	Cell line		BNIP3L		Apoptosis	[75]
	Xenograft				Apoptosis	[75]
	Metastatic			Intervenous protein injection	Growth inhibition	[89]
	Murine metastatic	VEGF	Caspase-3	Intra-tumoral plasmid injection	Apoptosis	[90]

Cancer type		Down-regulated	Up - regulated	IGFBP7 Introduction	Effect	Reference
Prostate	M12			Overexpressed	↑ Doubling time ↓Apoptosis sensitivity Epithelial Morphology change ↓ Colony formation	[102]
	M12 xenograft			Over-expressed	↓ Tumour size	[102]
Thyroid	N1M1	pERK1/2	p53 p21 cleaved PARP	Over-expressed	Apoptosis ↓Cell migration	[105]
	N1M1			Over-expressed	Reduced tumour growth	[105]

Table 1. IGFBP7 as a tumor suppressor in various cancer models. Summarized data from six different cancers, showing the effect of overexpression or inhibition of IGFBP7 on cancer cell growth both in vivo and in vitro, as well as signaling pathways affected.

Expression of IGFBP7 decreases with breast cancer progression. Normal breast tissues had very high IGFBP7 protein levels, such as luminal epithelial cells of normal lobules and ducts, as well as in benign proliferation of ducts consistent with fibroadenoma [45]. By immunohistochemical staining, IGFBP7 expression was detected in all normal and benign patient samples examined, with particularly strong staining in luminal epithelial cells of normal ducts, and acini or endothelial cells of blood vessels [81]. Intermediate to weak IGFBP7 staining was evident in hyperplastic breast tissue and DCIS specimens [81]. In addition, IGFBP7 was significantly upregulated in low grade ductal carcinoma in situ (DCIS) relative to high grade DCIS, as judged by CDNA microarray analysis. In invasive breast tumors, immunohistochemical analysis revealed that IGFBP7 is downregulated at the protein level [45]. IGFBP7 is downregulated in some breast tumors by loss of heterozygosity (LOH), and is also reduced by promoter methylation, both of which lead to increased tumor incidence and poor overall survival [45,82,83]. When DNA extracted from microdissected breast tissues was used with a microsatellite marker based method to determine allelic loss of the IGFBP7 locus in paired normal and invasive breast tissues, 50% of the informative samples from 30 matched pairs of normal and breast tumor tissues showed allele-specific LOH suggesting that the IGFBP7 gene was inactivated by deletions in at least a portion of each tumor [45]. A thoroughly characterized group of 106 invasive breast samples was surveyed using the tumor tissue microarray technique and immunohistochemistry [84]. Approximately 40% of tumors have low or no IGFBP7 staining suggesting that the gene or gene product was inactivated in a subset of invasive breast cancer samples [84]. Low IGFBP7 was associated with high cyclin E expression, retinoblastoma protein (pRb) inactivation, poorly differentiated tumors and higher stage. There was a significantly impaired prognosis for patients with low IGFBP7-expressing tumors. IGFBP7 also showed an inverse correlation with proliferation (Ki-67) in ER^- tumors [84].

IGFBP7 expression was examined in 32 primary patient breast tumors and matched metastatic counterparts (fig 4). Low levels of IGFBP7 expression were found in 25/32 primary tumors. Approximately half of these tumors had lower levels of IGFBP7 in their metastatic tumors compared to the matched primary tumor, indicating that loss of IGFBP7 confers a selective growth advantage for metastatic lesions [85].

In order to investigate the growth of human breast cancers in an in vivo model, 7 human primary tumors were implanted into human bone grafts under the right flank of human-bone NOD/SCID mice. Only triple negative breast tumors grew in these mice (table 2). One of the triple negative primary breast tumors was serially transplanted more than five times. Each serial transplant resulted in increased tumor uptake and shorter growth rate. The tumor latency was decreased by approximately half after the first re-implantation. Examination of IGFBP7 expression revealed that each serial transplant resulted in lower levels of IGFBP7 expression by qRT-PCR [85](fig 4). Comparing the xenografted tumor to the original primary patient tumor revealed an increase in the anti-human specific proliferation marker, Ki67 (42.03 ± 8.87 to 53.3 ± 3.6). These results again confirmed an inverse correlation between IGFBP7 expression and breast tumor growth as well as aggressiveness of the tumor.

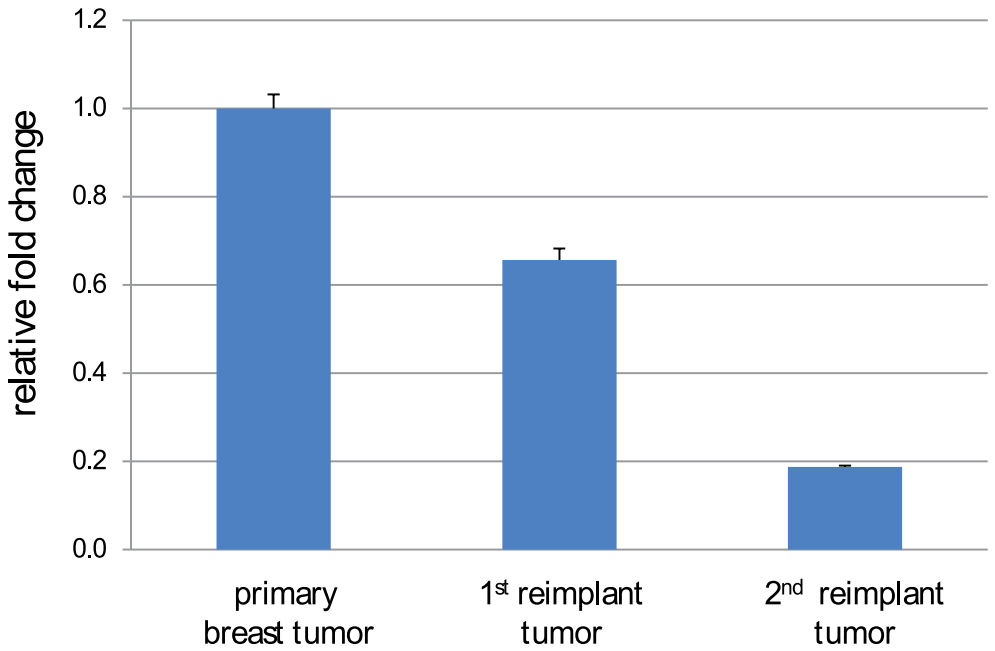

Fig. 4. Expression of IGFBP7 in primary and xenografted patient breast tumors by qRT-PCR. Quantitative PCR of IGFBP7 expression in primary and successively xenografted human breast tumors derived from first and second implantation into NOD/SCID mice. The data represent average values and standard error measurement from two triplicate samples, normalized against β-actin mRNA levels. The relative fold changes of the selected genes are obtained by dividing the expression levels of the re-implanted tumors by the expression levels in the primary patient tumors.

Patient	Age (Years)	Histo-pathological diagnosis	Grading	Estrogen /Progesterone receptor expression	ErB-2 expression	Node invasion (number positive/ number harvested)	Growth in hu-bone NOD/ SCID mice
HuP-1	67	Invasive ductal carcinoma	II/III	+/-	+	1/12	-
HuP-2	35	Metaplastic carcinoma	III	-/-	-	0/23	+
HuP-3	40	Invasive ductal carcinoma	II/III	+/+	+	0/2	-
HuP-4	48	Invasive ductal carcinoma	III	-/-	-	0/17	+
HuP-5	81	Invasive ductal carcinoma	II	+/-	-	0/1	-
HuP-6	50	Invasive and In-situ duct carcinoma	II	+/+	-	0/16	-
HuP-7	75	Invasive ductal carcinoma	I	+/+	-	4/15	-

Table 2. Characteristics of the human patient breast tumor tissues engrafted in hu-bone NOD/SCID mice.

The major traits of the engrafted human patient breast tumor samples (patient age, histopathological diagnosis, grading, estrogen/progesterone receptor expression, ErB-2 expression, node invasion) are indicated. The table also shows if the patient tumor samples were able to grow in the hu-bone NOD/SCID mouse model.

In order to transcriptionally characterize the colonization and aggressive behavior of engrafted patient breast tumors, microarray gene expression profiling was performed on breast tumors that were serially transplanted in the human-bone NOD/SCID mice. Genes were identified that were differentially expressed in the xenografted tumors by at least 1.5 fold compared to the primary patient tumors. There were 205 genes found to be differentially regulated in both HuP-2 and HuP-4 bone residing-breast tumors. Of the 129 known genes, 97 were expressed at higher levels and 32 at lower levels in the patient breast tumors colonized in bone. To narrow the spectrum of genes, 14 up-regulating and 18 down-regulating genes with bone colonization potentials are displayed (Table 3). Many of these gene identified have been previously associated with cancer function or metastatic activities such as cell viability, apoptosis and oncogenic transformation. IGFBP7 was identified as one of the genes that were downregulated in the xenografted tumors.

Gene	Fold Differences		Description	Identified cancer involvements
	HuP-2	HuP-4		
Down-regulated				
MTBP	7.11	1.94	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein	p53 regulator, Metastasis and cell proliferation suppressor
PARK7	5.30	3.14	Parkinson disease (autosomal recessive, early onset) 7	Negative regulator of PTEN, cell survival & aggressiveness
TOB1	1.79	6.12	Transducer of ERBB2, 1	Anti-proliferative protein
SDCBP	2.01	5.64	Syndecan binding protein (syntenin)	Cell adhesion & protein trafficking
CD24	2.12	11.29	CD24 molecule	Breast cancer stem cell marker & associated with bone metastasis
IL1R1	4.27	2.44	Interleukin 1 receptor, type I	Mediate cytokine induced immune & inflammatory response
PDLIM5	2.60	1.70	PDZ and LIM domain 5	Negative factor of oncogenic activity in neural tumor
HLA-DRA	2.15	6.33	Major histocompatibility complex, class II, DR alpha	Tumor immunosurveillance
PRKACB	3.11	3.93	Protein kinase, cAMP-dependent, catalytic, beta	Cell proliferation & differentiation
UBE2I	5.55	1.63	Ubiquitin-conjugating enzyme E2I	Suppressing p53 functions via RPA2 activity
IGFBP7	3.82	2.00	Insulin-like growth factor binding protein 7	Tumor suppressor & cell proliferation
ITM2B	1.94	2.28	Integral membrane protein 2B	Cell survival
ADAMTS12	1.61	3.43	ADAM metallopeptidase with thrombospondin type 1 motif, 12	Prevents tumorigenic effect of HGF
SPIN	1.56	3.35	Spindlin	Cell cycle regulation
PTPRF	1.86	2.32	Protein tyrosine phosphatase, receptor type, F	Regulation of epithelial cell-cell contact and cell growth
UHRF1BP1L	2.54	2.34	UHRF1 (ICBP90) binding protein 1-like	Regulate VEGF gene expression & tumor angiogenesis
TRMT5	2.20	1.87	TRM5 tRNA methyltransferase 5 homolog	Methylation
NR3C1	1.62	2.29	Nuclear receptor subfamily 3, group C, member 1)	Signaling and transduction
Up-regulated				
EIF5A	2.78	1.79	Eukaryotic translation initiation factor 5A	Cell viability & senescence
PCNXL2	2.81	1.79	Pecanex-like 2	Tumorigenesis in colorectal carcinoma
CD1C	3.11	1.54	CD1c molecule	Mediate immune responses to tumors
CSF1R	3.71	5.70	Colony stimulating factor 1 receptor	Metastasis & cell invasiveness
RPS5	1.99	1.99	Ribosomal protein S5	Cell differentiation and apoptosis
GOT2	2.10	1.74	Glutamic-oxaloacetic transaminase 2, mitochondrial	Serum GOT correlated with cancer and metastatic disease
RABL4	3.95	1.64	RAB, member of RAS oncogene family-like 4	Ras-related putative GTP-binding protein
RPS14	2.39	2.02	ribosomal protein S14	Haploinsufficiency disease gene
KLF8	1.57	1.53	Kruppel-like factor 8	Oncogenic transformation & EMT, downstream of FAK
DGKQ	1.82	2.28	Diacylglycerol kinase, theta 110kDa	Signal transduction pathways
AP2S1	3.23	1.60	Adaptor-related protein complex 2, sigma 1 subunit	Clathrin adaptor complex associated with plasma membranes

PALM	1.58	1.83	Paralemmin	Cell shape control
RPS8	1.87	1.76	Ribosomal protein S8	Up-regulated in astrocytoma and pancreatic cancer
POLR2J	3.61	2.07	DNA directed RNA polyermase II	Enzyme & transcription

Table 3. Schematic representation of microarray analysis from xenografted tumors compared to primary tumors. To identify genes with bone colonization potential, xenografted tumor tissues were harvested for microarray analysis. Fold changes are obtained by dividing the gene expression levels in the xenografted tumors by the expression levels in the primary patient tumors. 205 genes are at least 1.5 fold differentially expressed in both HuP-2 and HuP4 bone residing breast-tumors compared with their primary patient breast tumors. A representation of genes whose expressions in xenografted tumors were at least 1.5 fold down-regulated from primary patient tumors (18 of 157 genes), or upregulated from primary patient tumors (14 of 48 genes) are shown.

The increased expression of IGFBP7 in senescent versus proliferating normal HMECs [34], prompted the evaluation of potential antiproliferative capabilities of IGFBP7 in breast cancer cells. In order to test this theory, IGFBP7 was overexpressed by retroviral vector in the ER/PR$^+$ IGFBP7$^-$ MCF-7 breast cancer cell line. IGFBP7-transduced MCF-7 breast cancer cells showed a significant reduction in cell growth compared to parental IGFBP7 negative MCF-7 cells. When further analyzed, cells had arrested at the G0-G1 phase of cell cycle upon IGFBP7 expression. IGFBP7 was found to induce senescence rather than apoptosis [86].

ER/PR-negative breast cancers are the most aggressive and hardest to treat. In order to examine whether restoration of IGFBP7 could inhibit triple negative breast cancer cell growth, IGFBP7-overexpressing cells were engineered using a pSec-Tag2 plasmid in MDA-MB-468, a triple negative breast cancer line with barely detectable levels of endogenous IGFBP7, that is also tumorigenic in mice [87]. The vector contained a C-terminal c-*myc* epitope for detection with an anti-*myc* antibody, and a polyhistidine (6x*his*) tag for rapid purification with nickel-chelating resin and detection with an anti-*his*(C-term) antibody (fig 3). Western blots of conditioned medium from stable IGFBP7-transfectants revealed two bands in response to IGFBP7 staining, a 38 kD band seen also in cell lysates, and a weaker, smaller 29 kD band. N-terminal sequencing revealed that both bands are IGFBP7 gene products [85]. The 38 kD band corresponded to the full length protein minus the signal sequence, whereas the smaller 29 kD band was cleaved after amino acid lys^{97}, suggesting cleavage by the enzyme matriptase [39,85](fig 2, 3). IGFBP7 overexpression in MDA-MB-468 cells reduced cell growth and migration compared to parental MDA-MB-468 cells. Similarly, conditioned medium from IGFBP7 overexpressing breast cancer cell lines also lowered the growth of MDA-MB-468 cells. In order to examine the mechanism of IGFBP7-mediated growth inhibition, the effect of IGFBP7 overexpression on the MAP kinase pathway was analyzed. IGFBP7 overexpression inhibited the phosphorylation of MEK-1/2 and ERK-1/2 compared to parental MDA-MB-468 cells [85](fig. 5). These results are consistent with those observed in melanoma studies, whereby IGFBP7 is thought to act through autocrine and paracrine pathways to inhibit BRAF-MEK-ERK signaling resulting in induction of senescence or apoptosis [75].

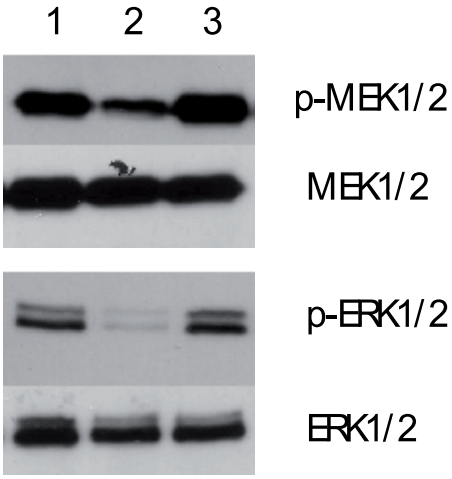

Fig. 5. Effect of IGFBP7 overexpression on the MAP kinase signaling pathway. Western blotting using equal amounts of protein from total cell lysates from MDA-MB-468 (lane 1), MDA-MB-468/IGFBP7 (lane 2), and empty vector control (lane 3) cells were examined by western blotting with antibodies to pERK-1/2,ERK-1/2, pMEK-1/2, and MEK-1/2.

The effects of IGFBP7 mediated growth inhibition were also examined *in vivo*. Parental MDA-MB-468 breast cancer cells and the IGFBP7-overexpressing variant were injected into NOD/SCID or NSG mice. Examination of tumor growth revealed a significant inhibition of tumor growth from the IGFBP7 overexpressing MDA-MB-468 cells (fig 6). Tumors were considerably smaller in the presence of IGFBP7. Immunohistochemistry and qRT-PCR of revealed the expression IGFBP7 in tumors derived from IGFBP7 overexpressing cells, confirming continual production of IGFBP7 *in vivo* during the duration of the experiment, which suggested that IGFBP7 was responsible for tumor growth suppression [85].

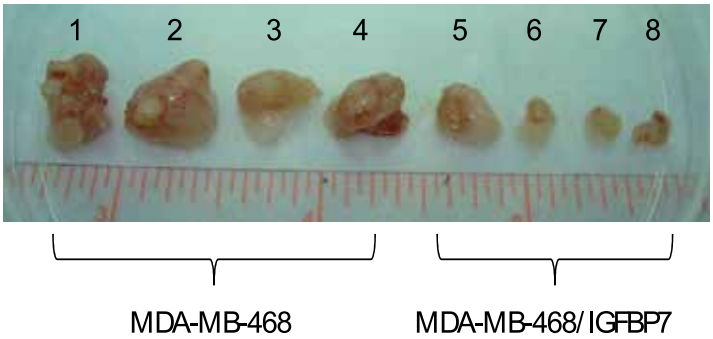

Fig. 6. Effect of IGFBP7 overexpression on breast tumor formation in vivo. $5x10^6$ MDA-MB-468 cells or MDA-MB-468/IGFBP7 cells were injected into NSG or NOD/SCID mice. After 36 days, tumors were removed and analyzed.

Melanoma

IGFBP7 was shown to be a tumor suppressor in melanoma studies, in that loss of IGFBP7 expression was critical step in melanoma development [75]. Activating BRAF mutations are found at a high frequency in melanomas (50-70%)[88]. In normal melanocytes, IGFBP7 is expressed at low levels. Following expression of the activating BRAFV600E mutation in melanocytic nevi, IGFBP7 is upregulated and induces senescence [75]. Melanoma cell lines harboring the activating BRAFV600E mutation, did not express IGFBP7, due to epigenetic silencing through promoter methylation of IGFBP7 [75,89]. Upon exposure to IGFBP7, BRAFV600E-positive melanoma cells underwent apoptosis. BRAFV600E expression in melanoma cells results in hyperactivation of the BRAF-MEK-ERK pathway. IGFBP7 treatment blocked cellular proliferation in part through inhibition of this pathway. Specifically, the phosphorylation of MEK by BRAF was prevented by upregulation of the RAF inhibitory protein (RKIP) by IGFBP7 through autocrine/paracrine pathways [75]. The apoptotic pathway induced by IGFBP7 involved the upregulation of BNIP3L, a proapoptotic BCL2 family protein. Furthermore, systemically administered IGFBP7 markedly suppressed the growth of BRAF-positive melanomas in xenografted mice, also through induction of apoptosis [75]. Epigenetic silencing of IGFBP7 is even more pronounced in human metastatic samples [89]. In a mouse model of metastatic melanoma, where mice were injected via tail vein with the highly metastatic BRAFV600E-positive malignant melanoma cells A375M-F*luc*, IGFBP7 systemic administration suppressed tumor growth and increased survival [89]. Another group demonstrated that intratumoral injection of IGFBP7 in the form of the plasmid, pcDNA3.1-IGFBP7, promoted stable expression of IGFBP7, and suppressed the growth of the murine malignant melanoma cell line, B16-F10, by inducing apoptosis. Caspase 3 levels were increased and VEGF levels were decreased in the pcDNA3.1-IGFBP7 treated group [90].

Colorectal cancer

In the normal colon, IGFBP7 expression varies from the basal compartment to the surface epithelium. Epithelial cells at the surface contain very strong IGFBP7 expression, whereas IGFBP7 staining was much weaker at the crypt base, which indicates that IGFBP7 expression is stronger in the differentiating areas of the colonic epithelium. Interestingly, IGFBP7 expression is actually increased in colorectal cancer. In colon carcinoma, IGFBP7 expression is strongest in the well differentiated colorectal adenocarcinoma, while weakly expressed in poorly differentiated colorectal adenocarcinoma [91]. IGFBP7 expression was correlated with differentiation, low grade tumor, and better prognosis. Cell differentiation and apoptosis are considered a result of normal colonocyte terminal differentiation in vivo. Introduction of IGFBP7 into colon cancer cells induced a more differentiated morphology. Upregulation of several colonic epithelial cell differentiation markers, such as AKP and CEA occurred with reintroduction of IGFBP7 [91]. This study identified IGFBP7 as a potential key marker associated with colon cancer differentiation.

The inhibition of IGFBP7 expression in colon cancer cell lines was shown to be due to aberrant DNA hypermethylation of the CpG island in exon 1 of IGFBP7, specifically in the promoter region [92]. Reactivation of IGFBP7 by 5-aza-dC treatment inhibited colon cancer cell proliferation in a dose dependent manner [93]. Demethylation restored p53-induced IGFBP7 expression[94]. Epigenetic inactivation of IGFBP7 appears to play a key role in tumorigenesis of CRCs with CpG island methylator phenotype (CIMP) by enabling

escape from p53-induced senescence [94]. Cell cycle was arrested, as cells accumulated in G2/M phase. 5-aza-dC treatment also increased the percentage of cells undergoing apoptosis. Cell migration and invasion were also reduced after treatment with 5-aza-dC [93]. The authors argue that demethylation increased the expression of tumor suppressor proteins, specifically IGFBP7, which was involved in the 5-aza-dC induced growth inhibitory effects.

A more direct effect of IGFBP7 as a tumor suppressor in colon cancer was shown in a subsequent study. Colorectal carcinoma cells, RKO and CW2, transfected with pcDNA3.1-IGFBP7 showed reduced proliferation. Cells were arrested in G1 phase of cell cycle (15% increased compared to control cells). The expression of E-cadherin and β-catenin were reduced in IGFBP7-transduced CW2 cells. Migration was not affected. A senescence like phenotype was induced, as judged by increased SA-β-Gal activity, together with increased p53 and reduced pRB expression [95]. Cellular senescence is a barrier to cancer, preventing cells from unlimited proliferation [96,97]. This study suggested that IGFBP7 is an important molecule that triggers senescence through two important pathways, the p53-dependent pathway and the p16/p21-pRB pathway [95].

IGFBP7 was also shown to inhibit colon cancer tumor growth. Overexpression of IGFBP7 in the human colon cancer cell line, DLD-1, reduced its tumorgenicity *in vivo* [98]. Anchorage independent growth was also reduced. IGFBP7 expression increased cell adhesion of DLD-1 cells to laminin-5 and fibronectin [98]. In a separate study, two human CRC cell lines, one with an activating BRAF mutation (HT29) and the second with an activating KRAS mutation (SW-620), when xenografted into nude mice, were significantly growth inhibited upon systemic IGFBP7 treatment [89].

Proteomics was used to identify proteins associated with IGFBP7 in CRC. Six proteins were downregulated upon IGFBP7 reintroduction in colon cancer RKO cells, one of which was heat shock protein (HSP) 60 [99]. The authors focused on HSP60, as a key protein involved in IGFBP7-mediated growth inhibition, since it is overexpressed in CRC tissue and involved in proliferation and inhibition of apoptosis. They argue that one mechanism by which IGFBP7 overexpression inhibits growth of CRC cells, is through downregulation of HSP60.

Prostate cancer

IGFBP7 expression is found in primary cultures of prostate epithelial cells, and within the conditioned media from these cells. Peripheral nerves and stromal components associated with prostate tissue were strongly positive for IGFBP7 [100]. IGFBP7 protein and mRNA expression was up-regulated by IGF-I, TGF-β, and retinoic acid in the nontumorigenic prostate epithelial line, P69, derived by immortalization of human primary prostate epithelial cells with simian virus-40 T antigen. IGFBP7 was undetectable by northern blot from malignant prostate lines such as LNCap, DU145, and PC-3 cells, and M12 cells (the tumorigeneic and metastatic subclone of P69) [101,100]. There was a significant loss of detectable IGFBP7 mRNA in metastatic prostate tissue [28]. Re-expression of IGFBP7 in the human prostate cancer cell line, M12, results in an increase in cell doubling time, a decrease in colony formation in soft agar, a marked change in epithelial morphology along with an increased sensitivity to apoptosis, and finally decreased tumor formation and size *in vivo* [102]. In order to identify genes upregulated by IGFBP7 expression in prostate epithelial cells, a cDNA array analysis of IGFBP7-overexpressing M12 was performed, identifying SOX9, a transcription factor associated with differentiation [103]. The overexpression of

SOX9 in M12 cells seemed to recapitulate the effects seen with overexpression of IGFBP7 alone, suggesting that SOX9 is at least partly responsible for the growth inhibitory effect of IGFBP7 on prostate cancer cells. Another group used similar techniques and identified another transcription factor, manganese superoxide dismutase (SOD-2), which they argue was at least in part responsible for the growth inhibitory effects of IGFBP7 in prostate cancer cells [104]. Whether these transcription factors were indeed part of the anti-proliferative mechanism of IGFBP7, or merely a consequence of IGFBP7 overexpression in M12 cells remains to be determined.

Thyroid cancer

In accordance with prostate, colon and breast cancer, IGFBP7 expression is also significantly downregulated in thyroid cancer tissue samples compared to normal thyroid tissue [105]. IGFBP7 is epigenetically silenced by promoter hypermethylation in PTC-derived NIM1 thyroid tumor cell line. NIM1, along with most other thyroid cancer cell lines, carries the BRAFV600E mutation. Restoration of IGFBP7 in NIM1 cells by cDNA transfection resulted in growth inhibition, reduced colony formation in soft agar, and decreased migration capability in wound healing assay. Furthermore, tumor growth was inhibited upon injection in nude mice [105]. Examination of the mechanism governing IGFBP7 mediated growth inhibition revealed that IGFBP7-expressing NIM1 cells were impaired in cell cycle progression, manifesting cell cycle arrest in G1. The G1 arrest was associated with a strong decline in phospho-ERK levels, and an upregulation of p53 and p21 tumor suppressors. IGFBP7 expression alone resulted in increased apoptosis, as judged by increased cleaved PARP, which was even more pronounced upon exposure to the TRAIL, a proapoptotic agent effective in NIM1 cells [105]. These results suggest that IGFBP7 is a tumor suppressor in thyroid carcinogenesis.

Hepatocellular carcinoma (HCC)

A strong antitumor activity against HCC has been demonstrated for interferon (IFN)-based combination therapy (IFN-α/ 5-FU therapy) [106-116]. However continuous exposure to IFN-α can result in IFN-resistant HCC cells. IGFBP7 was identified by microarray analysis as one of the most significantly downregulated genes in IFN resistant clones. Parental PLC/PRF/5 cells transfected with short hairpin RNA for IGFBP7 showed IFN-α resistance. IGFBP7 transfection into IFN-resistant HCC cells restored IFN sensitivity [106]. These results suggested that IGFBP7 could be a novel marker to predict clinical outcome to IFN-α/5-FU therapy.

A recent report studied PLC/PRF/5 cells treated with shRNA directed towards IGFBP7. They found that in the absence of IGFBP7 expression, the cells grew more rapidly, phospho-ERK was significantly increased, and apoptosis was decreased, as compared to the parental IGFBP7 expressing cells [117]. They found that apoptosis was decreased as a result of decreased expression of proapoptotic proteins, *SMARCB1* and *BNIP3L* by qRT-PCR. Furthermore, upon suppression of IGFBP7 expression, cell cycle progression was increased, concomittently with increased cyclin D1 and cyclin E, and decreased p27. IGFBP7 reexpression in an HCC line that had very low IGFBP7 levels resulted in growth inhibition and decreased invasive ability. IGFBP7 downregulation was also significantly associated with tumor progression and postoperative poor prognosis in resected human HCC samples [117]. These studies identify IGFBP7 as a tumor suppressor and also an independent significant prognostic factor in HCC.

Lung cancer

Expression of IGFBP7 in lung cancer cell lines using RT-PCR revealed decreased expression of IGFBP7 compared to controls, and 42 out of 90 patients with primary lung tumors exhibited negative staining of IGFBP7 by immunohistochemical analysis [118]. There was a significant correlation between DNA methylation of exon/intron 1 region and IGFBP7 downregulation. When a p53 expression vector was transfected into lung cancer cell lines, it could only induce expression of IGFBP7 in the unmethylated cell line, but not in the methylated cell lines, suggesting that IGFBP7 might be regulated by p53 in lung cancer cell lines.

Squamous cell carcinoma of the head and neck (SCCHN)

A study found that a single nucleotide polymorphism (G to A) in the IGFBP7 promoter region was significantly associated with a reduced risk of SCCHN, when analyzed in a hospital-based case-control study of 1065 SCCHN patients and 1112 cancer-free control subjects. Upon analyzing reporter gene constructs, the G to A allelic change at -418 of the IGFBP7 promoter had increased promoter and DNA binding activity, suggesting increased IGFBP7 protein expression [119].

Although IGFBP7 has been shown to function as a tumor suppressor in a wide variety of cancers, a few studies suggest that IGFBP7 has an opposite effect, ie. promoting cancer growth. These cancers include the blood cancer, leukemia, and the brain cancer, glioblastoma.

Glioblastoma

IGFBP7 is a selective biomarker of glioblastoma (GBM) vessels, strongly expressed in tumor endothelial cells and vascular basement membrane [120]. IGFBP7 was strongly expressed in GBM specimens but not nontumor brain tissue. Moreover, statistical analysis showed that expression of IGFBP7 correlated inversely with overall GBM survival rates. Inhibition of IGFBP7 expression using siRNA transfection in a glioma cell line inhibited cell growth [121]. Addition of IGFBP7 to cell culture medium stimulated cell proliferation. IGFBP7 also promoted glioma cell migration, through downregulation of AKT phosphorylation and enhanced ERK1/2 activation [121]. IGFBP7 expression in brain endothelial cells was found to be upregulated by secreted factors from GBM cells through TGF-β1/ALK5/Smad2 signaling pathway, which has been implicated in angiogenesis [122].

Acute leukemia

Overexpression of the human gene BAALC (brain and acute leukemia, cytoplasmic), was shown to be associated with inferior outcome and chemotherapy resistance in adult patients with cytogenetically-normal acute myeloid leukemia (CN-AML), T cell-acute lymphoblastic leukemia (T-ALL) and B-precursor acute lymphoblastic leukemia (B-ALL)[123,124,125,126,127]. IGFBP7 was strongly correlated with BAALC-expression, implicating IGFBP7 in acute leukemia [128]. Aberrent expression of IGFBP7 in adult leukemia was correlated with chemotherapy resistance and inferior survival. Addition of IGFBP7 to leukemic cell lines inhibited cell growth without induction of apoptosis or senescence, suggesting a role of IGFBP7 in contributing to drug resistance through reduced sensitivity to cytostatic drugs [128]. Aberrently increased levels of IGFBP7 were found in

CSF from children with acute lymphoblastic leukemia, implicating IGFBP7 with a more aggressive subtype of ALL [129]. IGFBP7 was also aberrantly overexpressed in the majority of AML at diagnosis and upon relapse, but not at remission stage [130]. Thus, IGFBP7 was shown to play a positive contributing role in the interaction between leukemia cells and the microenvironment, which may promote the leukemic cells' adhesion, invasion, and migration.

While the data observed in studies of leukemia and glioblastoma portray IGFBP7 in a negative role with respect to cancer, the vast majority of data from studies of solid tumors are in disagreement with these conclusions. It is possible that cell signaling pathways that result in senescence or apoptosis due to IGFBP7 are not present or functional in hematopoietic or glioma cells.

4. Conclusions and perspectives

IGFBP7 has been shown to have tumor suppressive function in breast and other cancers. When examining the summarized data in Table 1, a common thread appears. Overexpression of IGFBP7 leads to inhibition of growth both *in vitro* and *in vivo*, increased expression of apoptotic markers (caspases, cleaved PARP), senescence associated proteins (*i.e.* p21, p27, p53), and decreased expression of proteins associated with proliferation (p-ERK). IGFBP7 appears to affect signaling through the MAP kinase pathway in many tumor models, including breast cancer. OIS may be a mechanism of tumor suppression by IGFBP7. The breast cancer cell lines used in our study, MDA-MB-468 cells, have a mutated PTEN, disregulating the PI3K pathway [131]. OIS can be triggered not only by the activation of oncogenes but also by the loss of tumor suppressor genes, such as PTEN. By upregulating proteins that counteract proliferation, such as cyclin dependent kinase inhibitors, *ie.* p21, which we have shown to occur upon IGFBP7 addition to breast cancer cells, the combined effect can lead to OIS [132]. Our model for the role of IGFBP7 in breast cancer inhibition depicts the entrance of IGFBP7 full length or cleaved IGFBP7 (through matriptase) into the cell, where signals are propagated to the nucleus, leading to the upregulation of expression of cyclin dependent kinase inhibitors, such as p21 and p27 (fig 7). This together with an already hyperstimulated MAP kinase pathway due to oncogenic mutations such as RAS, leads to MAP kinase pathway inhibition, growth arrest, and senescence, as suggested by the conflicting signal model of senescence[132].

The strong link to breast cancer outcome suggests that IGFBP7 may not only be a good prognostic indicator for malignant disease progression, but also a useful surrogate marker for monitoring therapeutic responses in the treatment of breast cancers. Senescence has been shown to be a method of halting tumor growth by many standard chemotherapeutic drugs [133]. Preliminary results indicate that senescence may be one mechanism by which IGFBP7 inhibits breast cancer cell growth in our system. Inhibition of breast cancer growth in vivo and in vitro together with induction of senescence indicates that IGFBP7 could be further developed as a potential drug to treat breast cancers. The fact that IGFBP7 has growth inhibitory effects when expressed in triple negative breast cancer cells, *i.e.* MDA-MB-468, provides an exciting opportunity to bring to the clinic a potential drug for hard to treat breast tumors.

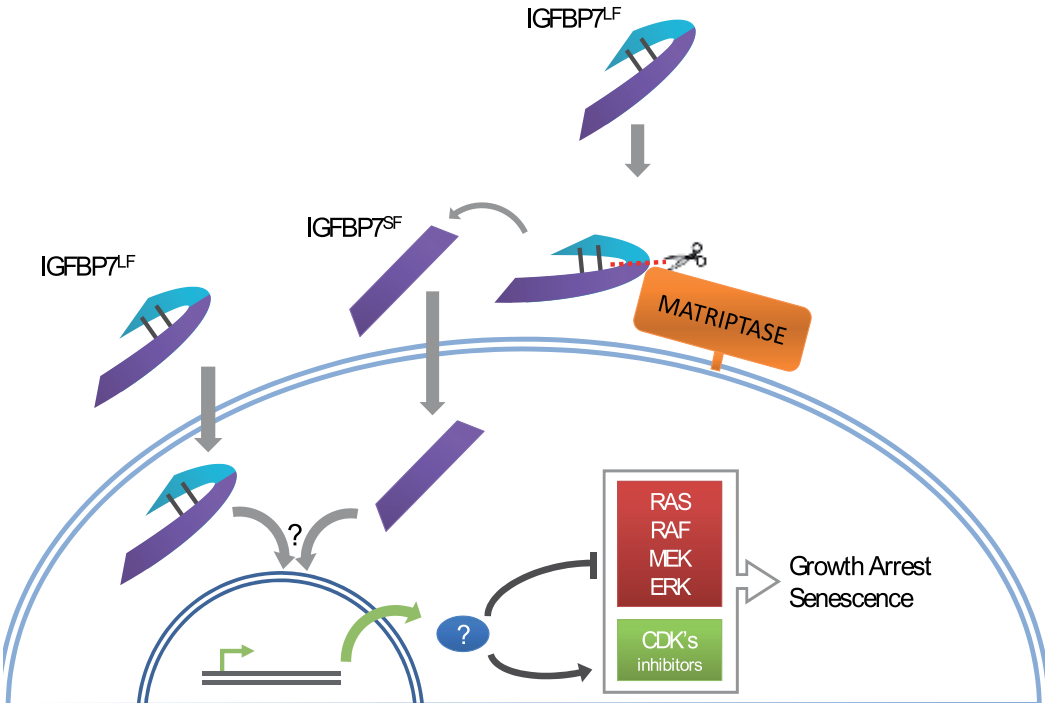

Fig. 7. Model for IGFBP7-mediated inhibition of breast cancer cell growth. IGFBP7 full length (FL) is cleaved by cell surface matriptase to short form (SF). Both forms enter breast cancer cells through an as yet unknown receptor, followed by signal propagation to the nucleus, which leads to upregulation of expression of cyclin dependent kinase (CDK) inhibitors, such as p21 and p27. This ultimately leads to growth arrest and senescence.

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4

Breast Cancer: Classification Based on Molecular Etiology Influencing Prognosis and Prediction

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1. Introduction

Cancer is a group of diseases that leads to uncontrolled cell division and eventually forms a lump or mass called a tumor. They are classified and named after the part of the body where the tumor originates. Breast cancer begins in breast tissue, which is made up of glands for milk production, called lobules, and the ducts that connect lobules to the nipple. The remainder of the breast is made up of fatty, connective, and lymphatic tissue. On the basis of origin, it is of two types (i) ductal and (ii) lobular. Ductal carcinoma constitutes 80-90% and lobular carcinoma constitutes 10-20% breast cancer cases.

Breast cancer is one of the most frequently diagnosed cancers in women worldwide, comprising 16% of all female cancers cases. It is estimated that this disease will affect one in eight females in America during their lifetime. It is estimated that occurrence of female breast cancer is 28% of cancers from all sites in U.S.A, and the relative risk of ever developing breast cancer is 0.125 (1 in 8) (American Cancer Society, 2009). Although breast cancer is thought to be a disease of the developed world, a majority (69%) of all breast cancer deaths occurs in developing countries (WHO Global Burden of Disease, 2004) and relative survival is poor in underdeveloped and developing countries (Coleman et al., 2008). The relative risk of developing breast cancer in the lifetime of women in the developed and developing countries is 0.048 (1 in 21) and 0.018 (1 in 56) respectively. In India, breast cancer is the leading cancer among women (Fig. 1) and the relative risk is 0.033 (1 in 30) (NCRP, 2008).

2. Risk factors of breast cancer

Every woman is at risk for developing breast cancer. Several relatively strong risk factors for breast cancer that affect large proportion of the general population have been known for some time. However, the vast majority of breast cancer cases occur in women who have no identifiable risk factors other than their gender and age (Kelsey & Gammon, 1990). The other established risk factors are previous family history, age at first full-term pregnancy, early menarche, late menopause, genetic and breast tissue density. These factors are not easily modifiable and classified under unmodified factors. However, other factors associated with

increased breast cancer risk are postmenopausal obesity, hormone replacement therapy (HRT), alcohol consumption, and physical inactivity, no breast feeding are modifiable and classified under modified factors. The relative risk of various factors responsible for breast cancer are shown in Table 1 (Hulka & Moorman, 2001).

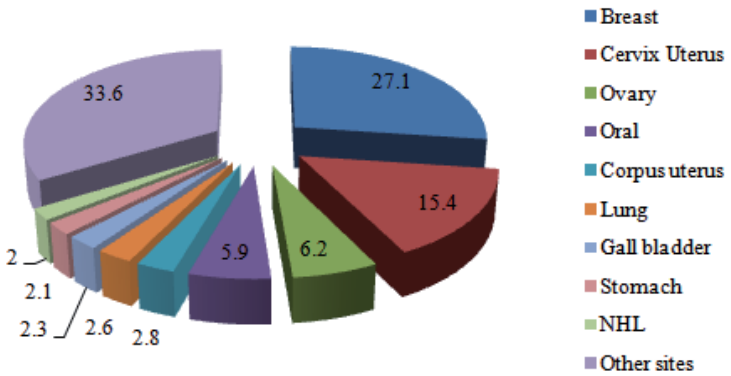


Fig. 1. Demographic profiles of cancer cases in Indian females. Based on 2004-2005 data for Bangalore, Barshi, Bhopal, Chennai, Delhi, Mumbai, Ahmedabad and 2005 data for Kolkata.

3. Classification of breast cancer

3.1.1 Histopathological classification

Each breast has 15 to 25 sections called lobes, formed by groups of lobules, the milk glands. Each lobule is composed of grape-like clusters of acini (also called alveoli), the hollow sacs that make and hold breast milk. The lobes and lobules are connected by thin tubes, called ducts that deliver milk to nipple (Fig. 2). The pink or the brown pigmented region surrounding the nipple is called areola. Connective and fatty tissue fills the remaining space in between the lobes and ducts. The most common type of breast cancer is ductal cancer. It is found in the cells of the ducts. Cancer that starts in lobes or lobules is called lobular cancer. It is more often found in both breasts than other types of breast cancer. Rarely breast cancer

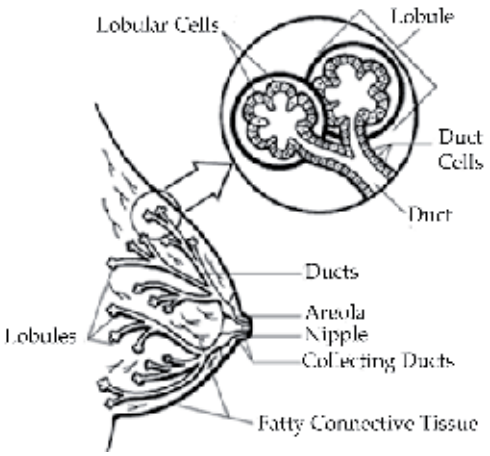


Fig. 2. Anatomy of female breast.

can begin in the connective tissue that's made up of muscles, fat and blood vessels. Cancer that begins in the connective tissue is called sarcoma. It accounts for less than 5% of all soft tissue sarcomas and less than 1% of breast cancer (Moore and Kinne, 1996). Phyllodes tumor and angiosarcoma are two common forms of sarcoma. Cancers are also classified as non invasive (in situ) and invasive (infiltrating). The term in situ means "in its original place" and refers to cancer that has not spread past the area where it initially developed. Invasive breast cancer has a tendency to spread (invade) to other tissues of the breast and/or other regions of the body. A less common type of breast cancer is inflammatory breast cancer characterized by general inflammation (red and swollen) of the breast (Fig. 3). The different types of invasive cancers, their frequency and percentage survival is shown in Table 1.2.

Relative Risk	Factor
>4.0	Female Age (65+ vs. <65 years, although risk increases across all ages until age 80) Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2) Two or more first-degree relatives with breast cancer diagnosed at an early age Personal history of breast cancer High breast tissue density or 75% dense
2.1-4.0	Biopsy-confirmed atypical hyperplasia One first-degree relative with breast cancer High-dose radiation to chest High bone density (postmenopausal)
1.1-2.0 Factors that affect circulating hormones	Late age at first full-term pregnancy (>30 years) Early menarche (<12 years) Late menopause (>55 years) No full-term pregnancies No breast feeding Recent oral contraceptive use Recent and long-term use of HRT Obesity (postmenopausal) Personal history of endometrial or ovarian cancer
1.1 -2.0 Other factors	Alcohol consumption Height (tall) High socioeconomic status

Hulka BS, and Moorman PG 2001. Maturitas 2008; 38:103-113

Table 1. Factors that increase the Relative Risk for Breast Cancer.

Invasive ductal carcinoma is the most common breast cancer and it accounts more than 75% of breast cancer cases. Most are invasive ductal carcinoma (IDC) not otherwise specified (IDC NOS), and remaining IDC includes Inflammatory breast cancer, medullary carcinoma, metaplastic, apocrine and tubular carcinoma. Medullary carcinoma accounts <5% of breast cancers diagnosed, and takes its name from its color, which is close to the color of brain

tissue, or medulla. It is an invasive breast cancer that forms a distinct boundary between tumor tissue and normal tissue. Metaplastic breast cancer is a form of invasive ductal cancer, meaning that it forms in the milk ducts and then moves into other tissues of the breast. Metaplastic breast carcinomas constitute a heterogeneous group of neoplasms, accounting for less than 1% of all invasive mammary carcinomas (Reis-Filho et al., 2005), such as squamous (skin) or osseous (bone) cells. The other groups of invasive breast cancers are invasive lobular carcinoma, adenoid cystic carcinoma, micropapillary carcinoma, mucinous carcinoma (formed by the mucus-producing cancer cells), etc as shown in Fig. 3.

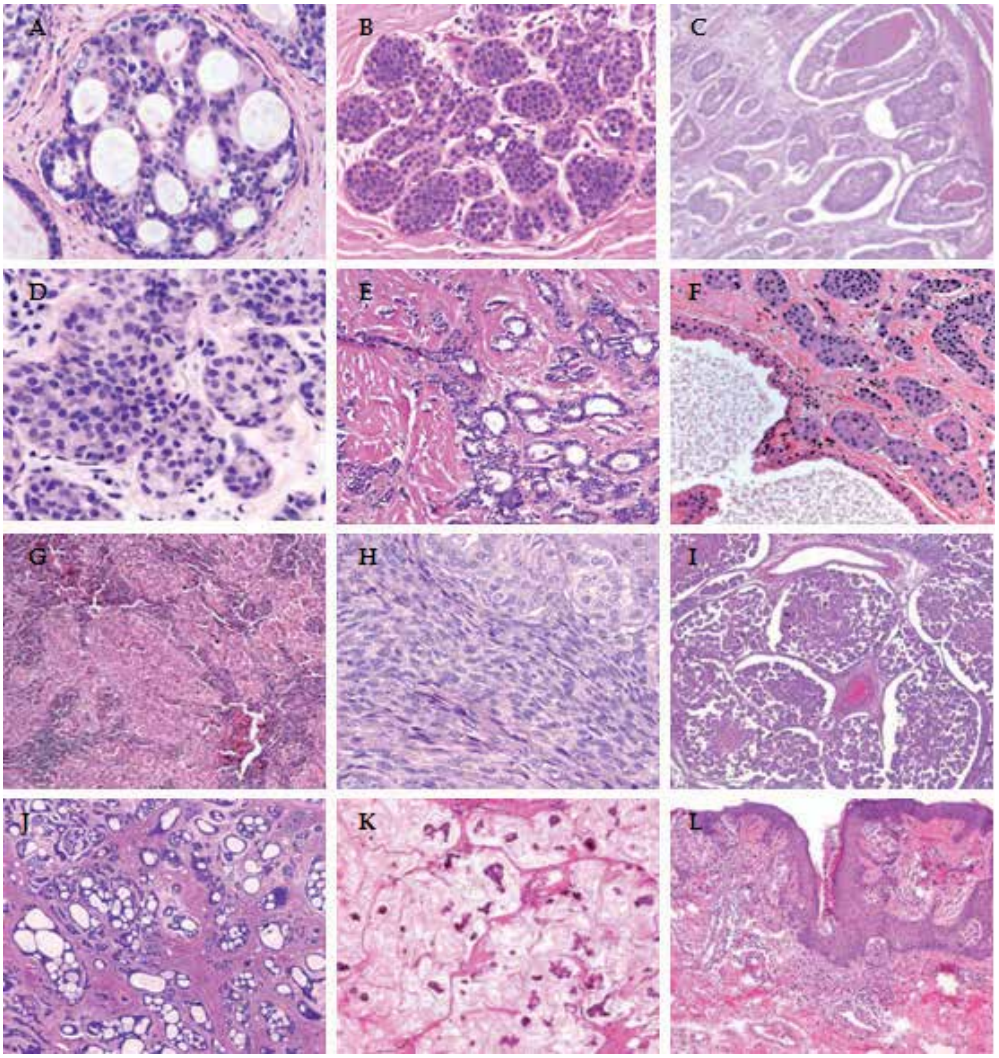


Fig. 3. Histology of breast carcinoma. Breast carcinoma is classified into Ductal (A), Lobular carcinoma (B) and Inflammatory carcinoma. (C). It can be further classified into non-invasive (A-B) and invasive carcinoma (C-L). Invasive cancer includes Inflammatory (C), Invasive lobular (D), tubular (E) apocrine (F), medullary, (G) metaplastic (H), micropapillary, (I) adenoid cystic (J), mucunous carcinoma (K), and paget disease (L).

Histopathological type of invasive breast carcinoma	Frequency (%)	10-year OS (%)
Invasive ductal carcinoma not otherwise specified (IDC NOS)	50-60	35-50
Inflammatory carcinoma	1-6	30-40
Apocrine carcinoma	1–4	Like IDC NOS
Medullary carcinoma	5–7	50–90
Metaplastic carcinoma	<5	Unknown
Micropapillary carcinoma	1-2	Unknown
Tubular carcinoma	1–2	90–100
Invasive lobular carcinoma	5–15	35–50
Adenoid cystic carcinoma	0.1	85–100
Mucinous carcinoma	<3	85–95
Neuroendocrine carcinoma	2–5	Unknown
Mammary Paget disease	1-4	40-50

Table 2. Frequency and outcome of histological types of invasive breast cancer.

3.1.2 Molecular classification

Breast cancer is a clinically heterogeneous disease. Histologically similar tumors may have different prognosis and may respond to therapy differently. It is believed that these differences in clinical behavior are due to molecular differences between histologically similar tumors. DNA microarray technology, Immuno-histochemistry (IHC), Fluorescent in situ hybridization (FISH), and quantitative reverse transcription polymerase chain reaction (RT-PCR) are ideally suitable techniques to reveal molecular differences among the same or different groups of histopathological specimens. Each of these molecular techniques has the potential for proper prognosis and prediction of human cancers, including breast. IHC was developed more than 30 years back and it is used for classification of breast cancer into ER positive and ER negative tumors. FISH was developed 20 years back and is used to classify breast tumors into *HER-2* amplified or non amplified categories. Breast cancer cells generally overexpress estrogen receptor (ER)/ progesterone receptor (PR), and human epidermal growth factor-2 (HER-2) receptor for breast tumor formation and progression. Thus, breast cancer can be classified into three sub-groups (i) ER/PR positive (ii) ER negative or HER-2 positive and triple negative (ER, PR and HER-2 negative) on the basis of receptor status. The classification of breast cancer on the basis of ER status improves the prognosis and clinical outcome of ER+ tumors as ER+ cancer cells depend on estrogen for their growth, and the treatment of patients with anti-estrogen agents (e.g. tamoxifen) will inhibit the effect of estrogen and thus improves the treatment outcome. Generally, HER-2+ had a worse prognosis, however HER-2+ cancer cells respond to drugs such as the monoclonal antibody, trastuzumab, (in combination with conventional chemotherapy) and this has improved the prognosis and pathological complete response significantly (Chang et al., 2010). Triple-negative breast cancer is a high risk breast cancer that lacks the benefit of specific therapy that targets these proteins. It can be categorized in basal subtypes (Rakha et al., 2007). It is found in 10-20% of breast cancer cases and mostly diagnosed in younger women with BRCA1 and BRCA2 mutations (Dent et al., 2007; Dawood et al., 2009). The rate of recurrence is very high, and it reaches its peak within first 3 years and then declines after that. Patients with triple negative breast cancer are most likely to die within 5 years than

patients with other breast cancers. All deaths due to breast cancer in patients' with triple-negative cancer occurred within 10 years of diagnosis.

A novel molecular classification of breast cancer based on gene expression profiles segregates breast cancer into four types (i) luminal, (ii) basal, (iii) HER-2 and (iv) normal type (Perou et al., 2000; Sotiriou et al., 2003; Tamimi et al., 2008) (Fig. 4).

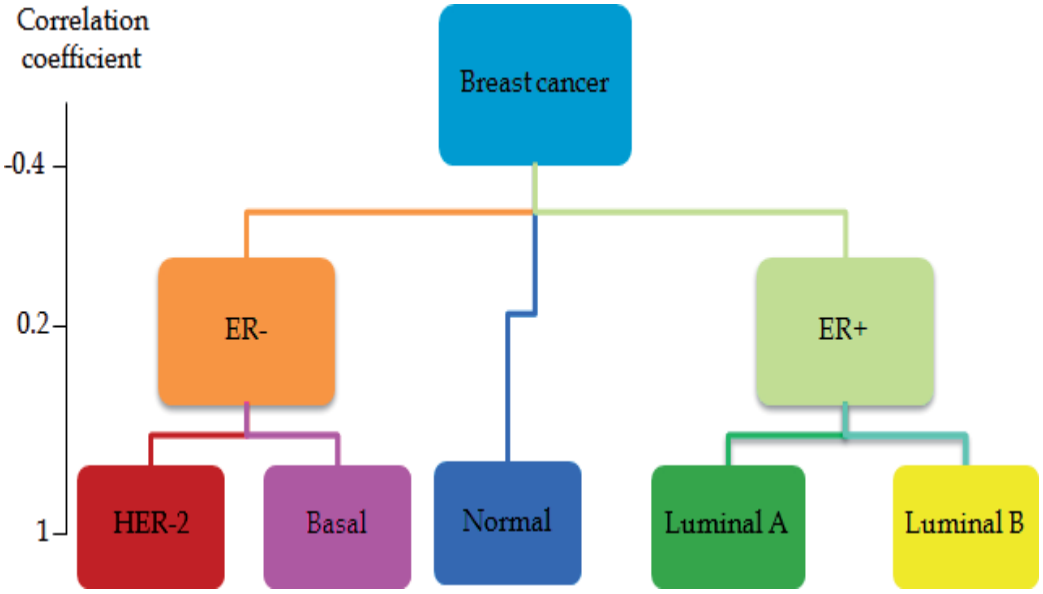


Fig. 4. Dendrogram of breast cancer. The tumors were separated into two main groups mainly associated with ER status as analyzed by hierarchical cluster analysis generated by using gene profile data. The dendrogram is further branched into smaller subgroups within the ER+ and ER- classes based on their basal and luminal characteristics: HER-2 subgroup, dark red; basal-like 1 subgroup, pink; luminal-like A subgroup, green; luminal-like B subgroup, yellow; and normal-like breast subgroup, blue.

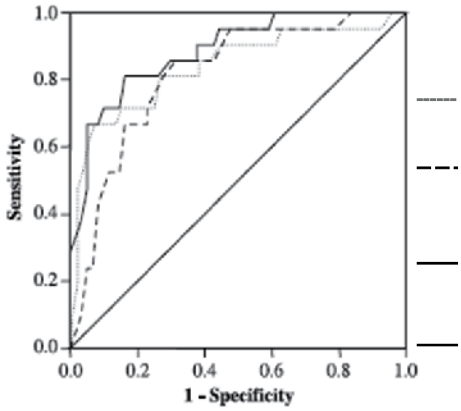
Luminal express keratin 8/18, ER, GATA binding protein, X-box binding protein 1, annexin XXXI, cytochrome P450 and basal type express keratin 5, keratin 17, integrin β4, matrix metalloprotease 14, laminin α3, basonuclin and mutated *TP53* gene. Luminal type is further classified into luminal A and luminal B. Luminal B expresses HER-2 along with ER where as luminal A doesn't express HER-2. HER-2 subtype express ERB-2/HER-2, growth factor receptor bound protein 7, TNF receptor-associated factor IV, GRB 7. Normal–breast-like group showed the highest expression of many genes known to be expressed by adipose tissue and other non-epithelial cell types. These tumors also showed strong expression of basal epithelial genes and low expression of luminal epithelial genes. It expresses CD36 antigen collagen type I, glycerol 3 phosphate dehydrogenase I, lipoprotein lipase A, alcohol dehydrogenase 2 (Sorlie et al., 2001). The molecular subclasses show difference in clinical outcome as per as overall survival (OS) and relapse free survival (RFS) is concerned as shown in Table 1.3. There was a significant difference in overall survival between the subtypes with basal and HER-2 is as associated with worse outcome and shortest survival time.

Molecular types of breast carcinoma	Frequency (%)	5-year OS+ (%)	5-year RFS* (%)	10-year OS (%)	10-year RFS (%)
Luminal A	50-60	85-95	80-90	75-85	75-85
Luminal B	5-10	70-80	65-75	55-65	54-64
Basal	10-20	63-73	60-70	57-67	45-55
ERB-2	10-20	55-65	15-20	45-55	15-30
Normal-like	10-15	84-94	80-90	75-85	72-82

Table 3. Breast cancer outcomes in molecular types of breast cancer.RFS: The percentage of people without any further symptoms of breast cancer during the interval elapsed between the date of breast surgery and the date of diagnosed further episode of breast cancer, whether the breast cancer was classified as a recurrence or second primary, and whatever the histology. OS: The percentage of people survived during the interval elapsed between the date of breast surgery and the date of breast cancer-related or un-related death (documented from hospital records).

4. Clinical outcomes of breast cancer in association with clinical, histopathological and molecular classification

Breast cancers can be classified by different schemata. Classification aspects include clinical (age, tumor, node), histopathological (grade, ER and HER-2 status, ductal, lobular, invasive) and molecular (normal-like, luminal, basal, HER-2) values. Every aspect influences treatment response and prognosis as shown in Table 2 and Table 3. The true prognostic or predictive value of the various molecular classes is unknown because there is a strong correlation between molecular class and conventional histopathologic variables (ER status, grade). For example, in one study, all luminal-type cancers were ER-positive and 63% of these were also low or intermediate grade, in contrast to 95% of basal-like cancers that were ER-negative, 91% of which were high grade (Pusztai et al., 2003). These associations partly explain the different clinical outcome observed in different molecular classes. Rouzier et al. studied the pathological outcomes of different molecular subclasses of breast cancer patients. They obtained tumor tissue biopsies from 82 patients with newly diagnosed breast cancer before they were given a commonly used chemotherapy (Taxol/5-fluorouracil, doxorubicin, and cyclophosphamide). Patients with basal-like and erbb-2+ subgroups were found to have the highest rates (45% each) of a pathological complete response (CR), while only 6% of luminal tumors had a complete response. Among the normal-like cancers, no response was seen (Rouzier et al., 2005). None of the 61genes associated with pathologic CR in the basal-like group were associated with pathologic CR in the HER-2+ group, which suggest that the mechanisms of chemotherapy sensitivity may vary across the subtypes. As molecular classification was not independently associated with pathologic CR, the predictive accuracy of the logistic regression models including (a) clinical + pathologic variables, (b) clinical variables + molecular classification, and (c) clinical + pathologic variables + molecular class (Fig. 5) was measured by constructing Receiver Operating Characteristics curve.



Source of the curve	AUC	95% CI	p
Clinical and pathological	0.84	0.73-0.95	<0.001
Clinical variables and molecular classification	0.82	0.72-0.92	<0.001
Clinical, pathological and molecular classification	0.89	0.81-0.97	<0.001
Reference line			

Rouzier R et al. Clin Cancer Res 2005;11: 5678-5685
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Fig. 5. Receiver Operating Characteristic curves for logistic regression models. Three different prediction models were compared including clinical plus histopathologic variables (model 1), clinical variables plus molecular classification (model 2), and clinical plus histopathologic plus molecularclassification (model 3). All three models were similarly done.

The three models yielded similar area under curve (AUC). This indicates that the molecular class alone can replace histopathological characteristics (estrogen receptor, HER-2 status, or grade) for prediction of pathologic CR but provides little additional information when these characteristics are included. The basal-like and HER-2 tumors were predominantly high nuclear grade and the basal-like tumors were almost all estrogen receptor negative and 80% of HER-2 molecular class expresses HER-2. These characteristics are known to be associated with higher likelihood of pathologic CR to preoperative chemotherapy (Rouzier et al., 2002; Abrial et al., 2005; Gennari et al., 2008). Because of this association, incorporation of molecular class into a logistic regression–based predictor of response didn't improve the prediction accuracy compared with using routine clinical and pathologic variables only. Therefore, it is likely that more focused gene signature–based predictors will need to be developed through supervised outcome prediction methods that are differentially expressed between cases of pathologic CR and residual disease.

5. Screening and detection of breast cancer

Screening uses test/techniques to check people who might have that disease (breast cancer) and to allow it to be treated at an early stage when a cure is more likely. Breast cancer screening is done by mammography (low dose x-ray technique to visualize the internal structure of the breast). On average, mammography will detect about 80-90% of the breast cancers in women without symptoms. Testing is somewhat more accurate in postmenopausal than in premenopausal women (Michaelson et al., 2002). It can reduce breast cancer mortality by 20-30% in women over 50 yrs old in high-income countries when the screening coverage is over 70% (IARC, 2008). MRI, or magnetic resonance imaging, is a technology that uses magnets and radio waves to produce detailed cross-sectional images of the inside of the body. MRI does not use x-rays, so it does not involve any radiation

exposure. Breast MRI is not recommended as a routine screening tool for all women as MRI screening results in more false positives results. However, it is recommended for screening women who are at high risk for breast cancer, usually due to a strong family history and/or a mutation in genes such as BRCA1 or BRCA2. It is also used for gathering more information about the suspicious area found on mammogram and ultrasound and also used for monitoring recurrence after treatment. Positron emission tomography (PET) scan creates computerized images of chemical changes that take place in the tissue. PET scans may play a role in determining whether a breast mass is cancerous. However, PET scans are more accurate in detecting larger and more aggressive tumors than they are in locating tumors that are smaller than 8 mm and/or less aggressive. They may also detect cancer when other imaging techniques show normal results. PET scans may be helpful in evaluating and staging recurrent disease. Clinical breast examination (CBE) is recommended for average risk asymptomatic in the age group of 20-30 to observe any changes in shape, texture, and location of lumps (situated in skin or deeper tissues). The breasts should also be inspected for skin changes (e.g., dimpling, redness) and asymmetry. The area under both arms will also be examined. CBE is also an opportunity for a woman and her health care provider to discuss changes in her breasts, early detection testing, and factors in the woman's history that might make her more likely to develop. All women should become familiar with both the appearance and feel of their breasts to detect any changes and report them promptly to their physician. A woman who chooses to perform breast self-exams (BSE) should receive instructions and have her technique reviewed by a health care professional who performs clinical examinations. Finding and reporting breast changes early offers women the best opportunity for improving breast cancer treatment and reducing breast cancer deaths. Mammotome® is a vacuum assisted breast biopsy that uses image guidance such as stereotactic x-ray, ultrasound, MRI and/or molecular imaging to perform breast biopsies. Mammotome offers a full array of tissue markers to mark the biopsy site for follow-up observations. There have been no reports of serious complications resulting from the Mammotome breast biopsy system. Ductal lavage is another screening and investigational technique for collecting samples of cells from breast ducts for analysis under a microscope. A saline (salt water) solution is introduced into a milk duct through a catheter (a thin, flexible tube) that is inserted into the opening of the duct on the surface of the nipple. Fluid, which contains cells from the duct, is withdrawn through the catheter. The cells are checked under a microscope to identify changes that may indicate cancer or changes that may increase the risk for breast cancer. The procedure is used to identify precancerous cells, called atypical cells. Ductal lavage is currently performed only on women who have multiple breast cancer risk factors to detect breast cancer before it starts. Ductal lavage appears to have low sensitivity and high specificity for breast cancer detection, possibly because cancer-containing ducts fail to yield fluid or have benign or mildly atypical cytology (Khan et al., 2004).

6. Breast cancer treatment

Breast cancer treatment depends on stage, age, hormonal and receptor status. Most women with breast cancer will undergo some type of surgery. Surgery is often combined with other treatments such as radiation therapy, chemotherapy, hormone therapy, and targeted therapy.

6.1 Surgery

Most patients with breast cancer have surgery to remove the tumor mass from the breast. The types of breast cancer surgery differ in the amount of tissue that is removed with the tumor, depending on the tumor's characteristics, whether it has spread (metastasized), and patient's personal feelings. Some of the lymph nodes under the arm are usually taken out and looked under a microscope to see if they contain cancer cells. Breast-conserving surgery or lumpectomy is done to remove the cancer cells but not the breast itself. Lumpectomy is almost always followed by about 5 to 7 weeks of radiation therapy. A woman who chooses lumpectomy and radiation will have the same expected long-term survival as if she had chosen mastectomy (Fisher et al., 2002). Simple or total mastectomy includes removal of the entire breast. Modified radical mastectomy includes removal of the entire breast and lymph nodes under the arm, but does not include removal of the underlying chest wall muscle, as with a radical mastectomy. Both lumpectomy and mastectomy are often accompanied by removal of regional lymph nodes from the axilla, or armpit, to determine the involvement of lymph nodes and spreading of the disease. Axillary lymph node metastasis is the most important prognostic factor for the disease-free and overall survival. Patients with multiple unfavorable risk factors such as positive axillary lymph nodes, high nuclear grade, young age and large tumor showed poorer local control and disease-free survival than patients without any risk factors, and so more aggressive treatment is required for these patients. Adjuvant radio-, chemo-, or targeted therapy has improved the prognosis of patients with higher risk factors (Lee & Chan, 1984; Kim et al., 2005).

6.2 Radiation therapy

Radiation therapy is a cancer treatment that uses high-energy x-rays or other types of radiation to destroy cancer cells remaining in the breast, chest wall, or underarm area after surgery, or to reduce the size of a tumor before surgery (Early Breast Cancer Trialists' Collaborative Group, 2000). There are two types of radiation therapy. External radiation therapy uses a machine outside the body to send radiation toward the cancer. Internal radiation therapy uses a radioactive substance sealed in needles, seeds, wires, or catheters that are placed directly into or near the cancer. The way the radiation therapy is given depends on the type and stage of the cancer being treated. Using traditional clinical and pathological factors, patients can be classified into subgroups by the risk of loco-regional recurrence. In the high-risk groups the absolute benefit of irradiation is larger. However, the patients are over-treated in every subgroup. Substantial proportion of the patients remains free of loco-regional recurrence even in the absence of irradiation, and some patients develop loco-regional recurrence despite postoperative irradiation. Molecular subtypes on the basis of receptors may provide sufficient information to allow accurate individual risk assessment to identify patients who might benefit from receiving post mastectomy radiotherapy (PMRT). A significantly improved overall survival after PMRT was seen only among patients of luminal subtypes. No significant overall survival improvement after PMRT was found among patients with basal and ERB2 subtypes (Fig. 6). There was also smaller improvements in loco-regional recurrence of breast cancer in basal and ERB2 subtypes as compared to luminal A and luminal B (Kyndi et al., 2008). Hence, the improvement in survival resulting from the use of irradiation is more related to the prevention of local recurrences. Post-irradiation local recurrence increases the risk of mortality, but with good prognostic factors (<4 positive nodes, tumor size <2 cm, Grade 1

malignancy, ER- and PR-positive, HER-2-negative) the 10-year survival is 80-90% (Fodor, 2009).

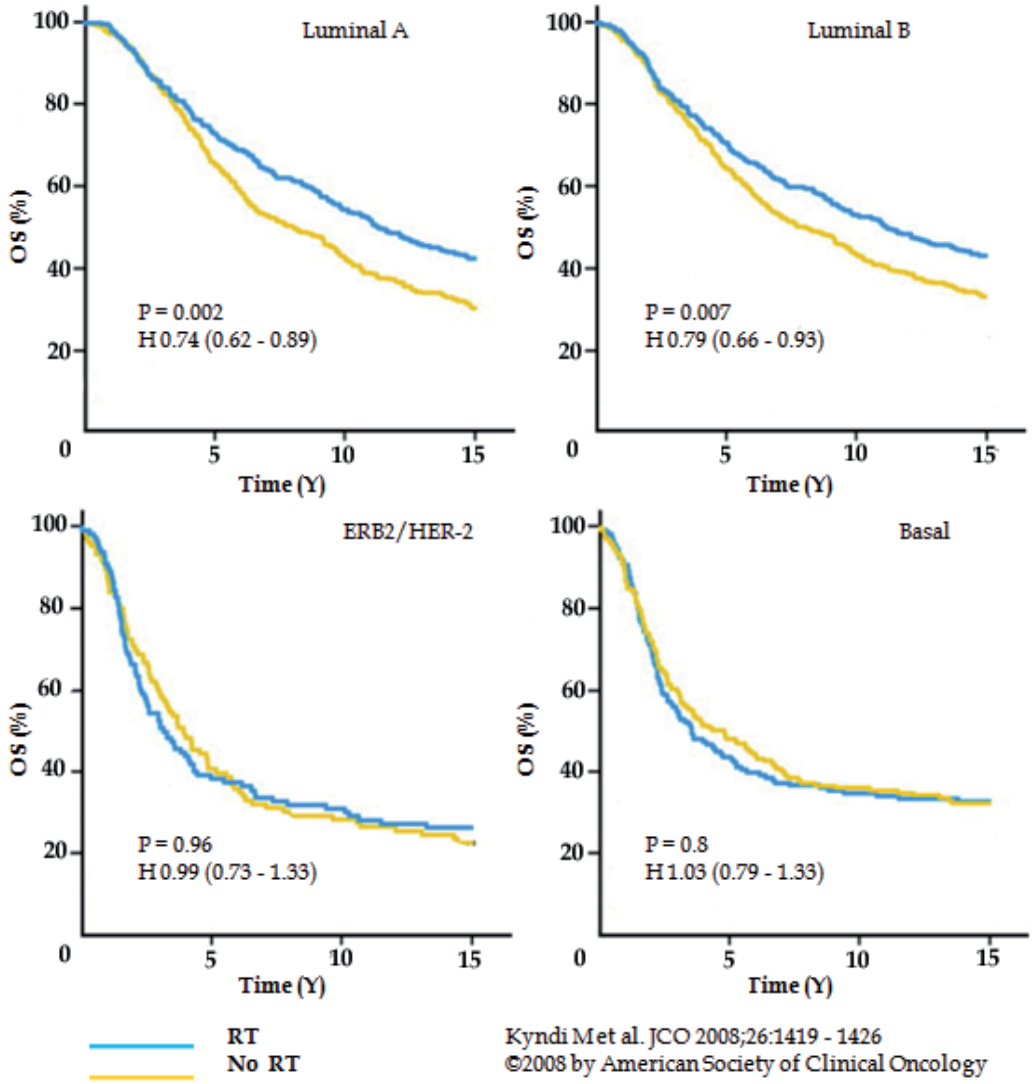

Fig. 6. Overall survival (OS)% of different molecular subtypes of breast cancer patients after receiving post mastectomy radiation therapy (RT). P values and 95% CI of Hazard (H) ratios are shown.

6.3 Chemotherapy and molecular targeted-therapy

Chemotherapeutic drugs are applied in neoadjuvant settingsto shrink the size of tumor that has metastasized and also in adjuvant settings to delay the further growth and spread of the tumor. It is found that combinations of drugs are more effective than just one drug alone for breast cancer treatment. The most common drugs recommended to be used in combination in early breast cancer are cyclophosphamide, methotrexate, 5-fluorouracil (CMF

combinations), doxorubicin (Adriamycin), epirubicin, paclitaxel (Taxol), and docetaxol (Taxotere). Although the benefit and clinical outcome of chemotherapy is dependent on clinical and histopathological parameters, but there are a percentage of cases that behave in an unexpected manner, even if the clinical and pathological parameters indicate the opposite (Gonzalez-Angulo et al., 2007). The introduction of hormonal receptor status to the classical clinical parameters improved the clinical outcome (Goldhirsch et al., 2003). The chemotherapeutic drugs are designed to target the specific molecular markers (molecular targeted therapy) overexpressed in cancer tissues. The presence of ER is correlated with a better prognosis, predicting response to hormonal therapies such as tamoxifen and aromatase inhibitors. But still 15-20% of breast cancer patients with ER+ have recurrent disease. It's the luminal B subgroup of previously classified ER+ tumor that is irresponsive to tamoxifen treatment as they co-express EGFRs and shows poor relapse-free survival (RFS) and over-all survival (OS). Thus over-simplified classification based on ER status required additional molecular makers for sub-classification for optimal treatment. The molecular portraits based on gene profiling divides breast carcinomas into luminal (A and B), basal, HER-2 and normal like. Basal and HER-2 types normally overexpress EGFR and HER-2 respectively. EGFR and HER-2 is overexpressed in 17-30% and 20-30% respectively in breast cancer. Both EGFR and HER-2 is associated with poor prognosis and worse clinical outcome. Basal like subtypes are more aggressive and less responsive to conventional chemotherapy and expected to benefit from EGFR-targeted therapies. Tyrosine kinase inhibitors (TKI) (ZD1839, ZD6474) in combined with anthracyclines (doxorubicin, epirubicin) or taxanes based regimens will improve the clinical outcome of the basal subtypes. HER-2 might serve as a marker for tissue HER-2 status, especially for the prediction of benefit from trastuzumab and/or chemotherapy regimens (anthracyclines) (Sandri et al., 2004). Although the molecular profile of the tumor is a major determinant of disease progression and response to treatment, other factors including chemo- sensitvity or resistivity may be of considerable importance. It is found that for 100 node-negative, premenopausal women receiving chemotherapy according to standard criteria, at 5 years 3 are cured by chemotherapy, 83.50 would have been alive without chemotherapy and 13.50 die despite chemotherapy. With application of molecular profiling to predict the outcome (for the same 100 people), the number treated would be reduced to 39.05 (allowing for a false-positive rate equivalent to that seen in the van 't Veer study (van 't Veer et al., 2002), resulting in an increase in the proportion cured (from 3 out of 100 to 3 out of 39 or 8%). If it were possible to predict chemo-responsiveness, it is possible that the number receiving chemotherapy would reduce further from 39.05 to 29.20 (allowing for a false-positive rate equivalent to that seen in the van't Veer study). In this scenario, the proportion cured by chemotherapy would be 3 out of 29.20 (10.16%) (>3-fold increase in survival rate using chemotherapy), and the number of women treated has been reduced by 70.80%. Thus it is found that molecular profiling will enhance the survival benefit of chemotherapeutic regimens, which will be further improved applying the knowledge of chemo-responsiveness as shown in Fig. 7. If accurate determination of chemo-sensitivity were achieved by observing the set of genes responsible for treatment response, the overall number receiving cytotoxic treatment unnecessarily would decrease, and the overall survival benefit derived, per person treated, increase accordingly, as shown in Fig. 7. However, the absolute survival benefit of patients diagnosed with breast cancer would be unaffected and would be improved with more molecular subtypes along with the development of specific agents targeting particular biomarkers (molecular targeted therapy).

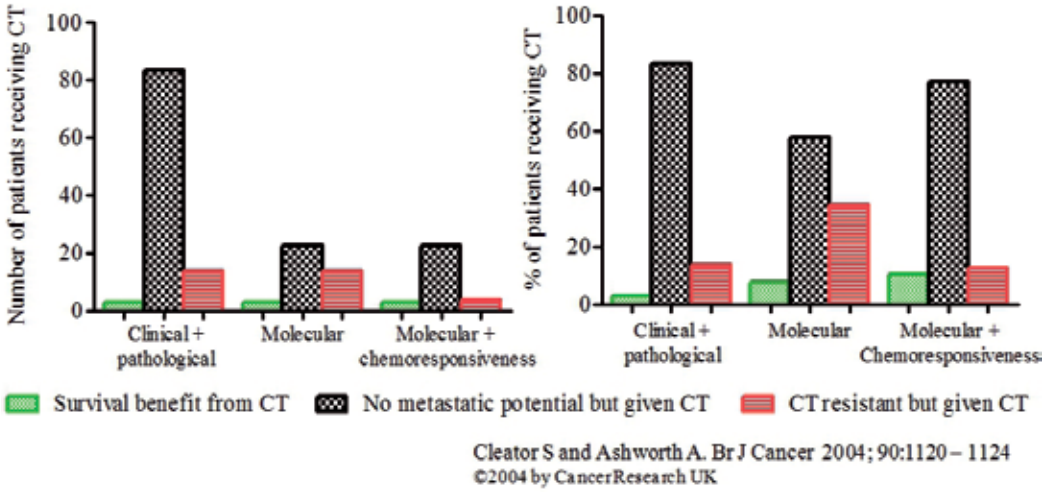

Fig. 7. Model for the effect of molecular profiling on breast cancer. The data shows numbers of premenopausal women with node negative breast cancer receiving chemotherapy (CT), and associated benefit at 5 years. 100 node-negative, premenopausal women receiving chemotherapy according to standard criteria, at 5 years showed survival benefit, no benefit and breast cancer specific death. The two bar graph represents absolute survival benefit and % survival benefit of breast cancer patients receiving chemotherapy. Note that in neither figure has consideration been given to the false-negative rate inherent in molecular profiling. It has been assumed that all deaths occurring were breast cancer related.

7. Conclusion

Adjuvant chemo- and radiotherapy improves survival of patients but it is being increasingly recognized that the benefit is not equal for all patients of breast cancer. Molecular characteristics of the cancer affect sensitivity to chemo- and radiotherapy. In general, ER- (Basal and HER-2) is more sensitive to chemotherapy than ER+ (Luminal A and Luminal B) breast cancer where as ER+ is more sensitive to radiotherapy than ER- breast cancer. The prognostic predictions made by traditional histopathological based models and molecular based models are discordant in about 30% of the cases (van de Vijver et al., 2002), suggesting that one of these methods may be superior to the other or at least that the information they capture is complementary. Corollary to this, it is found that when both the type of classifications are combined (histopathological and molecular), it yield better prognostic values as observed in Fig. 6. It is currently unknown whether genomic tests based on molecular signatures yield a more accurate risk prediction than conventional models. A better prognostic test based on molecular classification with the knowledge of chemo-responsiveness could lead to a reduction in overtreatment of low-risk individuals who are falsely assigned to high-risk category by clinical variables. Such a test could also lead to better overall survival by correctly identifying high-risk individuals who might currently miss out on systemic therapy. Even if molecular classification do not prove to be better than clinical models in prognosis and prediction outcome of breast cancer, inclusion of their results, as additional variables, in current models could improve prognostic predictions.

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5

Remarks in Successful Cellular Investigations for Fighting Breast Cancer Using Novel Synthetic Compounds

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1. Introduction

Breast cancer is one of the most life threatening risks in women's life. In spite of considerable progress in its understanding and challenges, treatment is not yet the correct word to apply on this disease and losing life is the most foreseeing adventure in many patients. Although new gene therapy based approaches are looking for the cure of breast malignant cells, but using cytotoxic agents is currently the main chemotherapy approach to fight this problem. Effective chemotherapy treatment of breast cancer requires targeting the pathways that support the cell growth and proliferation. A good *in vitro* investigational model is essential to understand the process of carcinogenesis, risk and hazard mechanism of carcinogens, protection from carcinogens, mode of action and efficacy of novel and even in practice chemotherapeutic agents. The main part for any of these laboratory models is suitable cell lines to properly address the problem and goal of investigation.

Estrogen Receptor (ER) is considered to cause different growth responses in ER-positive, normal, preneoplastic and neoplastic cells (DuMond et al., 2001; Roy & Cai, 2002; Welshons et al., 2003). One of the most significant researches in cancer treatment has been based on designing and studying the ER-antagonism effects of molecules on cells. This is important to select suitable cell lines for *in vitro* drug discoveries studies. Table 1 shows a list of epithelial breast cell lines with different expression in estrogen receptor.

Intracellular enzymes responsible for the different consequences of receptors stimulations and signaling cascades are also under big considerations in fighting breast cancer cells. Dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate-NADP+ oxidoreductase) is an example of pivotal importance in biochemistry and medicinal chemistry. DHFR catalyzes the reduction of folate or 7,8-dihydrofolate to tetrahydrofolate and intimately couples with thymidylate synthase (TS). Reduced folates are carriers of one-

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Cell line	Suitable growth media	Kinds of receptor	Oncogene considerations
ZR-75-1	RPMI-1640 & 10% FBS	Estrogen receptor	
MCF-7	DMEM:F12 & 10% FBS	-Estrogen receptor	
UACC-3199	Leibovitz's L-15 & 10% FBS	-Epidermal growth factor receptor expressed -Estrogen receptor negative, -Progesterone receptor negative	
HCC1954	RPMI-1640 & 10% FBS	-Estrogen receptor -Progesterone receptor	her2/neu + (over expressed)
HCC1500	RPMI-1640 & 10% FBS	-Estrogen receptor -Progesterone receptor	Negative for expression of Her2-neu, positive for expression of p53
HCC70	RPMI-1640 & 10% FBS	-Progesterone receptor negative	Negative for expression of Her2/neu, positive for expression of p53
HCC1008	DMEM:F12 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Positive for expression of Her2-neu, positive for expression of p53
HCC1143	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Negative for expression of Her2/neu, positive for expression of p53
HCC38	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Negative for expression of Her2/neu, positive for expression of p53
UACC-893	Leibovitz's L-15 & 10% FBS	-Estrogen receptor negative -Progesterone receptor negative -P glycoprotein negative	The cells exhibit a 20 fold amplification of the HER-2/neu oncogene sequence
HCC1395	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Negative for expression of Her2/neu, Positive for expression of p53
HCC1419 & HCC202	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Positive for expression of Her2/neu, Negative for expression of p53
HCC1806 & HCC1599	RPMI-1640 & 10% FBS	-Progesterone receptor negative, -Estrogen receptor negative	Negative for expression of Her2-neu, Negative for expression of p53
HCC1937	RPMI-1640 & 10% FBS	-Estrogen receptor negative -Progesterone receptor negative	BRCA1 (mutated, insertion C at nucleotide 5382), Negative for expression of Her2-neu, Negative for expression of p53

Cell line	Suitable growth media	Kinds of receptor	Oncogene considerations
HCC2157	ACL-4 medium & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Positive for expression of Her2-neu , Positive for expression of p53

Table 1. List of breast cell lines with different expression in estrogen receptor.

carbon fragments; hence they are important cofactors in the biosynthesis of nucleic acids and amino acids. The inhibition of DHFR or TS activity in the absence of salvage leads to 'thymineless' death.

There are some other enzymes which came into special consideration in cancer development, particularly in the breast cancer. Cyclooxygenase-2 is an example that over expresses in several epithelial tumors including breast cancer. Preclinical evidence favors an anti tumor role for COX inhibitors in breast cancer because there is a clear relationship between tissue prostaglandin levels in human breast tumors and the development of metastasis and patient survival (Arun & Goss, 2004). Selective COX-2 inhibitors can prevent mammary tumors from developing cancer in experimental animals. Celecoxib (a COX-2 inhibitor) has proven to minimize the progression of carcinogen-induced mammary tumors (Arun et al., 2001). A good cell line to clearly address alterations in above mentioned systems is also critical for challenging breast cancer cells *in vitro*.

A trustable measurement approach to detect results of the application of under-investigation agents on cells is very much important. Different methods have been applied to investigate cell alterations and ultimately cell death resulted from cancer chemotherapy and cytotoxic agents. Each of them has advantages and disadvantages in different situations and for different purposes. Misuse of any of these methods for the detection of the cytotoxicity of different agents on different cell lines is one of the main problems of many publications for years. These techniques usually look at the viability, morphology and/or biochemical function of various cellular functions. Table 2 lists some of the most popular methods used to measure the cytotoxicity of agents in cellular experiments.

A precise and accurate investigation is one that selects the best possible measurement method on the best possible cell line in the most optimal situation for the best possible conclusion. Cellular investigations to look for new anti-breast cancer agents rely on these bases. MCF-7 proves to be a suitable model cell line for breast cancer investigations worldwide. This is a well known breast cancer cell line derived from a 69 years old Caucasian female. MCF-7 cell line presents most of characteristics of differentiated mammary epithelium tissues including those of expressing estradiol and estrogenic receptors features (Brandes & Hermonat, 1983). Here, we are summarizing some of our results using this cell line to search for novel anti-breast cancer agents, with emphasis and conclusive remarks on the good laboratory practice.

2. Targeting estrogen receptors

Estrogens are known to play an important role in the regulation of the development and maintenance of the female reproductive system, in particular of the uterus, ovaries and breast. Moreover, estrogens are involved in the growth and/or function of several other tissues such as bone, liver, brain, and the cardiovascular system (Ciocca & Roig, 1995).

Method	Measurement criteria	Sample methodology references
Vital dyes (Methylene blue, Trypan blue, Phenol red, …)	Cell membrane integrity	(Shirazi et al., 2005; Shokrzadeh et al., 2006)
Clonogenic assay, cell numbers	Cellular proliferation	(Shirazi & Eftekhari, 2004; Shirazi et al., 1996)
MTT and XTT	Function of mitochondrial enzymes	(Shirazi et al., 2004; Tamaddon et al., 2007)
Thymidine assay, Bromodeoxyuridine	Cellular DNA synthesis	(Hammers et al., 2002; Maghni et al., 1999; Raaphorst et al., 1998; Yokochi & Gilbert, 2007)
Blotting techniques	DNA, RNA and Protein synthesis machinery	(Ko et al., 1993; Singh et al., 2008; Skliris et al., 2002)
Flowcytometry	Population based cell cycle analysis, Individual cell content and biophysical status	(Lukyanova et al., 2009; Niknafs & Shirazi, 2002; Skliris et al., 2002;)
Light and electron microscopes	Cellular morphology and structural features	(Lukyanova et al., 2009; Russo et al., 1977; Vic et al., 1982)

Table 2. Different popular methods to measure cellular alterations after exposure to cytotoxic agents.

Figure 1 represents the general effects of estradiol (as a proliferative estrogen receptor stimulant agent) and tamoxifen (as an estrogen receptor blocking agent) on the growth curve of MCF-7 cell line. To obtain this, 50,000 cells were seeded in four series of cell culture petri dishes and incubated in phenol red-free RPMI media supplemented with 10% fetal bovine serum for 7 days. From the beginning, three different series of petri dishes were selected for the experiments; estradiol was added into the media of one series, tamoxifen was added to the media of the second series and a mix of these two agents was added to the third series of petri dishes. Cells in each perti dish were counted for seven consecutive days as the presentation of cell proliferation in control, estradiol exposed, tamoxifen exposed, and affected by both of estradiol and tamoxifen agents. As is seen in figure 1, estradiol has a significant effect to promote the growth of MCF-7 breast cancer cells compared to the control cells. MCF-7 cells, however, are arrested for at least five days before being able to start a significant proliferation after the exposure to the estrogen-blocking agent of tamoxifen. This block is effective enough to prevent the stimulating effect of estradiol when cells are exposed to both agents simultaneously. This experiment would further emphasize on the stimulating effect of estrogen receptors in breast cancer progression.

Several studies have established that estrogens are predominantly involved in the initiation and proliferation of breast cancer. Lots of efforts are now being devoted to block estrogen formation and action as an anticancer strategy (Clemons & Goss, 2001; Jensen et al., 2001; Nelson et al., 2009). This has led to the development of compounds termed Selective

Estrogen Receptor Modulators (SERMs), which function as estrogen agonists in some tissues (bone, brain and the cardiovascular system) but as antagonists in others (uterus and breast). Estrogen action is mediated through two Estrogen Receptor (ER) subtypes, ERα and ERβ, which have distinct target tissue distributions and functional activities (Gustafsson et al., 2003; Matthews & Gustafsson, 2003; Välimaa et al., 2004). ERα is predominantly found in the uterus, bone, cardiovascular tissue, and liver and is the predominant ER expressed in breast cancer. ERβ is expressed in many tissues including prostate, breast, vascular endothelium, and ovary. The precise function of ERβ and its role in breast is not clear (Fox et al., 2008; Novelli et al., 2008). Recent studies indicate that ERβ expression may have a potential protective effect on normal cells against ERα induced hyperproliferation (Bardin et al., 2004).

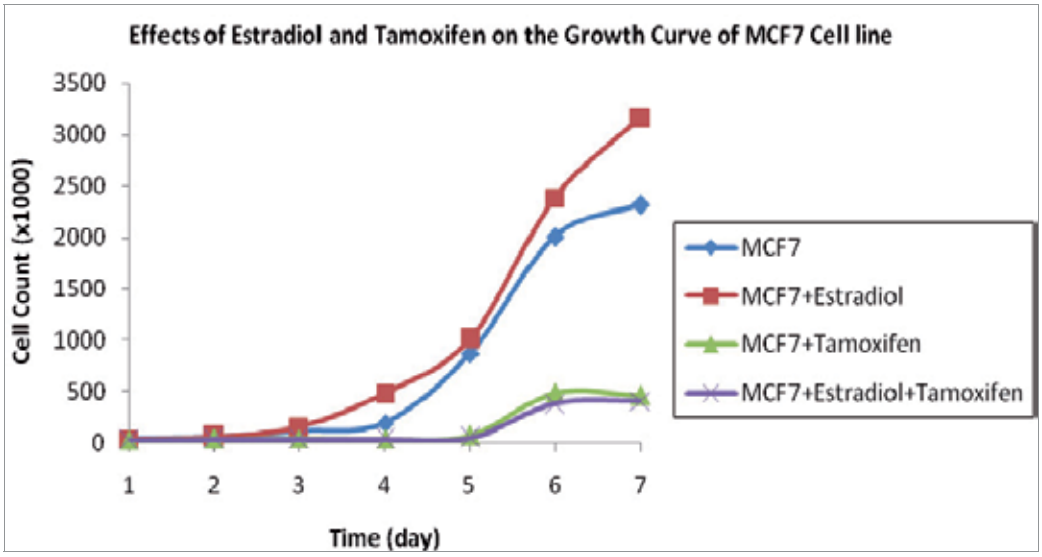

Fig. 1. Stimulation and inhibition of MCF-7 breast cancer cell line exposed to estradiol, tamoxifen and mix of these two agents for 7 days in phenol red-free RPMI media incubated in 37°C and 5% CO_2 humified incubator.

Estrogen receptors can bind a variety of steroidal and non-steroidal ligands. Tamoxifen was the first SERM approved for the treatment of breast cancer (Jordan, 1988). The search for better SERMs has driven efforts to increase the chemical diversity of these compounds, especially the non-steroidal ones (Meegan & Lloyd, 2003). Figure 2 shows the structures of tamoxifen and other known SERMs such as ralolxifen and rasofoxifen.

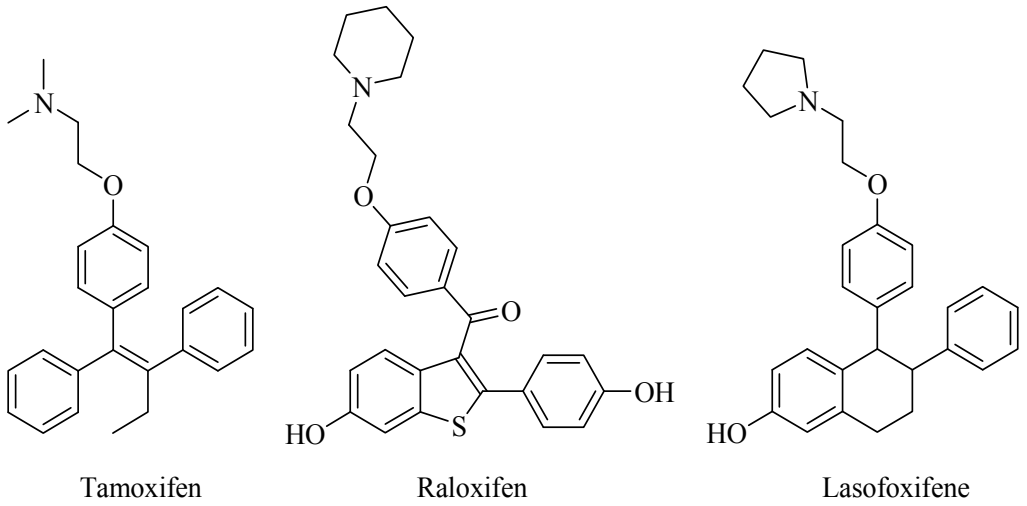

Fig. 2. Chemical structures of some known Selective Estrogen Receptor Modulators (SERMs).

Structure-activity relationship (SAR) studies and molecular modeling studies center lead to the design of novel structures containing 1,2,3-triarylpropenone scaffold to act as potential SERMs and anti breast cancer agents with a unique structure as is shown in Figure 3.

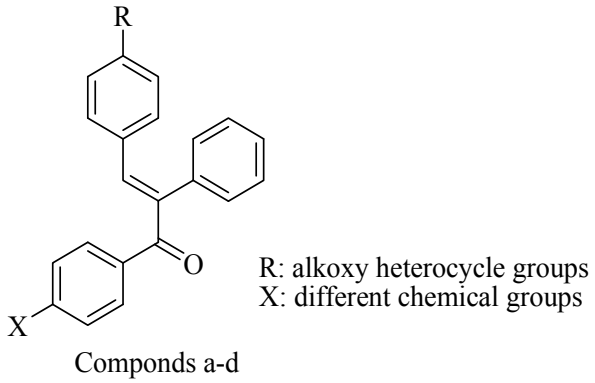

Fig. 3. The general model of 1,2,3-triarylpropenone scaffold as a novel potential SERMs and anti breast cancer agents**.**

The compounds a to d have been synthesized and undergone biological evaluations in an *in vitro* cellular system using MCF-7 breast cancer cell line as the model. The anti-proliferative activities of these compounds were determined using MTT assay. To do so, a ten thousands cells were seeded in phenol red-free RPMI-1640 medium supplemented with 10% FBS in each well of 96-well micro culture plates and incubated for 24 hours at 37 ºC in a 5% CO_2 incubator. Different concentrations of each compound were added to the wells with respective vehicle control for 72 hours. Media were then removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well. Formazon crystals were dissolved in 200 µL of DMSO after 4.5 hours incubation and the dye

absorbance for each well was measured at 540 nm. A comparison of absorbance in each well containing different concentrations of each compound to the control wells could easily represent the number of live cells in that well as a result of the cell mitochondrial function (Zhu et al., 2006).

The results of anti-proliferative MTT assays of compounds a to d on MCF-7 breast cancer cells are shown in the graphs below (Figure 4). Start point (time 0) is shifted in each set of figures for a better clarification of the shape and trends of graphs in case of compounds a, b and d. These graphs show the comparative cytotoxic and antiproliferative effects of all of these compounds on MCF-7 cell line.

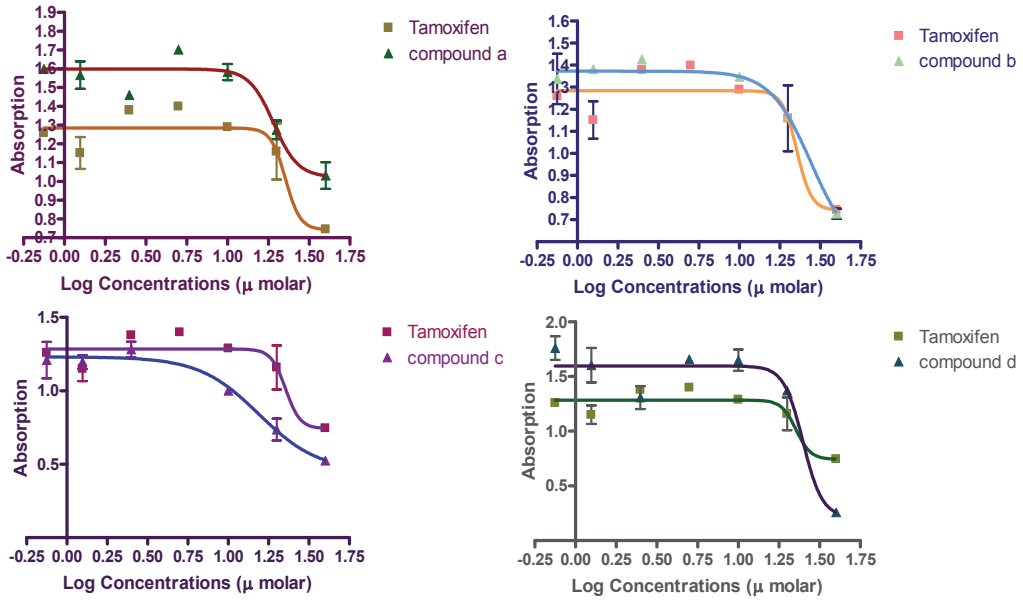

Fig. 4. Cytotoxic effects of tamoxifen and compounds a to d on MCF-7 cell line presents a comparable antiproliferative effects on cancer cells.

Estrogen receptor binding studies were carried out for the compounds with ERα and ERβ using a fluorescence polarization procedure to prove the stimulatory and inhibitory mechanism being through the estrogenic receptors (data not shown). The compounds were active on ERα at nanomolar concentrations and on ERβ at micromolar concentrations. Therefore compounds a to d selectively bind to ERα.

Interestingly, clonogenic assays on MCF-7 cell line after exposure to these compounds fail to present solid and reliable growth inhibitory effects. Figure 5 shows some graphs resulted from the same exposure strategy of above-mentioned compounds on MCF-7, but using the clonogenic methodology to compare the results. A clear weakness is evident in these graphs preventing from any conclusive interpretation of results. We will further discuss this finding at the end of this chapter.

3. Targeting COX-2 enzyme

There is considerable evidence to suggest that prostaglandins play an important role in the development and growth of cancer. The enzyme cyclooxygenase (COX) catalyses the

conversion of arachidonic acid to prostaglandins (Abou-Issa et al., 2001). There has been a considerable amount of interest in recent years to take advantage of COX inhibitors specifically COX-2 inhibitors in prevention and treatment of malignancies (Talley et al., 2000; Zarghi et al., 2006). Majority of COX-2 inhibitors belong to a class of diaryl heterocycles that possess vicinal diaryl substitution attached to mono, bicyclic or tricyclic central rings (Penning et al., 1997; Prasit et al., 1999; Riendeau et al., 2001).

As a part of ongoing program to design new types of selective COX-2 inhibitors, our center has synthesized novel COX-2 inhibitor derivatives having a new tricyclic central ring scaffold and different substituents at the N-3 as is shown in figure 5.

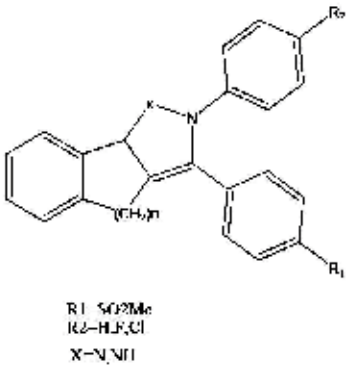

Fig. 5. Central structure of novel COX-2 inhibitors.

The nature and size of substituent attached to N-3 influenced both selectivity and potency for COX-2 inhibitory activity. Two different compounds of C1 and C2 with different N-3 substituent have been applied to MCF-7 cell line for the evaluation of anticancer effects, using clonogenic assay. MCF-7 cells were seeded for the clonogenic assay in 12-well plates at 150 cells per well for 24 hours. These cells were then exposed to C1 and C2 derivatives for 24 hours. Media was then changed to fresh media without these compounds and plates remained in incubator for couple of days until most of colonies in the control wells contained more than 50 cells. Media was then excluded and cells were fixed with 96% ethanol and stained using trypan blue. Plates were washed and percentages of colonies in different wells were compared to controls (Shirazi et al., 2005).

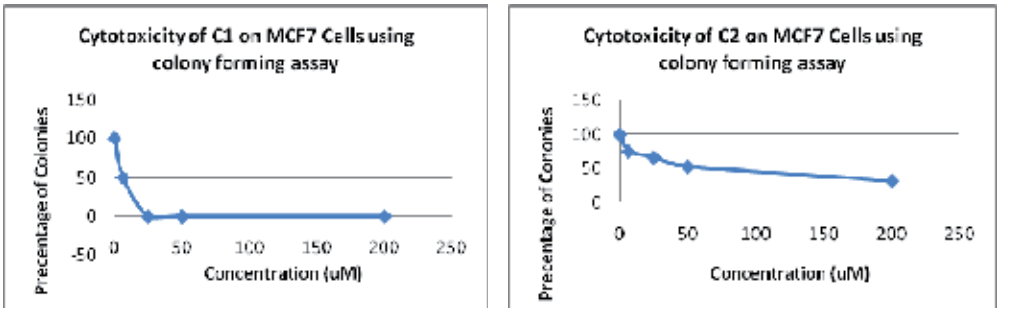

Fig. 6. Cytotoxicity of two novel COX-2 inhibitors of C1 and C2 on MCF-7 cell line using clonogenic assay.

As is shown in figure 6, both compounds have acceptable cytotoxicity effects with C1 being stronger. However, the same experiment has been conducted using the same cell line and the same concentrations of C1 and C2 compounds but using MTT assay. MTT failed to present any cytotoxicity for these compounds on MCF-7 cell line as is shown in figure 7.

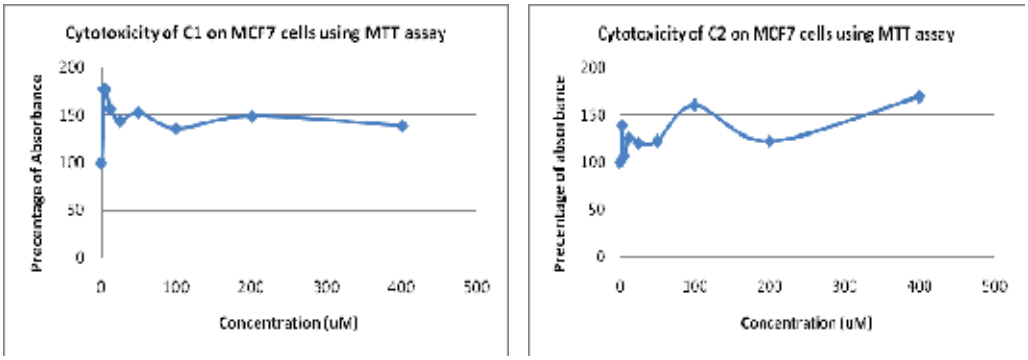

Fig. 7. MTT based cytotoxicity measurement of two novel COX-2 inhibitors of C1 and C2 on MCF-7 cell line.

Failure of one experiment using a technique in spite of success for the other technique in acquiring result is a considerable phenomena in cellular investigation on cytotoxic agents and will be discussed later on in this chapter.

4. Targeting dihydrofolate reductase (DHFR) enzyme

Inhibitors of DHFR are classified as either 'classical' or 'non-classical' antifolates. The 'classical' antifolates are characterized by a *p*-aminobenzoylglutamic acid side-chain in the molecule and thus closely resemble folic acid itself. Methotraxate (MTX) is the most well known drug among the 'classical' antifolates. Compounds classified as 'non-classical' inhibitors of DHFR do not possess the *p*-aminobenzoylglutamic acid side-chain but rather have a lipophilic side-chain. MTX serves as an antimetabolite, which means that it has a similar structure to that of a cell metabolite, resulting in a compound with a biological activity that is antagonistic to that of the metabolite, which in this case is folic acid (Barnhart et al., 2001; Takemura et al., 1997).

New, more lipophilic antifolates have been developed in an attempt to circumvent the mechanisms of resistance, such as decreased active transport, decreased polyglutamation, DHFR mutations and so on (Assaraf, 2007; Gangjee et al., 2006; Takemura et al., 1997). In a series of synthesized compounds for this purpose in our center the pyrimidine ring remained (figure 8) and the side-chain attachment at the position 2 was replaced with different substituent.

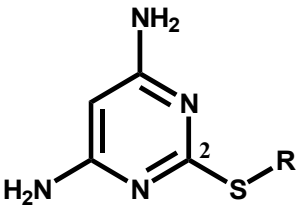

Fig. 8. The central structure of novel DHFR inhibitors.

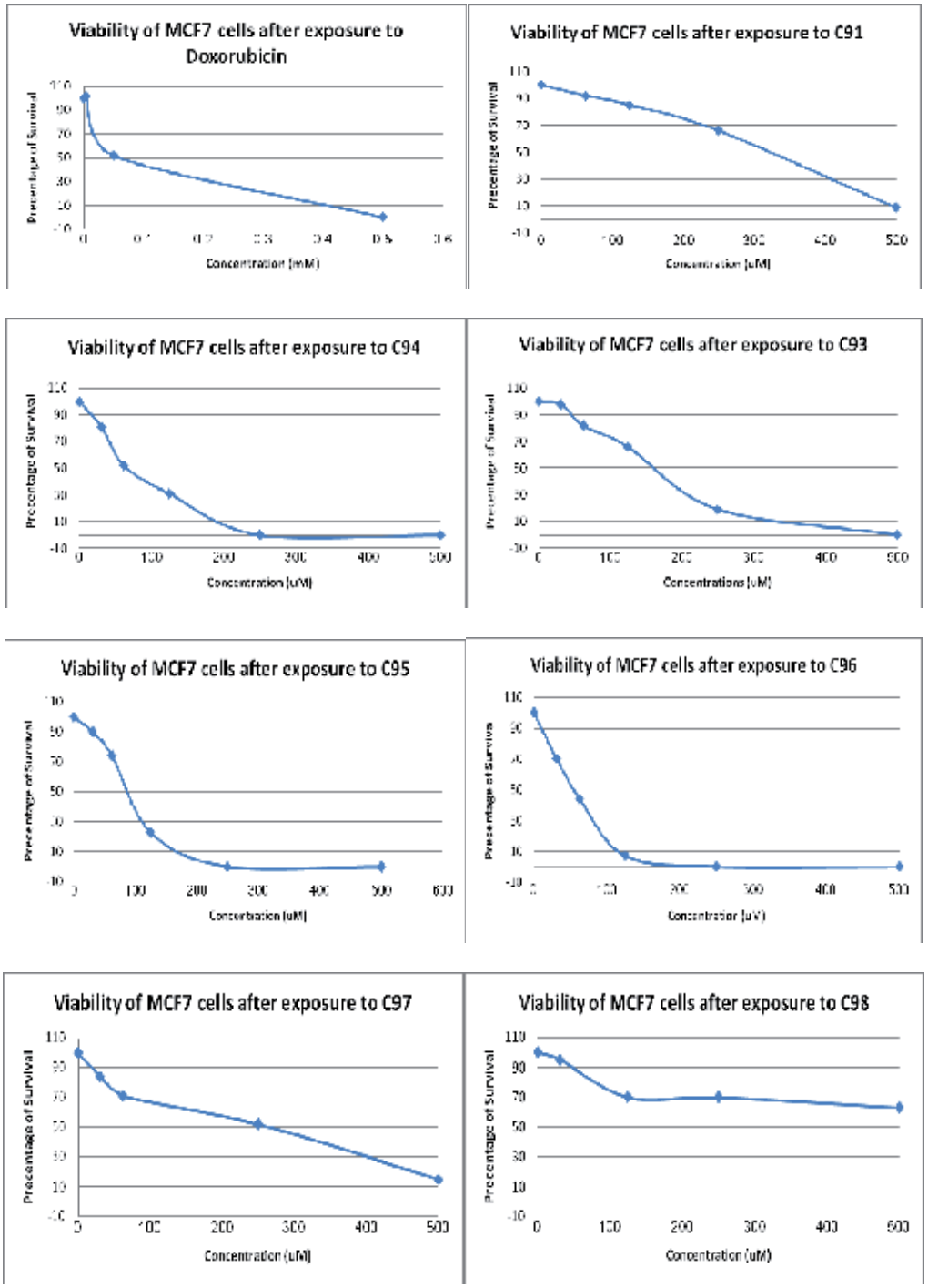

Fig. 9. Cytotoxicity measurement of seven selected DHFR inhibitors on MCF-7 cell line resulted from clonogenic assay.

These modified antifolates differ from the traditional 'classical' analogues by increased potency, greater lipid solubility, or improved cellular uptake. Although being very effective as inhibitors, problems still remain with respect to the issue of toxicity due to the lack of selectivity (Cody et al., 2003; Graffner-Nordberg et al., 2004; McGuire, 2003). To evaluate the cytotoxic potency of these compounds, we have used the clonogenic assay. MCF-7 cells were plated in 6-well plates (200 cells/well) for 24 hours before treatment with the test compounds to allow the attachment of cells to the wells surface. Seven different concentrations of each compound, doxorubicin (as reference), and 0.5% DMSO (applied solvent to dissolve the compound) were added to the monolayer cells in triplicates. The plates were then incubated for 10 days at 37 ^{o}C in atmosphere of 5% CO_2. The media were removed after 10 days and the colonies were stained with a solution of 0.5% crystal violet in ethanol for 10 minutes and the number of colonies containing more than 50 cells was counted under microscope. The relation between the number of the colonies (as a percentage to the control containing 0.5% DMSO) and the concentrations of each compound were plotted to get survival curve of the tumor cell line and IC_{50} values were calculated. Cellular viability test results for some examples of this series of novel DHFR inhibitors are presented in figure 9.

5. Discussion

Human mammary gland adenocarcinoma MCF-7 cell line (ATCC HTB-22™) is proven to be a good breast tissue model for anticancer drugs investigations in our experiments. However, selection of a suitable cell line is only a part of a successful and meaningful *in vitro* cellular examination of potential anticancer agents. Many different factors might very much influence the final outcome of the evaluation of a medication in a cellular experiment, among them are the cell culture media and its components during the time of drug exposure and afterward, exposure time, drug solvent, volume of drug solution to be added to the cell culture media, the proper use of agonists and antagonists for the purpose of elaborations on the results and making a meaningful conclusion, methodology of cellular viability assessment, and the most important factor; the personnel who run the experiment. We are not going to extensively discuss all of these parameters and their specific influences on the final result and conclusion, but the limited examples presented in this chapter may be sufficient to raise awareness for a good cellular practice.

The importance of a suitable protocol for the measurement of survival percentage (live versus death) of cells is underestimated in many of experiments. Selection of the method in many instances is easily a matter of facility, budget and distributing companies' advertisements in the region. However, one should notice that for many known and unknown reasons, various methods of MTT, XTT, SRB, fluorescence dye staining and so on might work or not for different experiments. The main reason might well be the cellular measurement criteria for any of these methods. One should keep in mind that although mitochondria is the heart of cellular energy system, but MTT and XTT experiments would only measure the functionality of a mitochondrial enzyme (Cody et al., 2003; Marshall et al., 1995; Scudiero et al., 1988) and would not necessarily reflect the cell viability. The same is very much true for many of staining methods e.g Annexin V which is an indication of cell membrane flip-flop that would most properly occur during the process of apoptosis (Kolodgie et al., 2003; Van Heerde et al., 2000). Both of these methods are extensively used for the measurement of the cytotoxicity of many different agents. The chemical structure of

under investigation compound, its solvent, its cellular site of action, the exposure time, the lag time from the beginning of exposure to the start of measurement, and even the selection of cell line might dramatically alter the final survival curve. Methotrexate is a good example of MTT limitation in cytotoxicity measurement (Haber et al., 1993) and colleagues have shown that MTT protocol is not able to assess the cytotoxicity of this anticancer agent on various cells including MCF-7. Our experiments on other novel DHFR inhibitors have also proven the same conclusion when MTT results were not conclusive while the clonogenic g assay could easily provide a meaningful dose-response result. Figure 10 shows a comparison of MTT versus clonogenic assay for the measurement of methotrexate as well as some other novel DHFR inhibitors. As is shown in this figure, clonogenic assay was more successful in determining the LD_{50} of these compounds in MCF-7 cell line, but not the MTT protocol. Alteration of the exposure time and lag time between the addition of drugs and start of MTT assay, media components and calculation method were not helpful to provide a conclusive survival curve using this method (Data are not shown).

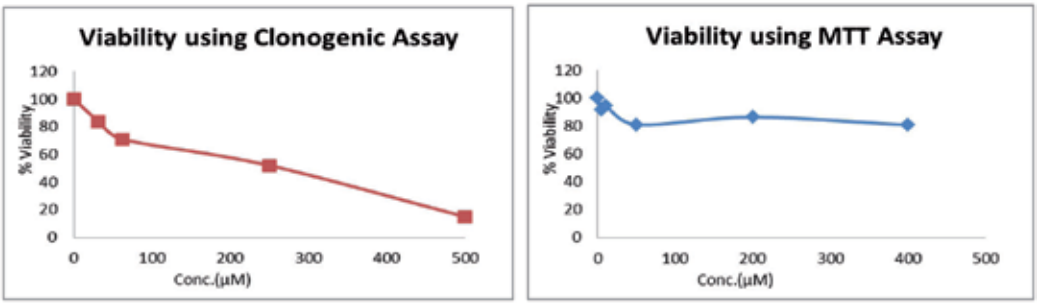

Fig. 10. Comparison of clonogenic versus MTT assays for the measurement of methotrexate and some other novel DHFR inhibitors.

Clonogenic assay is usually considered as a final answer for drugs cytotoxicity because of its long waiting time to acquire result. A minimum of five to six cellular doubling times to look at results in clonogenic assay might well overcome all cellular adventures of arrest, repair, detoxification and exertion pumps influences on drug cellular mortality which might affect the result of cross sectional measurement methods like MTT and Annexin V. Figure 7 is another example of the limitation of these type of experiments in some instances in comparison with the clonogenic assay. Clonogenic assay, however, would surprisingly fail to present a meaningful graph of cytotoxicity after exposure to some compounds.

There are many different mechanisms which might cause these differences in the result of the viability measurement using different methodologies. Cellular target of the test compound and the cellular repair system are two of the most possible explanation. Rosenberg confusion about the effects of electric field on the cells resulted in cisplatin identification and later use as a very important and most used anticancer drug in many different kinds of malignancies including the breast cancer (Rosenberg et al., 1969). Cells in Rosenberg set up did not die, rather changed shape and remained alive for a long time (Rosenberg, 1985, 1977). Cisplatin, like many other anti-mitotic agents, does not kill cells right after exposure. Its principle mechanism of action is on the DNA and thus while stopping DNA synthesis and cell proliferation, won't affect the mitochondrial action and cell membrane integrity. That is why, while the thymidine assay and cell cycle progression

based techniques like the flowcytometery, as well as proliferation based measurements like the clonogenic assay present good results, cell membrane integrity and mitochondrial enzyme function based assays have a significant lag time before the presenting of measurable alterations. A successful cellular repair event during this lag time may change the final conclusion dramatically. One needs to be aware of these possibilities in interpretation of cytotoxicity test results. Figure 11 represents the measurement of cisplatin cytotoxicity effect on MDCK cell line using MTT assay. As is shown in this figure, a 48 hours exposure time difference is needed to acquire a reasonable survival curve using this method.

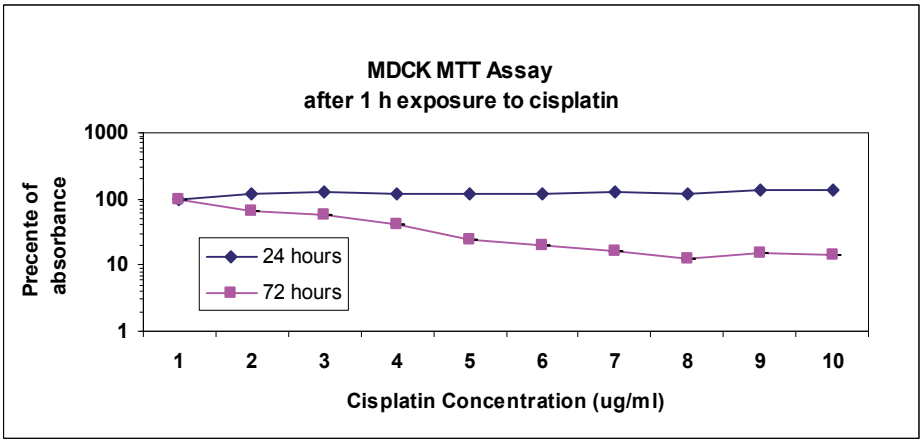

Fig. 11. The lag time required to get a good MTT result on the cytotoxicity of cisplatin on MDCK cell.

A discrepancy analysis to measure the cytotoxicity of many of novel anticancer drugs developed in our center under the same condition on the same cell line using two different methods of clonogenic assay and the neutral red assay, did not show agreement with a clear horizontal line and 95% confidence interval of about 1. It would further prove the importance of the selection and application of a suitable survival measurement system in the analysis of various anticancer candidates.

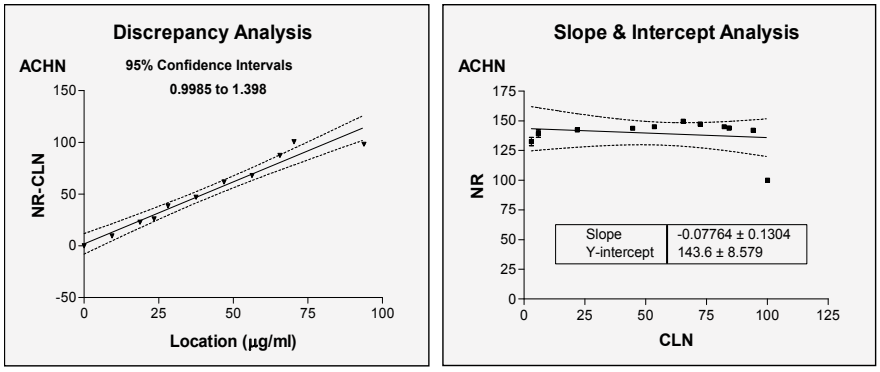

Fig. 12. A comparison of clonogenic versus neutral red assay for the measurement of cell survival after exposure to various novel anticancer drugs, using a discrepancy analysis method.

A good cellular practice on anticancer drugs requires the best selections of cell line and model system, the best matched measurement methodology, and the most optimized lag time to look at the result for acquiring the most precise and accurate conclusion.

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6

Breast Cancer from Molecular Point of View: Pathogenesis and Biomarkers

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1. Introduction

1.1 Breast cancer and risk factors

Breast cancer is the most common female cancer, the second most common cause of cancer death in women, and the main cause of death in women ages 40 to 59 (1). It has been reported that mortality rate from breast cancer has been significantly greater in women whose cancer was first diagnosed during pregnancy compared with those who had never been pregnant (2). Nowadays, many women all over the world faced the challenge of living with breast cancer. The lifetime probability of developing breast cancer is one in six overall (3). High prevalence of breast cancer and high mortality rate of women who stricken by, appoint it among the most challenging subjects in the area of experiments. The two major types of breast cancer risks are objective and subjective factors. Objective breast cancer risk is defined as an estimated chance for bearing breast cancer based on scientifically established risk factors for the disease and is predictive of resultant health outcomes. Subjective breast cancer risk is identified as an individual's realization of her chance for getting breast cancer based on her own cognitive appraisal and is affected by depressive conditions. Objective BC risk had a limited but significant relationship with immune response and natural killer cell activity (NKCA), whereas Subjective risk was highly associated with psychological distress but was not associated with NKCA also the results are still controversial (4).

Many factors including prenatal conditions, diet, physical activity, estrogen exposure, body mass index, depression and quality of life have been mentioned as breast cancer risk factors. A positive family history is the main risk factor. Diet with high amounts of alcohol, fat, caffeine and red meat is a positive risk factor for bearing breast cancer, whereas phytoestrogens and high amounts of calcium/vitamin D can be effective to reduce it (5,6).

Hormonal conditions stand among the most important factors. Prolonged exposure to and higher concentrations of endogenous estrogen; which is controlled and modulated by menarche, pregnancy, and menopause; increase the risk of breast cancer. Testosterone level has also showed some parallelism with higher rate of breast cancer in some studies, although not in all of them. Younger age of menarche and older age of first full-term pregnancy are associated with a higher risk of breast cancer. The data about the effects of oral contraceptives on breast cancer risk are controversial. Some studies show an increased risk of breast cancer in oral contraceptive users, whereas in some other researches, no significant difference was seen. The two newer researches didn't give any data which show

that oral contraceptives cause any increase in breast cancer risk. Long term use of post-menopausal hormone therapy is associated with higher risk of breast cancer. In contrast, short-term HT appears not to increase the risk significantly, although it may make mammographic detection more difficult. Environmental toxic agents such as Organochlorines include polychlorinated biphenyls (PCB's), dioxins, and organochlorine pesticides such as DDT are weak estrogens with high lipophilic properties and as a result, can store in adipose tissues. Some studies suggest that exposure to these chemicals will increase the risk of bearing breast cancer, however the data are controversial and more researches should be done.

Age and gender are among the strongest risk factors for breast cancer. Breast cancer occurs 100 times more frequently in women than in men. Incidence rates increase with age until about the age of 45 to 50.

Ethnic difference is another factor affecting breast cancer prevalence. For example, in United States, breast cancer is more common among whites. Much of these differences arise from lifestyle factors and social conditions. Furthermore, there are marked variations in breast cancer incidence and mortality among countries Women with higher educational, occupational and economic level are at greater risk because of their reproductive pattern including age of parity and age of first birth. Ethnic differences in estrogen and progesterone receptor subtypes have been also determined as important factors that affect the probability of breast cancer (7). In a Multiethnic Cohort Study, various status of estrogen receptor (ER)/progesterone receptor (PR) including ER-/PR-, ER+/PR+, ER-/PR+ and ER+/PR- have been reported and ER/PR status varied significantly across racial/ethnic groups even within the same tumor stage. Compared to whites, the high prevalence of hormone receptor-negative tumors in African-American women may contribute to their high breast cancer mortality (8).

2. Breast cancer classification

Nowadays, beside conventional use of grade, histology, and immunohistochemical analysis, changes in gene expression during bearing tumors are used as an instrument to classify breast cancer. Molecular profiling make us capable for better understanding of breast cancer, more precision in determining subtypes and better prediction of clinical outcome and response to therapy. New instruments like microarray kits provide the possibility for simultaneous studying of the expression of thousands of genes in a breast cancer cells and finding out the Gene expression profile. Future applications will take the same approach to proteins (proteomics), genome-wide germline variability (single nucleotide polymorphisms), or cellular metabolism (metabolomics). Based on these methods, several distinct breast cancer subtypes have been identified including two main subtypes of estrogen receptor (ER)-negative tumors and basal-like and human epidermal growth factor receptor-2 (HER2)-enriched, and two subtypes of ER-positive tumors including luminal A and luminal B. These subtypes differ markedly in prognosis and in the therapeutic targets they express.

The luminal cancers, luminal A and luminal B, so called because they are characterized by expression of genes also expressed by normal breast luminal epithelial cells, have overlap with ER-positive breast cancers. There are also several subtypes characterized by low expression of hormone receptor-related genes (ER-negative), one of which is called the "HER2-enriched" subtype (previously called HER2+/ER-) and another called the "basal-like"

subtype. The basal-like subtype is named because it expresses many genes characteristic of normal breast basal epithelial cells.

3. Luminal subtypes

The name "luminal" derives from similarity in expression between these tumors and the luminal epithelium of the breast; they typically express luminal cytokeratins 8 and 18. These are the most common subtypes, make up the majority of ER-positive breast cancer, and are characterized by expression of ER, PR, and other genes associated with ER activation.

3.1 Luminal A and luminal B traits

High expression of ER-related genes, low expression of the HER2 cluster of genes, and low expression of proliferation-related genes are the two main characters of Luminal A tumors. This kind has the best prognosis of all breast cancer subtypes. Whereas luminal B tumors have relatively lower (although still present) expression of ER-related genes, variable expression of the HER2 cluster, and higher expression of the proliferation cluster.
Luminal B tumors carry a worse prognosis than luminal A tumors. Unfortunately, this subtype has high probability of recurrence.

3.2 HER2-enriched subtype

The HER2-enriched subtype (previously the HER2+/ER- subtype) is characterized by high expression of the HER2 and proliferation gene clusters, and low expression of the luminal cluster. For this reason, these tumors are typically negative for ER and PR, and positive for HER2. It is important to note that this subtype comprises only about half of clinically HER2-positive breast cancer. The rest have high expression of both the HER2 and luminal gene clusters and fall in a luminal subtype. Promotion in HER2-directed therapy has improved the poor prognosis of this subtype.

3.3 Basal-like subtype

The name of “basal-like” subtype comes from the similarity in gene expression to that of the basal epithelial cells. This subtype shows lower expression of the luminal and HER2 gene clusters. Therefore, these tumors are typically ER-, PR-, and HER2-negative on clinical assays. Because of this reason, the name "triple negative" is also used to describe them. However, while most triple negative tumors are basal-like, and most basal-like tumors are triple negative, there is significant inconsistency (up to 30 percent) between these two classifications. Although any subtype can be triple negative on clinical assays, an interesting subtype found in non-basal triple negative breast cancers is the more newly described claudin-low subtype, which is uncommon but interesting because of its expression of epithelial-mesenchymal transition genes and characteristics reminiscent of stem cells (9).
Recently, many studies have focused on finding molecular pathways that play some roles in breast cancer pathogenesis. Mutation in oncogenes, pro-oncogenes and tumor suppressor genes has been remarked as potential elements in breast cancer. DNA amplification (mostly in proto -oncogenes, growth factors and their receptors) and DNA deletion (in tumor-suppressor genes) are repeatedly observed in breast tumors. Berouk him et al. found 76 amplifications and 82 deletions in 243 breast tumors, in regions containing new possible sensitive genes, such as MCL1 and BCL2L1 (apoptosis), Interleukin-1 receptor-associated

kinase1 (IRAK1), TNF receptor associated factor (TRAF) 6, IKBKG which codes NF-kappa-B essential modulator (NEMO) protein and IKBKB which codes inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β) protein in NK- kB signaling pathway. PIK 3CA, the gene encoding the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), is mutated in about 20 – 30% of breast tumors. TP53 mutations are found in about 30 – 35% of cases (10).

Two newly identified genes, BRCA1 (Breast Cancer gene A1) and BRCA 2 (Breast Cancer gene A2), have been identified and categorized as human tumor suppressor genes. Mutations in these two genes have been found in the majority of hereditary breast cancer cases. Until the age of 70 women with mutated BRCA1 or BRCA2 genes faces to 45-85% increase in the risk of developing breast cancer. Several studies have demonstrated that patients with mutation in BRCA1 usually bear triple-negative kind breast tumors. In contrast, pathologic characteristics of BRCA2-mutant cases did not seem to be very different with non-carriers. Both these two genes play important roles in DNA repair in a common pathway. BRCA 1 is necessary for mammary stem cell differentiation, a function that could explain its tissue-specificity.

Mutations usually result in dysregulation of signal transduction pathways. Increased expression of specific receptor tyrosine kinases (RTKs) has been implicated in the genesis of a significant proportion of sporadic human breast cancers. Increased activity of some of tyrosine kinases can result in aberrant cell proliferation. This phenomenon may result in cell transformation. For example, amplification and overexpression of neu/erbB2 proto-oncogene is observed in 20–30% human breast cancer, and is inversely correlated with the survival of the patient.

The epidermal growth factor receptor (EGFR) family is a member of growth factor receptors which consists of four members: EGFR, ErbB2/Neu, ErbB 3, and ErbB 4. Increase ErbB2 expression, has been further associated with poor clinical outcome, is observed in 20 – 30% of sporadic breast tumors. The main reason is ErbB2 gene amplification (11). Increased level of tyrosine phosphorylated ErbB3 has been also reported. The important point is that ErbB3 is a bridge which links the phosphatidyl inositol-3 kinase (PI-3K) signaling molecule to Neu which has attracted much attention because of its potent transforming properties. This oncogene activates a number of common signaling pathways by providing specific binding sites for a variety of signaling molecules that include either Src Homology 2 (SH2) or phosphotyrosine binding/interacting domains. Co-expression of ErbB2 and ErbB3 RTKs is usually observed in common tumor progression (11,12).

Mammary epithelial expression of Polyoma virus middle T (PyV mT) antigen, another tyrosine kinase involved in murine mammary tumorigenesis and metastasis, results in the rapid induction of multifocal metastatic mammary tumors. Since these tumors occur during early steps of mammary gland development and involve whole of the gland, expression of PyV mT will result in transformation of the primary mammary epithelium. This molecule is also associated with many signaling pathways via Src Homology 2 (SH2) or phosphotyrosine binding/interacting domains (13).

It has been shown that Activated growth factor receptors can interact with integrin receptors and control their biological function in cancerous cells. An example is the stimulation of a6ß1 integrin through association with activated members of the EGFR family which conversely results in activation of EGFR family phosphorylation. Induction of tumor by the PyV M T oncogene is also dependent on the presence of functional ß1-integrin. Lack of functional ß1-integrin makes tumor cells unable to enter the cell cycle. Although, these

tumor cells are unable to proliferate, There are still viable and bears pathological tumor dormancy. Interesting point is that inhibition of integrin-mediated FAK signaling will also shows the similar pathological features. ß4integrin, other member of integrin family, has shown a clear role cell proliferation and invasion through association with Erb B2. Not all integrins, however, have a role in bearing cancer. Deficiency in ß3 or/an d ß 5 integrins did not produce much difference in tumor growth, tumor numbers or lung metastasis in the PyV MT mouse model , only a little increase in tumor onset was observed. Taken together, these observations give promising data for targeting integrin receptors and their associated signaling pathways as a new treatment of breast cancer (11).
Activation of the phosphatidyl inositol-3 kinase is also important in mammary tumor progression. Association of PI-3K links to PyV mT through its binding to phosphotyrosine residues (Tyr 315/322) within the PyV mT coding sequences. Association with Neu happens through recruitment to ErbB3 (ErbB, is derived from the name of a viral oncogene to which these receptors are homologous: Erythroblastic Leukemia Viral Oncogene). Activation of PI-3K and resultant production of phosphoinotide-3 lipids stimulates several members of serine kinase family. The final of these cascades will be the stimulation a number of antiapoptotic signaling molecules such as nuclear factor-kB (NF- κB) (14,15)

4. Role of NF- κB

Because of the wide range of activities of transcription factor NF- κB in apoptosis and cell survival and cell proliferation pathways as well as cell adhesion and angiogenesis it plays a remarkable role in tumorigeneses.
Regulatory influence of NF- κ B on the expression of various tumor-promoting molecules such as MMP, cycloxygenase 2, inducible nitric oxide synthase, chemokines, and inflammatory cytokines explain its significant effect on bearing cancer. NF- κB increased the expression of these molecules, all of which enhance tumoral cell invasion and angiogenesis. Other aspect of the role of NF- κB in tumorigeneses includes increasing expression of proto-oncogenes such as c- myc and cyclin D1 which directly stimulate proliferation. (14)

4.1 Adapter proteins

Adapter proteins do not exert any kinase activity, but they regulate protein – protein interaction and help the formation of protein complex which participate in signal transduction pathways. GRB2-associated-binding protein 2 (Gab2) is one of the adapter proteins which is overexpressed in breast cancer. It promotes signaling pathways by recruiting SH2 containing proteins such as PI3K, Shc, and Shp2 downstream of tyrosine kinase receptors. Although elevated expression of Gab2 in the mammary epithelium is unable to induce tumor development, it has been shown that tumor onset time will decrease in presence of Gab2 (16,17)

4.2 Activation of the Ras signaling pathway

Activation of the Ras signaling pathway is commonly observed in mammary tumor progression. Adapter proteins such as Shc and Grb2 create some specific complexes with activated forms of Neu and PyV mT. The co-operation of Grb2 and Shc with these activated oncoproteins will result in stimulation of Ras signaling. In contrast to PyV mT, which signals to Ras only through its association with Shc, Neu can activate Ras through Grb2, Shc

and several other unidentified adapter proteins. Resultant phenomenon of Ras activation will be the recruiting of a number of downstream effector molecules including PI-3K, Raf serine kinase, GRB associated-binding protein (GAP) and Ras-related protein (Ral) (16). Figure 1 presents an overview of Ras/MAPKs signaling pathway.

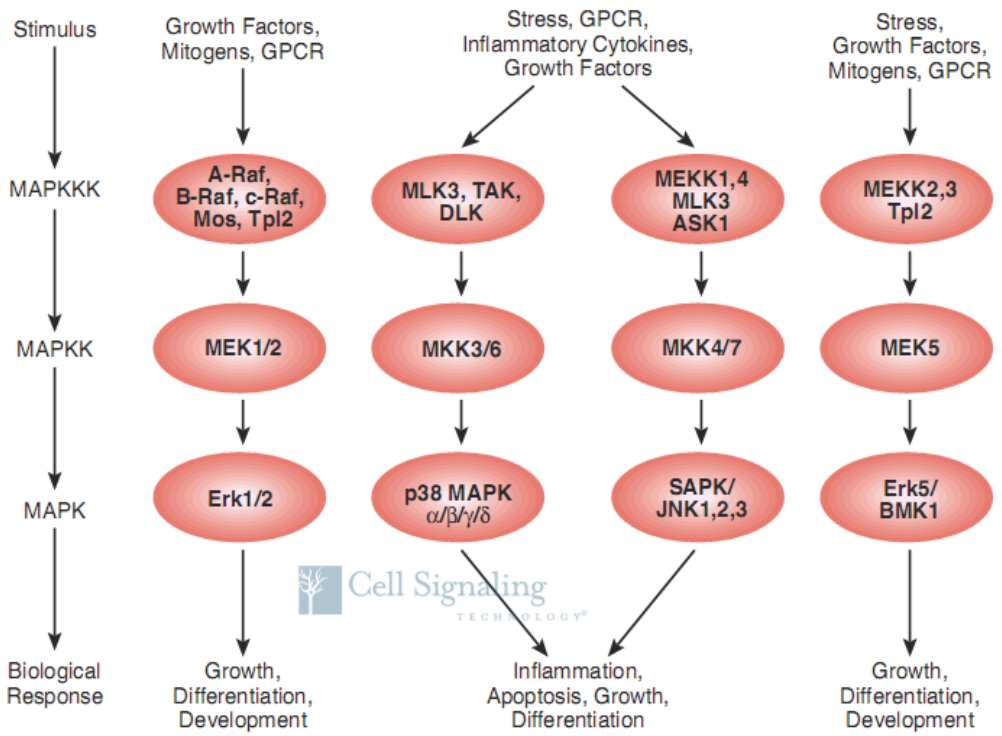

Fig. 1. MAPKs cascades Mitogen-activated protein kinases (MAPK) are a family of Ser/Thr protein kinases widely conserved among eukaryotes and are involved in many cellular programs such as cell proliferation, cell differentiation, cell movement, and cell death. MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli. [Source: Pathway diagram reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).]

5. Dysregulation of cell cycle

Dysregulation of cell cycle can also results in malignant cell proliferation and Tumorigenesis. Cyclin D1, for example, has been reported to be overexpressed in human breast cancer (18). Observation has been confirmed in MMTV-Ras and MMTV-Neu mice deficit in Cyclin D1. Tumor development completely stops in these animals which show the critical role of Cyclin D1 in Ras-Neu transformation pathway..Although overexpression of

Cdc25b make mammary glands hyperplasic and more sensitive to carcinogenic chemicals, it does not directly induce tumorigeneses. Recently, inhibitor of nuclear factor kappa-B kinase (IKK a, a responsible kinase for activation of NF-k B, was identified as a necessary factor for Cyclin D1-associated epithelial proliferation in MMTV-Neu (but not in MMTV- Ra s) mice (11).

5.1 The role of extracellular matrix (ECM) enzymes

In addition to integrin family, which has discussed above, the role of other extracellular matrix (ECM) enzymes such as cathepsins and plasmin in tumorigensis and metastasis has attracted much attention (19,20)

Matrix metalloproteinases (MMP) are a family of matrix degrading enzymes associated with tumor progression, metastasis, and poor prognosis. A tumor cell must degrade the surrounding stroma to reach blood vessels. That's why it is thought that these degrading enzymes control the primary step in invasion and metastasis. The roles ofMMP2, MMP3,MMP7 and MMP9 have been established (21,22).

urokinase-type plasminogen activator (uPa) is another extracellular degrading enzyme which cleaves plasminogen into plasmin. The latter can degrade ECM directly or indirectly via activating MMPs. PyV MT -associated lung metastasis shows remarkable decrease was in plasminogen-deficient mice as well as in uPa-deficient mice (11,23).

5.2 Mutations in tumor suppressor genes

Transforming growth factor-ß **(TGF- ß)** is a secreted cytokine which induces s growth arrest in normal epithelium. It interacts with the TGF- ß type II receptor (T ß RII) which followed by recruitment and phosphorylation of TGF- ß type I receptor (Tß RI) and activation of downstream signaling cascade. The cytostatic effect of TGF- ß is also seen on early tumor progression and is mediated through the regulation of both apoptosis and cell proliferation. However, TGF-ß signaling increases lung metastasis in some transgenic mouse models. Breast carcinomas are well known for overexpressed TG F- ß. Induct ion of TGF- ß 1 after tumor initiation do not exert much effect on proliferation of tumor, but remarkably increase the lung metastasis. These data support the hypothesis that that TGF -ß 1 may no longer perform an inhibitory role in established tumors (24).

Another important tumor suppressor associated with mammary tumor development is p53. p53 is well-known for its involvement in a variety of cancer types. P53 gene is one of the most altered tumor suppressor genes in human breast cancer, such that around 50% of all breast cancers include mutated form of p53 gene (25).

It has been reported that Insulin-like Growth Factor (IGF) may have effect in breast cancer progression. It has been showed that Retinoic Acid (RA) mediate their inhibitory effects on cell growth of cancerous human breast cancer cells "MCF7" via selective reduction of Insulin Receptor Subtype-1 (IRS-1) and its activity which results in the selective down-regulation of IP3-kinase/AKT. High levels of Irs-1 in human breast tumors correlate with elevated incidence of disease recurrence. Although the insulin receptor substrates (IRS) were primarily identified, as the name implied, as a substrate for the insulin receptor (IR), Nowadays it has been known that these adapter proteins, are involved in activation of downstream pathways of several growth factor receptors such as insulin-like growth factor-1 receptor (IGF-1R), vascular endothelial growth factor receptor (VEGF-R), cytokine

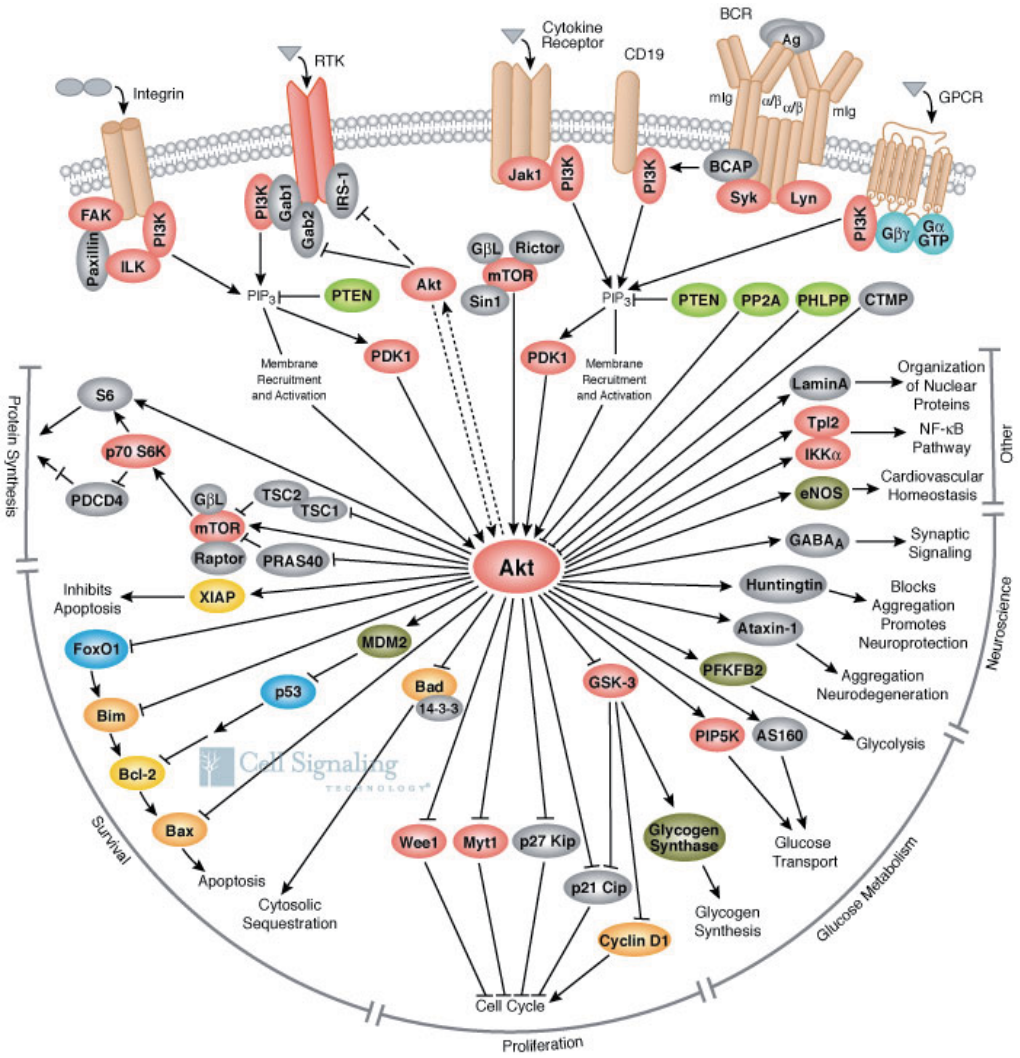

Fig. 2. PI3K / Akt Signaling.The Akt cascade is activated by receptor tyrosine kinases, integrins, B and T cell receptors, cytokine receptors, G protein coupled receptors and other stimuli that induce the production of phosphatidylinositol 3,4,5 triphosphates (PtdIns(3,4,5)P3) by phosphoinositide 3-kinase (PI3K). These lipids serve as plasma membrane docking sites for proteins that harbor pleckstrin-homology (PH) domains, including Akt and its upstream activator PDK1. There are three highly related isoforms of Akt (Akt1, Akt2, and Akt3) and these represent the major signaling arm of PI3K. For example, Akt is important for insulin signaling and glucose metabolism, with genetic studies in mice revealing a central role for Akt2 in these processes. Akt regulates cell growth through its effects on the mTOR and p70 S6 kinase pathways, as well as cell cycle and cell proliferation through its direct action on the CDK inhibitors p21 and p27, and its indirect effect on the levels of cyclin D1 and p53. Akt is a major mediator of cell survival through direct inhibition of pro-apoptotic signals such as Bad and the Forkhead family of transcription factors. T lymphocyte trafficking to lymphoid tissues is controlled by the expression of adhesion factors downstream of Akt. Figure 2 presents a

general map from the role of AKT and the signaling crosstalk which discussed above. In addition, Akt has been shown to regulate proteins involved in neuronal function including GABA receptor, ataxin-1, and huntingtin proteins. Akt has been demonstrated to interact with Smad molecules to regulate TGFβ signaling. Finally, lamin A phosphorylation by Akt could play a role in the structural organization of nuclear proteins. These findings make Akt/PKB an important therapeutic target for the treatment of cancer, diabetes, laminopathies, stroke and neurodegenerative disease. [Source: Pathway diagram reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).]

receptors, and some members of the integrin family. Interestingly, loss of either IRS -1 or IRS -2 did not show similar consequence on developing lung metastasis. Metastasis will increase in IRS -1-deficient tumors, IRS-2-deficient tumors shows decreased lung metastasis. It is thought that a compensatory mechanism which upregulate IRS-2 expression is involved in the increased metastasis seen in IRS-1-deficient tumors. These results are very similar to the ones seen with Akt1 and Akt2, where Akt1 was shown to inhibit invasion and metastasis while Akt2 perform in an opposite way. RA influence occurs at post-translational level by increase in ubiquitination and serine phosphorylation of IRS-1. The latter is protein-kinase C (PKC)-dependent, since PKC inhibitors block the process. Activation of PKC-δ by RA has also been reported. Activation of PI3K/PDK/Akt cascade also decreases sensitivity of MCF7 cells to anticancer drugs. Induction of Bcl-2 may contribute to this resistance (26,27). Figure 2 offers a comprehensive diagram which shows the role of PI3/Akt cascade in cellular functions. As it is seen, this pathway plays a vital role in cell proliferation and cell survival. Therefore, logically it is predictable that any signal disregulation in this cascade will be a risk factor for uncontrolled cell proliferation and malignancy.

In one of the recently-performed experiments, the increasing influence of estradiol (E2) on expression level of iNOS in breast cancer cell line T47D were identified as a result for resistance to tamoxifen. In these cells, administration of oligomycin-2 deoxy glucose (2DG) enhanced tamoxifen antiproliferative effects, which may be due to exacerbated ATP depletion following tamoxifen and oligomycin-2DG co-administration. Oligomycin-2DG neither changed iNOS expression nor affected its attenuated expression due to tamoxifen exposure, suggesting that ATP depletion-mediated sensitivity to tamoxifen is apart from iNOS (28).

6. Breast cancer stem cells

Recently, cancer stem cells (CSCs) have attracted a lot of attentions and some roles have been determined for estrogen and progesterone by affecting these cells. It has become clear that the normal and malignant breast contains stem cells (SCs) that play an essential role in the normal development o f the breast and are likely to play a significant role in the genesis and growth of human breast cancer. The CSC hypothesis introduced tissue-specific Stem Cells (SCs) and/or their early progenitors as the main causes of the malignant behavior of cancer. These cells are undifferentiated and, as a result, have the ability to divide into two daughter cells. But, division is asymmetrical and will cause an identical clone of the mother cell and another cell which can divide and fully differentiate into new cell line. This latter daughter cell is named a Progenitor. Physiological functions of breast SCs include producing the early milk ducts and the surrounding stroma at puberty and repair of damaged tissue and renovation the lost ductal and stromal cells during adult life.

In contrast to their progenitor and differentiated offspring, breast SCs are very long life and thus influences of the effect of chemicals and radiation. Since breast CSCs escape from the control of surrounding microenvironment, they are able to bear malignant progenitor offspring. The result will be the production of malignant daughter cells that create the bulk of the tumor.

As a rare phenomenon, some of breast CSCs are quiescent and, as it is expected, will be spared by current cancer therapies whose targets are rapidly divided cells (29-32)

6.1 Role of estrogens and progestins

It has been suggested that hormone therapy or oral contraception may increase the risk of breast tumor development because of proliferation of existing quiescent tumor cells. The estrogen receptor-alpha (ERa) has an important role in normal breast cell development. Genetic alterations in the ER a gene locus might therefore have important effects in breast carcinogenesis. Polymorphisms can also cause even more increase in estrogen-associated breast cancer risk. At least three polymorphisms, i.e. the G478T, A908G, and C975C have been put in this category (33).

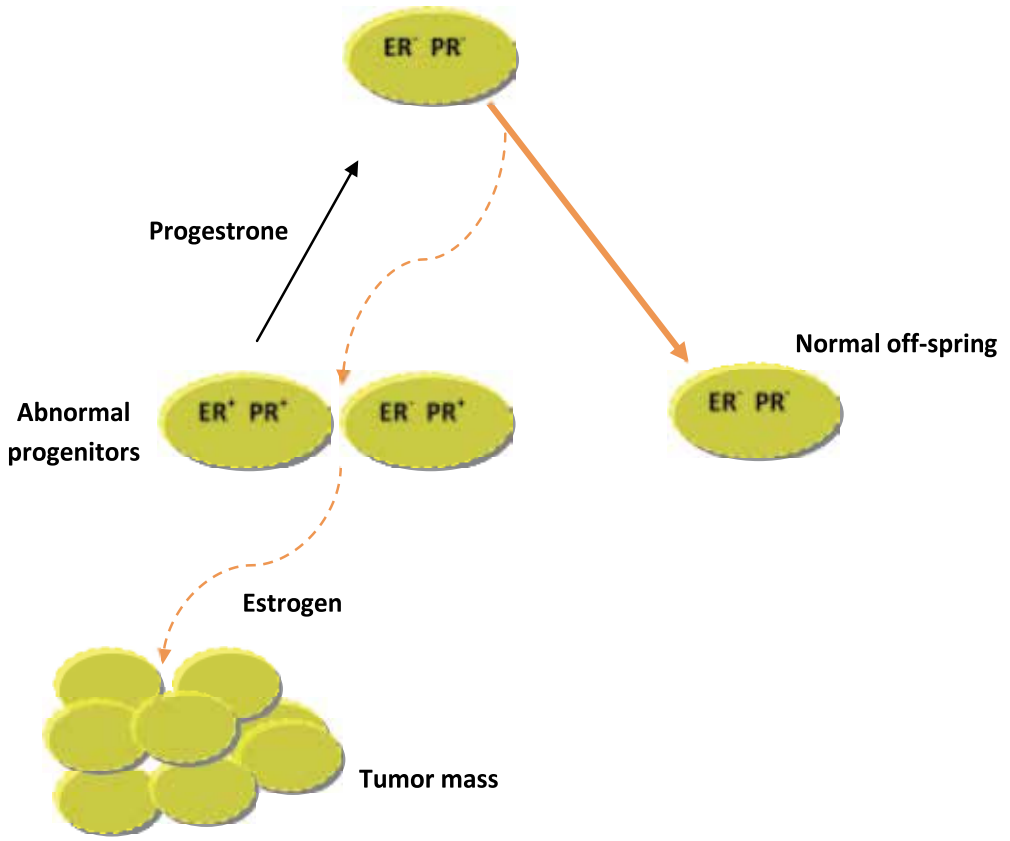

Fig. 3. Effect of Estrogens and progestines on breast CSCs. CSCs divide into abnormal off-spring which can differentiate to all types of breast tumoral cells

Progestins, on the other way, are able to upregulate growth factor and cytokine receptors at the cell surface. They are also involved in regulation of several intracellular effectors including Stat 5, and by potentiating mitogen-activated protein kinase (MAPK) and Janus kinase activities by increasing the levels and altering the subcellular compartmentalization of them at cytoplasmic level. Furthermore, growth factor-regulated nuclear transcription factors may have synergistic effect with PRs' agonists to regulate the function of key genes which are involved in breast cancer. (34)

Recently, the influence of estrogen, progesterone, and progestins on breast CSCs and their progeny has been found out. As it has been demonstrated in figure 3, although most of breast CSCs are estrogen receptor negative and progesterone receptor negative, some intermediate progenitor forms own hormone receptors, especially progesterone receptor. Progesterone and progestin specially work on these breast cancer stem intermediate forms, inducing them to return back to a more primitive breast CSC forms, thus increasing the pool of malignant SCs (29). These cells escape the microenvironment control. Estrogens, on the other hand, induce the proliferation of these abnormal progenitors, resulting in breast tumor. Figure 3 summarize this hypothesis.

7. P-glycoproteins and breast cancer resistance protein (Bcrp)

P-glycoproteins and breast cancer resistance protein (Bcrp) also play important roles in resistance and therapeutic outcome of breast cancer therapy and mutations in MDR genes (which codes p-glycoproteines) and influence the risk and resistance to treatment. Many drugs are substrates for this transporters and the reduction in their access to tissues can result in increase in metastasis and drug resistance. From glycoprotein family, glycoprotein non-metastatic B (GPNMB, also named as Osteoactivin) enhances breast cancer metastasis in an *in vivo* mouse model. It also has been studied as a prognostic indicator of recurrence. The data suggested this glycoprotein as a novel therapeutic target in breast cancer. GPNMB usually express in basal/triple-negative subtype of breast cancer and is associated with poor outcome (35).

Fetuin-A is another glycoprotein which its role in mammary tumorigenesis has been studied.

It is a serum component protein which forms approximately 45% of non-collagenous glycoproteins which is synthesized by the liver and excreted into plasma. It is a conserved member of the cysteine protease inhibitors which contains the TGF-β receptor II homology 1 domain (TRH1). As a result, it is able to compete with epithelial cells for TGF- β. The possible sequestration of TGF β by fetuin-A could affect TGF β signaling in breast epithelial cells as previously reported for intestinal epithelial cells. Fetuin-A shows reduced incidence of mammary tumors for breast cancer by more than 60% and increases tumor onset. Another tumor-enhancer property of fetuin-A is its stabilizer effect matrix metalloproteinases in the extracellular matrix.

Consequently, they can drive the "tumor islands" to invade the stroma metastasize to other organs. Stronger TGF-ß signaling in the absence of fetuin-A exert suppressor effect on cell proliferation through increase in is ARF-p53 expression, whereas the sequestration of TGF-ß by fetuin-A, results in reduction of its signaling in epithelial cells and inactivation of ARF-p53 which is parallel with shortening the latency of mammary tumorigenesis and implications of breast cancer development (36).

7.1 Astrocyte Elevated Gene-1

Some newly reported show that elevation in expression level of astrocyte elevated gene-1(AEG-1, also known as Metadherin and lyric) in human breast cancer dramatically

enhanced cell proliferation and their ability of anchorage-independent growth of breast cancer cells. These proliferative effects were significantly related to attenuation of two key cell-cycle inhibitors, p27Kip1 and p21Cip1, via Akt/ FOXO1 signaling pathway. FOXO1 is a transcription factor belonging to the Forkhead box-containing class O (FOXO) subfamily. Many biological functions have been shown to be related with FOXO1 including cell-cycle control, differentiation, stress response and apoptosis (37). FOXO proteins could act as tumor suppressors through induction of CDK inhibitors, including p21 Cip1, p27Kip1and p57 (38). Overexpression of *AEG*-1 increases migration and invasion of human glioma cells because of the presence of a lung-homing domain which facilitates breast tumor metastasis to lungs. Recent observations indicate that *AEG*-1 play this role by activating NF-κB pathway. Our recent observations indicate that, AEG-1 facilitates IκBa degradation, resulting in an increase in NF- κB DNA binding activity and NF- κB promoter activity in reporter assays These valuable findings are strengthen the idea which recommend *AEG*-1 as a crucial regulator of tumor progression and metastasis (39).

Another considerable role attributed to AEG-1 is mediating a broad-spectrum chemoresistance. In vitro and in vivo studies showed that knocking down AEG-1 makes several different breast cancer cell lines more sensetive to paclitaxel, doxorubicin, cisplatin, 4-hydroxy cylco phosphamide, hydrogen peroxide, and UV radiation mediated by the pro-survival pathways such as PI3K and NFκ B, or through other downstream genes of MTDH/AEG-1 that directly regulate chemoresistance. AEG-1 has also resulted in chemoresistance neuroblastoma and prostate cancer. In fact, MTDH/AEG-1does not affect the uptake or retention of chemotherapy a. Instead,

it enhances chemoresistance by increasing cell survival after chemotherapy. Data gathered from Microarray analysis of breast cancer cells showed reduction of expression of chemoresistance genes ALDH3A1, MET, HSP90, and HMOX1, and increased expression of pro-apoptotic genes BNIP3 and TRAIL after MTDH/AEG-1knocking down. Among these genes, ALDH3A1 and MET were established to partially be associated with the chemoresistance role of MTDH/AEG-1 in MDA-MB-231 breast cancer cells. Some other genes also contribute to chemoresistance including drug-metabolizing enzymes for different chemotherapeutic agents, such as dihydropyrimidine dehydrogenase (DPYD), cytochrome P4502B6 (CYP2B6), dihydrodiol dehydrogenase (AKR1C2), and the ATP-binding cassette transporter ABCC11 for drug efflux (40). Roles of MTDH/AEG-1 have been simplified in figure 4.

There are some studies which suggest that Activated protein C (APC), an anticoagulant serine protease, is related to cell survival, cell migration, angiogenesis and breast cancer invasion. APC recruits EPCR, PAR-1, and EGFR in extracellular matrix in order to increase the invasive properties of MDA-MB-231 cells. Other mechanisms include activation of matrix metalloprotease (MMP) -2 and/or -9 and activation of ERK, Akt, and NF-κB (but not the JNK) pathways. APC does not employ the endogenous plasminogen activation system to increase invasion (41).

7.2 Role of STAT family

The Stat (Stands for signal transducer and activator of transcription) family of proteins are latent cytoplasmic transcription factors which are involved in cytokines signaling pathways. They are necessary for normal cell growth, survival, differentiation, and motility. STAT proteins need activation through tyrosine phosphorylation, which leads to dimerization via conserved structural features phosphotyrosine-SH2 (Src homology domain 2) of two Stat molecules. Fallowing activatin, Stats transport to the nucleus, where they bind to the

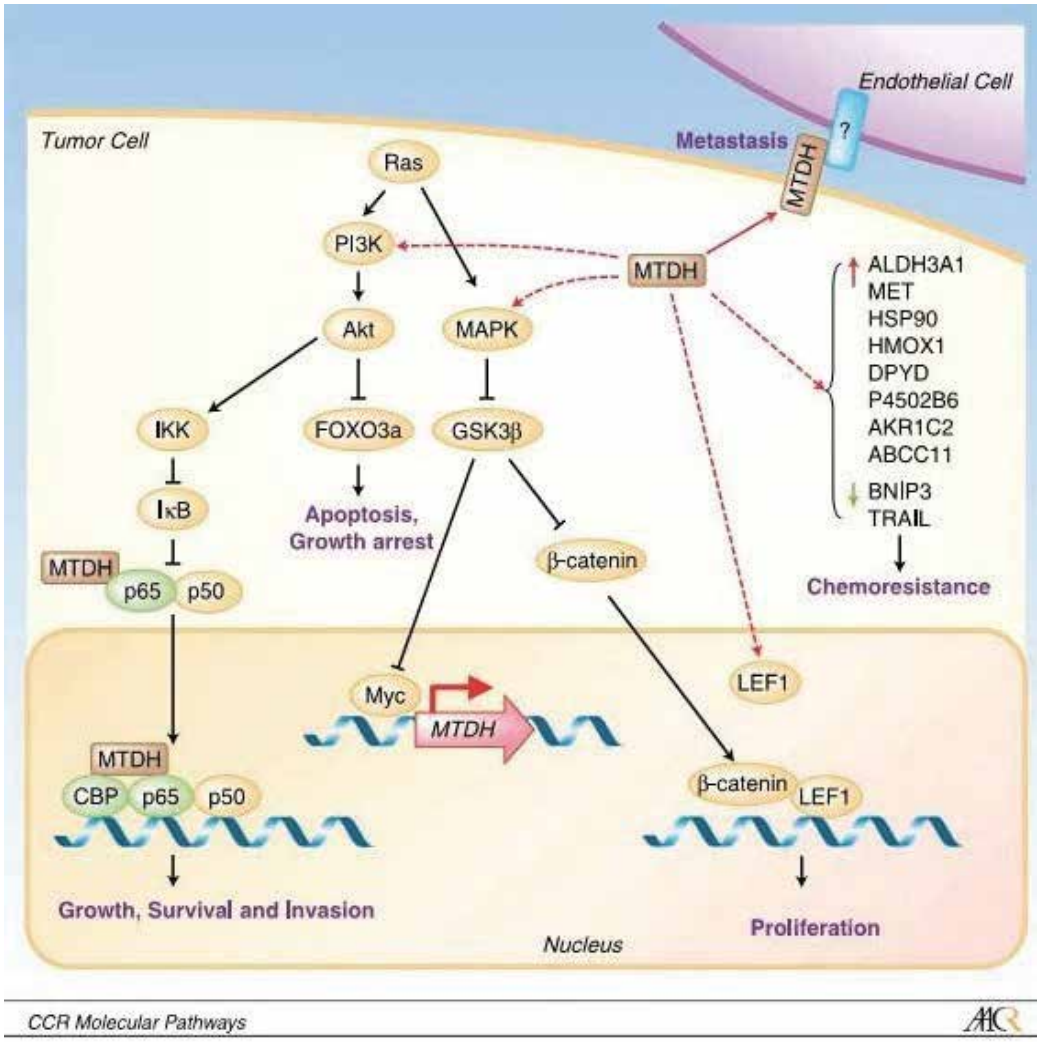

Fig. 4. MTDH/AEG-1 promotes tumor progression through the integration of multiple signaling pathways. Oncogenic Ha-Ras increases *MTDH/AEG-1* expression through the activation of the PI3K/Akt pathway, which phosphorylates and inactivates GSK3β, and subsequently enhances the stabilization and binding of c-Myc to the *MTDH/AEG-1* promoter. MTDH/AEG-1 can activate AKT, NFκB, and Wnt/β-catenin pathways to promote proliferation, survival, and invasion. Activation of NFκB signaling is in part mediated by the direct interaction of MTDH/AEG-1 with p65 and CBP, a general transcriptional co-activator. MTDH/AEG-1 activates the Wnt/β-catenin pathway through increasing the activity of MAPK kinases ERK and p38, which phosphorylates GSK3β and stabilized β-catenin. Furthermore, MTDH/AEG-1 increases the expression of LEF-1, a transcriptional cofactor for β-catenin. The prometastasis function of MTDH/AEG-1 is mediated by the interaction of the LHD of MTDH/AEG-1 with an unknown receptor in endothelial cells. The broad spectrum chemoresistance function of MTDH/AEG-1 is mediated by a number of downstream genes that promote the resistance to multiple

chemotherapeutic agents. Proteins with direct interactions with MTDH/AEG1 are shown in green. Dotted line indicates pathways yet to be fully validated or characterized. [Source: Figure 1 from Ref. 40] With permission

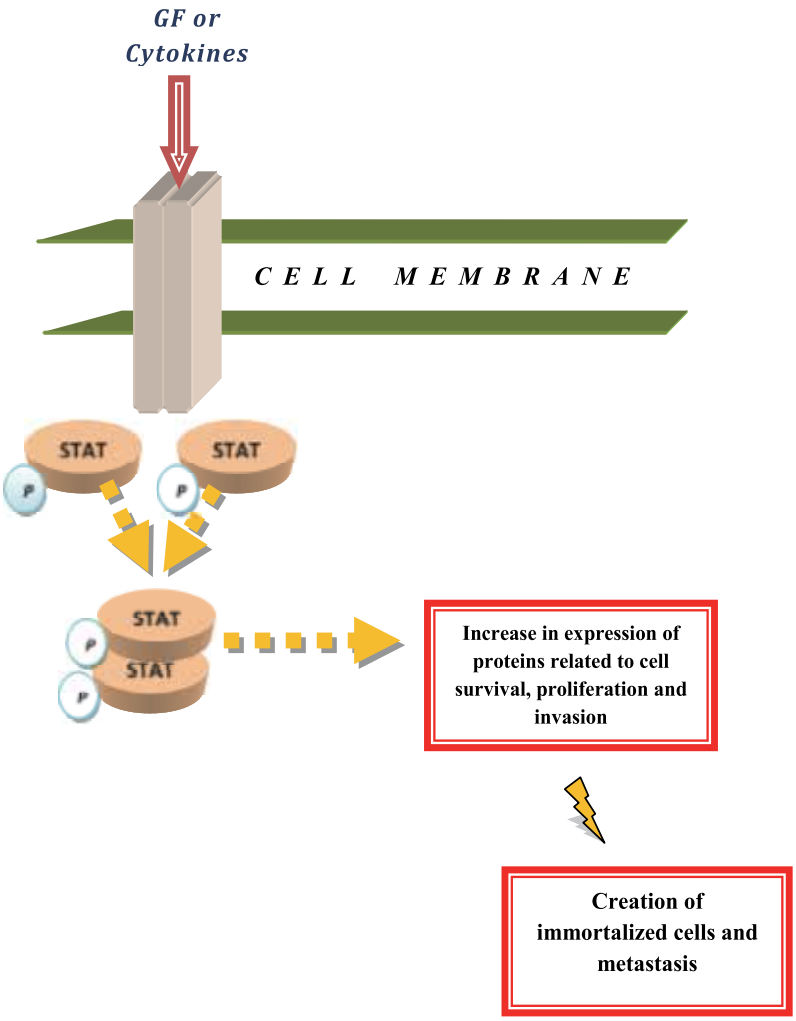

Fig. 5. Role of Stat3 signaling pathway to cancer metastasis. Activatin of STAT3 happens by recruitment to phosphotyrosine motifs within complexes of growth factor receptors (e.g., epidermal growth factor receptor), cytokine receptors (e.g., IL-6 receptor), or non-receptor tyrosine kinases (e.g., Src and BCR-ABL) through their SH2 domain. Stat3 is then phosphorylated on a tyrosine residue by activated tyrosine kinases in receptor complexes. Phosphorylated Stat3 forms homodimers and heterodimers and translocates to the nucleus. In the nucleus, Stat3 dimers bind to specific promoter elements of target genes and regulate gene expression. The Stat3 signaling pathway regulates cancer metastasis by regulating the expression of genes that are critical to cell survival, cell proliferation, invasion, angiogenesis, and tumor immune evasion.

promoter of target genes and activate their transcription. Dimerized status of STATs is transient in normal non-transformed cells. But in transformed cancerous cells, Stat proteins in particular, Stat3 are found in a permanent active dimerized manner. Activated form of STAT3 has been found in more than 50% of primary breast tumors and tumor-derived cell lines. It has been reported that expression of a constitutively active form of Stat3 (Stat3C) is sufficient for promoting cellular transformation and bearing an immortalized breast cell line. Since the IL-6/gp130/Jak signaling pathway has a crucial role in Stat3 activation in human breast cancer, blockade of this pathway may be an important therapeutic plan in breast cancer therapy (42). Role of STAT3 has been shown in figure 5

As it is mentioned above, dysregulation protein expression can result in increased metastatic properties of breast cancer. As a fact, reduction in cell adhesion and increased cell motility is necessary for tumor metastasis. Therefore, cell adhesion molecules have roles in promoting and inhibiting metastasis. Specific families of adhesion molecules including selectins, integrins, lectins, and cadherins have been established to be associated with metastasis (43-47). The cells have to pass the basement membrane to reach the surrounding vessels and spread to other sites. This process involves proteolysis and motility and need proteolytic enzymes to work. Three major categories of proteolytic enzymes including the matrix metalloproteinases (48), serine proteinases, and cathepsins (discussed above) are implicated in metastasis. Cell motility is another factor which cells need to be able to metastasize to other tissues. Several factors are necessary for cellular motility, including the autocrine motility factor, autotaxin, and hepatocyte growth factor (HGF). HGF will result in developing more as well as larger axillary lymph node metastases (24).

Chemoattractants and their corresponding receptors are the other factors affecting metastasis rocess. Osteonectin (a glycoprotein secreted by osteoblasts in bone, initiating mineralization and promoting mineral crystal formation) engages breast and prostate cancer cells to bone. Recently presented data indicate that chemokine receptors CXCR4 and CCR7 express in breast carcinoma cells predisposed for metastasis to lymph nodes and bone (24).

Metastasis-associated protein 1 (MTA1) mRNA expression is parallel to metastatic potential. Function of the MTA1 gene product in tumor progression and metastasis is still unknown, although it is thought that MTA1 is found in the chromatin remodeling histone deacetylase complex (24).

Osteopontin was identified as a metastasis associated gene. Osteopontin appears to be useful for prognosis in that elevated plasma levels and immunohistochemical staining of tumor cells are found in metastatic breast cancer patients. It is important, however, to note that not all studies show correlations. For example, immunohistochemical staining showed no correlation with lymph node involvement or histological grade (24).

8. Metastasis suppressor genes

8.1 E-cadherin

E-cadherin (a member of the cadherin superfamily of Ca^{2+}-dependent adhesion cell surface molecules, expressed predominantly in epithelial tissues) has been demonstrated to correlates negatively with the potential of tumor invasion. Reduction and/or loss of E-cadherin expression in carcinomas will result in increased tumor metastasis because of the reduction in tumor cell adhesiveness and increased cell motility (49)

Tissue Inhibitors of Metalloproteinases

The role of metalloproteinases (TIMPs) is inhibiting the activity of matrix proteinases (MMPs). As a result, they suppress tumor metastasis. An interesting paradox is that increased TIMPs are associated with progression to metastatic disease in some studies. One proposed explanation is that the balance between MMPs and TIMPs is important than the expression of each protein (50).

8.2 Maspin

Maspin (belonging to the serpin family of serine protease inhibitors) is a tumor suppressor gene which has been established to be involved at least in breast and prostate cancer. Loss of maspin expression has been established during immunohistochemical studies (51).

8.3 Kai1

Kangai 1 (from Chinese kang ai meaning anticancer) or Kai1 is a member of the Transmembrane-4 superfamily of adhesion molecules and is involved in lymphocyte differentiation and function. It was originally described as a metastasis suppressor in prostate cancer but its role has been established as a general suppressor of the metastatic phenotype in many cancer types including breast cancer, although KAI1 does not affect primary tumor growth (52).

8.4 BRMS1

Breast cancer metastasis-suppressor 1 (BRMS1) decreases metastatic potential of tumor cells, although tumorigenicity do not affected. The mechanism underlying BRMS1 tumor suppression is not yet known, but some data suggest that this role may be mediated by enhanced immune recognition, altered transport, and/or secretion of metastasis-associated proteins (53).

8.5 MKK4

This gene encodes a dual specificity protein kinase that belongs to the Ser/Thr protein kinase family. This kinase is a direct activator of MAP kinases in response to various environmental stresses or mitogenic stimuli. It has been shown to activate MAPK8/JNK1, MAPK9/JNK2, and MAPK14/p38, but not MAPK1/ERK2 or MAPK3/ERK3. This kinase is phosphorylated, and thus activated by MAP3K1/MEKK (54).

8.6 Role of micro-RNAs

A newly opened window in cancer studies is the discovery of microRNAs (mi RNAs). It has been noticed that alteration of non-coding genes, including miRNAs is related to cancer pathogenesis. Mi RNAs modulate the expression of many genes through cleaving mRNA molecules or inhibiting their translation. As a result, they are involved in a variety of physiological and pathological processes, including development, differentiation, cellular proliferation, programmed cell death, cancer initiation and metastasis. It is important to note that a single miRNA can influence the expression of hundreds of proteins. Early studies showed that compared to normal breast human tissues, miRNAs are extensively deregulated in breast tumors. MiRNAs exert their influences at several steps of tumor development and metastasis. Cancer cell adherence, migration, invasion, motility, and angiogenesis are all affected by these modulators. “Metastamir” is the name which has been applied for the class of miRNAs which are involved in metastasis associated processes.

Profiling of metastamirs in human breast cancer has been resulted in to find the new molecular mechanisms in metastatic process. Significant increase in expression of some of miRNAs has been identified in breast tumors and some others have shown some correlation with biopathological features such as Her2, ER and PR status, tumor stage, and response to treatments. The most important miRNAs involved in different steps of developing breast tumor are miR-335, miR-17/20, and miR-146 (involved inmicroenvironment modification), let-7, miR-200 and miR-30 (BCSC phenotype formation); miR-21,miR- 12 6, miR-373, and miR-520 (local invasion), miR-7, miR-661 and miR-17/20 (survival in vasculature) and miR-200 and let-7 (proliferation at distant sites).
Chemoresistance is also affected by miRNAs. Some miRNAs which play some roles in this step are miR-125b, miR-21, and miR -128. The mechanisms underlying miRNAs dysregulation in breast cancer development, whether dysregulated miRNA is a cause or consequence of pathological and many other questions remain to be explored (55). Some of the most important miRNAs have been mentioned in table 1.

miRNA involved	Protein inhibited	Function influenced
miR-7	EGFR	Anoikis resistance
miR-30	Ubc 9	Anoikis resistance
miR-520	CD 44	Local invasion
miR-373	CD 44	Local invasion
miR-21	Bcl-2	Colonization
miR-145	IRS-1	Colonization
miR-17/20	Cyclin D1	Colonization
MiR-205	VEGF	Angiogenesis
MiR-9	E-cadherin	Angiogenesis

Table 1. miRNAs and their function in cancer

9. Biomarkers

Identifying biomarkers in early stages of breast cancer as helpful instruments for increasing breast cancer survival has opened an important window in researches. Immunohistochemical testing of tumor samples for estrogen receptor(ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER 2) is a common method which is widely used (56,57). Biomarkers in biological fluids are more useful because they don't need biopsy and invasive methods. Four metabolic biomarkers including Homovanillate, 4-hydroxyphenylacetate, 5-hydroxyindoleacetate and urea have been shown to be different in urine samples of cancer subjects, compared to control group (58). The

intraductal sampling including samples of nipple aspiration, ductal lavage, and duct endoscopy, is newly used for direct access to the microenvironment surrounding the breast cells that are undergoing malignant transformation (59).

Serum antigens and autoantibody profiling is another approach for early detection and diagnosis of breast cancer. Elevation in level of two antigens, CA 15-3 and CA 27.29, has been reported. Another way is detection of serum autoantibodies against tumor suppressor genes. Studying the changes appeared in level of several autoantibodies instead of only one antibody appears preferable to achieve more accuracy.

BRCA1/2 mutation or functional losses are the other markers will likely serve as a useful predictive biomarker for diagnosis as well as of response to treatment with PARP inhibitors.

REGγ (also known as PA28γ, PSME3 or Ki antigen) is a member of the REG or 11S family of proteasome activators which bind to 20S proteasome and facilitate the related degradation of its intracellular protein substrates. REGγ is one of the potential markers in breast cancer whose expression is associated with breast cancer development and the presence of ER, CerBb-2 and lymph node metastasis. It has been reported that REGγ could facilitate the growth of breast cancer cells. Abnormal high expression of REGγ has been observed in breast cancer and its metastatic lymph nodes (60).

BCL2 has been introduced as an independent biomarker for prognosis of all types of early-stage breast cancers. Immunohistochemical studies have been introduced BCL2 expression as a new diagnostic instrument in breast cancer studies although further work should be done to ascertain the exact way to apply BCL2 testing for risk estimation and to find a standard protocol for BCL2 immunohistochemistry (61).

Ki-67, MI, PCNA, and LI have been reported as markers for poor prognosis, although the most important one has not been established yet (62). Serum associated tumor markers have been newly introduced for breast cancer diagnosis. Carbohydrate antigen (CA) 15-3 and carcinoembryonic antigen (CEA) are the most well-known markers. The noticeable point is that the elevation of CA 15-3 between 4 and 6 weeks after initiation of a new therapy, i.e. spurious early rise (surge), indicates poor prognosis. However, American Society of Clinical Oncology (ASCO) guidelines don't recommend CA 15-3 alone as a marker for either diagnosis or detection of early recurrence of breast cancer. CEA expression level has been not also confirmed as a marker for diagnosis or routine surveillance after primary therapy. The ASCO recommend CEA level measurement as supplementary information (63).

Overexpression of cathepsin B (CTSB) - which is involved in proteolytic pathways that lead to the degradation of ECM proteins - and caveolin-1 (cav-1) - which is correlated with increased expression of RhoC and resultant increase in cell motility and invasion - have been established in Inflammatory breast cancer (IBC) compared to non-IBC tissues. Furthermore, CTSB expression level has shown a significant positive correlation with the number of positive metastatic lymph nodes in IBC (and not in non-IBC patients). IBC is the most invasive and fatal form of primary breast cancer, the 3-year survival rate for this kind of breast cancer is 40% which compared to 85% for non- IBC, is very poor. Distinct clinical features of this form include a rapid onset, erythema, edema of the breast and a "peaud' orange" appearance of the skin. High metastatic behavior, rapid invasion into blood and lymphatic vessels and formation of tumor emboli within these vessels are also major characteristics of IBC which make this form the most dangerous kind of breast cancer (64).

MTDH/AEG-1overexpression or genomic amplification can also be used as biomarker to identify subgroups of patients with requirement for more aggressive treatment, although more studies should be done (40).

PKC (a family of serine/threonine kinases involved in several cellular signaling pathways including proliferation, differentiation, apoptosis, and migration) is a marker associated with poor prognosis of breast cancer. Although most breast cancers are PKCa -negative, the small PKCa-positive ratio shows more aggressiveness (65).

S100A4 protein expression appears to be elevated in early and advanced stages of breast cancer compared to normal breast, although its role in different stages of breast cancer seems to be complex. Compared to early stage, S100A4 protein has been observed to down regulate in more advanced stages of breast cancer (66).

Aldehyde dehydrogenase 1 (ALDH1) tumor cell expression is an independent predictor of BRCA1 mutation status. Since BRCA1 related breast cancers consist of increased cancer stem cell components, these hereditary tumors shows significantly elevated expression of ALDH1. ALDH1 positive population of breast cancer cells show high tumorigenic capacity through serial passages *in vitro*, compared with A LDH1 negative population. ALDH1 tumor cell expression has been introduced as an independent predictor of BRCA1 mutation status. Furthermore, ALDH1 might be useful as a BRCA1 biomarker and therapeutic target (67). High saturated to monounsaturated fatty acid ratio measured in blood is another indicator associated with breast cancer risk. Low activity or reduced expression of stearoyl-CoA desaturase-1 will result in a decreased breast cancer risk. The suppression of stearoylCoA desaturase expression leads to reduction of cell proliferation and invasion in vitro, and impairs tumor formation and growth which could not be overcome by use of exogenous monounsaturated fatty acids. Since high saturated to monounsaturated fatty acid ratiois related to the activity of this enzyme, it can be used as a new marker to assume breast cancer risk, although more studies should be done.

Since SCD-1 expression is regulated by dietary and lifestyle factors, new nutritional strategies for cancer prevention could be focused on SCD1 function (68).

Newly introduced Metastamirs assume to be useful biomarkers for prediction of progression and prognosis of breast cancer and in identification of the novel targets for therapeutic intervention in future breast cancer diagnosis and treatment (55).

Taken together, our knowledge about molecular pathways involved in breast cancer and prognostic and diagnostic markers are much more than before, although many works remain to be done.

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Part 2

Breast Cancer and Microenvironment

7

Novel Insights Into the Role of Inflammation in Promoting Breast Cancer Development

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1. Introduction

In the past decades the major focus of cancer research has been the transformed tumor cells itself, while the role of cellular microenvironment in tumorigenesis has not been widely explored. Several studies have demonstrated the ability of stroma to regulate the growth and differentiation state of breast cancer cells, and the invasive behaviour, and polarity of normal mammary epithelial and breast carcinomas are influenced by tumor microenvironment, immune and stromal cells (Bissell, et al., 2002, Radisky & Radisky, 2007, Tlsty, 2001, Tlsty & Hein, 2001). In addition, genetic abnormalities, such as loss of heterozygosity, occur not only in cancer cells, but in stromal cells as well (Kurose, et al., 2002, Kurose, et al., 2001, Moinfar, et al., 2000).

It is believed that a better understanding of the tumor microenvironment could help render more accurate diagnostics or assist in predicting tumor aggressiveness (i.e., bad prognosis) thus facilitating the design of personalized treatments.

By the end of the nineteenth century, the English surgeon S. Paget suggested the idea that, in order for breast cancer to develop, a specific "seeding" process must occur and, for this primary onset to metastasize to a specific distant organ, particular stromal features would be required postulating his "seed and soil" hypothesis (Paget, 1889). His work greatly contributed to somewhat earlier observations by T. Langhans who first used the word stroma to describe the connective tissue, vessels and other components between tumors (Langhans, 1879) and to the theory postulated by R. Virchow suggesting a possible origin of cancer at sites of chronic inflammation (Balkwill& Mantovani, 2001). A century later, researchers such as B. Mintz and K. Illmensee in general, as well as M. Bisell, in breast cancer in particular, pointed to the tumor milieu as an essential component of neoplasias, not only for cancer evolution but also for cancer instigation (Mintz & Illmensee, 1975; Lochter & Bissell, 1995). Together these and additional findings had painted a broad picture of the complexity of tumor microenvironment, where diverse stromal cells interact with

each other and with the cancer cells playing important roles in tumorigenesis (Soto & Sonnenschein, 2004; Egeblad et al., 2010).

It is clear now that metastatic tumors represent the greatest threat to cancer patient mortality. Indeed, when breast cancer is diagnosed early and metastases are not present, 5-year survival is >88%; however, if metastases are also present, long-term survival is significantly diminished (~10%) (Jemal, et al., 2011). Thereby, the major cause of mortality of breast cancer and different types of cancer is due to metastasis to distant organs, such as lung, bone, liver and brain (Lu & Kang, 2007). A notable feature of this process is the variation in metastatic organ tropism displayed by different types of cancer (Chambers, et al., 2002, Fidler, 2002). A classic view has proposed that purely mechanical factors regulate the fate of blood-borne metastasis tumor cells (MacDonald, et al., 2002); however, this does not fully explain the non-random distribution and distinct pattern of metastasis in each tumor type (Lu & Kang, 2007). However, tumor microenvironment has also shown an important role in the regulation of this process (Valdivia-Silva, et al., 2009). A number of different molecules present in the microenvironment have been associated to the metastasis of breast cancer, among them, chemokines, which have been associated with regulation of cell migration and invasion of tumor cells into specific organs (Muller, et al., 2001, Zlotnik, 2006). Chemokines are a superfamily of chemotactic cytokines characterized by their ability to induce directed migration of leukocytes, during haematopoiesis, lymphoid organ development, and in disease (Sallusto, et al., 2000); their expression may be inducible, primarily by pro-inflammatory cytokines such as TNF-a and IL-1-b (Ben-Baruch, 2003). Chemokine receptor expression in many cancer cells have shown to be a non-random process (Shields, et al., 2007, Zlotnik, 2006) and to have a role in organ-specific metastasis: for example, CXCR4 expression and metastasis to lung, bone and lymph nodes (Muller, et al., 2001), CCR7 to lymph nodes (Shields, et al., 2007), CX3CR1 to brain (Mourad, et al., 2005), CCR9 to liver and small bowel (Amersi, et al., 2008, Letsch, et al., 2004), and CCR5 and CXCR2 to lung, liver, vessel endothelial cells and bone (Gross & Meier, 2009, Keeley, et al., 2010, Miller, et al., 1998).

Here, we will discuss the ability of the chemokines to affect tumor cell–microenvironment interactions, increasing the invasive behaviour and metastasis, confirming the importance of the host inflammatory response that may differ between tumor types, disease stages, and/or many other host factors; and the role of stromal contribution of the inflammatory microenvironment to cancer progression and metastasis.

2. Inflammatory mediators as regulator of breast cancer development and metastasis

The link between inflammation and cancer has been observed over 150 years ago when Rudolf Virchow noted that cancers tend to occur at sites of chronic inflammation. Indeed, epidemiological studies indicate that inflammatory and infectious diseases are often associated with an increased risk of cancer (Coussens & Werb, 2002). The microenvironment of tumors mimics that of tissues during the height of an inflammatory response to injury (Joyce & Pollard, 2009). However, unlike the organized morphology of normal tissue, and the ultimate resolution of the inflammation that occurs during healing, tumors exist in a state of chronic inflammation characterized by the presence of cancer cells, immune cells, aberrant vascular cells, and the persistence of inflammatory mediators, such as cytokines and chemokines.

The presence and significance of leukocyte infiltrates in developing neoplasms is now undisputed (Allen, et al., 2007, Moser & Loetscher, 2001, Moser & Willimann, 2004). It has been demonstrated that leukocyte infiltration in developing tumors is one of the host´s main immune mechanisms to eradicate malignant cells. However, while some leukocytes certainly have this potential, i.e., cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (Luster, 1998), other leukocyte cell types, most notably innate immune cells, i.e., mast cells (MCs), immature myeloid cells, granulocytes, and macrophages, instead potentiate tumor progression (Baggiolini, et al., 1997, Chen, et al., 2006, Joyce & Pollard, 2009), and enhance neoplastic cell survival. Upon entry into the neoplastic microenvironment, infiltrating leukocytes become alternatively activated and manifest a pro-tumor phenotype as defined by activation of cellular programs involved in immune tolerance and tissue remodelling (Mishra, et al., 2011, Strieter, et al., 2006). During premalignant progression, a consequence of alternative activation of leukocytes is promotion and elaboration of a microenvironment rich in extracellular matrix (ECM) remodelling proteases, and increased presence of pro-survival, pro-growth and pro-angiogenic factors that further enhance proliferative and invasive capacities of neoplastic cells (Li, et al., 2007, Orimo, et al., 2005). Such pro-tumor inflammatory microenvironments promote not only malignant conversion and development of solid tumors, but also dissemination of neoplastic cells into blood vasculature by driving invasive capacity of malignant cells, expansion of angiogenic vasculature, and neoplastic cell entry into blood vessels (and lymphatics) (Keeley, et al., 2010).

Breast carcinomas are highly infiltrated by different types of host leukocytes, including primarily T cells, and monocytes that differentiate into tumor-associated macrophages (TAM) at the tumor site (Ben-Baruch, 2003, Crowther, et al., 2001). The presence of the cellular infiltrate in breast tumors was initially regarded as evidence for the potential activity of immune mechanisms against the growing neoplasm. As explained above, several studies suggest that T-cell antitumor responses are impaired in advanced stages of breast carcinoma, and there is no definite conclusion regarding the efficacy of T-cell-dependent immune mechanisms, or regarding the correlation between the type of T-cell infiltration and tumor progression in most subtypes of breast carcinoma (Hsiao, et al., 2010). The only exception is the relatively infrequent type of medullary carcinoma, in which favourable prognosis was correlated with intensive lymphoid infiltration (Hadden, 1999). In contrast to T lymphocytes, large evidence suggests that high levels of TAM are correlated with poor prognosis in breast carcinoma. Many studies have shown a positive relationship between high levels of TAM and lymph node metastases, and suggested that the density of TAM is associated with clinical aggressiveness (Crowther, et al., 2001, O'Sullivan & Lewis, 1994). Again, the potential contribution of TAM to tumor elimination, in view of several potential antimalignant activities that may be exerted by these cells, such as antigen presentation, cytotoxicity, or/and phagocytosis, was contradictory with the promalignant activities of TAM in breast carcinoma. These promalignant activities of TAM are the result of their ability to express numerous tumor-promoting mediators, such as growth factors for breast tumor cells, angiogenic molecules, ECM degrading enzymes, inflammatory cytokines, and chemokines (Balkwill & Mantovani, 2001, Colotta, et al., 2009). In addition, TAM might contribute to tumor progression by the release of reactive oxygen intermediates, which may induce mutagenic changes that could result in increased DNA damage and generation of new subtypes of cancer cells within the tumor (Colotta, et al., 2009). A major TAM-derived

inflammatory cytokine shown to be highly expressed in breast carcinomas is tumor necrosis factor alpha (TNF-a) (Leek, et al., 1998), which is a multifactorial cytokine. Tumor necrosis factor alpha was first isolated as an anti-cancer cytokines more than two decades ago (Aggarwal, 2003). However, these effects may depend on multiple factors, such as estrogen therapy and the expression of members of the epidermal growth factor receptor family. The fact that TNF-a activities vary under different physiological conditions and in a cell-type-dependent manner contributes to a sense of ambiguity regarding its antitumor effects (Kanoh, et al., 2001, Offersen, et al., 2002). A number of reports indicate that TNF-a induces cellular transformation, proliferation, and tumor promotion (Balkwill & Mantovani, 2001, Li, et al., 2007). A interesting study reported that human TNF-a is more effective than the chemical tumor promoters okadaic acid and 12-O-tetradecanoylphorbol-13-acetato in inducing cancer (Komori, et al., 1993).

The number of cells expressing TNF-a in inflammatory breast carcinoma has been correlated with increasing tumor grade and node involvement (Ben-Baruch, 2003, Leek, et al., 1998). Furthermore, patients with more progressed tumor phenotypes were shown to have significantly higher TNF-a and IL-2 serum concentration (Tesarová, et al., 2000). The tumor-promoting functions of TNF-a may be mediated by its ability to induce pro-angiogenic functions, to promote the expression of matrix metalloproteinases (MMP) and endothelial adhesion molecules, and to cause DNA damage via reactive oxygen, the overall effect of which is promotion of tumor-related processes (Garg & Aggarwal, 2002).

In addition, several inflammatory interleukins have been linked with carcinogenesis and tumor progression. Among these, IL-6 and IL-1 have been widely studied in breast carcinoma. In different types of cancer, IL-1 promotes growth and confers chemoresistance (Arlt, et al., 2002, Woodworth, et al., 1995). Furthermore, IL-1 secretion into the tumor milieu also induces several angiogenic factors from tumor and stromal cells that promotes tumor growth through hyperneovascularization (Zhou, et al., 2011). IL-6 may act as a paracrine growth factor for multiple myeloma, non-Hodgkin's lymphoma, bladder cancer, colorectal cancer, and renal carcinoma (Angelo, et al., 2002, Landi, et al., 2003, Okamoto, et al., 1995, Voorzanger, et al., 1996). However, contradictory studies suggested that elevated levels of IL-6 might contribute to breast cancer progression (Karczewska, et al., 2000, Kurebayashi, 2000). Initial analyses regarding IL-1b indicated that its levels were significantly higher in invasive carcinoma than in ductal carcinoma *in situ* or in benign lesions, implying that elevated levels of IL-1b are directly correlated with a more advanced disease (Jin, et al., 1997). Of interest is the fact that the two cytokines (IL-6 and IL-1b) and TNF-a are interrelated and may act in an additive manner, suggesting that these three cytokines form a network of related factors that may affect tumor cell progression in a cooperative manner.

Cyclooxygenase (COX)-2, an inducible enzyme with expression regulated by NF-kb, mediates tumorigenesis. COX-2, the inducible isoform of prostaglandin H synthase has been implicated in the growth and progression of a variety of human cancers, and its expression can be induced by various growth factors, cytokines, oncogenes, and other tumor factors. IL-1 has been reported to upregulate COX-2 expression in human colorectal cancer cells via multiple signalling pathways (Liu, et al., 2003). COX-2 is expressed at an intermediate or high level in epithelial cells of invasive breast cancers (Chang, et al., 2005, Half, et al., 2002). Expression of COX-2 in breast cancer correlates with poor prognosis, and COX-2 enzyme inhibitors reduce breast cancer incidence in humans. COX-2 overexpression has also been found in the mammary gland of transgenic mice induced mammary cancer (Kundu & Fulton, 2002).

Hypoxia is also an important cellular stressor that triggers a survival program by which cells attempt to adapt to the new environment. This primarily involves adaptation of metabolism and/or stimulation of oxygen delivery. These cell-rescuing mechanisms can be conducted rapidly by a transcription factor that reacts to hypoxic conditions, the hypoxia-inducible factor-1 (HIF-1a) (Semenza & Wang, 1992). HIF-1a stimulates processes such as angiogenesis, glycolysis, and erythropoiesis (Jiang, et al., 1996) by activating genes that are responsible for these processes. Cancer cells are able to survive and proliferate in extreme microenvironmental conditions and show changes in oncogenes and tumor suppressor genes. Hypoxia and HIF-1a have been implicated in carcinogenesis and in clinical behaviour of tumors. Upregulation of HIF-1a was noted during breast carcinogenesis (Bos, et al., 2001) especially in the poorly differentiated pathway. Hypoxia is related to poor response to therapy in various cancer types. In invasive breast cancer, high HIF-1a concentrations were associated with poor survival in lymph node-negative patients (Bos, et al., 2003). As prognosis in breast cancer is closely related to proliferation rate (van Diest & Baak, 1991) and poorly differentiated tumors usually exhibit high proliferation and HIF-1a overexpression, the prognostic value of HIF-1a might well be explained by a close association between HIF-1a and proliferation. Additionally, HIF-1a has shown to be a master regulator for surviving hypoxia interacting with cell cycle-related proteins. High concentrations of HIF-1a are associated with overexpression of p53 and markers of proliferation during the late SG2 phase of the cell cycle (Bos, et al., 2004).

3. Role of chemokines and their receptors in breast cancer progression and metastasis

While most evidence presented above suggests that proinflammatory cytokines and enzymes play an important role in mediating tumorigenesis, and tumor progression, the molecular mechanisms of metastasis and its relationship with the organotropism of cancer cell remain unclear. However, recent studies focused on the chemokines and their receptors, and the different interactions with inflammatory cytokines in the tumor microenvironment have provided additional information that might better explain the non-random patterns of organotropism during metastasis, including atypical metastasis to rare organs (Franco-Barraza, et al., 2010, Valdivia-Silva, et al., 2009).

Chemokine activities in different malignancy including breast cancer are mediated primarily by their ability to induce chemotaxis of leukocytes, endothelial cells, and/or the tumor cells. Chemokines induce migration of leukocyte subpopulations to tumor sites that may promote antitumor activities (such as Th1 cells or natural killer cells), while other chemokines are responsible for large quantities of deleterious tumor-associated macrophages (TAM) at tumor sites (Allavena, et al., 2008, Ben-Baruch, 2008, Soria & Ben-Baruch, 2008) as discussed above. Moreover, specific chemokines upregulate endothelial cell migration and proliferation, and promoting angiogenesis, whereas other chemokines have powerful angiostatic properties (Strieter, et al., 2006, Struyf, et al., 2011). Another very important activity of chemokines is induction of tumor cell invasion and migration, thereby playing key roles in dictating site-directed metastasis formation (Ben-Baruch, 2008, Zlotnik, 2006). Chemokines and their receptors can execute such multifaceted roles in malignancy because cells of the tumor microenvironment, and in many cases also by the tumor cells themselves express them. As such, they can affect through autocrine pathways the ability of

the cancer cells to express tumor-promoting functions, and can also act in paracrine manners on host cells, thereby influencing their roles in malignancy.

Breast cancer metastasis is the result of several sequential steps and represents a highly organized, non-random and organ selective process dependent on intricate stroma-stroma interactions at the target organ (Ben-Baruch, 2006, Lu & Kang, 2007), causing high mortality by invasion of vital organs, such as bone, lung, brain and liver. Important evidence suggests that chemokines have an important role in regulating trafficking and metastasis (Bagley, et al., 2010). Indeed, breast cancer cells express chemokine receptors in a non-random manner, and these observations pointed to several chemokine/ receptor pairs that control cell–cell migration (Zlotnik, 2008). Association of chemokine receptors with various cancers including breast carcinoma has been widely documented (Ali & Lazennec, 2007, Karnoub & Weinberg, 2006, Koizumi, et al., 2007, Ruffini, et al., 2007). Accumulative evidence, in particular from clinical retrospective studies, presents a compelling picture indicating that the experimental evidence derived from *in vitro* experiments and animal models pointing to a pivotal role of chemokine receptors in cancer metastasis. CXCR4 and CCR7 are the most widely expressed in many different cancers, and the expression of CXCL12 and CCL21, their specific ligands, respectively, are highest in lung, liver bone marrow for the first one and lymph nodes for both (Nevo, et al., 2004, Schimanski, et al., 2008). Additionally, the expression of CCR7 in patients with several types of cancer has an excellent correlation with the ability of the tumor to spread to the lymph nodes (Takanami, 2003, Wang, et al., 2005). Other chemokine receptors may participate in the regulation of metastasis of specific cancers and in tumor progression. CX3CR1 is involved in homing metastasis to brain for glioblastoma and breast cancer (Andre, et al., 2010, Lavergne, et al., 2003) and to bone and bone marrow endothelial cell for prostate cancer (Shulby, et al., 2004). CCR9/CCL25 axis was found in melanoma (Letsch, et al., 2004), ovarian cancer (Johnson-Holiday, et al., 2007), prostate cancer (Singh, et al., 2004), nasopharyngeal carcinoma (Ou, et al., 2006), acute lymphoblastic leukaemia (Annels, et al., 2004) and probably breast cancer (Johnson-Holiday, et al., 2011); most of the cases are related to metastatic lesions in the gastrointestinal tract included the liver. Additionally, elevated expression levels of CXCR2 and CCR5 and their ligands, CXCL8 and CCL5, respectively, in breast carcinoma and other neoplasias were significant associated with increased malignancy, advance disease, early relapse and poor prognosis (Ben-Baruch, 2006, Yaal-Hahoshen, et al., 2006). Moreover, it has been demonstrated that tumor cells can generate autocrine gradients of ligands of chemokine receptors (i.e., CCR7) that guide their migration in direction of a physiological level of interstitial flow towards functional lymphatics, even if lymphatic endothelial cells are absent; although the effect is greatly amplified when both flow and cells are present (Shields, et al., 2007). This data suggests that the chemokine–chemokine receptor interaction is of particular importance in the metastatic destination of many cancers.

However, a couple of questions are very important to make in this point: Is the chemokine receptor expression in cancer cells constant? Or might the tumor microenvironment or inflammation regulate the chemokine receptor expression in cancer cells? Interestingly, these questions, which are product of logic thinking on the tumor microenvironment, were not made until recently by our group (Valdivia-Silva, et al., 2009). Indeed, the chemokine receptor expression has not been thoroughly studied under inflammatory conditions.

Although there are reports demonstrating that tumor and leukocytes increase expression of chemokines and cytokines during disease progression, it is not clear what are the chemokine

receptors involved in regulation of metastasis. Most of the previously reported studies had focused in analysing chemokine receptors expressed in different neoplasias without evaluating their phenotypic changes and functionality during the progress of the disease (Ben-Baruch, 2008). However, it has not been clearly demonstrated any type of regulation of the microenvironment in these changes. Finally, the chemokine receptors expressed under non-stimulated conditions by cancer cells were considered biomarkers to specific homing to organs, but it does not explained atypical metastasis of cancer to rare organs (Charalabopoulos, et al., 2004, Johnson, 2010, Kilgore, et al., 2007, Saisho, et al., 2005).

Within the tumor microenvironment, chemokines and their receptors play different roles in modulating several functions as described above, and through these processes, help to define the progression of the cancer. Stromal, and immune cells, including leukocytes differentiating into tumor-associated macrophages (TAM) at the tumor site, express numerous promoting factors, such as growth factors, angiogenic mediators, extracellular matrix-degrading enzymes, inflammatory cytokines, and more chemokines (Polyak & Kalluri, 2010). Interestingly, pro-inflammatory cytokines like IL-1, IL-6, IFN-g and TNF-a, which are important modulators of chemokine receptors expression in different tissues, have demonstrated to regulate their expression in cancer cells in a non–random manner (Valdivia-Silva, et al., 2009). Similar to cytokines regulate for CXCR4 and CCR5 in astrocytes (Croitoru-Lamoury, et al., 2003), CXCR2 in human mesangial cells(Schwarz, et al., 2002), and CX3CR1 in smooth muscle cells (Chandrasekar, et al., 2003), synovium (Nanki, et al., 2002), and different epithelial cells (Fujimoto, et al., 2001, Matsumiya, et al., 2001); different doses and times of exposition allowed the expression of specific type of chemokine receptor in several breast cancer cell lines and the change of their phenotypes into more invasiveness ones (Franco-Barraza, et al., 2010).

We have analysed the human breast carcinoma MCF-7 cell line as a model of pre-invasive stage to demonstrate the regulation by an inflammatory microenvironment on chemokine receptor expression and functionality (Valdivia-Silva, et al., 2009). The comparison of the expression of CXCR4, CX3CR1, CXCR2, CCR9 and CCR5 at the transcriptional, protein, and functional levels under two different *in vitro* conditions (basal versus cytokine- stimulation) showed clearly the regulation of the specific cytokine over specific chemokine receptor, independently of the genetic background of MCF-7, which presents very low levels of these receptors under basal conditions. This was also observed in the highly metastatic MDA-MB-231, MDA-MB-361 and in the poorly metastatic T47D breast cancer cell lines; although the levels of expression observed after cytokine stimulation were different than those obtained in the MCF-7 cell line. A direct suggestion of these results, affirms that basal expression of a given chemokine receptor is not by itself a good marker of homing or aggressiveness and is subject to change by the microenvironment. Another important outcome in that work was the absence of correlation between the functionality of the receptor and their expression (gen or protein). For example, an increase in CXCR2 expression in MCF-7 cell line does not correlates with an increase in the migration index. In contrast, CX3CR, induced by TNF-a, had a small but significant increase at the protein level, which had an impact on their chemotactic activity. A considerable increase of chemokine receptors was found in non-migratory cancer cells indicating that that chemokine receptor expression does not necessarily result in migration response to a chemoattractant ligand. It also suggests that only a fraction of the cells have the potential to form metastases and capable to invade different organs. In fact, genetic analysis of the MDA-MB-231 breast cancer cell line subpopulations, obtained from *in vivo* experiments, identified a gene set whose expression

pattern is associated to metastasis to bone but not adrenal medulla (Kirschmann, et al., 1999, Xu, et al., 2010). Interestingly this signature is retained through repeated passage of the metastatic cell population both in vitro and in vivo. Therefore, breast cancer cells with a defined tissue-specific metastatic ability pre-exist in the parental tumor cell population and may have a distinctive metastasis gene expression signature. Thus, these data suggested that inflammatory stimulation in the tumor microenvironment might affect cancer cells migration by different mechanisms. Importantly, not all cancer cell population, including cell lines, had the same behaviour under the same cytokine stimulation. Finally, other important finding in this study suggested that cancer cells require constant inflammatory stimuli by the microenvironment to trigger their invasive and metastatic activity, because of after a short time without stimuli (hours to days), the cells diminished their specific-stimuli chemokine receptor expression.

Altogether, these data allowed us to propose that exist sub-populations expressing different levels of chemokine receptor expression, which under a particular stimuli in the host microenvironment, change their expression levels and thus their aggressiveness. Then, atypical metastasis of breast cancer to others organs, which are relatively rare, could fall under this scheme. The biological inflammatory global response in the tumor microenvironment might be triggering the expression of different chemokine receptors and determining a new homing for these cancer cells. More broadly, these observations strongly support the overall model where chemokines determine the metastatic destinations of cancer cells (Fig 1.)

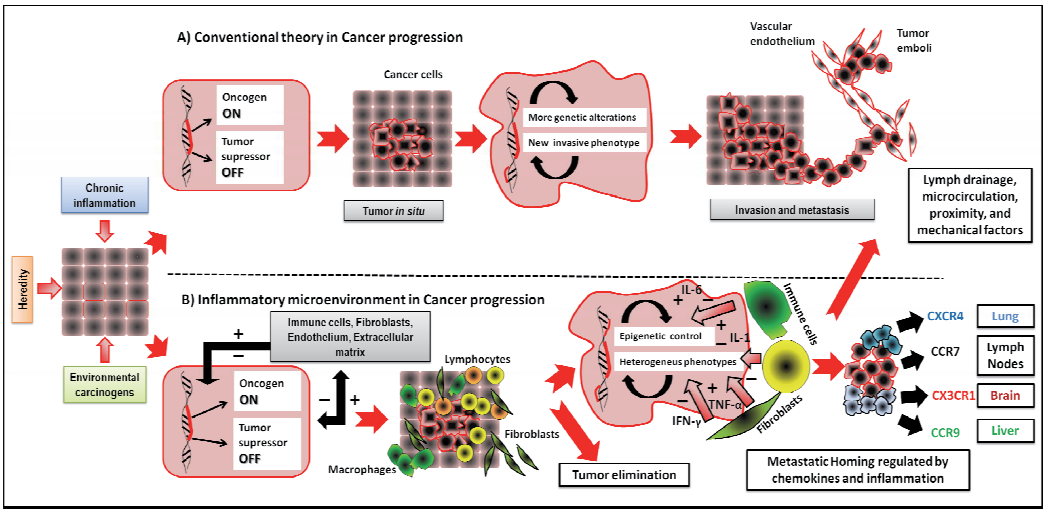

Fig. 1. Microenvironment and cancer progression.

Two theories have been proposed to explain this process, a conventional theory based on genetic alterations and a second view that involves participation of an inflammatory microenvironment. A) Initially, susceptible cells to different carcinogenic factors (e.g., genetic susceptibility obtained by inheritance) suffer specific DNA mutations that trigger tumorigenesis. The conventional theory is focused on the view that cancer progression is initially dependent on a sequence of genetic alterations and, finally, purely mechanical factors regulate the fate of blood-borne metastasis tumour cells (e.g. proximity,

microcirculation, direction of lymph or circulation drainage, etc.). B) A second view, based on the participation of an inflammatory microenvironment, takes into account constant interactions between tumor cells and surrounding cells during the different stages of cancer development. Therefore, the final response is the result of positive and negative effects and not only dependent on internal genetic changes in cancer cells but on interactions and epigenetic control of multiple inflammatory into molecules released into the tumor microenvironment. Therefore, the final metastatic homing, which is mediated by expression of chemokines and chemokine receptors, will be dependent on the deregulation of the host immune response

4. Targeting chemokines for breast cancer metastasis

As a consequence of studies focusing almost exclusively on cancer cells, nearly all of the currently used cancer therapeutic agents target the cancer cells that, due to their inherent genomic instability, frequently acquire therapeutic resistance (Rajagopalan, et al., 2003). In part due to frequent therapeutic failures during the course of treatment of advanced stage tumors, increasing emphasis has been placed on targeting various stromal cells, particularly endothelial cells, via therapeutic interventions. Since these cells are thought to be normal and genetically stable, they are less likely to develop acquired resistance to cancer therapy. Thus, isolating, and characterizing each cell type (epithelial, myoephitelial, and various stromal cells) comprising non-malignant and cancerous breast tissue would not only help us to understand the role these cells play in breast tumorigenesis, but would likely give us new molecular targets for cancer intervention and treatment.

There is now an abundant literature documenting the associations of chemokine receptors with various types of cancer (Zlotnik, 2006) and their importance to mediate the establishment or development of metastatic foci. In fact, some anticancer drugs currently in use -like Herceptin- may involve the downregulation of chemokine receptors as part of their mechanism of action (Li, et al., 2004). This would provide the ultimate validation of the hypothesis, and would also point to future opportunities for therapeutic intervention as we discussed below. Current therapies such as surgery, radiotherapy and chemotherapy are primarily concerned with destruction of cancer. Targeting chemokines and chemokine receptors will allow limiting angiogenesis or metastasis and may enable such therapies to act as chemotherapeutic agents alone or in synergism with conventional agents. The up-regulation of certain chemokine molecules in tumor as compared with normal cells offers a potential avenue—where cancer cells and their metastases can be specifically targeted. This selective destruction of cells is also pre-requisite of non-toxic treatment regimens.

Manipulation of the tumor microenvironment by treatment with chemokines can be used to recruit either immature dendritic cells for the initiation of anti-tumor responses or effector cells for cytotoxic responses. Intratumoral delivery of CCL21 using pox virus vaccine into established tumors derived from murine colon cancer line, CT26 results in enhanced infiltration of CD4 T cells which correlated with inhibition of tumor growth (Flanagan, et al., 2004). Non-immunogenic murine breast carcinoma is rejected after transducing cells with CCL19. The rejection of tumor was mediated by activated NK and CD4+ cells (Braun, et al., 2000). Adenoviral delivery of the CCL16 is able to inhibit growth of mammary tumors and prevent metastatic growth (Okada, et al., 2004). Importantly, in treatment involving delivery of chemokines to the tumor environment, there is a major problem of heterogeneity of the tumor cells. Chemokines may have dual effects, can be beneficial to one patient might be

harmful to another. However, this problem can be circumvented by chemokine typing every tumor prior to deciding on an appropriate therapy regime. They may be used as an adjunct to increase the efficacy of currently available therapies. Targeting specific chemokines can also modulate tumor infiltrating leukocytes or angiogenesis. High CXCL8 expression levels render tumor cells highly tumorigenic, angiogenic and invasive (Chavey, et al., 2007, Freund, et al., 2003, Freund, et al., 2004). In a murine model of breast cancer treatment with Met-CCL5, an antagonist of CCR1 and CCR5 led to a reduction in the total number of infiltrating inflammatory cells, in particular a decrease in macrophage infiltration and reduced growth of tumors (Liang, et al., 2004, Robinson, et al., 2003). The 7-transmembrane structure of chemokine receptors makes them attractive targets for small molecule inhibitors (Seaton, et al., 2009).

In summary, the exploration and manipulation of the chemokine network has just started and is likely to improve efficiency of current tumor therapies. However, since these chemotactic cytokines are also utilized in a plethora of normal interactions, caution is needed especially when extrapolating *in vitro* data into the clinical situation. Differences amongst tumor entities are obvious and the same chemokine/chemokine-receptor system seems to have divergent functions in different tumor entities. A more in-depth analysis of the real players in tumor immunosuppression, for example characterization of the subtypes of infiltrating immune cells and thorough analysis of the cytokine and chemokine milieu of primary tumors, will be necessary to pave the way for more efficient therapeutic interventions.

5. Tumor stroma: A permissive substrate for breast cancer development and progression

The stroma of carcinomas is an intricate ecosystem where heterogeneous cell populations coexist. This structural and functional connective tissue niche is inhabited by immune and inflammatory cells such as macrophages and monocytes, mesenchymal bone marrow-derived stem cells, endothelial and pericyte cells, lipocytes, additional smooth muscle cells and activated fibroblastic cells known as myofibroblasts, which are believed to be responsible for producing and maintaining the altered extracellular matrix (ECM) (Beacham & Cukierman, 2005; Li et al., 2007; Xouri & Christian, 2010). It is well accepted that the altered and excessive deposition of ECM, which is part of a process named desmoplasia, is directly associated with rapid progression and bad prognosis in carcinomas such as breast, pancreas, colon and prostate to name a few (Beacham & Cukierman, 2005; Arendt et al., 2010; Franco et al., 2010). In fact, we and others have suggested that stroma progression could be staged (analogously to classic tumor staging) into discrete stromagenic stages (Bissell et al., 2002; Mueller & Fusenig, 2002; Beacham & Cukierman, 2005; Quiros et al., 2008; Castello-Cros et al., 2009). Briefly, under normal (i.e., homeostatic) conditions, the breast stroma maintains the tissue architecture where a specialized ECM rich in collagen IV and laminin-1 known as basement membrane (BM) demarks a barrier between epithelium and the mesenchyme (Gudjonsson et al., 2002). A particular feature of the glandular epithelium in breast tissue is that both alveolar and ductal epithelial cells are not in direct contact with the BM. Instead, they are supported by a monolayer of myoepithelial cells that resides in between. Myoepithelial cells play an important role in supporting epithelial cell differentiation and controlling proliferation and cell polarity. These cells secrete the BM proteins and together with adjacent stromal fibroblasts maintain the integrity of this

specialized gland (Gudjonsson et al., 2002; Polyak & Kalluri, 2010). Under physiological conditions, a normal stroma preserves and drives regular breast tissue morphogenesis (Kuperwasser et al., 2004) and, at the same time, suppresses the transformation of epithelial cells thus preventing the development of breast carcinoma *in situ* (CIS) and inhibiting progression towards invasive cancer (Hu et al., 2008). Although not much information is available to describe the mechanistic events responsible for normal stroma prevention of carcinoma progression, recent data suggests that the tumor microenvironment lacks the regulatory mechanisms that are necessary to maintain a normal epithelial phenotype (Postovit et al., 2008). As shown by interesting work conducted by Mintz and Illmensee in 1975 where they observed that a normal embryo microenvironment is repressive of teratoma tumorigenesis (Mintz & Illmensee, 1975), more recent work by Postovit *et al* looking at specific human embryonic stem cells-secreted factors also concluded that embryonic microenvironments can control and sustain a normal behaviour of invasive tumor cells (Postovit et al., 2008). In summary, one could state that the normal stroma is a natural barrier or a non-permissive environment for tumor progression.

In an effort to understand premature events that occur during stroma progression (i.e., stromagenesis (Cukierman, 2009)), researchers have used animal models where they have shown stromal cells alterations at early stages of tumorigenesis. For example, prostate smooth muscle cells, known to support homeostasis and epithelium differentiation and considered to be analogous to normal myoepithelial cells in breast, have been shown to undergo alterations during early tumorigenesis (Wong & Tam, 2002). Similar to myoepithelial cells, smooth muscle cells are also lost in advanced stages of tumor progression, but prior to this they lower the expression levels of differentiation markers such as myosin, desmin, and laminin (Wong & Tam, 2002). This fact strongly suggests the advent of a discrete intermediate state between normal and activated stroma. To this end, the up-regulated expression of proteins, such as fibroblast activation protein, has been suggested as potential markers of this intermediate or primed stromal stage (Mathew et al., 1995; Huber et al., 2003; Santos et al., 2009). Another such molecule is tenascin-C, an ECM protein expressed in breast cancer at early stages of the tumorigenesis, which has been shown to have a diagnostic value (Adams et al., 2002; Guttery et al., 2010).

Once the stroma becomes activated, many histological features are evident. This stage is commonly described by pathologists as desmoplasia and is characterized by increased interstitial ECM-deposition. The desmoplastic ECM is believed to be produced by a highly proliferating fibroblastic and alpha-smooth muscle actin (α-SMA) expressing myofibroblastic cell population. It is common in many cancers including breast, and it can constitute up to 50% of the tumor mass (Kunz-Schughart & Knuechel, 2002a, b; Desmouliere et al., 2004). The altered architecture of the desmoplastic stroma reaction is characterized by the over expression of ECM proteins such as collagen I and differential spliced fibronectin isoforms such as EDA and EDB (Matsumoto et al., 1999; Desmouliere et al., 2004). The desmoplastic ECM is highly organized in a parallel fiber pattern, which is clearly oriented *in vivo* perpendicular to the tumor border (Provenzano et al., 2006). In fact, this particular feature of the tumor associated-ECM (TA-ECM) has been suggested to facilitate migration of breast cancer cells *in vitro*, in a β1-integrin dependent manner (Castello-Cros et al., 2009). Moreover, there is evidence to suggest that TA-ECM can induce a phenotypic switch upon naïve fibroblasts thus inducing a myofibroblastic (or activated) conformation (Amatangelo et al., 2005). To this end, in a xenograft model of human breast cancer, it was shown that

activated fibroblasts influence the local microenvironment to promote invasion (Orimo et al., 2005; Hu et al., 2008).

6. Tumor- or carcinoma-associated fibroblasts: A bad myofibroblastic influence

Fibroblasts are the main cellular component of the stroma and responsible for producing the mesenchymal (i.e., interstitial) ECM. These cells have been described as non-epithelial, non-inflammatory and non-vascular semi-differentiated connective tissue cells (Tarin & Croft, 1969). They are best known for their role in maintaining the tissue's integrity while they become quickly activated (e.g., myofibroblastic) and can modify the plasticity of the resident's tissue under conditions that alter the homeostatic equilibrium such as during wound healing, organogenesis, cancer and other pathological and inflammatory conditions (Kalluri & Zeisberg, 2006) . In fact, fibroblasts are known as tissue remodelers capable of renovating ECMs while, at the same time, facilitating access to ECM stored growth factors, such as transforming growth factor-beta (TGF-b), through a tightly regulated release and activation of matrix digestive enzymes such as matrix metalloproteinases (MMPs) (Jodele et al., 2006).

The fibroblastic cell population, known as carcinoma-associated fibroblasts (CAFs) or tumor-associated fibroblasts (Barsky et al., 1984), presents a myofibroblastic phenotype that is very similar to the one observed in activated fibroblasts during wound healing (Barsky et al., 1984). CAFs are the main stromal cell component of solid epithelial carcinomas (Shao et al., 2000). In addition to a characteristic, high proliferation rate and increased ECM deposition, the development of contractile cell features affects the physico-chemical characteristics of TA-ECM (Tomasek et al., 2002; Butcher et al., 2009; Cukierman & Bassi, 2010). Interestingly, CAFs are capable of establishing interactions with inflammatory, endothelial, and tumor cells by means of cytokines/chemokines secretions such as interleukin (IL)-1β, IL-6, CXCL-8, stromal derived factor-1 (SDF-1), also known as CXCL-12, and the monocyte chemotactic protein (MCPs/CCLs) among others (Silzle et al., 2004; Mishra et al., 2011). In an effort to find a discrete set of CAF specific markers, proteins such as γ- and α-SMA (Brouty-Boye et al., 1991; Kunz-Schughart & Knuechel, 2002b; Desmouliere et al., 2004; Xouri & Christian, 2010) specific isoforms of the actin binding protein palladin, (Ronty et al., 2006; Goicoechea et al., 2010; Gupta et al., 2011) as well as the intermediate filament proteins vimentin and desmin (Schmid et al., 1982) have been suggested. Furthermore, the specific breast cancer microenvironmental niche has been shown to contain increased levels of expression of ECM stabilizing (e.g., cross-linking) enzymes such as prolyl-4 hydroxylase (Orimo et al., 2005) and lysyl oxidase (Chang et al., 2005; Levental et al., 2009; Barry-Hamilton et al., 2010). Additional proteins have been shown to be specifically overexpressed at the tumor-associated stroma such as fibroblast activation protein (LeBeau et al., 2009; Lee, 2011), endosialin (Becker et al., 2008; Christian et al., 2008) S100A4 (Ambartsumian et al., 1996; Ryan et al., 2003; Katoh et al., 2010), and a plethora of MMPs, among others (Rasanen & Vaheri, 2010). In fact, some of these have already been proposed to serve as stromal monitoring or prognostic markers (Erkan et al., 2008; Gupta et al., 2011).

Nevertheless, this hardly consistent signature of myofibroblastic markers strongly suggests that the tumor stroma is a heterogeneous milieu (Sugimoto et al., 2006). The variety of

myofibroblastic phenotypes is also suggestive of the eliciting of different roles played by these cell populations at the tumor stroma. Interestingly, this heterogeneity could have been originated (i.e., differentiated) by the multiple cell lineages known to produce myofibroblastic CAFs. These are: local fibroblasts (Kalluri & Zeisberg, 2006), bone marrow recruited mesenchymal cells (Ishii et al., 2003; Goldstein et al., 2010), as well as endothelial and tumor (i.e., epithelial) cells (Petersen et al., 2003; Kalluri & Zeisberg, 2006; Zeisberg et al., 2007), among others. In all these cases, TGF-β has been closely associated with tumor-induced myofibroblastic activation or differentiation (Zeisberg et al., 2007; Hinz, 2010; Taylor et al., 2010). The myofibroblastic differentiation is a complex and not yet fully understood process that is believed to play a central role during breast tumorigenesis (Cukierman, 2004; McAllister & Weinberg, 2010). Even though a plethora of molecules has been implicated in regulating fibroblastic activation, the specific desmoplastic response in breast cancer is believed to be driven by four main groups of inducers; i) growth factors, ii) TA-ECM, iii) acute inflammation and iv) microenvironmental stress denoted by nutrient and oxygen depravation as well as low pH.

i. Specific growth factor presence at the tumor microenvironment may constitute the most studied aspect believed to trigger a myofibroblastic switch of the otherwise quiescent homeostatic fibroblasts. Determined mainly *in vitro* by an increment in proliferation rate, induction of α-SMA expression, and an up-regulation of ECM components, the growth factors most commonly implicated in this process are TGF-α, TGF-β, insulin-like growth factors I and II (TGF-I and TGF-II), the platelet-derived growth factor (PDGF), and the basic fibroblast growth factor (bFGF) (Beacham & Cukierman, 2005; Kalluri & Zeisberg, 2006; Rasanen & Vaheri, 2010; Xouri & Christian, 2010). Although many questions remain regarding specific triggers for breast cancer desmoplasia, work from Walker and Dearing implicated TGF-β_1, TGF-β_2 and TGF-β receptor as vital contributors of breast tumorigenesis associated with a stromal increment of fibronectin and tenascin in the tumor stroma (Walker & Dearing, 1992; Walker et al., 1994). Moreover, TGF-β known to induce myofibroblastic differentiation and to increase collagen I deposition during the wound healing process (Desmouliere et al., 2005), has also been implicated as a main factor in inducing breast cancer associated bone marrow-derived myofibroblasts differentiation (Goldstein et al., 2010). Similarly, PDGF has been shown to increase the breast myofibroblastic population by 30% while greatly increasing the amount of interstitial collagen I *in vivo* (Shao et al., 2000). In the context of epithelial to mesenchymal transition (EMT)-derived myofibroblasts, hepatocyte growth factor (HGF) and epidermal growth factor (EGF), in addition to the above-mentioned PDGF and TGF-β, have also been implicated (Mimeault & Batra, 2007; Kalluri & Weinberg, 2009).

ii. Breast TA-ECMs' features are known to become altered in both their molecular composition (Chen, S.T. et al., 2008; Levental et al., 2009; Ronnov-Jessen & Bissell, 2009) and their architectural characteristics (Provenzano et al., 2006). Together these two altered features can modulate tumorigenic behaviours of cancer cells and promote or delay the evolution of carcinomas in a permissive or restrictive manner (Ronnov-Jessen & Bissell, 2009; Cukierman & Bassi, 2010). In addition, it has been suggested that the physico-chemical characteristics of the ECM also affect the behaviour of mesenchymal cells (Discher et al., 2005). Fibroblasts are influenced by stromal stiffness, which exerts mechanical forces that modulate their cell behaviour. Thus, it has been demonstrated

that as the substrate stiffness increases, fibroblastic cells change exhibiting three discrete phenotypic switch stages: normal or naive fibroblasts, intermediate or proto-myofibroblastic and activated myofibroblastic (Hinz, 2010). The phenotype transition induced by the increased tension in the substratum is also accompanied by the maturation or elongation of focal adhesions, together with cytoskeletal changes known to build-up contractile stress fibers (Hinz, 2010). Interestingly, studies of normal breast revealed a relatively limp tissue composition (0.15 kPa, expressed in *E* values of a Young modulus) compared to the stiffer and highly desmoplastic ~4 kPa tissue that has been affected by breast cancer (Butcher et al., 2009). The altered (i.e., myofibroblastic) phenotype of fibroblasts is linked to the stiffer ECM during tumor progression as these cells are responsible for the production of the TA-ECM (Cukierman & Bassi, 2010). Indeed increments of mammographic density, suggesting excessive collagen deposition, have been associated with higher risk in breast cancer (Boyd et al., 1998). Moreover, increases in cross-linked collagen due to over expression of LOX together with patterned linearization of the TA-ECM and specific ECM receptor, integrin, clustering and enhanced phosphoinositide 3-kinase (PI3K) activity, have all been correlated with breast cancer progression (Levental et al., 2009). Additionally, it has been shown that the interstitial ECM can function as a reservoir for diffusible molecules, such as the above-mentioned TGF-β which is secreted by both stromal and tumor cells in its inactivated form (Wipff & Hinz, 2008), but can be both activated and released due to the intrinsic myofibroblastic forces that increase the tension of TA-ECM's fibrils (Wipff et al., 2007; Tenney & Discher, 2009).

iii. Recently, an inflammatory microenvironment has been suggested as the seventh hallmark of cancer (Colotta et al., 2009). This cancer hallmark is also believed to play an important role in desmoplasia as a fibroblast phenotypic-switch activator. To this end, it has been demonstrated that stromal inflammatory responses that result from wounding can trigger tumorigenesis (Arwert et al., 2010). The importance of an inflammatory component has also been suggested for the breast cancer stroma (Hu & Polyak, 2008), and its repercussion in inducing or promoting cancer aggressiveness and metastasis has been highlighted in numerous occasions (Pantschenko et al., 2003; Elaraj et al., 2006; Valdivia-Silva et al., 2009; Franco-Barraza et al., 2010; Goldberg & Schwertfeger, 2010). However, our current knowledge regarding fibroblastic responses to inflammatory cytokines in breast cancer remains relatively modest. Work conducted at the Polyak laboratory suggested that cytokines could participate in triggering a fibroblast phenotypic switch at the breast cancer microenvironment (Hu et al., 2009). This work and the work of others has opened up the possibility of targeting inflammatory cytokines for the treatment of neoplasias as in the case of COX-2 and arachidonic acid inhibitors (Chen, X. et al., 2006; Hu et al., 2009). In fact, in the kidney, it has been shown that collagen I regulates COX-2 expression in a pro-proliferative type of response (Alique et al., 2011). Interestingly, CAFs are known to promote inflammation in an NFκ-b dependent manner, suggesting a vicious cycle between inflammation and stromal activation during tumorigenesis (Erez et al., 2010). Moreover, it has been shown that CAFs effectively suppress anti-tumor inflammation while, at the same time, maintaining acute inflammatory (pro-tumor) conditions (Kraman et al., 2010).

As established before, the cytokine/growth factor TGF-β imparts a pleiotropic and decisive role in the promotion of the desmoplastic tumor microenvironment thus

supporting tumor progression (Yang et al., 2010). In addition, this same factor plays an additional important stromal role in inducing the expression of NADPH oxidase family protein, Nox4 (Bondi et al., 2010). Nox4 is a potent regulator of reactive oxygen species (ROS) (Barnes & Gorin, 2011) and has been shown to induce the accumulation of ROS in damaged tissues while transactivation of fibroblasts into myofibroblasts (Cucoranu et al., 2005; Rocic & Lucchesi, 2005). In breast cancer, the oxidative stress present at the tumor stroma is also considered to be an inductor for myofibroblastic differentiation, as recently shown in a JunD deficient mouse model, where the absence of this transcription factor allowed the accumulation of Ras-mediated production of ROS with the subsequent conversion of fibroblasts into myofibroblasts and shortening of the tumor free survival rate (Toullec et al., 2010).

iv. It is well known that as tumors progress increased regions of nutrient deprivation, low pH and low oxygen tension (hypoxia) are evident. Under these hypoxic stress conditions, breast cancer tissues are known to up-regulate the expression of hypoxia-inducible family (HIF) genes such as HIF-1α (Chen, C.L. et al., 2010). HIF proteins are known to participate in many cellular events such as angiogenesis, through the induction of vascular endothelium growth factor (VEGF), angiopoietin-2, PDFG and FGF (Allen & Louise Jones, 2011) which in turn can also activate stromal myofibroblastic differentiation in breast cancers (Shao et al., 2000). Finally, other molecules known to be induced by HIF-1α in carcinomas (and other fibrotic conditions) are the above mentioned ECM-cross-linkers (i.e., LOX) which have been associated with aggressive breast tumorigenesis (Chang et al., 2005; Levental et al., 2009; Barry-Hamilton et al., 2010).

7. Fibroblasts as moderators of signals at the tumor microenvironment

At the tumor microenvironment, intercellular communications resemble a social network emitting signals (either static or diffusible molecules) that in turn are collected, processed and emitted to additional cells. Using this analogy, it seems that CAFs play a decisive role during cancer progression acting as microenvironment signals moderators that sense extracellular signals and, after intracellular processing, emit new ones that in turn modulate both stromal and neoplastic neighbouring cells' behaviours (Bhowmick et al., 2004). In fact during cancer progression, CAFs constitute a very important source of the exogenous stimulants such as the above-mentioned TGF-β (Kalluri & Zeisberg, 2006). To this end, using an elegant humanized stromal reconstruction model of human breast cancer in mouse, Kuperwasser *et al* demonstrated that CAFs facilitate tumor development in a fibroblastic TGF-β- and HGF-dependent manner (Kuperwasser et al., 2004). Additionally, recent findings have demonstrated that epigenetic changes induced by mesenchymal cells on breast cancer cells that are regulated by the TGF-β/TGF- βR/Smad2 signalling axis provoke the silencing of critical epithelial genes resulting in the pro-tumorigenic EMT process (Papageorgis et al., 2010). To this end, in support of the above proposed vicious cycle effect, it is interesting to note that following quiescent fibroblasts transdifferentiation into CAFs, these cells support an invasive phenotype of mammary carcinomas where they secrete inflammatory cytokines (Powell et al., 1999; Buckley et al., 2001; Silzle et al., 2004) thus activating NF-κb and promoting EMT as well as promoting aggressiveness of breast cancer cells (Sullivan et al., 2009; Wu et al., 2009). An ever more complicated interplay between CAFs, cytokines and neoplastic cells has recently been proposed in breast cancers where,

due to the presence of an altered TA-ECM, an integrin-dependent activation of Src family kinases results in the increase of NF-κB activity which blocks the production of certain microRNAs such as Let-7. Under these conditions, IL-6 production is promoted resulting in the increased secretion of this pro-tumorigenic cytokine, which in turn induces or promotes a positive feedback in tumor cells (Iliopoulos et al., 2009). Moreover, activated myofibroblastic and cancer cells are known to remodel the stromal ECM by means of increased secretion of MMPs and urokinase-type plasminogen activator (uPA). These enzymes cleave the ECM molecules to release fragments that contain chemotactic properties called matrikines that activate leukocytes to also release inflammatory cytokines (Maquart et al., 2004; Silzle et al., 2004). For example, a special feature of MMP-2, -3 and -9 is that these proteases can increase the availability of IL-1b at the tumor microenvironment by cleavage of the pIL-1b (immature IL-1b) (Schonbeck et al., 1998). Also, analyses of co-cultures containing both breast cancer cells and CAFs have shown increases in stromal MMP-2 and MMP-9 expression (Singer et al., 2002). These observations concur with observations stemming from an immunohistochemical study where tissue arrays of breast cancer patients showed that intratumor stromal fibroblasts express MMP-2, -7, and -14, while fibroblast at the invasive front highly express MMP-9. What is more, this specific profile of stromal MMPs staining was found to be a predictor of future distant metastases occurrences (Del Casar et al., 2009). Another uncovered effect of released MMPs into the tumor stroma is the capacity of these molecules to promote a permissive environment that supports epithelial tumorigenic progression including the promotion of genomic alterations (Radisky, E.S. & Radisky, 2007). In the mammary glands of transgenic mice, the overexpression of MMP-3 has been shown to be sufficient to stimulate myofibroblastic presence, increased fibrosis, epithelial hyperplasia, and development of mammary carcinoma (Thomasset et al., 1998). What is more, mammary epithelial cells exposed to stromal MMP-3 showed activation of a genotoxic metabolic pathway, where the over expression of the spliced variant Rac1b produced DNA-damaging superoxide radicals and induced EMT (Radisky, D.C. et al., 2005). Interestingly, the epithelial genomic alterations induced by stromal MMPs *in vitro,* suggest a possible mechanism to understand the presence of areas with genomic imbalance patterns detected in histologically normal tissues adjacent to the tumor stroma (Ellsworth et al., 2004; Holliday et al., 2009).

8. Targeting fibroblasts as an anti-cancer therapy

Various aspects of the tumor microenvironment have been explored as putative therapeutic targets in the fight against cancer (Andre et al., 2010; Cukierman & Khan, 2010; Allen & Louise Jones, 2011). Since a desmoplastic reaction is an ECM component-rich substratum and some of the TA-ECM components are believed to be specific for discrete types of carcinomas, they constitute a promising basis for therapeutics (i.e., inhibitory functional antibodies). For example, in glioblastoma patients an iodine-131 radiolabeled anti-tenascin-C monoclonal antibody has produced encouraging results in phase II trials (Reardon et al., 2006). Similarly, the development of radioactive or bioactive molecules coupled to antibodies against TA-ECM specific EDB, the L-19 antibody, showed encouraging results when tested in various carcinomas (Kaspar et al., 2006). The TA-ECM has been considered as both a target as well as a means to attract anti-tumoral drugs. For example, as albumin binds efficiently to the TA-ECM protein osteonectin (also known as SPARC), known to be upregulated in a plethora of cancer stromas and often associated with bad prognosis (Tai &

Tang, 2008), paclitaxel delivered through nanoparticles conjugated to albumin (nab-paclitaxel) are being tested (Vishnu & Roy, 2010; Robert et al., 2011; Volk et al., 2011). Moreover inhibition of the serine protease activity of the CAF specific fibroblast activation protein has been suggested as a therapeutic target in a plethora of cancers including breast (Mersmann et al., 2001). In fact, antibodies against fibroblast activation protein induced a marked decrease in desmoplastic collagen I expression resulting in an increased (up to 70%) increment in chemotherapeutic drugs uptake (Loeffler et al., 2006). Therefore, it is not surprising that fibroblast activation protein has been suggested as a tumor targeting molecule for the delivery of peptide protoxins (amongst others) thus diminishing non-tumoral side effect toxicities (LeBeau et al., 2009).

Pro-inflammatory molecules have also been used as effective targets. For example TNF-α antagonists have been shown to have good results preventing disease acceleration in a considerable number of breast cancer patients (Madhusudan et al., 2004; Brown et al., 2008). The SDF-1α/CXCR4 chemokine axis has been proposed as a general target for anticancer strategies (Guleng et al., 2005), and recently a compound derived from marine organisms that blocks CXCR4 has been shown effective as well (He et al., 2008). Antibodies blocking the TGF-β signalling pathway have been developed and showed promising synergistic effects when added to known chemotherapeutics and, thus, have been regarded as anti angiogenesis-depending tumor stromal agents in breast cancer (Takahashi et al., 2001). Finally, it was recently shown that eliminating pro-tumorigenic macrophages in pancreas causes desmoplastic shrinkage and subsequent tumor stalling (Beatty et al., 2011). We believe that these types of treatments, together with similar novel ones, could provide increased hope in the common fight against breast cancers.

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8

Interleukin-6 in the Breast Tumor Microenvironment

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1. Introduction

Greater than 200,000 new cases of breast cancer cases were diagnosed in 2010 in the United States, with approximately 40,000 women succumbing to the disease (www.cancer.gov). Globally, an estimated 1.38 million new cases of breast cancer were diagnosed in 2008, with greater than 450,000 women succumbing to the disease (Jemal *et al.*, 2011). Despite our improved understanding of breast carcinogenesis, breast cancer remains the second most commonly diagnosed cancer in women behind non-melanoma skin cancer and the second leading cause of death in women behind lung cancer. These epidemiological statistics highlight the overwhelming clinical dilemma of breast cancer and emphasize the need for novel therapeutic targets and prevention strategies. Countless studies in the fields of mammary gland development and breast cancer have led to an appreciation of a breast tumor microenvironment that actively contributes to the heterogeneous nature of breast cancer. The current review will focus on the impact of IL-6 and STAT3 activation in the breast tumor microenvironment and subsequently present rationale for targeting the IL-6/STAT3 signaling pathway in this setting. IL-6 is a quintessential pleiotropic cytokine produced by a diverse number of cell populations, most of which can localize to the breast tumor microenvironment. Excessive IL-6 has been demonstrated in primary breast tumors and breast cancer patient sera and is associated with poor clinical outcomes in breast cancer. These clinical associations are corroborated by emerging preclinical data revealing that IL-6 is a potent growth factor and promotes an epithelial-mesenchymal (EMT) phenotype in breast cancer cells to indicate that IL-6 in the breast tumor microenvironment is clinically relevant. Numerous clinical reports have now demonstrated the safety and efficacy of IL-6 signaling antagonists in multiple diseases, which supports future investigations of these therapies in breast cancer.

Estrogen receptor-alpha (ERα) is a latent cytoplasmic ligand-activated transcription factor utilized by clinicians to subclassify the heterogeneous disease of breast cancer. ERα-positive breast cancer incidence increases up to age 51, the mean age of menopause, and continues to increase until age 80. Conversely, ERα-negative breast cancer incidence plateaus and even slightly decreases at age 51, while demonstrating an increase prior to age 50 comparable to that of ERα-positive disease. This discrepancy between the two incidence rates at menopause produces an inflection in the incidence rate of all breast cancer cases which has been termed Clemmesen's hook (Anderson and Matsuno, 2006). Whereas the prevalence of

ERα-positive cells within terminal duct lobular units of the breast of healthy premenopausal women has been reported at 7%, this number is estimated at 42% in postmenopausal women (Shoker *et al.*, 1999). In addition, approximately two-thirds of all breast cancers are diagnosed as ERα-positive, and 75% of postmenopausal breast cancers are ERα-positive (Macedo *et al.*, 2009). Progesterone receptor (PR) and epidermal growth factor receptor 2 (EGFR2; HER2; or ErbB2), a receptor tyrosine kinase involved in cellular proliferation, have also acquired much clinical attention following reports of dismal survival rates in "triple negative" (ERα-negative/PR-negative/HER2 not overexpressed) breast cancer patients. Triple negative breast cancer represents approximately 15 to 20% of all breast cancer cases and can only be treated with standard chemotherapy as it lacks current adjuvant therapeutic targets. Such breast tumors are highly proliferative with a high mitotic index, increased necrosis, elevated apoptosis, and typically are of higher tumor grade. *TP53* gene and p53 protein mutations as well as loss of the Rb tumor suppressor protein are common. Familial breast cancer patients with congenital BRCA1 mutations often present with triple negative breast cancer, as do relatively younger breast cancer patients and African American women. Currently, triple negative breast cancers are associated with a poor prognosis largely due to poor survival rates and early relapse. The fact that these breast tumors respond well if not completely to initial chemotherapy may seem counterintuitive, but enhanced invasiveness, consequent distant metastasis, and residual local recurrence eventually promote poor survival rates (Irvin and Carey, 2008).

Breast cancer most commonly metastasizes to bone, followed by lung, liver, and brain. Perhaps due to the heterogeneity across individual breast cancer cases, few prognostic molecular biomarkers have been demonstrated to accurately predict metastatic potential. One of the most important of these biomarkers is ERα, which is clinically exploited as a predictor of bone metastasis (Kominsky and Davidson, 2006). Whereas ERα-positive breast cancers have a strong tendency to metastasize to bone if at all (James *et al.*, 2003), their ERα-negative counterparts favor visceral organs such as lung and liver (Hess *et al.*, 2003). Primary mammary tumor cell dissemination has been quantified at 3 to 4 x 10^6 primary tumor cells in circulation per 24 hours per gram of tumor in a rat mammary carcinoma model, which exemplifies the inefficient nature of metastasis (Butler and Gullino, 1975). Although metastasis has been generally accepted as a relatively late event throughout cancer progression, recent work has revealed evidence of early primary tumor cell dissemination, thus refuting this paradigm (Klein, 2009). In particular, it has now been demonstrated that untransformed triple transgenic (doxycycline-inducible K-ras, MYC, and polyoma middle T antigen) mammary epithelial cells are capable of lung colonization when tail vein-injected into immunocompromised female mice on doxycycline. This work showed that untransformed "normal" mammary epithelial cells can colonize ectopic lung tissue, and upon oncogene activation, disseminated mammary epithelial cells within circulation or a foreign host microenvironment are capable of forming tumors at the ectopic site (Podsypanina *et al.*, 2008). Additionally, reports of bone marrow cytokeratin-positive epithelial cells in up to 48% of breast cancer patients without overt metastases also offer support for early primary tumor cell dissemination. Decreased survival in patients with such cells was demonstrated in all studies (Braun *et al.*, 2000; Diel *et al.*, 1996; Gebauer *et al.*, 2001; Pantel *et al.*, 2003; Vannucchi *et al.*, 1998). Furthermore, only 8% of these patients with bone marrow micrometastases exhibited cytokeratin-positive/Ki67-positive cells, suggesting that lack of overt bone metastasis may be due to disseminated tumor cell dormancy (Pantel *et al.*, 2003).

2. The breast tumor microenvironment

A normal epithelial tissue can undergo hyperplasia and acquire tumorigenic properties that promote the development of a benign, non-invasive solid tumor known as carcinoma *in situ*. Normal epithelial tissues and non-invasive carcinoma *in situ* tumors are separated from a supportive stromal compartment by an intact basement membrane. Ultimately, carcinoma *in situ* can progress to a malignant, invasive carcinoma, the most common form of human cancer. The panoply of published investigations between the fields of mammary gland development and breast cancer has led to an appreciation for a supportive non-epithelial mammary stroma that mechanically and biologically restrains tumorigenesis. However, tumors of the breast and other epithelial tissues obviously overcome these growth restraints and exploit this stroma to sculpt a vastly divergent tumor stroma. Tumor stroma is generally divided into four main components: tumor vasculature, inflammatory leukocytes, extracellular matrix (ECM) and soluble growth factors, and fibroblasts. Malignant carcinoma cells and tumor stromal cells bi-directionally communicate with one another through paracrine signaling and intercellular contacts in a disorganized ECM to constitute a tumor microenvironment. Tumor-associated fibroblasts (TAF), the predominant stromal cell population within the tumor microenvironment, acquire and sustain an "activated" phenotype that promotes tumor progression (Rasanen and Vaheri, 2010). TAF are capable of enhancing breast tumor growth and metastasis by means of promoting angiogenesis (Orimo *et al.*, 2005), epithelial-mesenchymal transition (EMT) (Martin *et al.*, 2010; Radisky *et al.*, 2005), and progressive genetic instability (Kurose *et al.*, 2001; Moinfar *et al.*, 2000). In contrast, a normal mammary microenvironment can act in a dominant manner to inhibit tumor growth and "revert" the malignant phenotype of breast cancer cells (Kenny and Bissell, 2003). While resident breast tissue fibroblasts can inhabit breast tumors as TAF, breast tumors also recruit distant cell populations that engraft within the breast tumor microenvironment where they actively contribute as TAF. For example, mesenchymal stem cells (MSC), a bone marrow-derived stromal cell population, home to breast cancer cell xenograft tumors and persist as TAF (Spaeth *et al.*, 2009).

3. Cancer-associated inflammation

Although highly characterized for their protective capacity against infection, inflammatory leukocytes also reside within the tumor microenvironment. In fact, various immune cells are capable of eliminating transformed cells and thus preventing tumorigenesis in a process termed immunosurveillance (Dunn *et al.*, 2004). Whereas acute inflammation may prevent tumorigenesis by promoting an immune response directed against transformed cells, chronic inflammation promotes tumorigenesis. Rudolf Virchow is credited with making the seminal link between chronic inflammation and cancer by noting that human tumor biopsies were often infiltrated with inflammatory cells (Balkwill and Mantovani, 2001). Leukocytes can be detected in non-malignant tumors and carcinomas, including breast cancer (DeNardo and Coussens, 2007), which suggests an ongoing antitumor immune response. Despite the infiltration of leukocytes such as cytotoxic T-cells and NK-cells, the persistence of a tumor demonstrates immune evasion and highlights the local and systemic immune suppressive state of the tumor microenvironment and the tumor-bearing host, respectively.

4. Interleukin-6: A quintessential pleiotropic cytokine

Interleukin-6 (IL-6) is an inflammation-associated cytokine and major inducer of C-reactive protein (CRP) throughout the acute phase inflammatory response. *IL6* gene expression is nuclear factor-kappaB (NF-κB)-dependent (Chauhan *et al.*, 1996) and produces a 26 kDa IL-6 protein product. First characterized as a T-cell-derived factor that induced proliferation, differentiation, and immunoglobulin production in B-cells, IL-6 was originally named B-cell stimulating factor-2 (BSF-2). It was later thought to be a novel interferon (IFN-β_2) due to studies demonstrating the ability of IL-6 to activate signal transducer and activator of transcription 3 (STAT3) (Kishimoto, 2006). Complementary DNA encoding the human IL-6 gene was subsequently cloned, and human IL-6 transgenic mice demonstrated a polyclonal IgG1 plasmacytosis phenotype (Suematsu *et al.*, 1989). Next, IL-6 knockout (IL-6$^{-/-}$) mice were generated and characterized. IL-6$^{-/-}$ mice underwent normal development, but adult animals exhibited reduced numbers of peripheral T-cells and impaired antiviral cytotoxic T-cell activity (Kopf *et al.*, 1994). In addition, IL-6 is a critical factor during hematopoiesis and subsequent lymphocyte differentiation and activation. Multiple diverse cell populations including fibroblasts, T and B-cells, monocytes, macrophages, endothelial cells, keratinocytes, astrocytes, and smooth muscle cells all have the potential to produce constitutive or inducible IL-6 (Kishimoto, 2006).

Depending on cellular context, IL-6 can signal through multiple kinase-dependent proliferation and anti-apoptosis pathways including the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-triphosphate kinase (PI-3K)/Akt pathway, and perhaps the most commonly evaluated in breast cancer, the Janus kinase (JAK)/signal transducer and activator of transcription-3 (STAT3) pathway (Hodge *et al.*, 2005). To do so, a plasma membrane-associated IL-6 receptor (IL-6R/CD126) homodimer first ligates two soluble IL-6 molecules, which leads to gp130 (CD130) homodimer ligation. Whereas IL-6R is only expressed on hepatocytes, osteoclasts, and most immune cells under normal physiological conditions, gp130 is a ubiquitous and promiscuous receptor involved in multiple cytokine signaling pathways (e.g., IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF)) (Rose-John *et al.*, 2006). To initiate classical JAK/STAT3 signal transduction, JAK are recruited to the intracellular domain of the gp130 receptor where they bind and autophosphorylate. Subsequent gp130 phosphorylation via activated JAK offers docking sites for STAT3 and other receptor-associated proteins. Once bound to the intracellular domain of gp130, STAT3 is specifically phosphorylated (pSTAT3) by adjacent JAK on a C-terminal tyrosine residue (Y705), which grants its disengagement from the receptor. Dissociation of pSTAT3^{Y705} from gp130 facilitates its homodimerization within the cytoplasm, and the pSTAT3^{Y705} homodimer translocates to the nucleus. There, pSTAT3^{Y705} binds to specific promoters whereby it initiates the transcription of multiple downstream target genes (Clevenger, 2004). Under normal physiological conditions, an inhibitory feedback loop maintains rapid and transient STAT3 activation. Following activation in normal cells, STAT3 induces suppressors of cytokine signaling (SOCS) and protein inhibitors of activated STATs (PIAS) expression. While SOCS-1 specifically inhibits JAK function, SOCS-3 binds the IL-6R complex to inhibit IL-6 signal transduction. PIAS-3 directly interacts with STAT3 to inhibit all STAT3 target gene expression (Kishimoto, 2006). In contrast, many human cancers, including breast cancer, exhibit constitutive STAT3 activity. Recent studies have demonstrated that unphosphorylated STAT3 (U-STAT3) accumulates in tumor cells with constitutively active

STAT3 where it forms a complex with NF-κB to activate a subset of NF-κB target genes (Yang and Stark, 2008).

Alternatively, IL-6 *trans*-signaling describes an IL-6 signaling pathway whereby an IL-6 soluble receptor (IL-6sR) binds IL-6 and subsequently ligates gp130 to stimulate STAT3 activation in cells that only express gp130. IL-6sR is naturally produced by either proteolytic cleavage of the membrane-bound IL-6R or alternative splicing of IL-6R mRNA (Rose-John *et al.*, 2006). Whereas IL-6 serum levels continue to increase with age, levels of serum IL-6sR rise until approximately age 70 at which time they gradually decline (Giuliani *et al.*, 2001). Furthermore, IL-6sR expression has been demonstrated in human breast cancer cell lines (Crichton *et al.*, 1996; Oh *et al.*, 1996; Singh *et al.*, 1995), suggesting that IL-6 *trans*-signaling mediates the effects of IL-6 in breast cancer cells. In contrast, an endogenous soluble gp130 (sgp130) specifically antagonizes IL-6 *trans*-signaling by exclusively ligating the IL-6/IL-6sR complex, thus having no effect on cells that express the membrane-bound IL-6R (Rose-John *et al.*, 2006) (Figure 1).

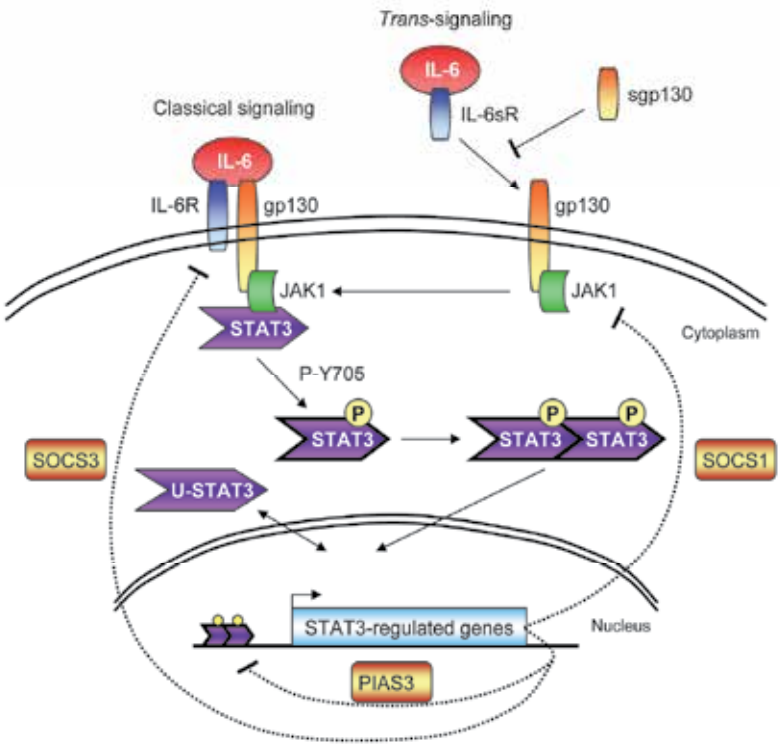

Fig. 1. The IL-6/STAT3 signaling pathway

5. Excessive IL-6 in human breast cancer

Aberrantly elevated IL-6 is associated with a poor prognosis in breast cancer (Bachelot *et al.*, 2003; Salgado *et al.*, 2003; Zhang and Adachi, 1999). Human breast tumors produce more IL-6 when compared to matched healthy breast tissue, and tumor IL-6 levels concurrently increase with tumor grade. In addition, increased serum IL-6 has been demonstrated in

breast cancer patients compared to normal donors and correlates with advanced breast tumor stage (Kozlowski *et al.*, 2003) and increased number of metastatic sites (Salgado *et al.*, 2003). Furthermore, a single nucleotide polymorphism (SNP) exists at position -174 in the IL-6 gene promoter region, noted as IL-6 (-174 G>C), with the following allele frequency in a Caucasion population: 36% G/G, 44% G/C, and 18% C/C. An inflammatory stimulus such as *Salmonella typhii* vaccination induced higher serum IL-6 in those individuals with the G/G allele (Bennermo *et al.*, 2004). Although the IL-6 (-174 G>C) SNP is not associated with increased risk of developing breast cancer (Gonzalez-Zuloeta Ladd *et al.*, 2006; Litovkin *et al.*, 2007; Yu *et al.*, 2009b), it is significantly associated with disease-free and overall survival in breast cancer patients (DeMichele *et al.*, 2003).

ERα is expressed in luminal subtype breast tumors (Perou *et al.*, 2000) and therefore associated with improved patient survival (Buyse *et al.*, 2006; Sorlie *et al.*, 2001). A clear and well-characterized inverse correlation exists between breast cancer ERα status and IL-6. In fact, ERα directly binds to NF-κB, thus preventing transactivation of *IL6* gene expression (Galien and Garcia, 1997), which demonstrates a direct mechanism for such a correlation. Furthermore, ERα-negative human breast tumors produce more IL-6 than tumors that express ERα (Chavey *et al.*, 2007), and IL-6 serum levels are higher in ERα-negative breast cancer patients compared to ERα-positive patients (Jiang *et al.*, 2000). Likewise, ERα-negative breast cancer cell lines produce autocrine IL-6 whereas ERα-positive breast cancer cell lines do not (Sasser *et al.*, 2007). Therefore, this strongly suggests that ERα-negative breast cancer cells would exploit both paracrine (i.e., stromal cell-derived) and autocrine IL-6 signaling, whereas ERα-positive breast cancer cells could only utilize paracrine IL-6 signaling. In addition, ERα-negative breast cancer patients, whose tumors produce more IL-6 than those that express ERα (Chavey *et al.*, 2007), showed no difference in survival between the G/G allele (higher inducible serum IL-6) and any C allele (lower inducible serum IL-6) at the IL-6 (-174 G>C) promoter SNP. In contrast, ERα-positive breast cancer patients with any C allele at the IL-6 (-174 G>C) promoter SNP demonstrated improved disease-free and overall survival compared to those with the G/G allele (DeMichele *et al.*, 2003).

6. IL-6 promotes breast cancer cell growth

Stromal fibroblasts isolated from multiple types of tumors (i.e., TAF) or cancers (i.e., CAF) are now appreciated as influential players in cancer progression and metastasis (Orimo and Weinberg, 2006). CAF derived from multiple cancer types, including murine mammary cancers, exhibit an activated, proinflammatory phenotype with increased IL-6 production (Erez *et al.*, 2010). Furthermore, work from our laboratory has demonstated that fibroblasts isolated from breast tissue and common sites of breast cancer metastasis such as bone and lung enhance the growth of breast cancer cells in an IL-6-dependent manner, and IL-6 is the major fibroblast-derived soluble factor that induced STAT3 activation in breast cancer cells (Sasser *et al.*, 2007; Studebaker *et al.*, 2008). MDA-MB-231 breast cancer cells are commonly utilized to model triple negative breast cancer and produce autocrine IL-6. MDA-MB-231 cells expressing a dominant negative isoform of gp130 lacked constitutively active STAT3 and exhibited impaired tumorigenicity in an orthotopic xenograft model (Selander *et al.*, 2004), thus suggesting that IL-6 may drive tumor progression in this model. In addition, STAT3 is estimated to be constitutively activated in more than half of primary breast cancers due to IL-6 signaling (Berishaj *et al.*, 2007).

Mesenchymal stem cells (MSC) are a bone marrow-derived fibroblast cell population that can be recruited to the breast tumor stroma, acquire a TAF phenotype, and produce high levels of IL-6. MSC enhance the growth of ERα-positive breast cancer cells, which do not express IL-6 or activated STAT3. In contrast, MSC have no effect on IL-6-producing ERα-negative breast cancer cells, which express constitutively activated STAT3. Moreover, ERα-positive breast cancer cells orthotopically co-injected with MSC or MSC conditioned medium and ERα-positive breast cancer cells that ectopically express IL-6 demonstrate enhanced xenograft tumor growth in the absence of exogenous 17β-estradiol (Sasser *et al.*, 2007). Similar differential growth enhancement was demonstrated *in vivo* with ERα-positive and ERα-negative breast cancer cells co-injected with MSC, which also promoted metastasis (Karnoub *et al.*, 2007). Interestingly, IL-6 has been reported to facilitate the recruitment of MSC to hypoxic breast tumor microenvironments (Rattigan *et al.*, 2010). Likewise, IL-6 secreted from breast cancer cells has been shown to contribute to a recently characterized phenomenon termed "self-seeding" in which aggressive circulating tumor cells engraft within their original xenograft tumor (Kim *et al.*, 2009). MSC have also been shown to mediate the self-renewal capacity of breast cancer stem cells, in part, through a reciprocal IL-6 loop (Liu *et al.*, 2010). Taken together, preceding evidence strongly suggests that IL-6 promotes breast cancer cell growth by activating STAT3, which culminates with the upregulation of proliferative oncogenes such as c-Myc and cyclin D1 and and growth factors such as IL-6, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) (Yu *et al.*, 2009a).

7. IL-6 promotes epithelial-mesenchymal transition in breast cancer cells

Normal polarized epithelial cells exhibit 'cobblestone' homophilic morphology and express E-cadherin, which is required for epithelial cell polarization, phenotype, and consequent homeostasis (Jeanes *et al.*, 2008). E-cadherin is a key prognostic molecular biomarker clinically utilized to predict the metastatic propensity of breast cancer. Whereas very few studies have failed to demonstrate E-cadherin as an independent prognostic biomarker in breast cancer patients (Lipponen *et al.*, 1994; Parker *et al.*, 2001), the overwhelming majority of relevant studies have revealed E-cadherin as one of the strongest predictors of patient survival. Specifically, impaired E-cadherin expression in human breast tumors correlates with enhanced invasiveness, metastatic potential (Oka *et al.*, 1993), and decreased breast cancer patient survival (Heimann and Hellman, 2000; Pedersen *et al.*, 2002). While appropriate E-cadherin function is essential to the maintenance of epithelial cell morphology, phenotype, and homeostasis, regulation of E-cadherin expression is of equal importance. *CDH1*, the gene that encodes E-cadherin, is located on human chromosome 16q22.1 (Rakha *et al.*, 2006) and is susceptible to inactivation by promoter hypermethylation, somatic mutation, or aberrant overexpression of repressive transcription factors including Twist, Snail, and Slug among others (Hirohashi, 1998). Likewise, E-cadherin loss of function can arise due to extracellular domain-specific proteolytic cleavage. Although uncommon, germline mutations of *CDH1* predispose individuals to hereditary diffuse gastric cancer (HDGC) syndrome, and a proportion of these patients present with other cancers, including breast cancer (Guilford, 1999).

E-cadherin was initially termed uvomorulin in mice and L-CAM in chicks following its discovery as a 120 kDa calcium-dependent trypsin-labile cell surface glycoprotein required for intercellular adhesion in mouse blastomeres (Hyafil *et al.*, 1981) and chick embryos

(Brackenbury *et al.*, 1981). It now represents the best studied member of the cadherin family of tissue-specific homophilic intercellular adhesion molecules. E-cadherin knockout studies have demonstrated early embryonic lethality due to impaired maintenance of epithelial polarity and failure to form an intact epithelium in E-cadherin$^{-/-}$ embryos (Larue *et al.*, 1994). E-cadherin is localized on the cell surface of epithelial cells, and each E-cadherin protein consists of an amino-terminal extracellular domain, a single-pass transmembrane segment, and a carboxy-terminal intracellular domain. Five calcium-binding repeated subunits comprise an extracellular domain that promotes homophilic interaction to ultimately form anti-parallel trans-E-cadherin dimers between adjacent cells (Guilford, 1999). The intracellular domain is comprised of a juxtamembrane p120-catenin binding subdomain and a C-terminal beta (β)-catenin binding subdomain. β-catenin, a potent transcription factor, binds E-cadherin and alpha (α)-catenin subsequently binds β-catenin. Although contentious (Weis and Nelson, 2006), it is generally acknowledged that α-catenin interacts with F-actin and thereby, facilitates the linkage of E-cadherin to the cytoskeleton. This E-cadherin-catenin-actin complex localizes to epithelial intercellular junctions called adherens junctions and is critical to epithelial cell adhesion, polarity, and morphology (Hartsock and Nelson, 2008). Furthermore, E-cadherin sequesters β-catenin at the cell surface as one means to inhibit β-catenin nuclear translocation and consequent expression of β-catenin responsive genes (Perez-Moreno *et al.*, 2003).

Another prominent role of E-cadherin is that of an invasion/metastasis suppressor protein. Upon loss of E-cadherin and subsequent dissociation of adherens junctions, epithelial cells acquire enhanced invasive capability (Behrens *et al.*, 1989). MDA-MB-231 cells, an ERα-negative breast cancer cell line, lack E-cadherin, whereas MCF-7 cells, an ERα-positive breast cancer cell line express high levels of E-cadherin (Kenny *et al.*, 2007), and MDA-MB-231 cells exhibit enhanced invasive capability compared to MCF-7 cells (Sommers *et al.*, 1991). Naturally, E-cadherin expression and consequent invasive capacity regulate the propensity of breast cancer metastasis. Multiple signaling pathways are activated following loss of E-cadherin protein, which promote transformed human breast epithelial cell metastasis in a xenograft model. Interestingly, Twist, a transcriptional repressor of *CDH1*, is induced upon loss of E-cadherin and is necessary for metastasis in this model. Furthermore, the E-cadherin binding partner, β-catenin, was shown to be necessary but not sufficient for the EMT phenotype induced following loss of E-cadherin (Onder *et al.*, 2008). Ectopic expression of murine E-cadherin in highly metastatic human MDA-MB-231 cells significantly reduced osteolytic bone metastases in a murine intracardiac dissemination model (Mbalaviele *et al.*, 1996). Likewise, aberrant cytoplasmic or diminished to negative E-cadherin immunostaining patterns are commonly detected in invasive poorly differentiated breast carcinomas compared to noninvasive well-differentiated breast carcinomas and are associated with increased probability of breast carcinoma metastasis (Oka *et al.*, 1993). The finding that distant metastases often express E-cadherin even in patients which exhibit primary breast carinomas which lack E-cadherin suggests that ultimate re-expression may be necessary for colonization of secondary tissues (Kowalski *et al.*, 2003; Saha *et al.*, 2007).

Loss of E-cadherin is a prerequisite for epithelial-mesenchymal transition (EMT), a highly conserved process which exemplifies the aberrant activation of an embryonic gene expression program during carcinoma progression. EMT is critical for multiple steps of developmental metazoan cellular morphogenesis as demonstrated in well-characterized

Drosophila and *Xenopus* models. Throughout embryonic development, EMT whereby epithelial cells give rise to more motile mesenchymal cells is essential for mesoderm and neural crest formation. Importantly, this is a transient process and mesenchymal-epithelial transition (MET) allows for cellular reversion (Yang and Weinberg, 2008).

Whereas EMT has been extensively studied for its essential role in embryogenesis, the concept of EMT-like cellular changes in human cancers has gained acceptance as a major mechanism to promote primary tumor cell invasion and subsequent tumor metastasis. A carcinoma cell must first detach from the primary tumor and invade through the basement membrane into the underlying tissue parenchyma to initiate the metastasic cascade. Although cancer-associated EMT was considered a controversial notion even in recent years (Tarin *et al.*, 2005), it has been demonstrated in multiple human carcinomas, including breast cancer (Cheng *et al.*, 2008; Heimann and Hellman, 2000; Moody *et al.*, 2005; Sarrio *et al.*, 2008), and is now recongnized as a putative mediator of tumor metastasis. An EMT phenotype including impaired E-cadherin expression with concominant induction of Vimentin, Alpha-smooth-muscle-actin, and/or N-cadherin is associated with the basal breast cancer subtype, suggesting that EMT may promote characteristic aggressiveness in these tumors and contribute to poor breast cancer patient survival (Sarrio *et al.*, 2008). Likewise, relatively noninvasive ERα-positive MCF-7 cells express E-cadherin, consistent with a characteristic epithelial phenotype, and are classified as luminal subtype, whereas highly invasive ERα-negative MDA-MB-231 cells lack E-cadherin and are classified as basal subtype (Blick *et al.*, 2008). Furthermore, ERα directly correlates with E-cadherin in primary human breast tumors (Ye *et al.*, 2010). While EMT may enhance carcinoma cell invasion and subsequent dissemination which would increase metastatic potential, it is not synonymous with metastasis in all models. For example, Lou, *et al.* demonstrated that EMT alone was insufficient for spontaneous murine mammary carcinoma metastasis (Lou *et al.*, 2008). Yet, Weinberg and colleagues described the promotion of metastasis with loss of E-cadherin and a consequent EMT phenotype in transformed human breast epithelial cells (Onder *et al.*, 2008).

Our laboratory has previously demonstrated that exogenous IL-6 exposure induced an EMT phenotype in a panel of human ERα-positive breast cancer cells, which included E-cadherin repression and concomitant induction of Vimentin, N-cadherin, Snail, and Twist. In addition, ectopic expression of IL-6 in ERα-positive MCF-7 breast cancer cells promoted an EMT phenotype and enhanced invasiveness. Likewise, MCF-7 cells with ectopic Twist expression exhibit an EMT phenotype (Mironchik *et al.*, 2005), autocrine IL-6 production, and constitutive STAT3 activation (Sullivan *et al.*, 2009).

8. Therapeutic targeting of the IL-6/STAT3 pathway

IL-6 levels are increased in human breast tumors and breast cancer patient sera, and excessive IL-6, both circulating and within the breast tumor microenvironment, is associated with poor clinical outcomes in breast cancer. STAT3, a critical downstream mediator of IL-6 signaling, is constitutively activated in more than half of human cancers and promotes the expression of proliferative, anti-apoptotic, immune suppressive, and pro-angiogenic target genes, which all potentiate carcinogenesis. Whereas the IL-6 signaling network has been targeted in numerous autoimmune diseases and cancers, this therapeutic strategy has yet to be clinically employed for breast cancer. Increased preclinical reports have revealed novel

mechanisms underlying IL-6/STAT3 signaling in breast cancer cells such as enhanced growth, induction of EMT, multidrug resistance, and recruitment of peripheral fibroblasts. Taken together, accumulating preclinical and clinical data emphasize IL-6 as a highly attractive therapeutic target in breast cancer. It is therefore imperative that more work be done to evaluate current therapeutics and develop novel agents that target IL-6/STAT3 signaling in breast cancer models.

Multiple strategies could be utilized to target the IL-6/STAT3 pathway, but first and most obvious would be anti-IL-6 neutralizing antibodies. One such anti-IL-6 monoclonal antibody is Siltuximab (CNTO 328). The safety and efficacy of Situximab has been demonstrated in preclinical studies and phase I/II clinical trials of diverse human pathologies and malignancies including Castleman's disease (van Rhee *et al.*, 2010), multiple myeloma (Hunsucker *et al.*, 2011; Voorhees *et al.*, 2007), prostate cancer (Cavarretta *et al.*, 2007; Cavarretta *et al.*, 2008; Dorff *et al.*, 2010; Karkera *et al.*, 2011), renal cell carcinoma (Puchalski *et al.*, 2010; Rossi *et al.*, 2010), non-small cell lung cancer (Song *et al.*, 2010), and ovarian cancer (Guo *et al.*, 2010). Furthermore, IL-6R can be targeted with tocilizumab, an anti-IL-6R monoclonal antibody that has shown promising results in IL-6-driven autoimmune diseases (Tanaka et al., 2011) and was recently approved by the FDA for the treatment of rheumatoid arthritis. The promiscuous IL-6 coreceptor, gp130, also has an endogenous soluble form (sgp130) that exclusively inhibits IL-6 *trans*-signaling, thus preserving classical IL-6 signaling. Therapeutic sgp130 would potentially be more targeted toward breast cancer cells, which generally lack membrane-associated IL-6R and therefore utilize IL-6 trans-signaling through IL-6sR. Recombinant soluble gp130 (sgp130-Fc) has been shown to inhibit murine colon carcinogenesis (Becker *et al.*, 2004), suggesting that it may prove effective in breast cancer as well. Finally, a growing number of non-selective kinase inhibitors and recent focus on specific JAK and STAT3 inhibitor development will provide further insight into the roles of JAK and STAT3 in breast cancer.

9. Conclusions

Breast cancer is a heterogeneous disease and thus, highly variable across individual patients. This heterogenicity arises not only due to the diversity of genetic and molecular aberrations in primary breast cancer cells but also due to the diversity of cellular populations that inhabit the breast tumor microenvironment. Although IL-6 levels are higher in breast tumors and patient sera, the precise source of this IL-6 remains elusive. Importantly, many breast tumor stromal cells provide a paracrine source of IL-6 for breast cancer cells within the breast tumor microenvironment. In addition, certain clinical subtypes of breast cancers and research models, such as ERα-negative primary breast cancers and ERα-negative breast cancer cell lines, produce excessive IL-6 (Figure 2). Therefore, ERα-negative breast cancer cells may supply the tumor microenvironment with IL-6 by means of autocrine IL-6 production to exacerbate the poor prognosis associated with this clinical subtype. It will be critical to determine the specific cellular source of breast tumor-associated IL-6 to advance our understanding of this pleiotropic cytokine in breast cancer progression and metastasis. Moreover, this knowledge will facilitate the validation and subsequent clinical utility of current and novel targeted antagonists of the IL-6/STAT3 signaling network in breast cancer.

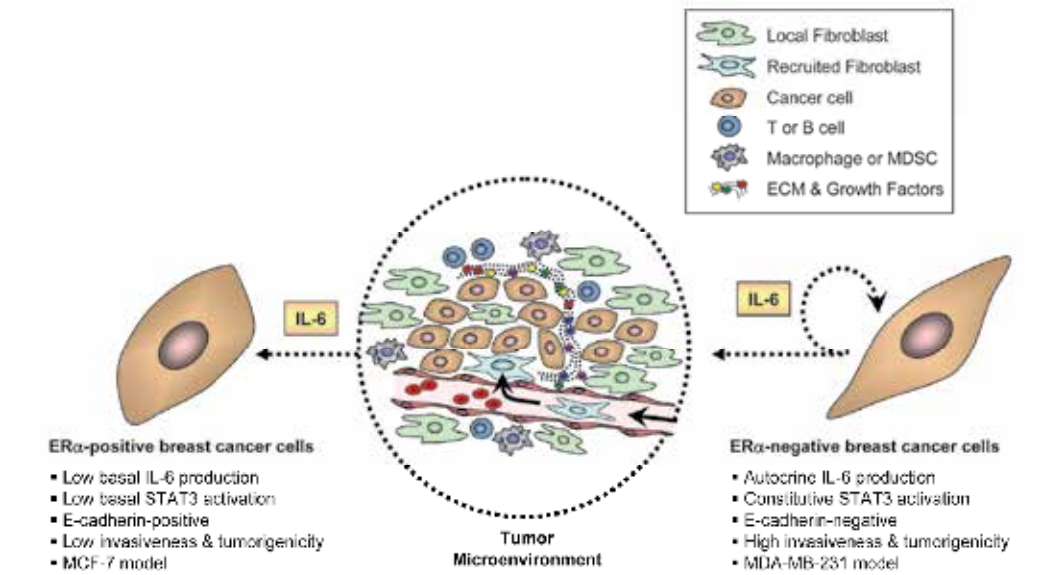

Fig. 2. Breast cancer cell ERα status dictates paracrine vs. autocrine IL-6 utilization.

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9

The Role of Fibrin(ogen) in Transendothelial Cell Migration During Breast Cancer Metastasis

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1. Introduction

Despite all the modern advances in treatment for breast cancer, metastatic disease remains the hurdle to surmount in curing breast cancer or, at least, in significantly reducing morbidity and mortality to improve long-term survival and quality of life. For over a century, inflammation and thrombosis have been linked to metastatic cancer (Boccaccio & Medico, 2006). In addition to being known for describing the factors leading to venous thromboembolism (alterations in blood flow, vascular endothelial injury, and hypercoagulability) as Virchow's triad, in 1863 Virchow noted a connection between chronic inflammation and cancer based on the recruitment of leukocytes to cancerous lesions (reviewed in (Balkwill & Mantovani, 2001)) (**Fig. 1**).

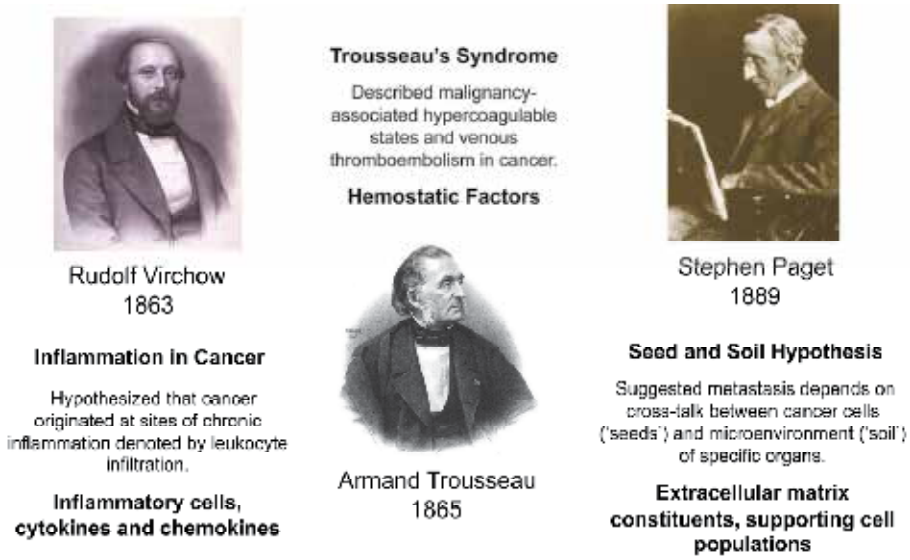

Fig. 1. The three faces of cancer metastasis. (Portraits obtained from public domain).

Rudolf Virchow, Armand Trousseau and Stephen Paget each provided valuable insight into the pathophysiology of invasive carcinomas—these theories still hold today to explain molecular mechanisms of cancer metastasis. Hypercoagulability is often diagnosed before identification of a coexisting malignancy, and is associated with increased thromboembolic risk (Sorensen et al., 2000). Armand Trousseau (Trousseau, 1865) (**Fig. 1**) identified and described the association between cancer and clot formation in 1865 and, shortly thereafter, self-identified these findings as a consequence of gastric cancer from which he later succumbed (Varki, 2007). Trousseau's Syndrome is associated with hypercoagulability and thromboembolic events in adenocarcinomas (Starakis et al., 2010). Another important contribution that has lead to better understanding of the mechanisms of cancer metastasis was provided by Stephen Paget in 1889 (Paget, 1889) when he propose the seed and soil concept of cancer metastasis (**Fig. 1**). By examining countless autopsy specimen from breast cancer patients, Paget determined that cancer cells, the "seed", had a preference to metastasize to distinct organs of the body based on favorable interactions with the stromal microenvironment, the "soil". As reviewed by Langley and Fiddler (Langley & Fidler, 2011), it is clear that cancer therapy is targeted to either the "seed" through chemotherapy with cytotoxic drugs or the "soil" by manipulating stromal contributions favorable to metastatic growth such as inhibiting angiogenesis.

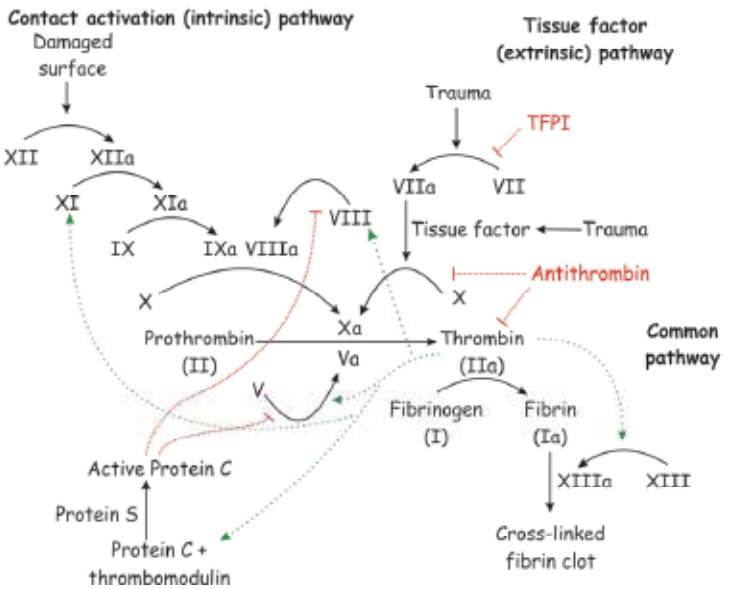

Fig. 2. Schematic view of intrinsic and extrinsic coagulation pathways.

Red lines denote pathway inhibitors of coagulation and green lines denote thrombin activation of hemostatic factors. (Reproduced from public domain image).

Appropriate activation of the clotting cascade is fundamental to arrest bleeding in response to vascular injury. The immediate response, known as primary hemostasis, involves vasoconstriction of blood vessels and activation and aggregation of platelets to form a plug at the site of vascular injury. Activated platelets release a panoply of stored constituents including: chemokines (IL-8) and growth factors such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and

transforming growth factor (TGF)-β; adhesive glycoproteins, including fibrinogen (Fg), fibronectin and von Willebrand factor; and lipid mediators such as lysophosphatidic acid, platelet-activating factor, leukotriene B_4, and thromboxanes. During secondary hemostasis, coagulation is activated either through the extrinsic pathway via tissue factor (TF)-Factor VII (FVII)/activated FVII (FVIIa) or the intrinsic pathway through Factor XII/FXIIa (**Fig. 2**). These pathways converge at the formation of the tenase complex that activates FX to FXa leading to thrombin activation. Thrombin cleaves soluble plasma Fg into fibrin monomers that form the insoluble fibrin clot after fibrin monomer polymerization and covalent crosslinking and stabilization by activated FXIII (FXIIIa). The fibrin clot provides a provisional matrix upon which injured endothelial cells adhere, proliferate and migrate to restore an intact endothelium lining blood vessels. Furthermore, fibrin and Fg provide a reservoir for sequestration of growth factors including FGF-2 (Sahni et al., 1998; Sahni et al., 1999), VEGF (Sahni & Francis, 2000), and TGF-β (Schachtrup et al., 2010), as well as an adhesive substrate for recruitment of leukocytes and stromal fibroblasts to aid in wound repair (Rybarczyk et al., 2003; Ugarova & Yakubenko, 2001). Normal wound repair is self-limiting as the provisional fibrin matrix is dissolved by various proteases, *e.g.*, plasmin, upon resolution of the vascular injury, reduction of inflammation and restoration of normal function (**Fig. 2**).

In the 1980s, however, Dvorak likened cancer progression to "wounds that never heal" in which Fg and fibrin also play prominent roles (Dvorak, 1986), and as reviewed by Coussens and Werb (Coussens & Werb, 2002). Several key steps in normal wound repair are also manifested during cancer progression (**Fig. 3**). As discussed above, a heighten state of coagulation occurs immediately after wound injury, and the release of chemokines and cytokines from activated platelets to recruit and activate proinflammatory cell types to the wound site amplify the inflammatory response system wide.

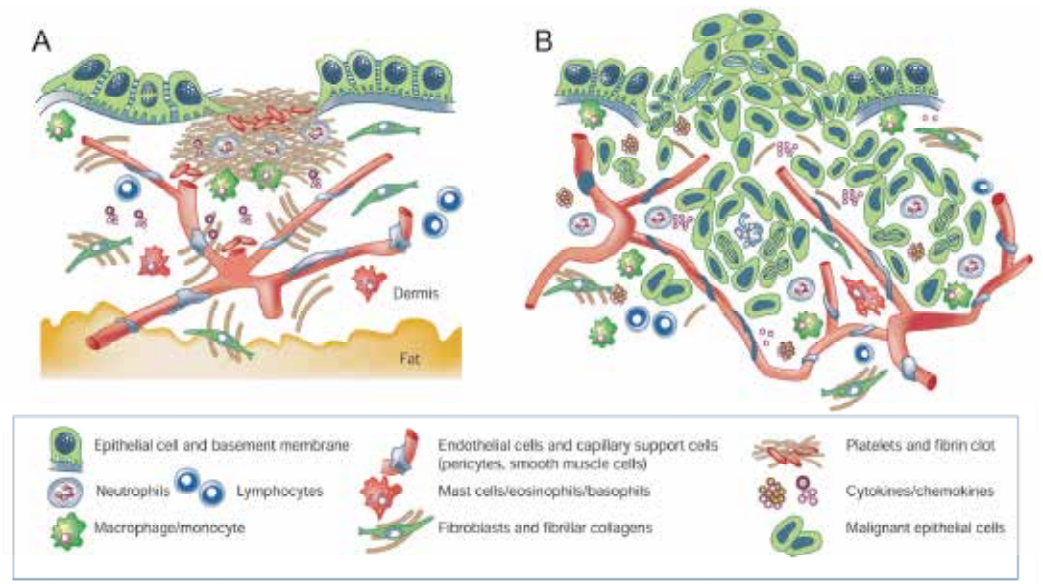

Fig. 3. Normal wound repair is depicted in panel A and mechanisms of wound repair left unchecked in cancer are depicted in Panel B. (Figure reprinted from (Coussens & Werb, 2002) with permission from Nature Publishing Group).

Systemic inflammation is best characterized by the innate acute phase response to injury or infection whereby the synthesis of a host of plasma proteins by the liver is altered to immediately respond to disruptions of homeostasis (Baumann & Gauldie, 1994). Of note, C-reactive protein and Fg are two positive (upregulated) acute phase proteins whose expression is also elevated in malignancies (Jones et al., 2006; Yamaguchi et al., 1998; Yigit et al., 2008). Coagulation and deposition of a provisional fibrin matrix occurs within minutes of vascular injury, and changes in expression of adhesion molecules on the surface of activated endothelium leads to the rolling and slowing of circulating leukocytes, firm attachment and the processes of diapedesis, *i.e.*, transmigration across the endothelial cell barrier into interstitial spaces. Neutrophils are the first proinflammatory cells to appear in the wound space where they release molecules to kill invading microorganisms and promote recruitment of stromal cells such as fibroblasts and endothelial cells to the wound space. Locally deposited growth factors promote cell proliferation and migration leading to the formation of granulation tissue over several days to a few weeks, which is the result of fibroblasts/myofibroblasts depositing extracellular matrix constituents (*e.g.*, collagens) and endothelial cells forming new blood vessels to facilitate would closure. In the case of cutaneous wounds, re-epithelialization begins to close the wound, the provisional fibrin matrix is dissolved, and infiltrating monocytes/macrophages clean up wound debris in preparation for matrix remodelling, deposition of a complete basement membrane (*e.g.*, laminin) and, over weeks to months, gradual restoration of the tensile strength of the tissue (Coussens & Werb, 2002). In contrast, the orderly array of signaling components that turn on and off cell migration, cell proliferation, and angiogenesis during wound repair goes array during cancer such that cell growth is unchecked, mechanisms of apoptosis are overridden and the stromal compartment is dramatically altered to perpetuate angiogenesis, tumor growth and cell migration to promote metastasis (**Fig. 3**).

Metastatic disease remains the prevailing reason for treatment failure and death from solid tumors including breast cancers. Only recently have three major areas of research outside the realm of the primary tumor cells themselves been considered viable for development of new therapeutic strategies to prevent the initiation, progression and metastasis of tumors. These include hemostatic factors, the tumor stromal microenvironment, and chronic inflammation. The blood coagulation protein Fg and its insoluble counterpart, fibrin, play central roles in inflammation, venous thromboembolism, and as components of the extracellular matrix. The goals of this chapter are three-fold: first, to review the current understanding of the roles of Fg and/or fibrin {commonly referred to as fibrin(ogen)} in cancer progression in general; second, to provide evidence that fibrin(ogen) likely plays a critical role in the metastatic spread of breast cancer; and third, to propose new therapies for treatment and future avenues of research to elucidate the molecular mechanisms that promote the phenotypic switch of breast epithelial cells to a metastatic cell phenotype.

2. Fibrin(ogen) in cancer progression

2.1 Hemostatic factors and vascular cells promote tumor metastasis

Molecules and cells linked to the prothrombotic state of Trousseau's syndrome that also facilitate cancer metastasis including thrombin, TF, selectins, platelets, endothelial cells and fibrin (Varki, 2007). It is well known that thrombin contributes to the severity of cancer progression by promoting tumor angiogenesis, cancer cell proliferation and metastasis by mechanisms other than just thrombin generation of fibrin (Nierodzik & Karpatkin, 2006).

Cell-associated TF expression by cancer cells correlates with disease severity and poor prognosis {reviewed in (Palumbo & Degen, 2007)}. Although tumor cell-associated TF expression is not required for the growth of primary tumors, it is necessary for their metastatic spread (Palumbo et al., 2007). Similarly, FXIII and Fg are important for the metastatic spread of tumor cells through both the circulation and lymphatic systems but not primary tumor growth (Palumbo et al., 2008; Palumbo & Degen, 2001; Palumbo & Degen, 2007; Palumbo et al., 2000; Palumbo et al., 2002; Palumbo et al., 2007; Palumbo et al., 2005). Moreover, FXIII, Fg and platelets are important substrates or cell targets for thrombin action demonstrating the critical role played by the hemostatic system in promoting cancer metastasis. Degen and colleagues suggest that tumor cell-associated TF mediates thrombin generation to support the early survival of micrometastases by at least two mechanisms: 1) the formation of platelet-fibrin microthrombi to protect newly formed micrometastases from natural killer (NK) cell-mediated cytotoxicity, and 2) by promoting mechanical stability of tumor cell emboli within vascular beds at distant metastatic sites (Palumbo et al., 2008; Palumbo & Degen, 2001; Palumbo & Degen, 2007; Palumbo et al., 2000; Palumbo et al., 2002; Palumbo et al., 2007; Palumbo et al., 2005).

2.2 Chronic inflammation is associated with cancer initiation and progression

Systemic inflammation is clearly linked with adverse prognosis in patients with cancer, and is characterized by elevated expression of pro-inflammatory mediators including interleukin (IL)-6 (Gao et al., 2007; Knupfer & Preiss, 2007). IL-6 is the major cytokine responsible for upregulation of specific plasma proteins in the liver during an acute phase response (Baumann & Gauldie, 1994), and also in chronic inflammation (Barton, 2001; Lin & Karin, 2007; Neurath & Finotto, 2011). IL-6 induces expression of target genes, including Fg, by activation of Stat3 (Duan & Simpson-Haidaris, 2003); Stat3 is often constitutively active in breast cancer, and tumor growth can become dependent on Stat3 signaling (Pensa et al., 2009). Both IL-6 and Fg levels are elevated in patients with advanced lung cancer (Yamaguchi et al., 1998). In breast cancer patients, serum IL-6 correlates with increasing numbers of involved sites, liver metastasis, and disease progression (Knupfer & Preiss, 2007; Salgado et al., 2003). In 2002, Drix et al demonstrated that IL-6, VEGF and D-dimer levels are elevated in patients with progressive breast cancer; these markers correlate positively with disease severity, and serum IL-6 is an independent prognostic factor in patients with metastatic disease (Dirix et al., 2002). Elevated levels of Fg, D-dimers, IL-6, VEGF and soluble P-selectin, an indicator of platelet activation, were also found in the plasma of breast cancer patients by Caine et al, who furthered demonstrated that IL-6 induces dose-dependent release of VEGF from platelets in vitro (Caine et al., 2004). Steinbrecher et al demonstrated a direct link between fibrin(ogen), elevated IL-6 levels and the development of inflammation-driven cancer using a mouse model of colitis-associated cancer (Steinbrecher et al., 2010). IL-6 serves as a marker to predict which patients will respond poorly to anti-endocrine chemotherapy (Zhang & Adachi, 1999), as a marker of tumor staging and a predictor of micrometastases (Ravishankaran & Karunanithi, 2011). IL-6 also induces VEGF expression (Cohen et al., 1996) and invasion and migration of breast cancer cells (Walter et al., 2009). Furthermore, overexpression of Her2 in breast cancer cells upregulates IL-6 leading to Stat3 activation and altered gene expression resulting in an autocrine feedback loop promoting cell survival (Hartman et al., 2011). Together, these reports substantiate the importance of fibrin(ogen) and inflammation in cancer metastasis.

2.3 Fibrin(ogen) functions as a bridging molecule in cell-cell interactions during coagulation and inflammatory cell trafficking

Excessive fibrin deposition is accompanied by local expression of proinflammatory mediators, vascular leakage, and inflammatory cell recruitment and activation, leading to amplification of the inflammatory response (Clark, 1996; Simpson-Haidaris & Rybarczyk, 2001; van Hinsbergh et al., 2001). Specific structural features of fibrin(ogen) modulate the functions of a variety of different cell types including endothelial, epithelial, leukocytes, platelets and fibroblasts (**Fig. 4**). Cell receptors that bind to fibrin(ogen) include: β3 integrins (αIIbβ3 and αvβ3) (Bennett et al., 2009); β2 integrins (CD11a/CD18 and CD11b/CD18) (Altieri et al., 1993; Flick et al., 2004; Lishko et al., 2004; Loike et al., 1991; Ugarova et al., 2003; Yakovlev et al., 2005); and β1 integrin, α5β1 (Asakura et al., 1997; Suehiro et al., 1997). Nonintegrin adhesion molecules that bind to fibrin(ogen) include intercellular adhesion molecule (ICAM)-1 (Languino et al., 1993; Pluskota & D'Souza, 2000), vascular endothelial (VE)-cadherin (Bach et al., 1998b) and heparan sulfate proteoglycans (HSPG) (Odrljin et al., 1996a; Odrljin et al., 1996b). Fibrin(ogen) also modulates a number of signaling molecules important in innate immunity. Fg-bound FGF-2 induces expression of uPA, uPA receptor and PAI-1, and fibrin(ogen) induce IL-8, MCP-1 or IL-1β expression in endothelial cells (Guo et al., 2004; Harley & Powell, 1999; Kuhns et al., 2001; Lee et al., 2001; Qi & Kreutzer, 1995; Ramsby & Kreutzer, 1994; Sahni et al., 2004). Fg and fibrin activate NF-κB and AP-1 (Guo et al., 2004; Sitrin et al., 1998), transcription factors critical for propagation of inflammation.

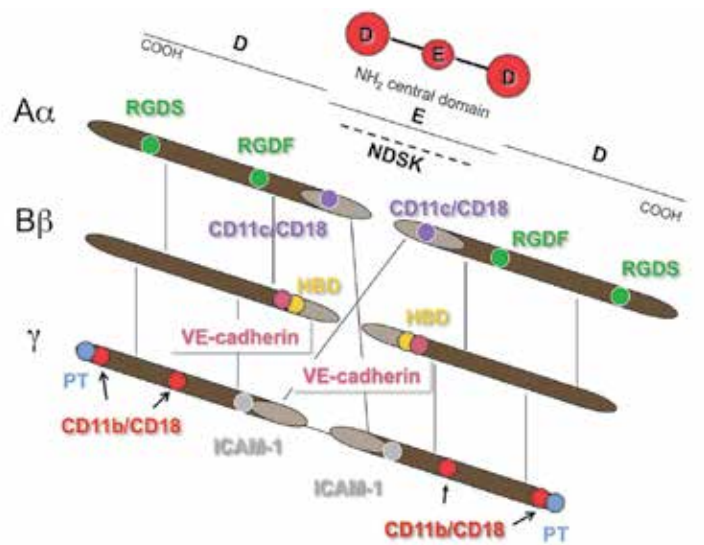

Fig. 4. Fibrin(ogen) enzyme and CNBr cleavage fragments and cell recognition domains.

Fg Aα, Bβ and γ chains are held together by 29 pairs of disulfide bonds (approximated by the vertical lines) with the N-termini of all six chains held together in the central domain. Electron microscopy studies indicate that the dimeric Fg molecule appears as a trinodular structure as depicted by the red ball and stick cartoon. Thrombin release of fibrinopeptides, FPA and FPB, from Aα and Bβ N-termini, respectively, produces soluble fibrin leading to fibrin polymerization into an insoluble gel stabilized by FXIIIA-mediated crosslinks between γ-γ and α-γ chains. Lines below the ball and stick cartoon denote N-terminal

plasmin cleavage fragment E and C-terminal fragments D. N-terminal disulfide knot (NDSK) (dashed line) is the minimal sequence of the central domain after CNBr cleavage and is structurally similar to plasmin E fragment. Residues on Fg for receptor-cell binding domains are: CD11c/CD18, $A\alpha^{17-19}$; integrin RGDF, $A\alpha^{95-98}$ and RGDS, $A\alpha^{572-575}$; ICAM-1, $\gamma^{117-133}$; CD11b/CD18, $\gamma^{190-202}$, $\gamma^{228-253}$ and $\gamma^{390-396}$; platelet (PT) binding, $\gamma^{400-411}$. The heparin binding domain (HBD) at β^{15-42} overlaps the VE-cadherin binding site. The first fibrin degradation products (FDPs) released by plasmin cleavage are the β^{15-42} domain and the C-terminal 2/3rd of the Aα chain, termed αC, which contain several cell binding domains.

2.4 Fibrin(ogen) in the stromal microenvironment in breast cancer

The tumor microenvironment is a complex entity composed not only of extracellular matrix (ECM) constituents including: i) growth factors; ii) cytokines and chemokines; iii) proteases; and iv) matrix glycoproteins, glycosaminoglycans and proteoglycans—but also diverse cell populations that influence the behavior of cancer cells including: v) immune cells such as lymphocytes, NK cells, dendritic cells, macrophages and neutrophils; vi) stromal fibroblasts/myofibroblasts, adipocytes and stem cells; and vii) cells of the vasculature including endothelial cells, pericytes and smooth muscle cells (reviewed in (Andre et al., 2010; Anton & Glod, 2009; De Wever et al., 2008; Deryugina & Quigley, 2006; Tlsty & Coussens, 2006; Ulisse et al., 2009)). Although activated inflammatory cells in the tumor microenvironment play important roles in cancer initiation, progression, angiogenesis and metastasis, they are not the most numerous. Cancer-associated fibroblasts, similar to myofibroblasts of healing wounds, are the most abundant stromal cells in the tumor microenvironment (Tlsty & Coussens, 2006), and contribute significantly to chronic inflammation by production of chemokines, cytokines, and pro-angiogenic factors and deposition of matrix constituents that support new blood vessel formation required for tumor growth, cell migration and metastasis (De Wever et al., 2008). Solid tumors need to develop their own blood supply for nutrient delivery and removal of toxic waste. Angiogenesis, the formation of new blood vessels from existing vasculature, requires activation of proteases leading to degradation of the basement membrane, endothelial cell sprouting and pericyte attachment for vessel stabilization. Cancer-associated fibroblasts play an important role in synchronizing these events (De Wever et al., 2008). Furthermore, the topography of the ECM mediates vascular development and regulates the speed at which cells migrate during angiogenesis (Bauer et al., 2009). Vascular endothelial cells play a pivotal role in regulating leukocyte recruitment during inflammation (McGettrick et al., 2007). In most cases, cancers exploit pro-inflammatory mediators and recruited inflammatory cells to benefit their own survival (Lorusso & Ruegg, 2008) (also as reviewed in (Simpson-Haidaris et al., 2010)).

Fg and fibrin deposition is found within the stroma of most solid tumors (Simpson-Haidaris & Rybarczyk, 2001), and elevated levels of plasma Fg and fibrin degradation products (FDPs) correlate positively with lymph node involvement and metastatic spread of colorectal, ovarian, lung and breast cancers (Sahni et al., 2009; Varki, 2007). Fibrin deposition at the tumor-normal host cell interface as well as in the stroma of primary tumors is well documented, and is thought to protect tumors from infiltrating inflammatory cells by acting as a barrier thereby preventing inflammatory reactions directed towards the tumor cells (reviewed in (Simpson-Haidaris & Rybarczyk, 2001)). The presence of D-dimer, a fibrin degradation product indicative of pathological fibrin formation and dissolution, correlates

with poor prognosis in most solid tumors including colon, prostate, lung and breast (Batschauer et al., 2010; Kilic et al., 2008; Knowlson et al., 2010). However, in some malignancies, including breast, evidence demonstrating deposition of fibrin within the primary tumor is lacking (reviewed in (Simpson-Haidaris & Rybarczyk, 2001)). Instead, abundant Fg deposition occurs in breast tumor stroma in the absence of thrombin generation (Costantini et al., 1991).

2.5 Cancer cells, including breast, synthesize and secrete fibrinogen

The origin of tumor-associated fibrin(ogen) and fibrin(ogen) degradation products has historically been thought to be from exudation of plasma Fg due to the increased vascular permeability and subsequent procoagulant or fibrinolytic activity at the tumor site (Rybarczyk & Simpson-Haidaris, 2000). However, because Fg deposition in the stroma, but not fibrin formation, is considered a hallmark of breast cancer (Costantini et al., 1991), we hypothesized that breast cancer cells were capable of endogenous synthesis and secretion of Fg. We demonstrated that human MCF-7 cells are capable of synthesizing Fg chains, although assembly of intact Fg is defective due to degradation of the Bβ chain (Rybarczyk & Simpson-Haidaris, 2000). In addition, we have shown that lung, prostate and breast cancer epithelial cells synthesize and secrete Fg that enhances FGF-2-mediated cell proliferation, assembles into the ECM and binds to cancer cell surface receptors (Rybarczyk & Simpson-Haidaris, 2000; Sahni et al., 2008; Simpson-Haidaris, 1997; Simpson-Haidaris & Rybarczyk, 2001). Others have shown Fg production in cervical (Lee et al., 1996) and intestinal (Molmenti et al., 1993) cancer cell lines. Expression array profiling studies confirmed that Fg genes are expressed in breast (Pentecost et al., 2005) and lung carcinomas (Tan et al., 2005) from patients. Thus, Fg synthesized by cancer cells promotes growth of the primary tumor and supports tumor-associated angiogenesis characterized by localized VEGF production and leaky vessels (Dvorak, 2006). The importance of VEGF in promoting tumor vascular permeability, angiogenesis and leakage of plasma Fg into the perivascular space to induce tumor stroma desmoplasia is well known. However, whether *tumor-associated fibrin(ogen)* contributes to permeability of tumor vessels and breast cancer metastasis is unknown.

2.6 Fibrinogen is an extracellular matrix protein

Although Fg is known for its hemostatic role, we showed that Fg, not fibrin, is a component of the insoluble fibrillar ECM of fibroblasts, alveolar epithelial cells, endothelial cells and breast epithelial cells (Guadiz et al., 1997; Pereira et al., 2002; Sahni et al., 2009; Simpson-Haidaris et al., 2010; Simpson-Haidaris & Sahni, 2010). Upon assembly into matrix fibrils, Fg undergoes conformational changes exposing the cryptic β^{15-42} epitope in the absence of thrombin cleavage or covalent crosslinking (Guadiz et al., 1997; Simpson-Haidaris & Sahni, 2010). When Fg is pre-established in the ECM of adventitial fibroblasts prior to wounding, increased cell proliferation and migration enhance wound closure (Rybarczyk et al., 2003), which is dependent on *de novo* protein synthesis (Pereira & Simpson-Haidaris, 2001) but independent of added growth factors, PDGF and FGF-2 (Rybarczyk et al., 2003). However, assembly of Fg into mature matrix fibrils of breast epithelial cells appears to correlate negatively with the increasing invasive potential of the cell (**Fig. 5**). We also determined whether the cryptic HBD in soluble Fg (Odrljin et al., 1996b) was accessible in matrix Fg using a specific MoAb (T2G1) (Kudryk et al., 1984). Whereas the T2G1 epitope (β^{15-21}) within β^{15-42} is not accessible for antibody binding in soluble Fg or Fg immobilized to a surface, the

results indicated that β^{15-21} is exposed on Fg assembled into matrix fibrils (Guadiz et al., 1997; Rybarczyk et al., 2003). Together these data suggest that matrix Fg possesses "fibrin-like" properties in the absence of fibrin polymerization and that Fg deposition rapidly changes the topology of the ECM to provide a surface for cell migration and matrix remodeling during wound repair. However, the mechanisms by which β^{15-42} modulates cell-cell or cell-matrix adhesion are not well understood.

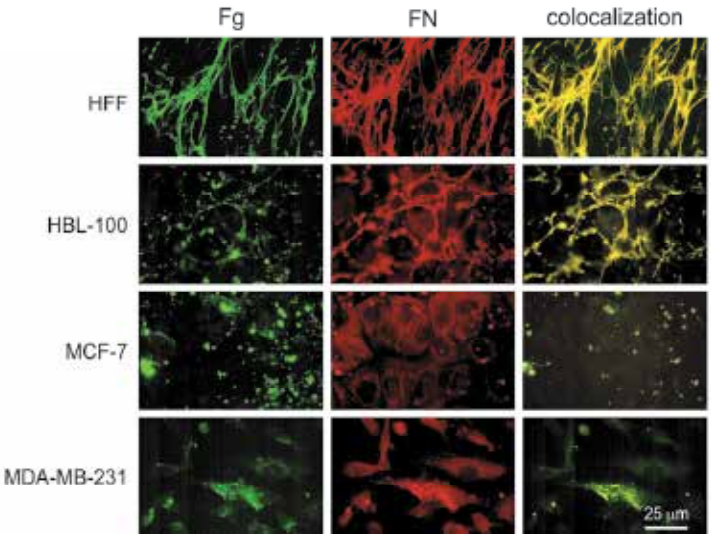

Fig. 5. Plasma fibrinogen assembles into mature matrix fibrils of nonmalinant cells (HFF and HBL-100) but poorly assembles in the matrix of malignant breast cancer cells (MCF-7 and MDA-MB-231). Primary human fibroblasts (HFF), a nonmalignant human breast cancer cell line (HBL-100) and two human breast cancer cell lines (MCF-7 and MDA-MB-231) were grown on gelatin-coated glass coverslips and treated with Fg conjugated to Oregon Green™ (30 µg/ml) for 24 hr. The cells were washed, fixed, stained with anti-fibronectin (FN) polyclonal antibodies followed by rhodamine-goat anti-rabbit secondary antibodies, and visualized by epifluorescence microscopy. Green fluorescence is Fg-specific and red fluorescence denotes FN staining. Colocalization of Fg and FN results in yellow fluorescence. The loss of FN in the more invasive cell lines (MCF-7 and MDA-MB-231) is likely an explanation for purified plasma Fg binding to the surface of cells but failure to assembly into mature matrix fibrils, as we have shown that assembly of Fg into an elaborate fibrillar ECM depends on the assembly of FN fibrils as well (Pereira et al., 2002).

3. Role of Fibrin(ogen) in breast cancer metastasis

3.1 Importance of Fg peptide β^{15-42} in Fg-endothelial cell interactions

Fibrin(ogen) β^{15-42} sequences support a diverse array of biological functions mediated by fibrin(ogen). Although the primary structure of fibrinopeptide B (FPB) is poorly conserved across species, the fibrin β^{15-42} domain is highly conserved, implying evolutionary conservation of function (Courtney et al., 1994). The β^{15-42} region constitutes a cryptic domain in soluble Fg that is exposed in fibrin after thrombin cleavage (Odrljin et al., 1996b). Both the HBD and overlapping binding site for VE-cadherin are localized to β^{15-42}. VE-

cadherin mediates homophilic cell-cell adhesion critical for the maintenance of barrier integrity of the endothelium. Disruption of VE-cadherin-mediated endothelial barrier function leads to altered vascular permeability found in a number of diseases including ischemia-reperfusion (IR) injury, inflammation, angiogenesis, and cancer growth and metastasis (discussed in (Sahni et al., 2009)). Exposure of β^{15-42} and binding by VE-cadherin is also required for endothelial capillary tube formation in fibrin gels (Bach et al., 1998a; Chalupowicz et al., 1995); portions of the third extracellular domain (EC3) of VE-cadherin constitute a fibrin β^{15-42} receptor (Bach et al., 1998b; Yakovlev & Medved, 2009). Newly exposed β chain residues, β15-GHRP-18, play a critical role in fibrin monomer aggregation during polymerization and clot formation during secondary hemostasis (Mosesson, 2005). Furthermore, exposure of the β^{15-42} domain mediates heparin-dependent fibrin binding to endothelial cell surfaces (Odrljin et al., 1996a); promotes endothelial cell adhesion and spreading (Bunce et al., 1992); promotes the release of endothelial cell-specific markers of endothelial activation (Ribes et al., 1989); and stimulates proliferation of endothelial cells, fibroblasts and cancer cells (Rybarczyk et al., 2003; Sahni et al., 2008; Sporn et al., 1995).

3.2 Fibrin β^{15-42} protects the myocardium from Ischemic-Reperfusion (IR) injury

A synthetic peptide of fibrin residues β^{15-42} has been implicated as a potential therapeutic agent to reduce tissue damage and scarring after a heart attack (Hirschfield & Pepys, 2003; Petzelbauer et al., 2005b; Roesner et al., 2007; Zacharowski et al., 2006; Zacharowski et al., 2007). Peptide β^{15-42} works by inhibiting leukocyte migration across the endothelium into heart tissue, which prevents excessive inflammation and tissue damage. Peptide β^{15-42}-mediated reduction of tissue injury depends on its ability to bind to VE-cadherin. Peptide β^{15-42} competes with FDP (*e.g.*, the plasmin E domain of fibrin as depicted in **Fig. 4**) for binding to VE-cadherin to prevent transendothelial cell migration (TEM) of leukocytes during myocardial IR injury (Petzelbauer et al., 2005b; Roesner et al., 2007; Zacharowski et al., 2006; Zacharowski et al., 2007). These published reports demonstrate the physiologic efficacy of fibrin β^{15-42} for treating IR injury. *However, the molecular mechanisms induced by fibrin(ogen)* β^{15-42} *binding to VE-cadherin to mediate enhanced paracellular permeability and whether fibrinogen-induced cancer metastasis involves binding interactions with fibrin(ogen)* β^{15-42} *have not been previously studied.*

3.3 Fibrin(ogen) β^{15-42} induces endothelial barrier permeability via VE-cadherin binding interactions

In a recent report (Sahni et al., 2009), we sought to determine whether fibrin(ogen) β^{15-42} binding to VE-cadherin induced endothelial cell permeability, and whether fibrinogen-induced cancer metastasis involves binding interactions between VE-cadherin and fibrin(ogen) β^{15-42}. Using transwell insert culture systems, we showed that Fg β^{15-42} and VE-cadherin binding interactions promote endothelial cell barrier permeability (Sahni et al., 2009) (**Fig. 6**). Peptides containing or missing residues β^{15-17} critical for β^{15-42} binding to VE-cadherin (Gorlatov & Medved, 2002) and neutralizing antibodies that bind to Fg β^{15-21} (T2G1) and VE-cadherin (BV9) (**Fig. 7A**) were used to induce or inhibit permeability. Fg induced dose-dependent permeability of human umbilical vein endothelial cells (HUVEC) and microvascular endothelial cells (HMEC-1) (**Fig. 6**), but not epithelial cell barriers (as shown in Fig. 1 in ref (Sahni et al., 2009)), which could be inhibited by neutralizing antibodies against β^{15-21} (T2G1) and VE-cadherin (BV9) and synthetic peptides (not shown).

However, the neutralizing antibodies (T2G1 and BV9) did not completely inhibit Fg-induced permeability (**Fig. 7B**), suggesting that additional cell recognition domains on Fg participate in fibrin(ogen)-induced vascular permeability.

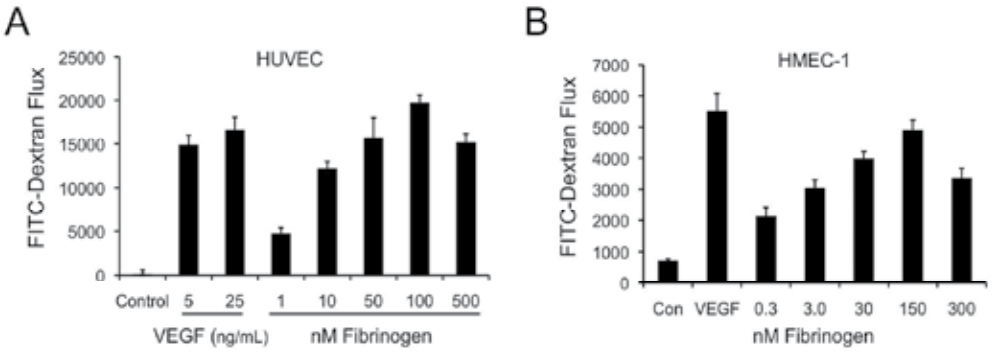

Fig. 6. Fg-induced EC permeability involves Fg β^{15-42} and VE-cadherin. Cells were grown to confluency on Millicell™ 24-well cell culture inserts. Panel 6A, HUVEC were left untreated (control) or treated for 15 min with increasing concentrations of Fg or VEGF as indicated. Panel 6B, HUVEC were treated with 30 nM of Fg plus 1 mg/ml FITC-Dextran for the times indicated. The FITC-Dextran flux to the bottom chamber was measured by fluorometry and the data presented as the mean relative FITC-Dextran Flux ± SEM. Data points were derived from 3 or more independent experiments with the total number of replicates per condition ranging from 6-13. (Reprinted from (Sahni et al., 2009) with permission). P-values can be found in ref (Sahni et al., 2009).

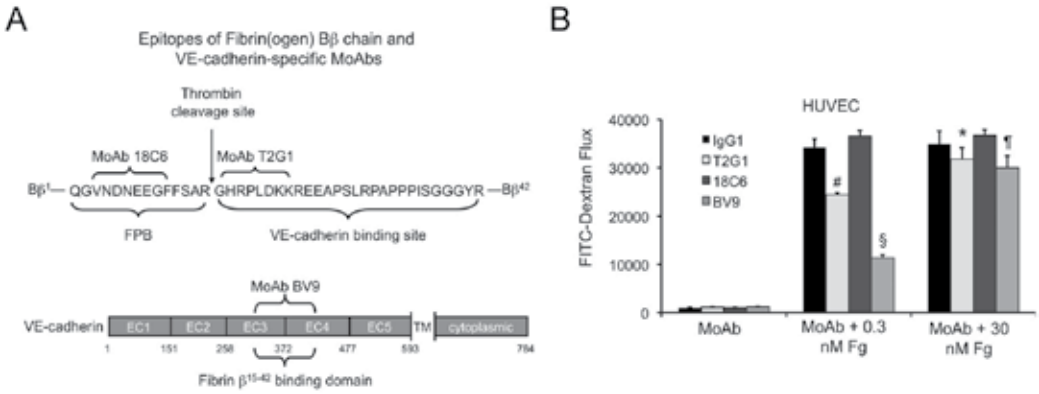

Fig. 7. Fg-induced EC permeability involves Fg β^{15-42} sequences and VE-cadherin. Panel 7A, schematics of the aminoterminus of the fibrin(ogen) Bβ chain and the domain structure of VE-cadherin are depicted. The arrow denotes the thrombin cleavage site for release of FPB. The 18C6 epitope maps to FPB, the T2G1 epitope maps to β^{15-21} and the VE-cadherin binding site on fibrin maps to β^{15-42}. The epitope of the VE-cadherin-specific monoclonal antibody BV9 maps to the third and fourth extracellular domains (EC3-EC4). The fibrin β^{15-42} binding site on VE cadherin maps to EC3 near the EC3-EC4 junction. TM, transmembrane domain. Panel 7B, all monoclonal antibodies used are IgG_1 isotype murine antibodies and

nonimmune IgG_1 was used for the control. Monoclonal antibodies were used at 3 nM in the absence of Fg, or with 0.3 nM or 30 nM Fg for 45 min. The data were plotted as the mean ± SEM of relative FITC-Dextran Flux and were obtained from three independent experiments with a total sample size of 6-9 per condition. (Reprinted from (Sahni et al., 2009) with permission). P-values can be found in ref (Sahni et al., 2009).

3.4 VE-cadherin binding domain of Fg (β^{15-42}) enhances transendothelial migration of malignant breast epithelial cells

Because plasma Fg promotes metastasis of some types of cancer and Fg β^{15-42} sequences promote endothelial cell permeability, we hypothesized Fg β^{15-42} sequences would play a role in promoting TEM of breast cancer cells. To test this hypothesis, breast cancer cells were labeled with a fluorescence cell-tracking dye (DiI) before they were mixed with increasing concentrations Fg. Breast cancer cells and Fg were allowed to pre-incubate for 15 minutes prior to addition to the upper chamber of a barrier monolayer of endothelial cells. After 45 minutes incubation, the relative number of breast cancer cells migrating to the underside of the transwell insert membrane were quantified by relative fluorescence and

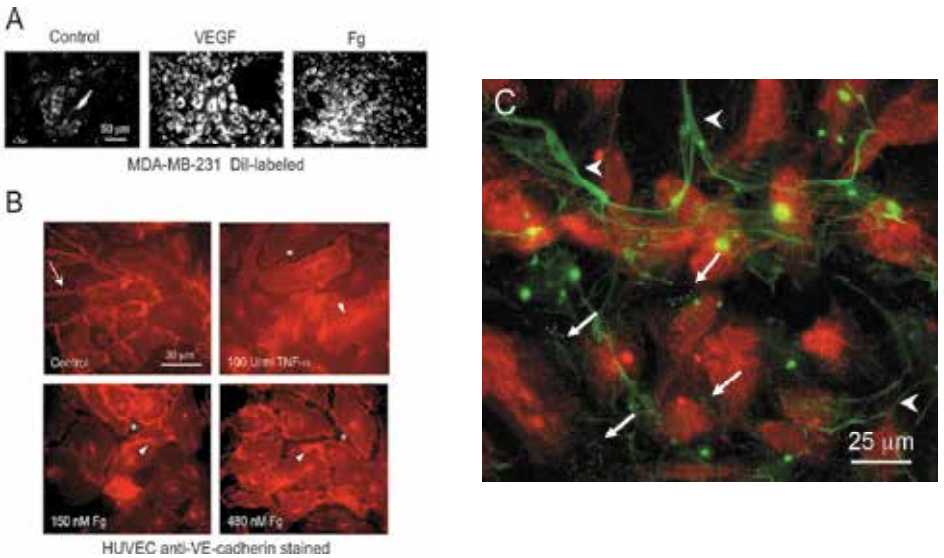

Fig. 8. Fg enhances TEM of malignant breast epithelial cells (Panel A), induces gap formation between adjacent endothelial cells (Panel B, asterisks), promotes intracellular relocalization (Panel B, arrowheads) of VE-cadherin at membrane cell-cell junctions (Panel B, Control, arrow), assembles into ECM (Panel C, arrowhead), and shows punctate, cell surface receptor-like binding between adjacent endothelial cells (Panel C, arrows). Cells in Panels A and B were treated as described in Section 3.3. In Panel C, endothelial cells were treated for 24 hours with purified plasma Fg conjugated to Oregon Green. Cells were fixed, permeabilized and stained with anti-FGF-2 (red fluoresence). After staining, the coverslip was mounted upside down on a microscope slide so that the basolateral aspect (bottom of cells) and the subendothelial ECM appear as the "top" of the cells. Matrix Fg and receptor bound Fg are shown in green fluorescence. Cover Figure ref (Sahni et al., 2009).

visualized by microscopy. VEGF was used as a positive control to induce endothelial cell permeability and TEM of breast cancer cells. The results indicated that TEM of both MCF-7 and MDA-MB-231 cells was increased in a Fg-concentration-dependent manner (see Fig. 3a of ref (Sahni et al., 2009)) and as visualized by immunofluorescence microscopy showing MDA-MB-231 cells adhered to the bottom side of the transwell filter (**Fig. 8A**).

To determine whether VE-cadherin and/or Fg β^{15-42} were involved in Fg-enhanced TEM of MDA-MB-231 cells, the assay was repeated in the presence of the neutralizing and control antibodies (as shown in Fig. 3c of ref (Sahni et al., 2009)). To determine whether Fg promoted gap formation between cells, confluent HUVEC were treated with 150 or 480 nM Fg or 100 Units/ml TNF-α, a known inducer of endothelial permeability and gap formation, for 30 minutes then cells were fixed, permeabilized and immunostained with an anti-VE-cadherin. Fg treatment induced gap formation between adjacent endothelial cells, and such treatment promoted the subcellular relocalization of VE-cadherin from the cell periphery as in control cells into the cytoplasm in Fg- and TNF-α-treated cells (**Fig. 8B**). Indirect evidence for Fg binding at endothelial cell-cell junctions was obtained by fluorescence microscopy. The data reveal that Fg binds to endothelial cell-cell junctions in a punctate pattern, consistent with cell surface receptor binding to the cell-cell adhesion receptor, VE-cadherin (**Fig. 8C, arrows**). Fg also assembles as part of the fibrillar subendothelial ECM (**Fig. 8C, arrowhead**). Taken together, the data in **Fig. 6-8** demonstrate that the VE-cadherin binding domain defined by residues 15-42 on the β-chain of human Fg induces permeability of endothelial but not epithelial cell barriers and enhances TEM of malignant breast cancer cells by a VE-cadherin-dependent mechanism. In contrast, the basal level of TEM of nonmalignant breast epithelial cells was not enhanced by Fg treatment (Sahni et al., 2009).

3.5 Fibrinogen potentiates endothelial cell permeability at low doses of VEGF

Both FGF-2 and VEGF bind to fibrin(ogen) at distinct sites with high affinity (Sahni & Francis, 2000; Sahni et al., 1998). Fg bound-FGF-2 potentiates endothelial cell proliferation over FGF-2 alone (Sahni et al., 2003; Sahni & Francis, 2004; Sahni et al., 2006; Sahni et al., 1999). Although Fg-bound VEGF remains active, it does not potentiate endothelial cell proliferation over VEGF alone (Sahni & Francis, 2000). Because Fg induces endothelial cell permeability through VE-cadherin binding interactions (Sahni et al., 2009) and VEGF binds to Fg (Sahni & Francis, 2000), we tested the hypothesis that Fg would potentiate VEGF-induced EC permeability (**Fig. 9**).

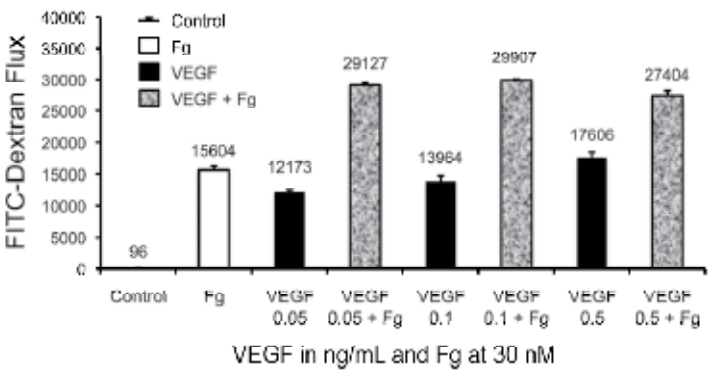

Fig. 9. Fg enhances permeability induced by low concentrations of VEGF.

The data indicate that 10 μg/ml (30 nM) Fg enhanced the flux of FITC-dextran to the bottom chamber of the transwell plate at low doses of VEGF (0.05 and 0.1 ng/ml); however, the additive effect on induction of endothelial cell permeability was lost at 0.5 ng/ml and higher concentrations of VEGF (**Fig. 9**). Fg-enhancement of VEGF-induced permeability is rapid and saturated within 5 min, whereas 5 ng/ml of VEGF is required to induce a similar amount of FITC-dextran flux as 30 nM Fg + 0.05 ng/ml, *i.e.*, 100-fold less VEGF. Studies by others suggest that low-dose VEGF mediates inflammation to promote cell survival of vascular and nonvascular cells such as those of the CNS, prior to induction of angiogenesis (Abumiya et al., 2005; Croll et al., 2004). Furthermore, VEGF colocalizes with exuded Fg at sites of edema in renal cell carcinoma (Verheul et al., 2010). Together with the aforementioned published data, our results suggest that Fg may regulate vascular permeability induced by low doses of VEGF without inducing EC proliferation—such a response would be conducive to fibrinogen induction of breast cancer cell TEM.

4. Summary, therapeutic strategies and future research to elucidate fibrin(ogen)-mediated mechanisms of breast cancer metastasis

4.1 Summary and therapeutic strategy using free peptide β^{15-42} to inhibit breast cancer metastasis as depicted in Fig. 10, Steps 1-11

Regardless of the subtype of breast cancer, once the primary tumor becomes established (*Step 1*), it needs to develop its own blood supply for nutrient delivery and removal of toxic waste (*Step 2*). Breast cancer cells produce VEGF, which initiates permeability of nearby blood vessels allowing plasma Fg to leak into the tumor stroma promoting desmoplasia and deposition of a provisional fibrin(ogen) matrix in the tumor microenvironment (*Step 2*). Alternatively, endogenous synthesis of Fg by breast cancer cells could induce cancer progression. Thus, the innate immune response is activated to defend the host against this neoplastic insult. Release of IL-6 systemically leads to increased production of plasma Fg and fibrin formation resulting in exposure of β^{15-42} and binding to VE-cadherin, a step critical for angiogenesis (Bach et al., 1998b; Martinez et al., 2001). Furthermore, VEGF binds to Fg and fibrin with high affinity (Sahni & Francis, 2000), which may be necessary for Fg to enhance VEGF-mediated endothelial cell permeability without potentiating endothelial cell proliferation. In contrast, VE-cadherin and VEGF receptor-2 form a signaling complex to promote endothelial cell proliferation (Carmeliet et al., 1999; Dejana, 2004; Esser et al., 1998).
Fibrin(ogen) potentiates FGF-2- but not VEGF-induced proliferation of endothelial cells, angiogenesis and cancer cell growth (Rybarczyk & Simpson-Haidaris, 2000; Sahni & Francis, 2000; Sahni et al., 2006; Sahni et al., 2008; Sahni et al., 1999; Simpson-Haidaris, 1997; Simpson-Haidaris & Rybarczyk, 2001). Furthermore, fibrin(ogen) enhances cell migration and cancer invasion through tumor stroma, and TEM, *i.e.*, intravasation of breast cancer cells into the blood stream (*Step 3*) (Roche et al., 2003; Rybarczyk et al., 2003; Sahni et al., 2009). Fg and fibrin can bridge between cells of the same or different kinds (Kloczewiak et al., 1983; Languino et al., 1995; Languino et al., 1993; Saito et al., 2002; Sriramarao et al., 1996) and form aggregates or tumor emboli coated with fibrin(ogen) (*Step 4*). Because the host immune system does not recognize fibrin(ogen)-coated tumor emboli (Palumbo et al., 2005), immune-mediated destruction of tumor cells does not occur and these tumor emboli travel through the circulation to sites favorable for metastatic growth (*Steps 5 & 6*) such as lung. To establish metastatic growth, tumor emboli need to leave the circulation and enter lung tissue (*Steps 7 and 8*) where they find a receptive niche (*Step 9*) to begin the process again. Tumor

cell proliferation and angiogenesis (*Step 10*) in lung results in metastatic disease (*Step 11*). We *hypothesize* that free peptide β^{15-42} will bind to VE-cadherin between endothelial cells to block endothelial cell binding to β^{15-42} on intact fibrin(ogen) found in the tumor stroma or tumor vessels, thereby inhibiting tumor-associated angiogenesis (*Step 2*), intravasation (*Step 3*), extravasation (*Step 8*), and angiogenesis at metastatic tumor sites (*Step 10*) (**as denoted by the lightening bolts at these steps in Fig. 10**).

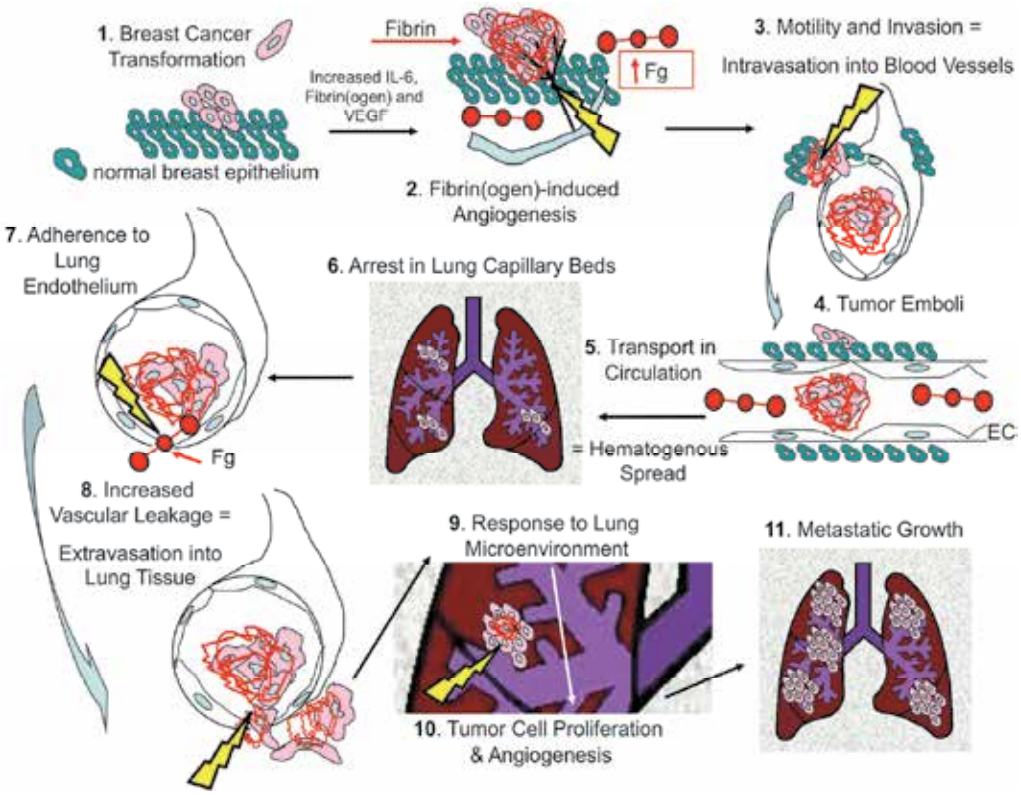

Fig. 10. Schematic summarizing role of fibrin(ogen) β^{15-42} in breast cancer metastasis and hypothesis development for employing free peptide β^{15-42} as a therapeutic strategy to treat metastatic breast cancers.

Successful demonstration of peptide β^{15-42} as an inhibitor of breast cancer metastasis and tumor-associated inflammation and angiogenesis *in vivo* would significantly impact breast cancer treatment in a timely manner. Peptide β^{15-42}, an endogenous fragment of fibrin, is already shown to be well tolerated in humans and effective in reducing damage to heart muscle after a heart attack in preclinical models of IR injury. However, until now, no one has proposed the use of peptide β^{15-42} as an inhibitor of breast cancer metastasis. A precedent and pipeline for production of viable therapeutics based on peptide β^{15-42} exists for treatment of damaged heart tissue, and Phase I and Phase II clinical trials are ongoing to test the safety and efficacy, respectively, of free β^{15-42} peptide for IR injury (Hallen et al., 2010; Petzelbauer et al., 2005a; Petzelbauer et al., 2005b; Roesner et al., 2007; Roesner et al., 2009; Wiedemann et al., 2010; Zacharowski et al., 2006). Therefore, the timeline for

successful translational to a therapeutic agent to treat metastatic disease in breast cancer patients with different subtypes of the disease would be significantly shortened. Moreover, even if the primary tumor develops its own blood supply before adjuvant therapy with peptide β^{15-42} is begun, we predict that free peptide β^{15-42} will prevent subsequent steps required for metastatic spread and growth of breast cancers. Another advantage to this therapeutic strategy is that peptide β^{15-42} functions outside the cell, precluding the need to deliver the peptide inside cells. Identifying molecular targets for therapeutic intervention of breast cancer metastasis, recruitment of inflammatory cells and angiogenesis will increase long-term disease-free survival and improve the quality of life for breast cancer patients.

4.2 Putative mechanisms whereby nonmalignant breast epithelial cells switch to a metastatic breast cancer cell phenotype responsive to fibrinogen induced TEM

A class of molecules found in the ECM, inside cells and attached to cell surfaces, called heparan sulfate proteoglycans (HSPG), contribute to breast cancer progression by promoting cancer cell proliferation, TEM, and tumor-associated angiogenesis (Koo et al., 2008). The ability to affect any one of these functions would help to reduce breast cancer metastasis; however, if all three of the functions could be targeted with one therapeutic approach, the morbidity and mortality due to metastatic breast cancer could be significantly reduced. Heparin is widely used as an anticoagulant, but it also inhibits HSPG-dependent mechanisms of cancer metastasis (Levy-Adam et al., 2005). However, anti-metastatic heparins that also inhibit blood coagulation are, therefore, not good candidates for widespread use to treat metastatic breast cancer due to bleeding complications. Thus, another molecular target to inhibit the prometastatic effects of HSPG but not inhibit coagulation is greatly needed. Spontaneous blood-borne and lymphatic metastasis of tumor emboli requires fibrin(ogen) (Palumbo et al., 2002). In addition to binding to VE-cadherin (Yakovlev et al., 2003), Fg β^{15-42} also binds to heparin and HSPG on endothelial cells with high affinity (Odrljin et al., 1996a; Odrljin et al., 1996b); however, a role for HSPG in Fg-mediated breast cancer metastasis has not been studied. Fg binding to heparin and HSPG involves residues β^{15-42}, and β^{15-42}-dependent fibrin binding to EC surfaces can be inhibited with heparin and heparan sulfate but not with chondroitin sulfate, indicating that Fg-β^{15-42} represents a HBD (Odrljin et al., 1996a; Odrljin et al., 1996b). The Fg HBD was later mapped to residues β^{15-57}, which includes the β^{15-42} VE-cadherin binding domain (Yakovlev et al., 2003; Yakovlev & Medved, 2009). In our recent publication (Sahni et al., 2009), we unexpectedly discovered that Fg enhanced TEM of only malignant breast cancer cells (MCF-7 and MDA-MB-231) but not nonmalignant breast epithelial cells (MCF-10A), suggesting inherent differences in the ability of cancer vs. normal breast epithelial cells to interact with fibrin(ogen). Because TEM of nonmalignant epithelial cells (MCF-10A) could not be enhanced in the presence of Fg (Sahni et al., 2009), we hypothesize that loss of HSPG from the surface of premalignant breast epithelial cells serves as a molecular switch to induce a highly aggressive, metastatic breast cancer phenotype (**Fig. 11A**). We plan to investigate this hypothesis in future studies.

Another mechanism to regulate Fg-enhanced TEM of malignant breast cancer cells is a gain in function of cancer-associated Mucin-1 (MUC1), which is a membrane-associated mucin expressed at low levels on the apical surface of normal polarized epithelial cells. MUC1 is a tumor-associated glycoprotein aberrantly expressed in >90% of breast cancers (Singh & Bandyopadhyay, 2007), promotes cancer cell proliferation and metastasis, and is associated

with poor survival (Hattrup & Gendler, 2006; Yuan et al., 2007). MUC1 is upregulated and hypoglycosylated in breast cancers. The polarized expression of MUC1 is lost on cancer cells such that it is expressed on the entire cell surface (Kondo et al., 1998; Moase et al., 2001; Wesseling et al., 1996; Yang et al., 2007). The MUC1 extracellular domain protrudes ~200 nm above the cell surface, whereas most cell surface receptors are ~35 nm long (Wesseling et al., 1996). When MUC1 is interspersed between adhesion molecules, it nonspecifically reduces cell-cell and cell-ECM interactions in vitro and in vivo, likely by steric hindrance caused by the extreme length and high density of the MUC1 at the cell surface (Wesseling et al., 1996) (**Fig. 11B**). MUC1 expression is found on MCF-7, MDA-MB-231, as well as other types of breast cancer cells, particularly on those isolated from patients with a highly aggressive subtype called inflammatory breast cancer (Alpaugh et al., 2002; Schroeder et al., 2003; Walsh et al., 1999); elevated expression of MUC1 contributes to lymphovascular tumor invasion of inflammatory breast cancer cells (Alpaugh et al., 2002).

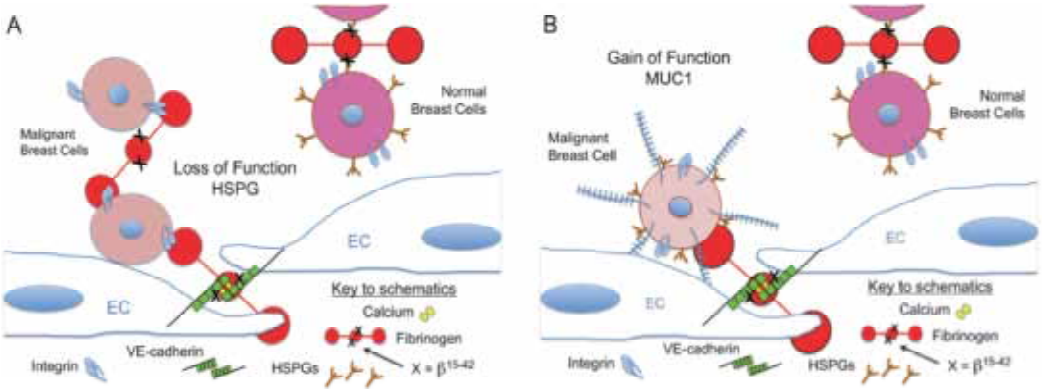

Fig. 11. Putative mechanisms whereby nonmalignant breast epithelial cells switch to a metastatic breast cancer cell phenotype responsive to fibrinogen-induced TEM. Panel A, schematic depicting loss of function due to release of cell-surface HSPG. Panel B, schematic depicting gain of function by overexpression of MUC1 leading to loss of polarity and cell-cell adhesion in breast epithelial cells.

We predict that Fg could bind to normal breast cell surface HSPG through Fg $\beta^{15\text{-}42}$, thus preventing Fg $\beta^{15\text{-}42}$ binding to VE-cadherin extracellular domain 3 (EC3) and inhibition of TEM. Enhanced heparanase expression and enzymatic digestion of HSPG in human tumors correlates with metastatic potential, tumor vascularity, and reduced postoperative survival of cancer patients (Vlodavsky et al., 2008). Heparanase-induced loss of breast epithelial cell surface HSPG during conversion of non- or pre-malignant to malignant breast cancers would allow Fg $\beta^{15\text{-}42}$ binding to VE-cadherin at cell-cell junctions to induce EC permeability. Fg would also bind to breast cancer cell integrins via binding sites on Fg C-terminal domains (**see Fig. 4**) then movement of VE-cadherin (induced by Fg binding to VE-cadherin as shown in **Fig. 8B**) in the endothelial cell membrane would induce paracellular transfer of Fg-bound breast cancer cells across the endothelial cell barrier to promote cancer metastasis. A precedent for this mechanism is already established; Fg binding to a counter adhesion molecule facilitates neutrophil TEM 20- to 30-fold (Languino et al., 1995). Overexpression of MUC1 could block accessibility of HSPG on breast cancer cells, which

would also prevent Fg β^{15-42}–HSPG binding interactions leaving Fg β^{15-42} available for binding to VE-cadherin. Alternatively, loss of cell surface HSPG and elevated expression of MUC1 may contribute to Fg-enhanced TEM of malignant compared to nonmalignant breast epithelial cells. These possibilities will be addressed by future experiments.

5. Acknowledgements

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10

Hyaluronan Associated Inflammation and Microenvironment Remodelling Influences Breast Cancer Progression

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1. Introduction

1.1 The breast microenvironment

The breast is an organ composed predominantly of glandular, fatty, and fibrous tissues. Glandular tissue is composed of ducts lined by luminal epithelial cells that secrete milk, and is surrounded by a layer of myoepithelial cells that contract to release milk. Myoepithelial cells produce proteases, growth factors and growth factor receptors that contribute to remodelling during breast tissue expansion. Each duct is enclosed by a laminin-rich basement membrane and embedded in extracellular matrix (ECM). Mammary gland ECM and is a mixture of fibrillar proteins such as collagens, laminins, fibronectin, and polysaccharides such as heparin sulphate, chondroitin sulphate and hyaluronan (HA). These collectively provide the mechanical and structural support required for maintaining mammary tissue architecture and for storage of the soluble regulatory molecules needed for tissue homeostasis, plasticity, and remodelling. ECM promotes both the differentiated, homeostatic integrity of mammary tissue and is also a key determinant in branching morphogenesis, response-to-injury and pathological processes such as neoplastic disease. The importance of the ECM in determining homeostatic vs. tumourigenic events was originally demonstrated three decades ago by Beatrice Mintz, who showed that marked embryonic carcinoma cells injected into blastocysts do not give rise to tumours but instead contribute to normal tissue architecture. The same cells injected into adult mice develop into tumours (Mintz and Illmensee, 1975). Components of the microenvironment that support tumour progression have since been identified. For example, chick embryos infected with Rous Sarcoma virus express the oncogene v-src in every cell but tumours develop only at sites of wounding due to the accumulation of TGF-β1 (Weigelt and Bissell, 2008).

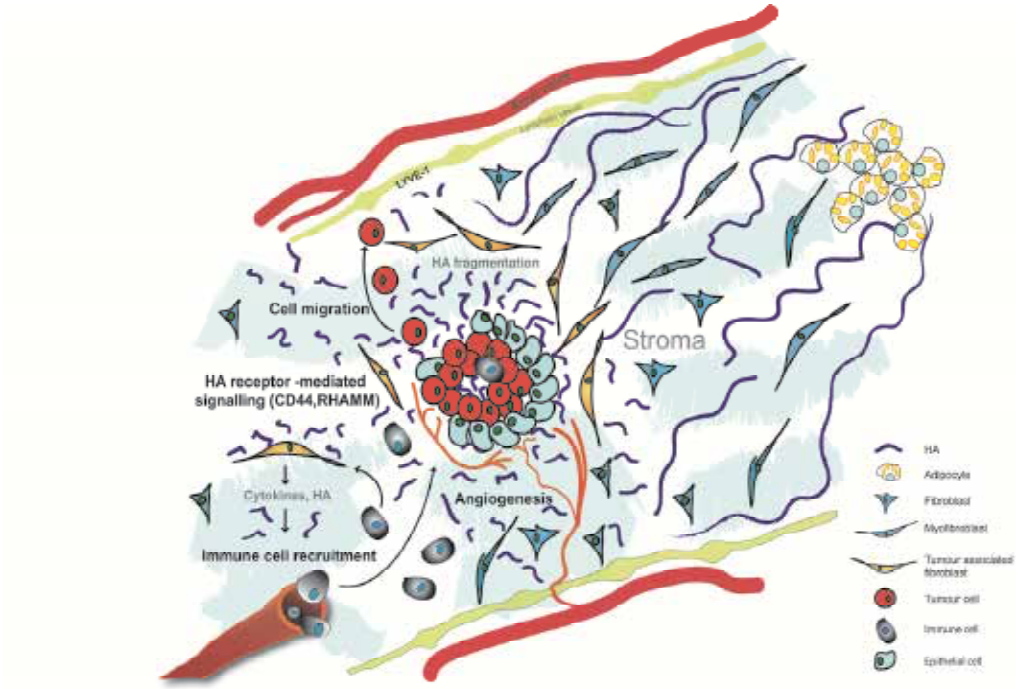

Fig. 1. Breast tumour microenvironment

Conversely, breast tumour cells can be reverted by blocking signalling through ECM receptors, including integrins (Turley *et al.*, 2008) and HA receptors such as RHAMM (Hall *et al.*, 1995). These and other studies have revealed a key role of ECM in initiating and sustaining breast cancer and introduced the novel concept that transformation can be a plastic rather than irreversible process. Specifically, increased HA accumulation in tumour cells or stroma is associated with poor outcome in Breast Cancer (BCA) (Tammi *et al.*, 2008). These studies predict that HA is an important component of ECM that determines a homeostatic vs. tumourigenesis "switch".

2. HA biology

2.1 Biochemical properties

HA belongs to the glycosaminoglycan group of polysaccharides composed of disaccharide units of a hexose linked to a hexosamine. It consists of repeating units of *N*-acetyl glucosamine and β-glucuronic acid (Fig. 2). The native polymer consists of up to 10^6 to 10^7 non-branching disaccharide units. The functions of HA within the ECM and cells depend upon its molecular weight, the type of cell, and the HA receptor(s) that target cells express. High molecular weight HA (e.g. >200 kDa) is a major biomechanical factor in ECM, which contributes to tissue hydration and elasticity by providing a template for the assembly of macromolecular complexes. A well known example is the "bottle brush" complex of aggrecan and link proteins, which provides the visco-elastic nature of synovial fluid. HA fragments provide signalling functions and are usually present during the ECM remodelling that is associated with morphogenesis or disease. Regulated synthesis and degradation are key factors in maintaining a delicate balance between structural (homeostatic) and signalling

(wound and disease) functions of HA (Itano *et al*., 2008, Jiang *et al*., 2007, Veiseh and Turley, 2011). BCA cells are particularly adept at producing and responding to HA fragments. BCA cells produce increased levels of HA by increasing HA synthase expression, rapidly fragmenting HA as a result of increased Reactive Oxygen Species (ROS) production, and increasing hyaluronidase expression and release, and increasing expression and display of HA receptors to elevate the response to these fragments (Simpson and Lokeshwar, 2008, Toole and Slomiany, 2008, Veiseh and Turley, 2011).

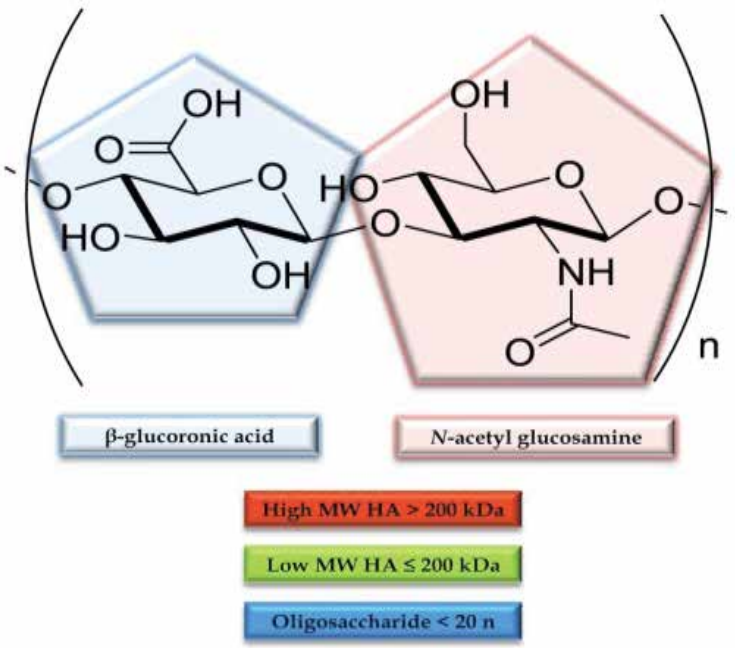

Fig. 2. HA structure and molecular weight ranges.

2.2 HA synthesis and tumourigenesis

HA is synthesized by three HAS isoforms, HAS1-3, which are located on different chromosomes but share from 57 to 80% sequence homology (Weigel *et al*., 1997, Lokeshwar and Selzer, 2008, Stern, 2008). The mature enzymes are multi-pass integral proteins, which are primarily located in the plasma membrane and catalyze polymerization of HA from the uridine diphosphate (UDP) sugars uridine diphosphate glucuronic acid (UDP-Glc-UA) and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAC). Synthesis and secretion of HA occur concurrently, allowing for the rapid production and release of large polymers into the ECM (Weigel *et al*., 1997). There is some evidence that HASs are resident in endosomes, ER and the perinuclear membrane although whether or not these produce intracellular HA is not yet clear (Karousou *et al*., 2010, Vigetti *et al*., 2010). HAS1 and 2 are widely expressed throughout the embryo while HAS3 expression is more restricted, for example, to developing tooth-forming neural crest cells and hair follicles. Genetic deletion of HAS2 is embryonic lethal in mice due to severe defects in cardiac tissue development, whereas targeted disruption of the HAS1 or 3 alleles results in fertile viable animals with only minor

aberrations in tooth and follicle development (Weigel and DeAngelis, 2007). It is not fully understood why only HAS2 is absolutely required for organogenesis, but it has been suggested that it produces high molecular weight tissue HA while the other HASs produce the smaller HA sizes (Itano *et al*., 1999). There are differences in the mechanisms by which HAS isoform expression and enzyme activity are regulated that may be relevant to their functions and essential or non-essential roles in organogenesis (Tammi *et al*., 2008).

BCA cells use several mechanisms to rapidly control the synthesis and release of HA, thereby modifying their ECM, including substrate availability, gene expression, posttranslational control of enzyme activity, and differential response to cytokines and ECM signalling. The availability of UDP sugars can profoundly influence the yield of HAS enzymes (Kakizaki *et al*., 2004). This has been demonstrated by the use of 4-Methylumbelliferone (4-MU), which depletes intracellular levels of UDP-Glc-UA (Kakizaki *et al*., 2004) by serving as a glucuronidation substrate. It blocks HA production and reduces BCA tumourigenicty.

The genomic plasticity and instability of cancer cells often leads to chromosomal aberrations that can result in both de-regulation of gene expression and allele duplication. Chromatin breakpoint analysis using a BCA line revealed significant chromosomal rearrangements close to the HAS2 gene. These result in de-regulation of HAS2 expression and significantly higher HAS2 mRNA levels in transformed cells compared to normal breast cells (Unger *et al*., 2009). Detailed *in vitro* and *in vivo* studies of BCA lines and xenografts have provided numerous insights into the effects of genetically modifying HAS expression levels on HA concentration within the tumour and peri-tumoural stroma. Antisense inhibition of HAS2 in MDA-MB-231 BCA cells delays proliferation via a transient arrest of the cell cycle (Udabage *et al*., 2005). Knockdown of HAS expression also results in significant alterations in genes associated with HA metabolism. CD44 and HYAL1 expression are both down-regulated in response to antisense inhibition of HAS2. *In vivo*, MDA-MB-231 cells expressing antisense HAS2 do not form tumours in nude mice after 12 weeks, whereas the parental cell line readily establishes both primary and secondary tumours during this time. This clearly implicates tumour cell HA as a significant driver of BCA formation. Elevated HA accumulation within BCA peri-tumoural stroma is also a prognostic factor and appears to promote a microenvironment suitable for BCA growth. For example, HAS2-/- fibroblasts transplanted with BCA cells into the fat pads of NOD/SCID mice fail to recruit macrophages and promote angiogenesis to the same extent as HAS2+/+ fibroblasts. This defect results in decreased tumour volume (Kobayashi *et al*., 2010).

The expression of all three HASs is controlled by growth factors and cytokines. However, there appear to be subtle differences in the response of each isoform that depend upon the cell type. For example, PDGF and TGFβ induce HAS2 expression in fibroblasts but HAS1 or 3 expression in synoviocytes and keratinocytes, respectively (Karousou *et al*., 2010). H-Ras transformation increases only HAS2 expression in 3Y-1 tumour cells, while transformation with v-src or v-fos increases both HAS1 and HAS2 expression in the same cells (Itano *et al*., 2004). Posttranslational modification of HAS, including phosphorylation by PKC, PKA, and the ERK/ErbB2 MAPK pathways (Goentzel *et al*., 2006, Itano and Kimata, 2008) as well as mono-ubiquitination (Karousou *et al*., 2010) also affects HAS activity. HAS3 serine phosphorylation is enhanced upon treatment with a PKC activator (Goentzel *et al*., 2006). All three HAS isoforms expressed by SKOV3 ovarian cancer cell line are phosphorylated by

ERK1,2 in response to treatment with Heregulin (Bourguignon *et al.*, 2007) and mono-ubquitination of K190 on HAS2 rapidly inactivates this enzyme (Karousou *et al.*, 2010).

2.3 HA fragmentation and its role in tumourigenesis

In addition to HAS1-3 expression, the amount and polymer size of HA are also affected by reactive oxygen species (ROS) and secreted hyaluronidases (HYALs), which fragment HA to various sizes. Significant levels of ROS can be generated during times of oxidative stress and these are considered critical in cancer initiation, promotion and progression (Karihtala *et al.*, 2007). ROS are produced in response to extracellular stimuli such as bacterial infections and environmental toxins, but can also be produced by cellular metabolism (Yu *et al.*, 2011). Five HYALs fragment HA: HYAL-1-3, PH-20 and HYAL-5. The HYALs differ in their cellular location and enzymatic properties. HYAL-1 and 2 are the major HYALs produced by somatic tissues whereas HYAL-3 is expressed mostly in bone marrow and testes. Both PH-20 and HYAL-5 expression are normally restricted to testes but PH20 is aberrantly expressed in BCA (Stern, 2008). HYAL-1 and 2 cooperate to degrade HMW HA in a coordinated fashion. HYAL-2, which is GPI anchored to the cell surface, degrades extracellular HA to fragments of 20 kDa, which are then taken up into endocytic vesicles. HYAL-1 present in the lysosome further degrades intracellular HA into tetrasaccharides (Tammi *et al.*, 2001, Stern, 2008, Simpson and Lokeshwar, 2008). Coordinated breakdown of HA by HYALs increases the rate of HA metabolism and this appears to be an important factor in tumourigenesis (Veiseh and Turley, 2011). For example, co-expression of HAS3 and HYAL-1 increases the aggressiveness and spread of prostate cancer cells compared to expression of either alone (Bharadwaj *et al.*, 2009). In BCA, HYAL-1 and HYAL-2 are often coordinately overexpressed compared to non-malignant breast tissue. Knockdown of HYAL-1, which is overexpressed in MDA-MB-231 and MCF-7 BCA lines, reduces tumour xenograft size (Tan *et al.*, 2010).

3. HA receptors detect oligosaccharides and fragments: Control of key signalling pathways by HA fragments

3.1 CD44

CD44 is a class I transmembrane receptor, which binds to HA via a link domain and is expressed by a variety of cells, including fibroblasts, endothelial and epithelial cells, smooth muscle, and haematopoietic cells. A vital role of CD44 is recruiting cells, including immune cells and fibroblasts, to sites of inflammation through HA-mediated signalling. Under homeostatic conditions, CD44 is in a low HA binding state, but during injury and tumourigenesis its binding affinity is increased and it mediates the inflammatory and tissue repair responses (Thorne *et al.*, 2004, Naor *et al.*, 2008). CD44 is expressed as many different isoforms due to extensive splicing in a region proximal to the transmembrane domain (Thorne *et al.*, 2004). The smallest CD44 isoform, CD44s (standard form), skips this variable region. The role of CD44s and variants in BCA progression is still controversial. For example, CD44s expression in $CD44^{low}$ MCF-7 human BCA cells results in xenograft metastasis to the liver (Ouhtit *et al.*, 2007) while CD44-/- mice develop more lung metastases than wildtype animals in response to polyomavirus middle T (Lopez *et al.*, 2005). Importantly, a recent study by Brown *et al.* (2011) demonstrated that CD44s expression is elevated and required for epithelial-mesenchymal transition of immortalized human mammary epithelial cells and for recurrence of HER2/*neu* induced murine mammary tumours (Lopez *et al.*, 2005). HA synthesis is elevated in CD44+ BCAs

compared to CD44- and both CD44+ and HER2+ BCAs are amongst the most aggressive and invasive subtypes of BCA with poor prognosis. Expression of variant exons, in particular exon v6, is associated with increased *in vitro* cell migration and invasion of human BCA cells (Herrera-Gayol and Jothy, 1999). Although CD44v6 expression has been correlated with multiple clinicopathological features (primary tumour size, axillary nodal status, histological grade and pTNM stage) it is not an independent prognostic factor (Ma *et al*., 2005). A study by Rys *et al*. (2003) found a correlation between the expression of CD44 v3 and the presence of BCA metastasis. Additionally, high CD44s expression correlates with increased disease free survival in node negative invasive BCA (Diaz *et al*., 2005). The controversies surrounding CD44 and its role in BCA progression may be caused by a limited number of patient samples in some of these studies, heterogeneity of BCA, and CD44 expression by cancer stem cells. The latter, in particular, has raised much recent interest in CD44 since several groups have identified CD44 as a potential marker for BCA stem cells. This is a highly tumourigenic population of cancer cells that, although only representing a small percentage of cells in the tumour, are thought to be responsible for tumour recurrence, metastasis and treatment failure. Aggressive BCA and BCA tumour progenitor cells have enhanced CD44 expression, associated with an increase in HA synthesis and CD44-HA binding affinity (Heldin *et al*., 2008).

In BCA cells, HA triggers CD44 interactions with a variety of signalling mediators involved in cell proliferation, migration and chemo-resistance. Ankyrin is a membrane-associated component of the cytoskeleton that is involved in regulation of cytoskeleton turnover and IP3 receptor-mediated regulation of intracellular Ca^{2+}. CD44-HA interactions induce CD44-ankyrin coupling and modify receptor-dependent Ca^{2+} mobilization (Bourguignon *et al*., 2008). CD44 also localizes ankyrin and IP3 receptor to lipid rafts, which are cholesterol and caveolin rich signalling microdomains in the plasma membrane (Fig. 3). The Rho GTPases, RhoA, Rac and CDC42, are key regulators of cell migration and HA stimulates RhoA in BCA cells. RhoA activity is regulated by RhoGEF, a guanine nucleotide exchange factor that forms a complex with CD44 in BCA cells. One of the downstream RhoA targets, ROK, phosphorylates the cytoplasmic domain of CD44 thereby increasing CD44-ankyrin interactions. Other targets of ROK are myosin phosphatase and myosin light chain, two important mediators of actin-myosin dependent membrane ruffling required for cell migration. HA also activates the PI3 kinase/AKT pathway: Gab-1 phosphorylation by ROK stimulates PI3 kinase and AKT activation, leading to increased cell proliferation, invasion and cytokine production (Bourguignon *et al*., 2008). Additionally, ROK phosphorylates and activates NHE1, a Na^{+}-H^{+} exchanger, causing intracellular and extracellular acidification leading to HYAL-2 driven HA degradation, ECM breakdown and tumour progression. CD44-HA interactions stimulate signalling through Rac1, another RhoGTPase, via the GEF Tiam1. In MDA-MB-231 cells, CD44-HA interactions also activate c-Src kinase resulting in activation and nuclear translocation of the transcription factor Twist, miR-10b expression and down-regulation of the tumour suppressor gene HOXD10 (Bourguignon *et al*., 2010 Toole, 2004). CD44 undergoes sequential proteolytic cleavages resulting in the release of its ectodomain from the cell surface and formation of a CD44 intracellular domain fragment, which is translocated to the nucleus, acting as a transcription co-regulator (Nagano and Saya, 2004). CD44 ectodomain cleavage is mediated by MT1-MMP and is stimulated by multiple factors, including HA fragments and TGF-β (Kuo *et al*., 2009, Sugahara *et al*., 2006) which, contribute to tumour cell migration and invasion (Fig. 3).

3.2 RHAMM/HMMR

Receptor for HA Mediated Motility (RHAMM/HMMR) belongs to a group of proteins that are found intracellularly as well as extracellularly. RHAMM does not contain a transmembrane domain or classical export signal and is likely exported through an unconventional mechanism that does not involve the Golgi/ER. RHAMM is expressed as multiple isoforms and one of these, an N-terminal truncation that lacks the first 163 aa residues, is transforming in mesenchymal cells (Hall *et al.*, 1995). On the cell surface, RHAMM interacts with HA and forms complexes with transmembrane receptors such as CD44, PDGFR, and RON (Maxwell *et al.*, 2008). Interestingly, CD44 surface display is reduced in mesenchymal cells isolated from RHAMM-/- mice, demonstrating functional interplay between these two HA receptors (Tolg *et al.*, 2006). RHAMM is elevated in most types of cancer in particular breast, ovarian, and prostate cancer, as well as in MM, AML and CML. In BCA, RHAMM is a tumour marker, novel susceptibility factor and prognostic factor for poor outcome (Maxwell *et al.*, 2008). Consistent with these clinical correlations, RHAMM has tumourigenic properties in experimental systems that have been linked to its ability to bind HA. In BCA cells, RHAMM/CD44/HA complexes sustain phosphorylation and activation of the Ras/MAPK (ERK1,2) signalling pathway, leading to BCA progression and constitutively high rates of motility and invasion (Hamilton *et al.*, 2007). The relationship between RHAMM and ERK1,2 activation has recently been confirmed in BCA samples where concomitant upregulation of phosphorylated ERK1,2 and RHAMM in tumour samples correlates with a high tumour grade (Ward C., in preparation). Intracellularly, RHAMM binds directly to tubulin and is involved in regulation of microtubule stability and turnover as a result of its association with ERK1,2. In mesenchymal cells, the absence of RHAMM increases microtubule stability resulting in reduced cell migration and aberrant mitotic spindle formation (Tolg *et al.*, 2010, Groen *et al.*, 2004). RHAMM interacts directly with ERK1, inferring that RHAMM may act as a scaffolding protein that directs ERK1 to its substrates including microtubule associated proteins that regulate microtubule stability (Tolg *et al.*, 2010). Interestingly, RHAMM expression is downregulated by p53, an important tumour suppressor gene, suggesting that RHAMM may be involved in p53 loss-induced tumour progression (Buganim and Rotter, 2008, Godar and Weinberg, 2008, Sohr and Engeland, 2008). RHAMM also acts on the BRCA1, pathway and may play an important role in BCA tumours arising from loss or inactivation of BRCA1 (Joukov *et al.*, 2006)

3.3 TLR2 and TLR4

Toll like receptors (TLR) are part of a cellular defence mechanism that is based on pattern recognition. TLRs recognize and bind bacterial lipopolysaccharides, DNA, and, in the case of TLR2,4, small HA fragments. In general, HA-TLR2,4 interactions control innate immunity through several mechanisms. For example, TLR 2,4 activation results in cytokine and chemokine release and leads to expression of metalloproteinases (MMPs) in immune cells (Voelcker *et al.*, 2008). Versican, which is associated with poor prognosis and relapse in BCA, interacts with HA polymers to form cord-like structures that link TLR2 on endothelial cells and fibroblasts. This, in turn, causes the secretion of pro-inflammatory cytokines (Theocharis *et al.*, 2010). HA-TLR2,4 interactions also stimulate NFκB signalling and activate TNFα. In BCA cells, TLR 2,4 interact with CD44 and act as co-receptors to stimulate signalling through HA and CD44 regulated pathways which may play a role in breast

tumour cell migration/infiltration. The human BCA cell line MDA-MB-231 expresses mainly TLR4, and siRNA mediated knock-down of TLR4 significantly reduces cell survival and expression of the cytokines Il-6 and Il-8, suggesting that TLR4 is a promising target for BCA therapy (Yang *et al.*, 2010).

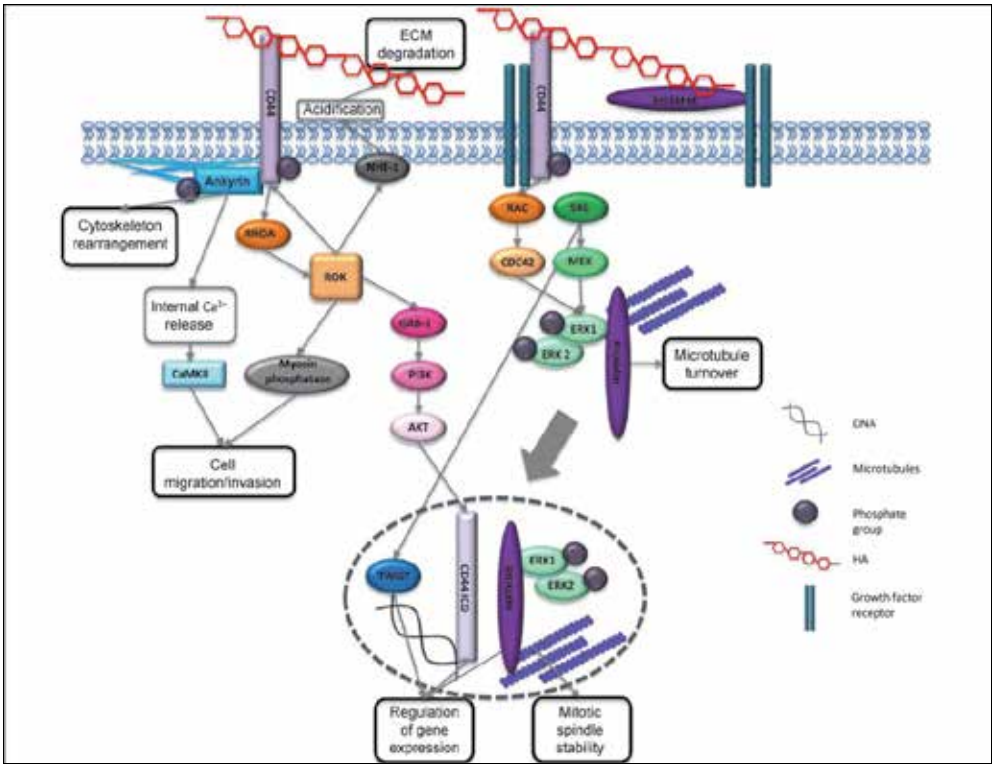

Fig. 3. HA initiates the signalling of RHAMM and CD44 regulated pathways, resulting in a variety of pro-tumourigenic outcomes.

3.4 LYVE-1

HA links the two main functions of the lymphatic system: draining of interstitial fluids and immune surveillance. These functions are achieved through its interaction with the receptor LYVE-1, present in lymphatic endothelia (Jackson, 2009). LYVE-1 is a type I integral membrane polypeptide that exhibits high homology with CD44 (Banerji *et al.*, 1999) and is a homeostatic HA receptor required for liver and lymphatic vessel formation. Its expression does not change as frequently in malignancy as HA receptors involved in response to injury, for example CD44 and RHAMM/HMMR. This does not rule out a role in injury and tumour progression however, as lymphangiogenesis is an important processes in both events, and elevated accumulation of HA in stroma results in lymphangiogenesis *via* signalling through LYVE-1 (Gale *et al.*, 2007).

To further demonstrate the association of LYVE-1 with tumour dissemination through the lymphatic system, (Du *et al.*, 2010) expressed LYVE-1 in COS-7 kidney cells and performed cell adhesion assays with the BCA cell line HS-578T which produces HA. These two cell lines had enhanced adhesion over the control cells, COS-7 not expressing LYVE-1. This

suggests that LYVE-1 plays a role in tumour cell adhesion which is dependent on HA-LYVE-1 interaction. Apart from its effect on tumour cell adhesion, LYVE-1 has also been proven to be a prognostic factor in tongue squamous cell carcinoma and decreased levels of LYVE-1 in the invasive front of tumours predicts cervical lymph node metastasis (Matsumoto *et al*., 2010).

4. HA expression and signalling in different cell types and its relationship to BCA

4.1 HA, inflammation, and the role of inflammatory cells in tumourigenesis

4.1.1 Macrophages

HA has a major role in macrophage biology during inflammation, wound repair, and tumourigenesis and at least part of the detrimental effects of HA accumulation during tumourigenesis is due to the activation of tumour associated macrophages (TAMs). For instance, TAMs preferentially traffic to stromal compartments formed within HA producing tumours (Kobayashi *et al*., 2010). Macrophages are classed into type 1 and 2 according to the adaptive immune polarization with which they associate. Type 1 macrophages are antigen-presenting cells which promote the cytotoxic response, resulting in tumour cell killing. Type 2 macrophages, however, are classically associated with tissue remodelling, angiogenesis, and scavenging/phagocytosis. TAMs are similar to type 2 polarized macrophages which have decreased or inhibited cytotoxic activity (Mytar *et al*., 2003). Kuang *et al*. (2007) found that overexpression of HAS2 was able to polarize macrophages towards a malignant TAM phenotype. Additionally, exposure to solid tumour cell culture supernatant elicits a pro-inflammatory response in monocytes and their subsequent TAM-like polarization, showing that the tumour cells themselves are responsible for the immunosuppressive macrophage phenotype observed in solid tumours (Kuang *et al*., 2007). The importance of TAM recruitment in BCA dissemination was additionally illustrated by CSF-1 null mice crossed with the MMTV transgenic mouse model of BCA. In these mice, a failure to recruit macrophages into the primary tumour results in delayed primary tumour invasion and metastasis to the lungs compared to wildtype MMTV mice. The addition of exogenous CSF-1 rescues macrophage recruitment and restores tumour and metastasis development to baseline levels (Lin *et al*., 2001). After injury, or during tissue inflammation, small fragments of HA associate with TLR4 and control macrophage cytokines and chemokines (Termeer *et al*., 2000). For example, BCA cell associated HA promotes the production of pro-inflammatory cytokines and chemokines, such as TNF-α and IL-12, as well as ROS, by TAMs, an effect which can be alleviated by either blocking CD44 receptors on monocytes, or by the addition of non-BCA cell associated HA (Mytar *et al*., 2001). HA regulation of pro-inflammatory cytokine production also occurs in monocytes pre-exposed to a variety of solid tumour cell types and culture supernatants, including the BCA line MCF-7 (Mytar *et al*., 2003, del Fresno *et al*., 2005), modulating the IRAK family of NFκB regulatory molecules, this further downregulating TNF-α and IL-12 production. HA-mediated CD44 cross-linking induces this activity and is prevented by the addition of exogenous HYAL (Mytar *et al*., 2003). TAMs are recruited and regulated in response to NFκB, whose activation is often HA-mediated through TLR4 (del Fresno *et al*., 2005) and NFκB overexpression results in tumour metastasis (Mantovani *et al*., 2007). Nitric oxide, which is the product of nitric oxide synthase 2 (NOS), is stimulated by hypoxia and CSF-1, among others, and is a signalling molecule integrated within the NFκB inflammatory pathway. NOS2 signals the upregulation of CD44,

c-Myc, MMP, and VEGF, which are all involved in promoting tumourigenesis. In BCA, NOS2 expression within tumour cells themselves is correlated with increased tumour grade and angiogenesis (Ambs and Glynn, 2011).

4.1.2 T Cells

T cells orient their cytoskeleton and migrate towards sites of inflammation, such as those present in a tumour microenvironment (TME), in a PKC-dependent manner as a direct result of CD44 crosslinking by HA (Fanning *et al.*, 2005). In BCA, CD8+ T cells are most predominant in advanced cancer stages where their presence in proliferating tumours is a good prognostic indicator. T cells are able to participate in either a Th1 or Th2 polarized immune response and, when polarized to a Th1 response, they express and secrete IFNγ, TGFβ, TNFα, IL-2, resulting in cytotoxic cooperation (T cells and M1). Th2 polarized CD4+ T cells secrete IL-4,5,6,10,13 which leads to an increase in B cell mediated immunity (DeNardo and Coussens, 2007). Because of the anti-tumour effects of T cells, the activation of cytotoxic T cells against HA receptors as immunotherapy in leukemias is currently undergoing clinical trials and will be discussed later in this chapter. On the other hand, the presence of CD4+ T cells correlates with disease progression and metastasis; however, it has been shown by different groups that CD4+ T cells are crucial for mounting an immune response against cancer. For example, tumour growth of EL4 lymphoma cells inoculated into mice is inhibited by the presence of dendritic cells primed against RHAMM protein. This interaction, however, is dependent on CD4+ T cells, as the effect of DC killing of the tumour is significantly reduced with a reduced CD4+ T cell population (Fukui *et al.*, 2006). Furthermore, Rakhra *et al.* (2010) showed that in ALL and B-cell leukemia, CD4+ cells were necessary for sustained tumour regression. In mouse models, inhibition of MYC or BCR-ABL rescues tumours from oncogene addiction; however, tumours regress in the presence of TSP-1 induced CD4+ T cells, and knockdown of TSP-1 impairs this ability (Rakhra *et al.*, 2010).

Regulatory T cells (Treg; CD4+/CD25+/FOXP3+) play controversial roles in tumour progression and can have both anti- and pro-tumourigenic effects, depending on the chemokines or cytokines produced and the type of solid tumour. Treg cells may be activated in an immunosuppressive manner, preventing cytotoxic immune responses, and allowing the tumours to evade immune attack. For example, in CLL, a large Treg population dampens specific CD8+ T cell responses against tumour associated antigens (Giannopoulos *et al.*, 2010). The same may be true for solid tumours. When coordinated, however, with a high T cell density, they may indicate good prognosis and inhibition of metastasis (Camus *et al.*, 2009, Carreras *et al.*, 2006).

4.1.3 B Cells

Immunoglobulin deposition by B cells in BCA stroma can be detrimental to disease progression and the accumulation of autoantibodies produced by B cells and deposited in the stroma correlates with poor prognosis (Fernandez Madrid *et al.*, 2005). An increase in serum IgG correlates with an increase in TAM numbers which, in turn, promotes angiogenesis in mouse mammary carcinoma, a process associated with poor clinical outcome. A proposed mechanism for the involvement of TAMs in B cell processes is the phagocytosis of IgG by macrophages. IgG engages Fcγ receptors, which stimulates VEGF secretion, increases angiogenesis and promotes tumour growth rate (Barbera-Guillem *et al.*,

2002). The majority of stromal B cells localize to perivascular regions within tumours and chronic B cell activation promotes tumours by recruiting macrophages and activating an innate immune response. However, the role of B cells in BCA progression is complicated since, for example, B cells may also recruit antigen presenting cells, such as CD8+ T cells and dendritic cells which help to eradicate neoplasms.

4.1.4 Dendritic cells and mast cells

Dendritic cells (DC) can also exhibit HA dependent characteristics that either promote or inhibit tumourigenesis. HA or chondroitin sulphate, in conjunction with CSF-1, activate DC from an immature to differentiated state via an NFκB regulated process, illustrating the importance of HA in eliciting an immune response (Yang *et al*., 2002). Pedroza-Gonzalez *et al* (2011) recently showed that human BCA produces thymic stromal lymphopoietin (TSLP) which induces expression of OX40L on DCs, polarizing them towards a Th2 inflammatory response. *In vitro* this drives the production of IL-13 and TNF by Th2 polarized T cells (Pedroza-Gonzalez *et al*., 2011). DC also become tumour insensitive and, as a result, do not mature and differentiate into cytotoxic cells. Furthermore, HA fragment build ups are at least partly responsible for preventing DC maturation in tumour bearing animals (Kuang *et al*., 2008).

In BCA, c-kit expression by mast cells, a protein which is usually only present in specific tissue types, such as germ cells, predicts primary tumour recurrence (Khazaie *et al*., 2011). However, an abundance of stromal mast cells in invasive BCA is associated with good prognosis (Rajput *et al*., 2008). The mast cell line HMC-1 expresses high levels of CD44s and, through an interaction with HA, adheres to stromal tissue (Fukui *et al*., 2000). Therefore, in both mast cells and DC, a CD44-HA interaction may result in anti-tumour responses.

4.2 HA regulation of a pro-inflammatory environment by non-immune cells

4.2.1 Breast cancer cells and their contribution to a pro-inflammatory environment

BCA cells secrete a variety of cytokines and chemokines which promote tumour progression. Studies by Tafani *et al*. (2010), showed that MCF-7 cells upregulate pro-inflammatory gene transcription and translation *in vitro*, and a pro-inflammatory gene expression profile can be seen in human BCA tumours even in the absence of an immune infiltrate. This illustrates that BCA cells themselves contribute to the pro-inflammatory/pro-tumourigenic TME. One or both of HER2 and ERα, which are often expressed on BCA cells, promote the expression and secretion of CXCL8 (IL-8) through the PI3K and ERK pathways. CXCL8 is a pro-angiogenic chemokine and secretion of CXCL8 by the MCF7 BCA line (which express both HER2 and ERα) is additive upon stimulation of both of these receptors (Haim *et al*., 2008). The pro-inflammatory chemokines CCL2 and CCL5 are also secreted by BCA cells (Ben-Baruch, 2003) and expression and secretion of all three chemokines requires HA fragment/CD44 interactions on TAMs, tumour associated fibroblasts (TAFs) and BCA tumour cells. Both CCL2 and CCL5 are monocyte-recruiting chemokines and their expression in BCA tumours is correlated with poor prognosis, and in the case of CCL2, pro-angiogenesis factors and vascular invasion (Soria and Ben-Baruch, 2008). TNF-α secretion by TAMs activates a positive feedback loop in BCA tumour cells, stimulating further secretion of growth promoting chemokines (Ben-Baruch *et al*., 2003). Eck *et al* (2009) also showed that conditioned media from BCA cells stimulates the expression of pro-inflammatory genes in normal mammary fibroblasts, polarizing them towards a TAF phenotype. Furthermore, TAF migration is increased, along with the secretion of MMP-1 and CXCR4 (IL-1/SDF-1 receptor), both of which are important factors in BCA progression (Eck *et al*., 2009).

4.2.2 HA/stromal fibroblast/epithelial cell interaction and tumour progression

To begin to define the role played by TAFs in tumour progression, Micke *et al.* (2007) conducted cDNA microarray analyses comparing the transcriptome of TAFs from basal cell carcinoma with normal dermal fibroblasts (Micke *et al.*, 2007). This study showed that TAFs overexpress multiple growth factors such as PDGF, EGF, and VEGF, chemokines such as SDF1 and CXCL12 and matrix proteins such as MMP11, LAMA2 and COL5A2. In fact, these TAFs are known to secrete IGF-2, FGF-7, TGF-β, leptin, and NGF, which bind to their cognate receptors on BCA cells to stimulate HA production (Szabo *et al.*, 2011). This then promotes expression of cytokines such as TGF-β that attract and stimulate TAFs to proliferate. This paracrine effect is a positive feedback mechanism, because proliferating TAFs secrete additional growth factors, cytokines, chemokines, and MMPs that sustain BCA transformation and promote BCA progression. Additionally, VEGF, produced by TAFs, and HA oligosaccharides induce angiogenesis. HA itself also impairs immune surveillance, and/or activates TAMs and neutrophils that have tumour enhancing potential. Overexpression of HAS in a non-transformed rat fibroblast, 3Y1, increases high MW HA production and the resultant pericellular HA coat provides cells with a proliferation advantage that is accompanied by loss of contact inhibition of growth. This is achieved through HA-mediated activation of PI3 kinase. Lower MW HA also increases proliferation in these cells but has no effect on the HA matrix (Itano *et al.*, 2002). TAFs affect not only BCA cells but also normal cells in which the tumour is embedded. For example, TAFs induce stem cell-like behaviour and aberrant differentiation in normal fibroblasts, which can affect BCA progression. TAFs promote the expression of stem-cell markers such as Oct4 and Sox2 in 3T3 cells (Szabo *et al.*, 2011) and stimulate trans-differentiation of normal fibroblasts into myofibroblasts when they are confronted with primary BCA cells.

4.2.3 HA, adipocytes and adipose tissue

Adipose tissue in mammary glands is important for its secretory and endocrinal functions as well as metabolism, energy homeostasis and stem cell compartment. Adipocytes contribute to the mammary tissue ECM and this effect is at least partly regulated by HA. There are not many studies that focus on HA and its relationship to adipocytes, however, the importance of this polysaccharide on adipose-stromal interactions in the breast tissue is becoming apparent. For example, HA increases the crosslinking of collagen-HA matrices, supports proliferation and differentiation of pre-adipocytes and induces a higher proportion of cycling cells (Davidenko *et al.*, 2010).

Chen *et al.* (2007) also showed that HA extends the lifespan, reduces cellular senescence and enhances differentiation potential of murine adipose-derived stromal cells (mADSCs) *in culture*. Collectively, these results provide preliminary evidence for a key role of HA in controlling the adipose component of the breast tissue and allude to a potential role of this regulation in BCA (Chen *et al.*, 2007).

5. HA regulates mammary cell functions that promote BCA progression

5.1 Cell migration

Considerable evidence indicates that HA fragmentation is required for immune cell trafficking, fibroblast migration, stem cell migration from niches to the wound site and endothelial cell migration during angiogenesis. For example, acellular hydrogel matrix composed of fibronectin and HA, which simulates a wound microenvironment, supports

proliferation, migration and spreading of human dermal fibroblasts *in vitro*. HA seems to regulate motility via a variety of mechanisms that include indirect and direct effects on the migrating cell population. An example of an indirect effect was provided by a study of the role of HA on fibroblast migration using a porcine skin wound model. The wound matrix, which contained HA, promoted cell migration and recruitment of fibroblasts. This was shown to be in part due to wounding produced HA, which promotes collagen fibril formation, thus indirectly affecting cell motility (Docherty *et al*., 1989). Direct effects of HA on cell motility can result from its structural properties and from its ability to activate motogenic signalling cascades such as ERK1,2 and PI3 kinase. Both of these effects have been related to an association of HA with cell surface receptors such as CD44 and RHAMM. For example, extracellular HA accumulation induces penetration of stromal cells by increasing turgidity and hydration or disrupting cell-to-cell junctions. These effects may be a result of interactions with CD44 and RHAMM (Itano *et al*., 2008). HA fragments bind to CD44 and/or RHAMM to induce activation of MAPK (ERK1,2) that results in enhanced BCA cell migration and invasion (Hamilton *et al*., 2007). Moreover, upon HA-mediated activation of PI3 kinase, increased HAS2 production induces faster migration in scratch wound assays (Itano *et al*., 2002).

5.2 Angiogenesis

Hypoxic conditions within tumours require neovascularisation of the microenvironment for the tumour to continue to grow and metastasize. Hypoxia, a condition often found within the TME, induces the activation, as seen by nuclear translocation, of either or both of NFκB and HIF-1α. This effect has been shown both *in vitro* in MCF-7 BCA cells, and *in vivo* (Tafani *et al.,* 2010). Invasion, migration, and proliferation of endothelial cells, as well as tissue remodelling, are essential processes during angiogenesis, which directly and indirectly help to promote tumour growth and metastasis. Necrotic cells, which have died as a result of hypoxia, also release chemokines that recruit macrophages and a pro-inflammatory response conducive to tissue remodelling. Hypoxia may produce ROS which in turn cause HA fragmentation and Noble *et al.* (1996) showed that NFκB transcription in macrophages is activated by HA fragments (Noble *et al*., 1996). Later, Rockey *et al.* (1998) were the first to show in hepatocytes that HA activation of NFκB induces NOS2 production, which can be synergistically increased in the presence of cytokines such as IFN-γ (Rockey *et al*., 1998). It has since been shown that HA fragments activate the NFκB pathway through TLR4 in both DC and macrophages (Termeer *et al*., 2002). Hypoxia induced activation of HIF-1α and NFκB induces pro-inflammatory gene expression and both mRNA and protein levels of inflammatory mediators such as RAGE, PTX3, NOS2, COX2, and CXCR4 are increased. Increased expression of CXCR4, which is the receptor for SDF-1, is seen on MCF-7 cells subjected to hypoxic conditions (Tafani *et al*., 2010). This increases the migratory and invasive capacity of these cells, which are usually non-invasive. In these same studies it was found that nuclear translocation of NFκB is at least partly dependent on HIF-1α, indicating that it may be under hypoxic regulation, as inhibition of HIF-1α decreases nuclear localisation of NFκB, and in turn RAGE and P2X7R expression, inhibiting cell invasion (Tafani *et al*., 2010).

In general, high MW HA inhibits angiogenesis while fragments promote angiogenesis. Overexpression of HA and HYALs has been linked to an increase in angiogenesis in several types of cancers including breast (Tan *et al*., 2010), bladder (Lokeshwar *et al*., 2000, Golshani

et al., 2008), prostate (Ekici *et al*, 2004, Bharadwaj *et al.*, 2007), and endometrial (Paiva *et al.*, 2005). Koyama *et al.* (2007) demonstrated that an increase in HAS2 expression by genetic modifications in a mouse model of BCA causes a higher incidence of adenocarcinoma accompanied by an increase in angiogenesis (Koyama *et al.*, 2007). An increase in HA by overexpression of HAS2 in transgenic mice induces a more aggressive BCA phenotype and an increase in blood and lymphatic vessels (Kobayashi *et al.*, 2010). In these tumours, the stromal cells also secrete a variety of pro-angiogenic factors. Furthermore, HA concentration in stroma and blood vessels is increased, as well as the amount of small HA fragments.
The pro-angiogenic effects of HA fragments result from the display of CD44 and RHAMM (Wang *et* al., 2011, Slevin *et al.*, 2007) on the surfaces of endothelial, BCA or leukocyte cells. and Interaction of HA fragments with these cells produces the factors required for stimulating endothelial cells to form new blood vessels. HA fragments stimulate endothelial cell proliferation, migration and tube formation. Increased expression of HYALs in conjunction with MMPs and Cathepsin-D induce a more invasive phenotype in the endothelial cell line ECV-304 as detected by matrigel invasion assay (Wang *et al.*, 2009). Additionally, pro-inflammatory cytokines, secreted by leukocytes activated by CD44-HA mediated interactions, stimulate endothelial cells to produce HA. When HUVEC cells are stimulated with IL-1B, TNF-α and β1, they secrete HA. CD44-HA interaction stimulates early morphogenic events, such as tube formation and proliferation in HUVECs (Wang *et al.*, 2011). Furthermore, HA works synergistically with macrophage recruitment to promote vascular formation and HA in the stroma promotes lymphangiogenesis at the invasive tumour front in BCA through the activation of endothelial LYVE-1 (Itano *et al.*, 2002).

6. HA and multi-drug resistance in BCA

Most tumours initially respond to chemotherapy treatment but later acquire resistance, resulting in treatment failure and tumour recurrence. Some mechanisms by which tumour cells acquire resistance include inhibition of apoptosis, stimulation of cell proliferation and enhanced expression and activity of drug export pumps, particularly ATP driven pumps (ABC transporters), which reduce the intracellular, and therefore active, concentration of several chemotherapeutic agents. HA fragments augment expression and activity of MDR1, a member of the ABC drug transporter family, in primary BCA cells (Toole and Slomiany, 2008). This HA induced upregulation involves the Akt/PI3 kinase signalling pathway and is CD44 dependent. CD44-HA interactions stimulate MDR1 expression via multiple signalling mechanisms including epigenetic gene expression regulation. CD44-HA binding results in activation of PKCε as well as increased phosphorylation and nuclear translocation of Nanog, a stem cell specific transcription factor. Moreover, interaction of Nanog with Stat-3 in the nucleus increases Stat-3 regulated gene expression, resulting in increased expression of MDR1. Activation of Nanog also results in production of the micro RNA miR-21 and down-regulation of PDCD4, a tumour suppressor protein (Bourguignon *et al.*, 2008, 2009). CD44-HA interaction increases an association between MDR1 and the cytoskeletal protein ankyrin, resulting in enhanced drug export (Bourguignon *et al.*, 2008). Additionally, CD44-HA interactions upregulate the expression of the histone acetyl-transferase, p300, inducing the acetylation of β-catenin and NFκB. This stimulates expression of MDR1 and the anti-apoptotic protein, Bcl-x_L (Bourguignon *et al.*, 2009). It is very likely that BCA tumours with high HA metabolisms are also highly resistant to treatment with drugs that can be exported by MDR1.

7. HA and receptor antagonists in clinical trials

Since it is evident that HA and its receptors play an important role in BCA and other tumours, it is unsurprising that reagents blocking HA metabolism are being assessed as therapeutic agents in certain types of cancer. In pre-clinical models, Kultti *et al.* (2009) demonstrated that the HAS inhibitor 4-MU (4-Methylumbelliferone) specifically depletes intracellular levels of UDP-Glc-UA (Kakizaki *et al*., 2004) by serving as a glucoronidation substrate in A2058 melanoma cells, MCF-7, MDA-MB-361 BCA cells, SKOV-3 ovarian, and UT-SCC118 squamous carcinoma cells. Additionally, Lokeswar *et al.* (2010) used 4-MU to block growth of human prostate cancer cell line xenografts in immunocompromised mice. 4-MU induces apoptosis in these tumours and also strongly inhibits cell proliferation, motility and invasion. These effects can be reversed by addition of HA, which demonstrates that, although 4-MU does not specifically block HAS and has other off target effects, its effects on tumour cell growth result from inhibition of HAS (Ekici *et al*., 2004).

HA has also proven to be a good adjunct therapeutic option *in vivo* in human cancers since it promotes targeting of active anti-cancer compounds. For example, when patients with Calmette-Guérin refractory bladder cancer were included in a Phase I clinical trial using Paclitaxel-HA (ONCOFID-P-B™) for treatment of their cancers, 60% of the patients treated exhibited a clinical response with minimal toxicity reported (Bassi *et al*., 2010). HA has been successfully used to carry/target other chemotherapeutics, thus reducing cytotoxic side effects of the active drug. Hyung *et al.* (2008) demonstrated the efficacy of HA-coated drug carriers by delivering doxorubicin to MDA-MB-231 and ZR-75-1 human BCA cell lines (Hyung *et al*., 2008). Similarly, after coating nanoparticles containing paclitaxel with HA, cytotoxicity is reduced while cellular uptake of the drug by S-180 sarcoma cell line is enhanced 9.5 fold *in vitro* and in a mouse model (He *et al*., 2009).

In light of fairly recent evidence for the display of CD44 on BCA tumour initiator cells, interest in developing CD44 targeted therapies has increased. Riechelmann *et al.* (2008) exploited the potential of CD44 in a Phase I clinical trial using an antimicrotubule agent (mertansine) and a monoclonal antibody to CD44v6 (bivatuzumab), (BIWI 1), to treat patients with recurrent or metastatic head and neck squamous cell carcinoma (Riechelmann *et al*., 2008). The response to the treatment was unexpectedly variable and the trials using these agents were stopped after one patient died of toxic epidermal necrolysis (Tijink *et al*., 2006). Targeting the HA binding ability of activated CD44 may result in decreased toxicity.

RHAMM peptide vaccination (e.g. R3, which is HLA-A2-restricted) has recently been assessed in PhaseI/II clinical trials for treatment of MM, AML, and CLL (Giannopoulos *et al*., 2010, Greiner *et al*., 2008, 2010, Schmitt *et al*., 2008). Additionally, vaccination with DC pre-stimulated against the same peptide has also undergone Phase I and II clinical trials for treatment of CLL (Hus *et al*., 2008). Vaccination with RHAMM peptide has the attractive advantage of very low toxicity because it is not expressed in healthy bone marrow tissue. RHAMM vaccination resulted in leukemic blast lysis, blast reduction in the bone marrow and avoided the need for blood transfusions for one patient. Furthermore, an immunological response, marked by an increase in T cell frequency, was observed in 70% of AML, MM, and MDS patients in an initial study (Schmitt *et al*., 2008). Subsequently, RHAMM peptide was shown to be non-toxic at high dosage (1000 μg/vaccination), however, there was no dose-dependent effect, indicating that RHAMM is an effective therapeutic target even at low levels (Greiner *et al*., 2010). A similar response was seen in CLL patients vaccinated with RHAMM peptide, as well as RHAMM peptide-stimulated DC.

Clinical response was correlated with an increase in CD8+ T cell proliferation and in some cases a decrease in Treg population. Interestingly, in B-CLL patients with clinical response to vaccination with stimulated DC cells, the CD8+ cytotoxic T cell and IL-12 anti-tumour response was increased, whereas the Treg cell population was decreased (Hus *et al*., 2008). In a Phase I study of CLL patients vaccinated with RHAMM peptide, there was no correlation between clinical response and Treg population dynamics (Giannopoulos *et al*., 2010). This strategy has not yet been used for BCA, although, as RHAMM is a prognostic marker for BCA. and overexpressed in many cases which currently do not have a specific targeted therapeutic option (e.g. basal subtype) and also given the magnitude of the response, along with such low toxicity, it is an approach which merits further consideration.

8. Conclusion

In summary, HA is a glycosaminoglycan that exerts a critical role in BCA progression by interacting with other ECM components and the tumour cells themselves. HA fragmentation induces inflammation and signalling that results in cancer and immune cell proliferation and migration, which can lead to poor outcome. The links between HA and cancer progression, as well as HA and inflammation have in some aspects been well established. Given the similarities in their signalling cascades and cellular processes, the relationship between HA stimulated innate immunity and the BCA microenvironment should be further considered.

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10. References

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Part 3

Breast Cancer Stem Cells

11

The Microenvironment of Breast Cancer Stem Cells

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1. Introduction

Ernst Haeckel first described the term "stem" as a concept for the evolution or organisms. For representation purpose he described the ancestor organism as a "stem" from which all the other organisms evolved. Arthur Pappenheim later adopted this concept in the context of cells, and he elegantly placed the "stem cell" in the centre in cartoon from which all the blood cells arise describing hematopoiesis (Ramalho-Santos and Willenbring, 2007).

The concept was carried forward and the term "cancer stem cell" was first coined in 1980 (Carney *et al.*, 1982) where the authors described the stem cell origin of lung cancer cells. The difficulty in isolation and the absence of specific markers of cancer stem cell stalled the research in this area. However a decade later Bonnet and Dick successfully isolated CSC in AML which then incited the development in the field of cancer stem cells (Bonnet and Dick, 1997). Their discovery was later supported by many groups, which also resulted in isolation of CSC from a variety of malignancies including solid tumors.

Now a large body of evidence suggests that cancer comprises of different population of cells with various tumorogenic potentials. The tumor cells follow a hierarchy, where the subset capable of self-renewal, generate the tumor heterogeneity and are called cancer stem cells (CSC). Very low number of these cancer stem cells generates tumors in immunocompromised mice whereas large number of non-CSCs fails to generate tumors.

CSCs have been characterized based on their ability to form colonies in soft agar and their ability to form spheres in serum free media. The generation of tumors in immunocompromised mice however remains the gold standard. Another characteristic of CSC is their ability to resist the action of common chemotherapeutic drugs which is attributed to higher expression of ABC transporters and their slow cycling nature. Further it has also been documented that these CSCs have activated signaling pathways as in the case of normal stem cells. Hence CSCs are distinct from other non-CSC in many respects.

Cancer stem cells have been isolated based on membrane markers. One of the characteristics is their ability to efflux the Hoechst dye. However this ability to efflux the dye is also attributed to membrane ABC transporter ABCG2. ABCG5 has been used as a cancer stem cell marker as it pumps out the drug doxorubicin. ALDH1 has the ability to convert retinol to retinoic acid, which has diverse role in cell physiology, and this activity is used as a marker for CSC. CD 44, CD 133, EpCAM and CD 90 are also abundantly expressed in CSCs and are used to isolate or enrich CSC (Visvader and Lindeman, 2008). A number of groups have isolated CSC based on these markers however a robust marker for CSC still remains to be identified.

1.1 Origin of CSC

A number of theories have been proposed for the generation of these CSCs. (1)CSC can originate from genetic/ epigenetic alteration of normal stem cells or from the progenitor cells. (2) They can be derived from somatic tumor cells by de differentiation or reprogramming into a stem- like cell (Visvader and Lindeman, 2008). (3) And recently it has been suggested that CSC can be generated from non-CSC through production secretary molecules (Iliopoulos *et al.*, 2011).

2. Breast cancer stem cells

The existence of cancer stem cells was first demonstrated in solid tumors by Al Hajj et al., where CSCs were identified from human breast cancer tissue using $CD44^+$ / $CD24^-$ Lin^- as cellular markers (Al-Hajj *et al.*, 2003). They isolated the cells from primary breast cancer or metastatic pleural effusions and injected them directly in to mice or after cellular sorting with the above mentioned markers. They found that $CD44^+$, $CD24^-$ were able to form tumors while $CD44^-$, $CD24^-$ were unable to form tumors in immunocompromised mice. Further they performed repopulation assays where they found that the tumorigenic population ($CD44^+$ / $CD24^-$ Lin^-) was able to give rise to phenotypic heterogeneity of the initial tumor. This suggested that the breast cancer stem cells undergo self-renewal and differentiation as in the case of normal stem cells. After this report a large number of studies identified CSC from various other malignancies (Curley *et al.*, 2009; Fang *et al.*, 2005; Kondo *et al.*, 2004; Liu *et al.*, 2007; Prince *et al.*, 2007; Singh *et al.*, 2004).

The normal stem cells reside in a distinct environment called the "stem cell niche". This stem cell niche consists of complex composition of ECM, soluble factors, stromal cells, immune cells which are responsible for maintaining the self renewal ability of stem cells. Similarly the CSCs also depend on similar environment, which may be altered in many ways. Moreover in some of the tumors, the tumor niche has been shown to have a protective role from genotoxic insults (Garcia-Barros *et al.*, 2003). Although much research has been done on understanding the cancer stem cells, very few studies have been carried out on understanding the microenvironment of breast cancer stem cells and their targeting. We believe that understanding the breast cancer microenvironment will offer easily tractable solutions to cancer therapy.

2.1 Role of microenviroment in mammary gland development

The breast tissue is composed of multiple cell types for proper functioning of tissue and the primary function of which is production of milk. During lactation milk is produced by the luminal epithelial cells and secreted in the hollow cavity. The luminal epithelial cells are surrounded by myoepithelial cells, which synthesize the basement membrane. Together the luminal epithelia and the myoepithelia form the milk duct. Different cell types whose function is to maintain the homeostasis surround milk duct. These cells include fibroblasts, leucocytes and endothelial cells.

The environment of epithelial cells plays a critical role in shaping their function. For eg. When the epithelial cells from breast tissue were placed on plastic, they were unable to produce milk and exhibited different phenotype as compared to the cells when plated in 3 dimentional reconstituted basement membrane (Matrigel) which led to proper function of epithelial cells (Howlett and Bissell, 1993; Petersen *et al.*, 1992). Hence proper cellular interaction and spatial localization of cells with the right constituents are required for

correct functioning of epithelial tissue. This was explained by the fact that invivo normal mammary gland are in contact with myoepithelial cells and not the basement membrane.
Further luminal epithelial cells display apical–basal polarity as demonstrated by MUC 1, ESA and occludin expression on the apical membrane and ß4 integrin on the basolateral membrane. However such a polarity is observed when luminal cells are grown in matrigel but not in collagen(Gudjonsson *et al.*, 2002). The polarity is restored when the myoepithelial cells are co- cultured with luminal epithelial cells even in collagen, which is mediated by laminin 1 secreted by myoepithelial cells. These studies demonstrate the role of 3D environment and is important for optimal function of epithelial cells.

2.1.1 Microenvironment of breast cancer cells

A large number of reports demonstrate that breast tumor progression is facilitated by stromal cells and that their presence is critical for survival of cancer cells. However it is also important to note that the normal mammary gland microenvironment has inhibitory effect on breast cancer progression (DeCosse *et al.*, 1973). This indicates that cancer cells can maintain their properties only in an abnormal microenvironment. One of the recent reports underlies the role of mesenchymal stem cells in amplifying the metastatic potential of weakly metastatic cells. Karnoub A et al mixed a weakly metastatic cell line MDA MB 231 with bone marrow derived human MSC and found that the metastatic potential of the cell line is dramatically increased (Karnoub *et al.*, 2007). To further understand the mechanism of this increase in metastatic potential they used a cytokine array to identify soluble factors. They found CCL5 release, which was induced by physical interaction between breast cancer cells and the MSC, and that it renders the breast cancer cells more metastatic.
Another seminal report by Kaplan et al demonstrate that bone marrow- derived hematopoietic progenitors may localize to future sites of metastasis and "prepare" the sites for the arrival and growth of disseminated cancer cells (Kaplan *et al.*, 2005). This has been proposed a new concept in metastasis, which is called the "premetastatic niche". The precise mechanism and the factors responsible for such localization of bone marrow derived hematopoietic progenitors is unclear however it appears to be derived from the serum (Kaplan *et al.*, 2005).
One of the extensive study in understanding the breast cancer microenvironment, Allinen et al. performed genome wide gene expression analysis of stromal cells (Endothelial cells, infiltrating leukocytes, fibroblasts, and myofibroblasts) and breast epithelial cells (luminal epithelial and myoepithelial cells) from normal, insitu carcinoma and invasive carcinoma. The authors found that alterations in gene expression takes place in all cell types however clonally selected genetic alterations are confined to tumor epithelial cells. Further there were consistent and significant alterations in myoepithelial cells from DCIS as compared to normal myoepithelial cells and many of these changes were in secreted proteins and cell surface receptors (Allinen *et al.*, 2004). This further underlines the importance of soluble factors in breast cancer progression.
Although a large amount of literature is present on microenvironment of breast cancer cells, there are few studies on cancer stem cell microenviroenment. This is ascribed to the age of this new field however research in this direction will significantly impact the therapy of breast cancer.

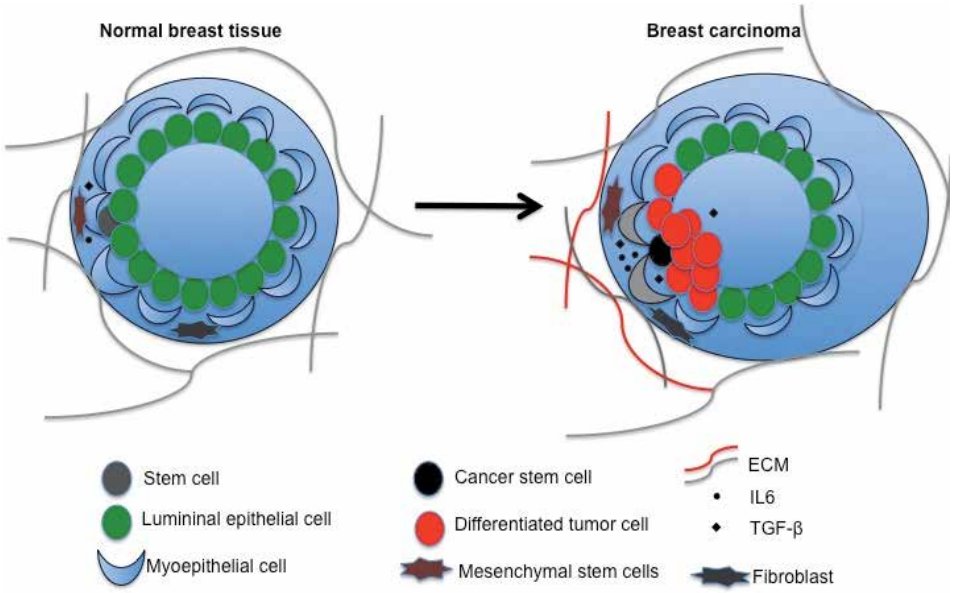

Fig. 1. Microenvironment of normal breast epithelium and breast cancer cells.

2.1.2 Influence of microenvironment on development of breast cancer stem cells

A limited number of factors have been studied to understand the interaction of microenvironment generated by tumors and its effect on development and maintenance of cancer stem cells. One of the widely studied environment which the solid tumors reside in, is hypoxia.

2.1.2.1 Hypoxia

It has been suggested that hypoxia contributes to the generation aggressive cancer by selecting tumor cells and results into growth of cells that can survive compromised levels of oxygen and nutrients (Graeber *et al.*, 1996). Further the growth of tumor results in hypoxic microenvironment, which is followed by periods of reoxygenation. Hence to mimic the invivo environment and to assess the fate of cells undergoing periods of hypoxia-reoxygenation Louie E etal., exposed breast cancer cells (MDA-MB-231 and BCM2) to cycles of hypoxia and nutrient deprivation. They discovered that after the first cycle of hypoxia a small fraction of cells survived and that repetitive exposure of the same cells to hypoxia and reoxygenation led to increased viability under hypoxia and to proliferate either as monolayer or tumor spheres. They also found increase in the number of cells expressing CD44+/CD24-/ESA+ cell surface markers, and hence the cancer stem cell content. Therefore repetitive cycling of hypoxia and re-oxygenation can increase the stem cell content of metastatic breast cancer cell lines indicating that microenvironment plays an important role in selectively increasing CSC (Louie *et al.*, 2010).

2.1.2.2 Stromal cells

Carcinoma associated fibroblasts (CAF)

For a long time scientist have primararily focused on epithelial component of breast cancer, however recently, the critical importance of tumor stroma has been realized. Literature

documents important interaction between mammary epithelia and the adjacent tumor stroma. One of the reports demonstrates that CAF increases the number of $CD44^{+}CD24^{-}$ cells in mammospheres, whereas normal fibroblasts (NFs) down-regulated it in mammospheres. They also demonstrate increase in the ability to form epithelial tumors in immunocompromised mice in presence of CAF. This indicates that CAFs can increase the cancer stem cell population in breast cancer (Huang *et al.*, 2010). Furthermore since, CXCR4 expression on carcinoma cells is known to correlate with a poor prognosis for several types of carcinomas (Balkwill, 2004), the authors assessed CXCR4 gene expression in mamosphere co cultured with CAF. They found increase expression of CXCR4 and it was speculated that increase in cancer stem cell population could be because of CXCR4 signaling (Huang *et al.*, 2010).

The normal fibroblasts on the contarary have a inhibitory effect on the tumor growth. For e.g Coculture studies using different mesenchymal cells and MCF10A and preneoplastic MCF10AT1-EIII8 mammary epithelial cells showed that fibroblasts derived from normal reduction mammoplasty inhibit or retard the morphological conversion and growth of MCF10A and EIII8 cells, whereas tumor derived fibroblasts evoke ductal-alveolar morphogenesis of both cell types (Shekhar *et al.*, 2001). Further caveolin-1 deficient (Cav1-/-) mammary stromal fibroblasts were shown to mimic the effects of human breast cancer associated fibroblasts as they show similar profile of RB/ E2F-regulated genes that are up-regulated and confer a poor prognosis with enhanced epithelial-mesenchymal transition (EMT) (Sotgia *et al.*, 2009).

Interestingly, genome-wide expression profiling of human breast cancer-associated fibroblasts and Cav-1 (-/-) mammary stromal fibroblasts indicates that they both show the upregulation of a number of ES-cell related genes and factors (Oct4, Nanog, Sox2 and Myc-target genes), indicating that they may behave like "cancer stem cells". Thus, the tumor stromal microenvironment may directly contribute to maintaining the "cancer stem cell" phenotype, leading to drug-resistance and treatment failure (Sotgia *et al.*, 2009).

Fibroblast synthesize growth and survival factors which are critical for the tumor. In breast cancer, stromal fibroblasts evolve with the tumor epithelial cells and assist the growth of tumor cells. Inspite of much known about role of stromal cells the mechanistic basis of such a requirement of fibroblast remains elusive. PTEN is a tumor suppressor and is a critical regulator of PI3K signaling whose activation is associated with activation of tumor stroma (Cully *et al.*, 2006). To understand the role of fibroblast in tumor formation Trimboli et al deleted PTEN from fibroblast in MMTV- ERBB2 mice model. They found that deletion of PTEN from fibroblast results in increase incidence and tumor load in the mice model. Extensive remodeling of ECM and increased recruitment of innate immune cells were some of the salient findings. Gene expression analysis revealed that PTEN deleted stromal fibroblasts consists of activation of Ets2 transcription factor. Further double transgenic mice having inactivation of Ets2 in mammary stroma reversed the increased malignancy caused by PTEN deficiency. These observations show the importance of the PTEN-Ets2 axis in stromal fibroblasts in the *MMTV-ErbB2* model in suppressing breast cancer growth and indicate the stromal pathway contributes to the complexity of human breast cancer stroma (Trimboli *et al.*, 2009).

Mesenchymal stem cells

Mesenchymal stem cells localize to the breast carcinoma and integrate into tumor associated stroma. A seminal report by Ling X et al., demonstrate that MSC overexpressing IFN-beta inhibit breast cancer growth and metastasis (Ling *et al.*, 2010). They demonstrate that MSC

are recruited to tumors and that IFN-beta inhibits tumor growth. (Ling X 2010). Such a reduction in tumor could also be attributable to decrease CSC content. Karnoub A et al., have shown increase in the metastatic potential of the breast cancer cells when they were mixed with bone marrow derived human MSC. Using a cytokine array they identified CCL5 is induced by physical interaction between breast cancer cells and the MSC, and that it renders the breast cancer cells more metastatic. These results indicate the importance of mesenchymal stem cells in rendering the cells more metastatic (Karnoub *et al.*, 2007).

2.1.2.3 Stromal factors

IL-6

IT has been documented that CSCs arise from mutant versions of normal stem cells. Alternatively, CSCs can also represent a stage in the path of transformation. CSCs are precursors of differentiated cancer cells (NSCCs), however CSCs can also be derived from NSCCs or can arise independently. The proportion of CSCs remains constant over multiple generations, but the basis of this phenomenon is unknown. Hence Iliopoulos D et al., assessed these issues using an inducible model of oncogenesis that MCF-10A cells which harbor a ligand-binding domain of estrogen receptor (ER-Src), a derivative of the Src kinase oncoprotein (v-Src) that is fused to the ligand-binding domain of the estrogen receptor. Treatment of these cells with tamoxifen (TAM) rapidly induces Src, results in transformation within 24-26 h. This property of the model helps in understanding the transition between normal and transformed cells. The authors then discovered that induction of CSC from non-CSC through activation of v-src. They also document that CSC formation depends on transformation however it is not required for transformation. Moreover because of the fact that breast CSCs have an enhanced inflammatory feedback loop compared with NSCCs, they treated the cells with IL6 which resulted in generation of CSC fron non-CSC (Iliopoulos *et al.*, 2011). This indicates the critical role of microenvironment as the CSC itself secrete IL6 which can maintain the stemness of a cancer cell population. Further the fact that macrophages and dendritic cells are potent IL-6 producers, which can be activated by molecular "danger" signals by cancer cells it is important to control the IL6 signaling to regenerate the CSC .

TGF beta

One of the elegant studies by Mani et al demonstrates the role of TGF beta in cancer stem cell through induction of EMT. The authors treated the immortalized HMEC cells with TGF beta which resulted in fibroblast like, mesenchymal like phenotype with concomitant downregulation of ephtielial markers like E-cadherin and upregulation of mesenchymal markers like vimentin, fibronectin and N-cadherin. Similar results were obtained through ectopic expression of TWIST or SNAI1. They further assessed the CD44 and CD24 population of these cells and found that CD44+ and CD24 ^{low} cells were increased which TGF beta treatment/ TWIST, SNAI1 expression. The rise in CD44+ and CD24 low population was accompanied by approximately 30-40 fold enrichment in mamosphere forming capability (Mani *et al.*, 2008). This was a clear demonstration of TGF beta induction of cancer stem cell population.

Yin X et al., showed that the activating transcription factor 3 (ATF3) is induced by TGF beta in breast cancer and is important for increasing the migration potential of the breast cancer cells. Further ATF3 can be induced by a number of stromal factors like TGF beta, IFN alpha, TNF alpha and hypoxia. And the fact that ectopic expression of ATF3 increases the cancer stem cell content of breast cancer cells (CD 24^{low}/ CD 44^{high}), it was hypothesized that tumor microenvironment has a significant effect in the development of cancer (Yin *et al.*, 2010).

2.1.2.4 Embryonic microenvironment

Four decades back it was documented that embryonic microenvironment can reprogram the cancer cells to a benign phenotype; however, the mechanisms underlying this phenomenon remains unclear (Hendrix *et al.*, 2007). The human embryonic stem cells (hESC) and cancer cells have various common features however hESC do not form tumors owing to the ability to differentiate in response to signals from the microenvironment. Normally the stem cell microenvironment or the stem cell niche controls the fate of the stem cells and that it provides the necessary constituents for maintaining homeostasis of tissue (Fuchs *et al.*, 2004). In cancer cells such control is lost and that restoring the niche may result in maintaining the homeostasis of growth and normal differentiation.

Hence to understand the mechanism Lynne-Marie Postovit et al (2006) developed an in vitro 3D model to investigate the capacity of hESC-derived factors to epigenetically influence metastatic cancer cells. They showed exposure of melanoma cells to a hESC microenvironment results in the reexpression of melanocyte-specific markers which are indicative of differentiation and a reduction in invasive potential.

Further (Lynne-Marie Postovit, 2006) they discovered that hESC microenvironments suppress the tumorigenic phenotype of human metastatic melanoma and breast carcinoma cells and that this effect is is brought about only by hESCs and not other stem cell types. Further they found that hESC microenvironment neutralize the aberrant expression of Nodal in metastatic melanoma and breast carcinoma cells and reprogram them to a less aggressive phenotype (Postovit *et al.*, 2006a; b). They also identified lefty which is sectreted by hESC (an inhibitor of Nodal signaling) as an important mediator of these phenomena. Hence the microenvironment of hESCs provides a previously unexplored therapeutic entity for the regulation of aberrantly expressed embryonic factor(s) in aggressive tumor cells (Postovit *et al.*, 2008).

3. Conclusion

CSC are rare cells and they are distinct from other bulk tumor cells. They generate the tumor and maintain the tumor hetrogenity. If the CSCs are elemiminated/differentiated to nonCSCs then cancer can be eradicated. The CSC niche maintains the CSC characteristics and increases the CSC potential, hence CSC niche offers a critical window treatment of cancer. Hence strategies that target the pathways critical for selfrenewal which are maintained through niche should be the focus of therapy. Notch, Wnt and Hedgehog pathways are known for maintaining self renewal of normal stem cells (Merchant and Matsui, 2010; Pannuti *et al.*, 2010; Takahashi-Yanaga and Kahn, 2010). These pathways offers targets in combination of other tumor specific markers for CSC targeting. For eg. Farnie, G et al., demonstrated that inhibiting notch signaling using gama secretase inhibitors in DCIS derived cells decreases their mamosphere forming efficiency (Farnie *et al.*, 2007). Further antibodies against the ECM Protein fibronectin receptor α4β1 integrin prevented the interaction of cancer cells with premetastatic niches and reduce the minimal residual disease (Kaplan *et al.*, 2005). Moreover antibodies to fibronectin and β1 integrin promoted epithelial phenotype of invasive breast cancer cells in organotypic three dimentional cultures (Sandal *et al.*, 2007). Hence when formulating such therapeutic modalities a combination of inhibitors/biomolecules which can efficiently inhibit the cancer stem cells self renewal should be considered.

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Involvement of Mesenchymal Stem Cells in Breast Cancer Progression

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1. Introduction

For many reasons, mesenchymal stem cells (MSCs) have lately received much attention. Their plasticity, their tropism for wounds and cancer, their ability to assist in tissue regeneration, their immunomodulary activities, their effects on cancer development and finally their usefulness as drug-delivery vectors made MSCs a prime target for many researchers worldwide. Many aspects of MSC functions have been covered by recent reviews (Beyer Nardi & da Silva Meirelles, 2006; Kidd et al., 2008; Klopp et al., 2011; Krabbe et al., 2005; Patel et al., 2008; Uccelli et al., 2008; Wislet-Gendebien et al., 2005; Yen & Yen, 2008). In this review, we are summarizing the current knowledge on the communication of MSCs with breast cancer cells and its consequences for breast cancer progression.

2. General aspects of MSC biology

2.1 What are mesenchymal stem cells?

Mesenchymal stem cells, also called multipotent mesenchymal stromal cells, were first described as stromal cells residing in the bone marrow (Friedenstein et al., 1966). They have stem cell-like characteristics (Caplan, 1991; Friedenstein & Kuralesova, 1971), a fibroblast-like appearance and features different from cells of the haematopoietic lineages. Those features include the ability to differentiate to osteoblasts, chondrocytes and adipocytes (Friedenstein et al., 1974; Noth et al., 2002; Pittenger et al., 1999). MSCs may also play a role in haematopoiesis, as MSCs have been shown to be involved in forming niches for the haematopoietic stem cells and to regulate the activities of these cells (Ehninger & Trumpp, 2011; Mendez-Ferrer et al., 2010; Omatsu et al., 2010; Sacchetti et al., 2007). MSCs are rare in the bone marrow. Only 1 of 34,000 nucleated cells in this tissue were determined to be MSCs (Wexler et al., 2003). Though much is known about MSCs today, there are still no specific markers available that clearly define a cell as an MSC. In 2006, the International Society for Cellular Therapy published a list of minimal criteria instead (Dominici et al., 2006) that are now commonly used to identify MSCs. Among these criteria are two functional features, the potential to differentiate to osteoblasts, chondrocytes and adipocytes as mentioned above and the ability to adhere to plastic. The latter feature allows the separation of MSCs from the other bone marrow cell populations, as cells of the haematopoietic lineages are non-adherent cells (Beyer Nardi & da Silva Meirelles, 2006). Other critieria used to characterize

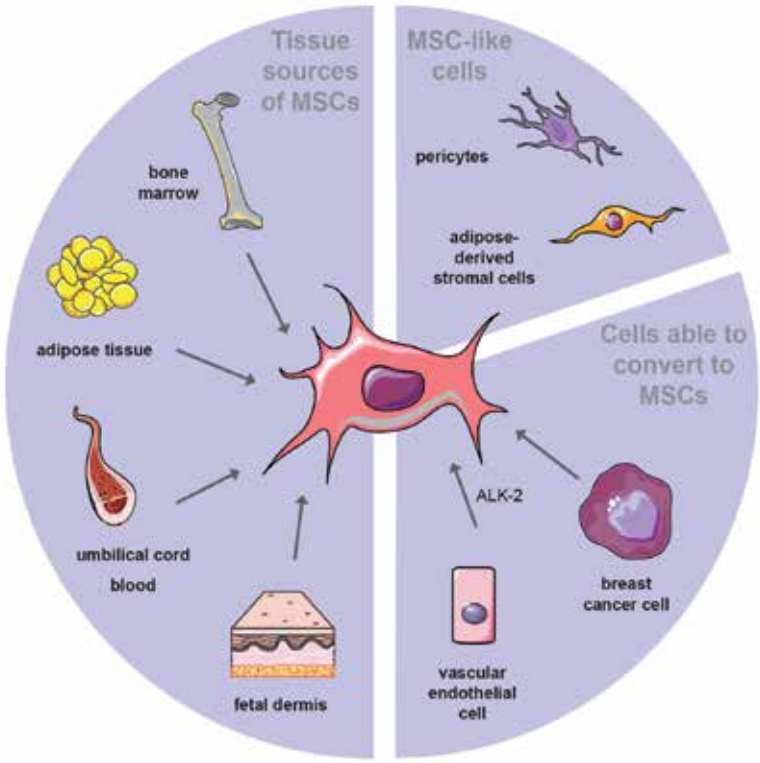

Fig. 1. Sources of MSCs. The cartoon depicts the different sources from which MSCs can be isolated (left), the cells that can convert to MSCs (right, bottom) and cells that display MSC-like features (right, top). Details are described in the text. ALK-2 = activin-like kinase-2.

MSCs are the expression profiles of certain proteins. MSCs express CD105 (endoglin), CD73 (ecto 5′-nucleotidase) and CD90 (Thy-1) and are deficient of CD45 (pan-leukocyte marker), CD34 (marker for primitive haematopoietic progenitors and endothelial cells), CD14 and CD11 (marker for monocytes and macrophages), CD79α and CD19 (marker for B-cells) and HLA-DR (MSCs not stimulated by IFN-γ). Bone marrow is not the only source of MSCs, other tissues are suitable to isolate MSCs as well (Fig. 1). Among these tissues are human adipose tissue (Zuk et al., 2002), umbilical cord blood (Sun et al., 2010), fetal dermis tissue (Qiao et al., 2008a), pancreatic tissue (Seeberger et al., 2006) and breast milk (Patki et al., 2010). More MSC sources are expected (Ding et al., 2011). Recently, menstrual blood and endometrium have been shown to contain MSCs. It is likely that most MSCs found in other tissues originated from the bone marrow. However, there is also evidence that some tissues, such as the adipose tissue, may produce their own MSCs (Bianco, 2011; Zhao et al., 2010). The MSC pool of a tissue may be expanded by dedifferentiation of differentiated cells (Fig. 1). This has been demonstrated for vascular endothelial cells that, under certain conditions, can undergo endothelial-to-mesenchymal transition to convert to MSCs (Medici et al., 2010). Some tissue-specific MSCs may be known for many years by other names (Fig. 1). Adipose-derived stromal cells or preadipocytes are likely to be MSCs residing in adipose tissue (Locke et al., 2011; Manabe et al., 2003; Zuk et al., 2002). Pericytes isolated from skeletal muscles or non-muscle tissues have recently be found to show the typical characteristics of

MSCs (Crisan et al., 2008). It is possible that MSCs from different sources may not be identical and may behave differently (Zhao et al., 2010). In fact, environmental conditions, such as the supply with growth factors or oxygen, have been shown to change the behavior of MSCs (Krinner et al., 2010; Sanchez et al., 2011). Even a population of MSCs derived from a single source may not be homogenous and may have different developmental potentials (Phinney, 2002). This hypothesis was confirmed by Wicha and his co-workers who demonstrated that MSCs from bone marrow contain at least two subpopulations, one that expresses and one that lacks the stem cell marker ALDH-1 (aldehyde dehydrogenase-1) (Liu et al., 2011). These two subpopulations behaved also functionally different (see 4.3).

2.2 Plasticity of MSCs

In addition to the ability to mature to osteoblasts, chondrocytes and adipocytes, MSCs are also capable of differentiating to fibroblasts (Mishra & Banerjee, 2011). This conversion may be of particular importance in cancer, where MSCs that colonize a cancerous lesion switch to a particular type of fibroblast-like cells, the carcinoma-associated fibroblast (CAF) (Mishra et al., 2008; Spaeth et al., 2009). This may have consequences for tumor progression (see 4.4). The differentiation potential of MSCs goes far beyond the ability to differentiate towards the mesodermal lineage (Uccelli et al., 2008). Differentiation of MSCs to cells of ectodermal and endodermal lineages have been demonstrated as well. E.g., MSCs derived from adipose tissue were shown to be able to differentiate to endothelial cells (Zuk et al., 2002), while pancreatic MSCs could become hepatocytes (Seeberger et al., 2006). In addition, MSCs from umbilical cord blood were shown to have the potential to switch to cells displaying features of neural cells (Li et al., 2005; Park et al., 2007; Tondreau et al., 2004), though, in some cases, the neural phenotype may have caused by fusions of MSCs with neurons (Krabbe et al., 2005; Wislet-Gendebien et al., 2005). Under certain conditions, MSCs can also become epithelial cells, such as lung or renal epithelium-like cells (Kale et al., 2003; Lin et al., 2003; Ortiz et al., 2003; Rojas et al., 2005).

3. Attracted to wounds and cancer

3.1 Tropism towards injured tissue: MSCs as “repair” cells

MSCs are believed to play an important role in wound healing. Chemokines and cytokines as secreted by inflammatory cells seem to chemoattract MSCs to injured tissues (Brooke et al., 2007). E.g., Kidd and his co-workers reported that, when inoculated into wounded mice, labeled MSCs were preferentially detected in wounds, whereas, in non-injured mice, MSCs settled in lung, liver and spleen (Kidd et al., 2009). MSCs are attracted to many types of organs after injury, such as heart after myocardial infarction (Barbash et al., 2003), kidney after glomeruli damage (Ito et al., 2001), injured muscles (Natsu et al., 2004), bleomycin-damaged lung (Ortiz et al., 2003) and brain after stroke (Chen et al., 2001; Mahmood et al., 2003). Interestingly, homing to the injured brain could be specifically blocked by an antibody directed to the chemokine MCP-1 (monocyte chemotactic protein-1)/CCL2 (Wang et al., 2002) suggesting that MCP-1/CCL2 is an important chemoattractant for MSCs. In the injured tissue, MSCs were found to help to regenerate this tissue. MSCs accomplish this goal partly by directly converting to those cells specifically needed to restore the function of the tissue. It is therefore tempting to consider the MSC as a general repair cell (Dittmer, 2010). Numerous reports support this hypothesis. E.g., bone marrow-derived MSCs were demonstrated to facilitate healing of injured muscles by differentiating to muscle progenitor

cells (Natsu et al., 2004). In bleomycin-injured lung, MSCs switched to a phenotype typical for lung epithelial cells (Ortiz et al., 2003; Rojas et al., 2005). In the damaged myocardium, bone marrow-derived MSCs converted to cardiomyocytes (Toma et al., 2002; Wang et al., 2001). In ischemically injured renal tubules, MSCs are able to become tubular epithelial cells (Kale et al., 2003; Lin et al., 2003). In kidneys after anti-Thy1 antibody-induced glomerulonephritis, MSCs have been shown to mature to mesangial cells (Ito et al., 2001). And in diabetic mice, MSCs induced the number of pancreatic islets to increase and enhanced insulin production (Hess et al., 2003; Lee et al., 2006). The affinity of MSCs to injured tissue can be utilized for therapy (Brooke et al., 2007; Tocci & Forte, 2003). MSCs can be used as vectors to deliver drugs to injured tissues. Examples are BDNF (brain-derived neurotrophic factor)- or insulin-secreting MSCs to improve recovery from stroke (Kurozumi et al., 2004) or to treat diabetes (Xu et al., 2007), respectively. MSCs have also been used in clinical trials (Herberts et al., 2011). Most of the clinical trials with MSCs were carried out to treat patients with heart disease (Prockop & Olson, 2007). In many cases, patients' conditions improved suggesting that MSCs have positive effects on tissue repair also in humans.

3.2 Tropism towards cancer: MSCs are attracted to breast cancer lesions

Given the fact that MSCs are entering wounds to facilitate tissue repair, MSCs are of great value to maintain body functions. However, the affinity of MSCs to wounds may be of disadvantage to people who are suffering from cancer. In support of the view that a tumor is a wound that never heals (Dvorak, 1986), MSCs were also found to be attracted to cancerous lesions (Kidd et al., 2009) where they may promote tumor progression. Importantly, wounds and cancers secrete a similar cocktail of inflammatory cytokines and chemokines (Kidd et al., 2008). Among them are MSC-attracting factors, such as the growth factors PDGF (platelet-derived growth factor) and IGF-1 (insulin-like growth factor-1), the cytokines IL-6 (interleukin-6) and IL-8 as well as the chemokines MCP-1/CCL2, RANTES/CCL5, MDC (macrophage-derived chemokine)/CCL22 and SDF-1 (stromal-derived factor-1)/CXCL12 (Dwyer et al., 2007,Ponte, 2007 #228; Kim et al., 2011; Liu et al., 2011). It was confirmed that MSCs express the corresponding receptors for these ligands, i.e. PDGFR (PDGF receptor), IGFR (insulin growth factor receptor), IL-6R, gp130, CXCR1, CCR2, CCR3, CCR4 and CXCR4 (Dwyer et al., 2007,Ponte, 2007 #228; Kim et al., 2011; Liu et al., 2011). The susceptibility of MSCs to chemoattractants can be enhanced by certain factors. E.g., TNFα (tumor necrosis factor α) was shown to increase the response of MSCs to certain chemokines by upregulating the expression of the receptors CCR2, CCR3 and CCR4 (Ponte et al., 2007). Many studies demonstrated that MSCs are attracted by tumors. In one study, the bone marrow of a mouse was replaced by the bone marrow from a transgenic mouse that expressed beta-galactosidase and MSC migration monitored from the bone marrow towards a prostate tumor xenograft (Ishii et al., 2003). It was found that X-gal positive MSCs colonized the tumor and differentiated to fibroblasts and endothelial cells. In a similar experimental setting, Direkze and co-workers could show that MSCs enter pancreatic insulinoma and convert to myofibroblasts (Direkze et al., 2004). Also breast cancer cells have been shown to chemoattract MSCs *in vitro* as well as *in vivo* (Dittmer et al., 2009; Dwyer et al., 2007; Goldstein et al., 2010; Klopp et al., 2007; Lin et al., 2008; Ling et al., 2010; Liu et al., 2011; Mishra et al., 2008; Pulukuri et al., 2010; Rattigan et al., 2010; Ritter et al., 2008; Zielske et al., 2009). Most breast cancer studies with MSCs were performed with luminal A-type

MCF-7 cells and mesenchymal (basal-B)-type MDA-MB-231 cells. In some investigations, also luminal A-type T47D, basal A-type MDA-MB-468, murine 4T1 breast cancer cells and primary human breast cancer were used. In all cases, breast cancer cells stimulated MSC migration. However, the chemoattractive potency differed among the different breast cancer cell subtypes. E.g., the highly invasive MDA-MB-231 cells were more potent than the weakly invasive MCF-7 cells in stimulating migration of MSCs *in vitro* and *in vivo* (Dittmer et al., 2009; Goldstein et al., 2010; Ritter et al., 2008). Hence, it seems that MSCs have a higher affinity to more aggressive tumors. It is well established that factors secreted by breast cancer cells are responsible for MSC attraction (Fig. 2). IL-6 is one factor that is secreted by breast cancer cells and acts as a chemoattractant for MSCs (Liu et al., 2011; Rattigan et al., 2010). In response to IL-6, MSCs not only enhance their migratory activity, but also secrete chemokines, such as CXCL7 (see 4.3) (Liu et al., 2011). Interestingly, hypoxic conditions as often found in tumors trigger breast cancer cells to produce more IL-6 which further enhances migration of MSCs (Rattigan et al., 2010). Hypoxia also affects MSCs directly in

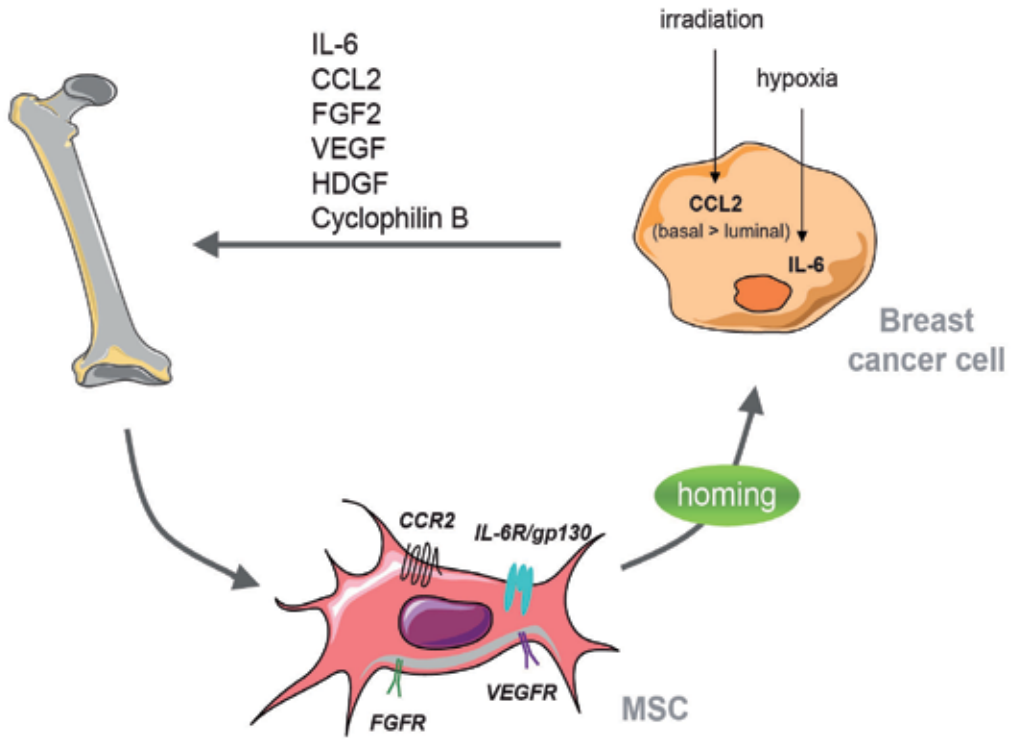

Fig. 2. Chemoattraction of MSCs to breast cancer cells. Breast cancer-derived cytokines and growth factors stimulate MSCs to migrate towards the tumor. Irradiation or hypoxia increase the CCL2 or IL-6 secretion, respectively, by breast cancer cells. Basal-type breast cancer cells seem to produce more CCL-2 than luminal A-type breast cancer cells. IL-6(R) = interleukin-6 (receptor), FGF(R) = fibroblast growth factor (receptor), VEGF(R) = vascular endothelial growth factor (receptor), HDGF = hepatoma-derived growth factor.

that it increases their proliferative activity and their expression of stem cell and differentiation markers (Grayson et al., 2006). Besides IL-6, breast cancer cell-derived FGF-2, VEGF (vascular endothelial growth factor), cyclophilin B and HDGF (hepatoma-derived growth factor) were demonstrated to induce migration of MSCs (Lin et al., 2003; Ritter et al., 2008). Another important tumor-derived chemoattractant was shown to be the chemokine MCP-1/CCL2 (Dwyer et al., 2007) which is recognized by MSCs via the receptor CCR2 (Lu et al., 2006; Wang et al., 2002). Interestingly, mesenchymal (basal B-type) MDA-MB-231 cells produce more MCP-1/CCL2 than luminal A-type T47D cells, which may explain why more aggressive breast cancer cells have a higher potential to stimulate MSC migration. In primary breast cancer, which contains both an epithelial and a stromal compartment, the stromal compartment seems to be the major source of MCP-1/CCL2 (Dwyer et al., 2007). Irradiation of tumors was found to increase the expression of MCP-1/CCL2 and, along with it, the potential to recruit MSCs to tumors (Zielske et al., 2009). This further supports the notion that MCP-1/CCL2 plays an important role in attracting MSCs to tumors. The efficiency of recruitment of MSCs to tumors may also depend on inherent features of MSCs. MSCs overexpressing uPA (urokinase plasminogen activator) have a higher ability to migrate towards breast and prostate cancer cells than their vector-treated counterparts (Pulukuri et al., 2010). Given their similar tropism to injuries and cancer (Kidd et al., 2009), MSCs are a promising tool for therapeutic intervention of cancer (Motaln et al., 2010) as much as they are for treating injuries. MSCs engineered to express anti-cancer drugs can be used as vectors to deliver toxic loads to tumor cells. In many studies with engineered MSCs, MSCs were forced to express TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a membrane protein that induces apoptosis of tumor cells, but not of normal cells (Walczak et al., 1999). Using mouse xenografts, it could be shown that TRAIL-expressing MSCs are able to eradicate many kinds of tumor cells, including glioma, cervival, pancreatic, colon and breast cancer cells (Grisendi et al., 2010; Loebinger et al., 2009; Menon et al., 2009; Sonabend et al., 2008; Yang et al., 2009). MSC-delivered TRAIL can induce apoptosis by upregulating caspase 8 (Grisendi et al., 2010). TRAIL-expressing MSCs were also able to attack metastatic breast cancer cells and to significantly reduce pulmonary metastatic load in mice (Loebinger et al., 2009). In contrast to recombinant TRAIL, which has a short half life in plasma, TRAIL-expressing MSCs allow prolonged TRAIL exposure (Grisendi et al., 2010). Other approaches use MSCs that were engineered to express IFN-β (interferon-β) or transduced with CRAds (conditionally replicating adenoviruses) (Dembinski et al., 2009; Ling et al., 2010; Stoff-Khalili et al., 2007). In another setting, MSCs were transfected with enzymes to locally convert a relatively non-toxic substance into a toxin. Examples are MSCs expressing HSV-TK (herpes simplex virus-thymidine kinase) which catalyses the conversion of the prodrug ganciclovir to a toxic compound (Conrad et al., 2011) and MSCs loaded with cytosine deaminase which induces the deamination of 5-fluorocytosine to the chemotherapeutic drug 5-fluorouracil (Kucerova et al., 2008; You et al., 2009). In both cases, the non-toxic prodrug was systemically administered to tumor-bearing mice. MSCs can also be engineered such that they boost immune responses to cancer cells. MSCs engineered to express Her2 (human epidermal receptor2), a receptor tyrosine kinase often overexpressed in breast cancer (Theillet, 2010), can act as antigen-presenting cells to induce an immune reaction against Her2-exposing breast cancer cells (Romieu-Mourez et al., 2010). However, it should be noted that caution should be exercised when using MSCs as therapeutic tools as

MSCs may be able to transform to sarcoma cells (Burns et al., 2008; Gjerstorff et al., 2009; Li et al., 2009; Mohseny & Hogendoorn, 2011; Riggi et al., 2008; Rosland et al., 2009). Currently, there is a debate about whether the MSC and not a primitive neuroectodermal cell is the cell of origin of Ewing's sarcoma (Lin et al., 2011).

3.3 Immunosuppression by MSCs: Consequences for wound healing and cancer progression

It is well established that MSCs act anti-inflammatory by modulating the activities of cells of the innate and the adaptive immune system (Rasmusson, 2006; Uccelli et al., 2008; Yagi et al., 2010). Among the affected cells are antigen-presenting dendritic cells, tumor cell-targeting natural killer cells, neutrophils and B- as well as T-lymphocytes. MSCs block antigen presentations by dendritic cells (Jiang et al., 2005; Ramasamy et al., 2007), inhibit the proliferation of activated T-lymphocytes (Bartholomew et al., 2002; Di Nicola et al., 2002; Krampera et al., 2003; Rasmusson et al., 2005), activate regulatory T cells (T_{regs}) that suppress T-effector cells (Aggarwal & Pittenger, 2005; Patel et al., 2010; Selmani et al., 2008), inhibit the activity of cytotoxic T-lymphocytes (Rasmusson et al., 2003) and block the proliferation of natural killer cells (Aggarwal & Pittenger, 2005; Sotiropoulou et al., 2006; Spaggiari et al., 2008). Direct and indirect interactions of MSCs with immune cells are made responsible for the anti-inflammatory activity of the MSCs (Uccelli et al., 2008). The indirect effects are mediated by a number of cyto- and chemokines as secreted by MSCs. Among them are TGFβ1 (transforming growth factor β1) which stimulates the proliferation of inhibitory T_{regs} (Patel et al., 2010), IL-6 shown to inhibit neutrophil proliferation (Raffaghello et al., 2008) and prostaglandin E2 that inhibits antigen presentation by dendritic cells as well as proliferation of T-effector cells (Aggarwal & Pittenger, 2005; Bartholomew et al., 2002; Di Nicola et al., 2002; Glennie et al., 2005; Jiang et al., 2005; Krampera et al., 2003; Ramasamy et al., 2007; Rasmusson et al., 2005; Selmani et al., 2008). In the mouse model, the anti-inflammatory effects of MSCs were also linked to increased phagocytosis and enhanced elimination of bacteria (Mei et al., 2010). However, due to differences in the anti-sepsis defense in mice and men, it is unclear whether these data allow the prediction of an MSC-induced anti-sepsis effect also in humans (Monneret, 2009). It is likely that, by down-modulating the immune response, MSCs prevent excessive inflammation in injuries. This is thought to be the second way by which MSCs facilitate regeneration of the injured tissue. While for that reason the anti-inflammatory effect of MSCs may be beneficial for a patient with an injury, it may be however detrimental to a cancer patient. By inducing local immunosuppression cancer-residing MSCs may help cancer cells to escape immune surveillance.

4. Communication between MSCs and breast cancer cells

4.1 The cytokine cocktail secreted by MSCs

MSCs secrete a plethora of cytokines and chemokines. In addition to the immuno-regulatory proteins, such as TGFβ1, IL-6 and prostaglandin E2, MSCs produce many other interleukins, including IL-7, IL-8 and IL-9, CC-type chemokines (CCL1, 2, 5, 8, 11, 15, 16, 20, 22, 26, and 27), CXC-type chemokines (CXCL1, 5, 6, 10, 11, 12, 13, and 16) and other factors, such as TIMP (tissue inhibitor of metalloproteases) -1 and -2, TNFα and β, PDGF A and B, G-CSF (granulocyte colony-stimulating factor), HGF (hepatocyte growth factor), VEGF and angiopoietin (Parekkadan et al., 2007). The syntheses of these factors can be further stimulated. E.g., IL-6 induces the expression of CXCL7, which further enhances the

expression levels of IL-6, IL-8, CXCL5 and CXCL6 (Liu et al., 2011). TGFα (transforming growth factor α) was found to stimulate MSCs to secrete more IL-6, IL-8, angiopoietin-2, G-CSF, HGF, VEGF and PDGF-BB (De Luca et al., 2010). TNFα forced MSCs to increase their expression of CXCL9, CXCL10 and CXCL11 (Shin et al., 2010). Exposure of MSCs to conditioned medium from tumor cells also stimulate expression of chemokines, such as CXCL2 and CXCL12 (Menon et al., 2007). Direct interactions of cancer cells with MSCs may as well contribute to the rise of chemokine secretion by MSCs. Direct contacts of MSCs with MDA-MB-231 breast cancer cells were found to strongly upregulate the production of RANTES/CCL5 (Karnoub et al., 2007).

4.2 Modulatory effects of MSCs on breast cancer cell function

MCF-7 cells are ERα-positive luminal A-type breast cancer cells that show many features of normal breast epithelial cells, including the formation of E-cadherin-based cell-cell interactions and the ability to generate multicellular 3D-aggregates that can mature to lumen-containing spheroids (dit Faute et al., 2002; do Amaral et al., 2010). Decreased expression or complete loss of E-cadherin has been linked to epithelial-mesenchymal transition (EMT) and increased cellular migration of breast epithelial cells as well as to metastasis (Cano et al., 2000; Chua et al., 2007; Mani et al., 2008; Onder et al., 2008). We and others have shown that MSCs negatively interfere with the E-cadherin status of MCF-7 cells either by downregulation of the full length protein (Fierro et al., 2004; Hombauer & Minguell, 2000; Klopp et al., 2010) or by increasing E-cadherin shedding as triggered by the transmembrane protease ADAM10 (a disintegrin and metalloprotease 10) (Dittmer et al., 2009). It is noteworthy that as few as one MSC per 500 MCF-7 cells was sufficient to induce E-cadherin shedding. E-cadherin shedding leads to extracellular E-cadherin fragments that may block E-cadherin-based cell-cell contacts by competing with membrane-bound E-cadherin proteins (Ryniers et al., 2002). Hence, both downregulation of E-cadherin expression and increased E-cadherin shedding may decrease the strength of E-cadherin-based cell-cell interactions. With intercellular adhesions weakened cellular migration may increase. In fact, MSCs have been shown to significantly enhance the migratory activity of MCF-7 cells (Dittmer et al., 2009; Rhodes et al., 2010). Also along with the destabilization of cell-cell contacts, disruption of the architecture of MCF-7 spheroids was observed. It is interesting that, despite these changes in the E-cadherin status, MSCs did not induce EMT of MCF-7 cells, as indicated by the failure of MSCs to stimulate the expression of mesenchymal markers, such as vimentin or snail (Dittmer et al., 2009; Klopp et al., 2010). However, in the luminal A-type T47D breast cancer cell line, MSCs not only downregulated E-cadherin levels, but also increased expression of vimentin, snail, twist and N-cadherin (Martin et al., 2010) suggesting that, under certain conditions, MSCs can induce EMT of breast cancer cells. MSCs were also shown to increase the proliferation of MCF-7 cells in a dose-dependent manner (Fierro et al., 2004; Klopp et al., 2010; Rhodes et al., 2010; Sasser et al., 2007a). These effects may be mediated by IL-6, VEGF and/or SDF-1/CXCL12 as secreted by MSCs (Fig. 3) (Fierro et al., 2004; Sasser et al., 2007b). MSCs or similarly IL-6 induced the phosphorylation of STAT3 (signal transducer and activator of transcription 3) on tyrosine-705 in MCF-7 cells (Sasser et al., 2007b). Incubation of MSCs with TGFα, a ligand of the EGFR (epidermal growth factor receptor), further stimulated the secretion of IL-6 and other factors (De Luca et al., 2010). This suggests that TGFα-primed MSCs would even be more effective in promoting proliferation of MCF-7 cells. MSCs also enhanced the tumorigenic activity of

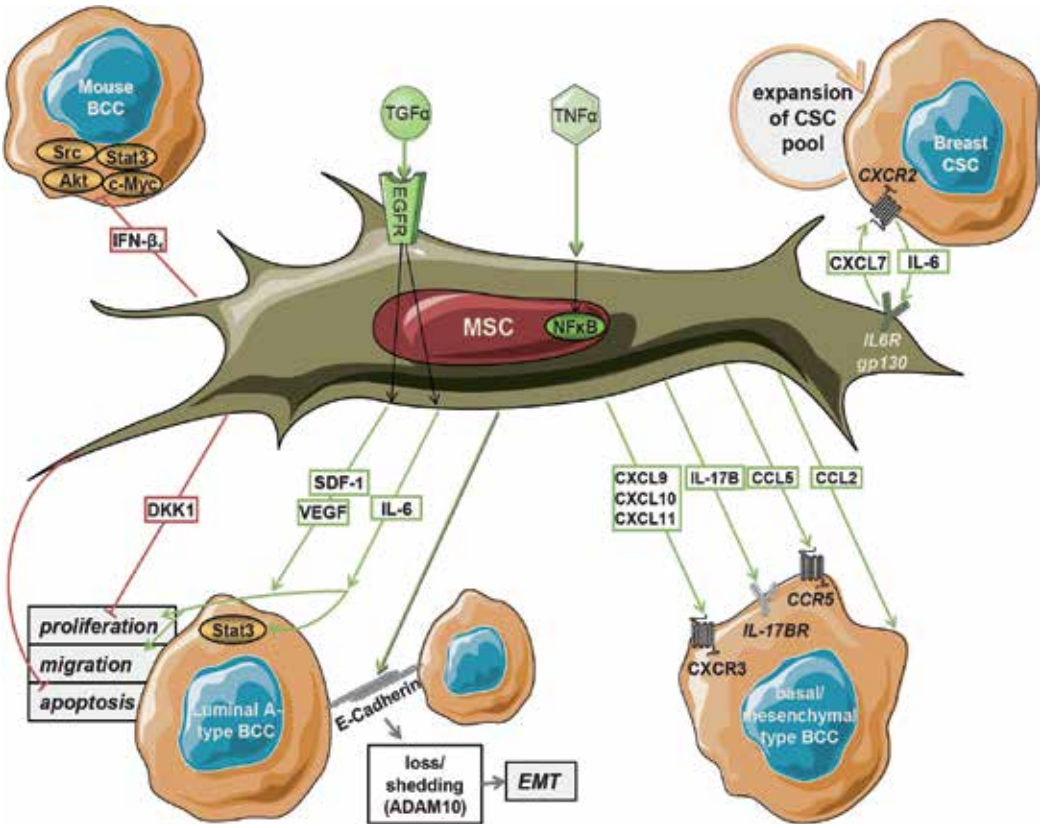

Fig. 3. Paracrine actions of MSCs on breast cancer cells (BCCs). The effects of MSCs on luminal A and basal/mesenchymal subtype BCCs, on murine BCCs and on cancer stem cells (CSCs) are separately displayed. IL-6/-17B(R) = interleukin-6/-17B (receptor), VEGF = vascular endothelial growth factor, SDF-1 = stromal-derived factor-1, DKK = dickkopf, INF-β_1 = interferon β_1, TGFα = transforming growth factor α, TNFα = tumor necrosis factor α, EGFR = epidermal growth factor receptor, NF-κB = nuclear factor kappa-light-chain-enhancer of activated B-cells, Stat3 = signal transducer and activator of transcription 3, ADAM10 = a disintegrin and metalloprotease 10, EMT = epithelial-to-mesenchymal transition.

MCF-7 cells. In mouse xenografts, MCF-7 tumor formation and growth were fostered by MSCs (Klopp et al., 2010). Though ERα-positive MCF-7 cells are dependent on estrogen for growth, MSCs may even trigger estrogen-independent proliferation of MCF-7 cells (Rhodes et al., 2009). The estrogen-independent growth may nevertheless be dependent on ER, as the proliferation-promoting effect of MSCs on MCF-7 cells was found to be blocked by ER-specific inhibitor ICI 182780 (Rhodes et al., 2010). It is thought that, by a yet unknown mechanism, MSCs activate ER which, in turn, stimulates the expression of SDF-1/CXCL12, a chemokine shown to trigger the proliferation of MCF-7 cells. Growth-stimulating effects of MSCs were also found on other ERα-positive breast cancer cell lines, including T47D, BT474 and ZR-75-1 (Sasser et al., 2007a) and may be dependent on similar mechanisms as those on MCF-7 cells. There are also two reports that show that MSCs are able to inhibit the proliferation of MCF-7 cells (Goldstein et al., 2010; Qiao et al., 2008a). Dickkopf-1 (DKK-1) secreted by MSCs and known to block differentiation and to promote proliferation of MSCs

by an autocrine mechanism (Pinzone et al., 2009) may be responsible for this effect (Qiao et al., 2008a). As an inhibitor of the Wnt/β-catenin pathway, DKK-1 was shown to downregulate β-catenin activity and, concomitantly, to reduce the expression of proliferation-promoting proteins c-Myc and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells) in MCF-7 cells (Qiao et al., 2008a; Qiao et al., 2008b). Why some studies showed stimulatory while others demonstrated inhibitory effects of MSCs on MCF-7 cell proliferation is not clear yet. Qiao et al. used human fetal dermal tissue as a source to isolate MSCs for their study (Qiao et al., 2008a). In this case, the different MSC sources may have accounted for the contradictory results. Since MSCs are a heterogeneous population (Uccelli et al., 2008), a different environment may drive the selection of a certain subtype of MSCs with features distinct to the bone-marrow MSC population. In particular, types and amounts of chemokines/cytokines these MSC populations secrete might be different. The importance of environmental conditions for the ability of MSCs to interfere with breast cancer functions is nicely demonstrated in a study that compared serum-exposed MSCs with serum-deprived MSCs (Sanchez et al., 2011). Serum-deprived MSCs were found to be more effective than serum-exposed MSCs in protecting MCF-7 cells from apoptotic death by secreting pro-survival factors. MSCs also modulate the functions of highly aggressive ERα-negative MDA-MB-231 cells (Fig. 3). Two studies demonstrated that MSCs increase the invasive and metastatic behavior of these breast cancer cells (Goldstein et al., 2010; Karnoub et al., 2007). In one study, this effect was found to be mediated by IL-17B (Goldstein et al., 2010). In the other study, the chemokine RANTES/CCL5 was shown to be responsible (Karnoub et al., 2007). Paracrine feedback loops between breast cancer cells and MSCs seem to be important for these effects. It could be shown that MDA-MB-231 cells stimulate the expression of RANTES/CCL5 in MSCs by secreting osteopontin which binds to MSC surface integrins which then leads to the activation of AP-1, a transcription factor able to induce the transcription of the RANTES/CCL5 gene (Mi et al., 2011). MCP-1/CCL2 is another chemokine whose secretion can be stimulated when MSCs are co-cultured with MDA-MB-231 cells (Molloy et al., 2009). MCP-1/CCL2 belongs to those chemokines that enhance the motility of MDA-MB-231 cells. Other migration-promoting chemokines are CXCR3 ligands CXCL9, CXCL10 and CXCL11 (Shin et al., 2010). These CXCL chemokines also increase the activity of Rho GTPases and the expression of MMP-9 (matrix metalloprotease-9). These chemokines may be of particular importance when MSCs are exposed to TNFα which was found to induce CXCL gene transcription through a mechanism involving NF-κB (Fig. 3). One group also demonstrated inhibitory effects of MSCs on MDA-MB-231 cells (Sun et al., 2009; Sun et al., 2010). According to their data, MSCs suppress the proliferative, migratory, tumor-initiating and metastatic activities of MDA-MB-231 cells and induce apoptosis of these cells by interfering with the AKT/mTOR (mammalian target of rapamycin) pathway. Different to the other investigations, these studies were performed with MSCs isolated from human umbilical cord blood or adipose tissue. Hence, as discussed above, source-dependent features of MSC isolates may be responsible for these contradictory results. Also murine metastatic 4T1 breast cancer cells were shown to be affected by MSCs (Ling et al., 2010). Using a syngeneic, immunocompetent murine model, Ling and colleagues demonstrated that murine MSCs enter 4T1 tumors to deliver IFN-β to the tumor. This factor then inhibited cancer growth by inducing the inactivation of STAT3, Src and AKT and by triggering the downregulation of c-Myc and MMP-2 (matrix metalloprotease-2). Interestingly, human MSCs engineered to

secrete IFN-β have also a suppressing effect on growth of MDA-MB-231 cells in mouse xenografts (Studeny et al., 2004). It may well be that the ratio of tumor-suppressing vs. tumor-promoting factors as secreted by MSCs determine whether MSCs promote or inhibit tumor growth. This ratio could be different among different MSC isolates.

4.3 MSCs, EMT and breast cancer stem cells

There is growing evidence that, in accordance with the hierarchical model of cancer development (Visvader & Lindeman, 2008), breast cancer is driven by cancer stem cells (CSCs) (Liu & Wicha, 2010). Breast CSCs are characterized by high expression of surface marker CD44 and low expression of CD24 (Fillmore & Kuperwasser, 2007). Another useful breast CSC marker is ALDH-1 (Ginestier et al., 2007). A recent study showed that MSCs increase the pool of CSCs in breast cancer lines, including MCF-7, SUM149 and SUM159 cells (Liu et al., 2011). Interestingly, bone marrow-derived MSCs themselves show also a hierarchical organization with only a minority of cells expressing the stem cell marker ALDH-1. And only those ALDH-1 positive MSCs were able to interfere with the CSC pool. Wicha and his co-workers showed that the MSC/breast cancer interaction generated a cytokine network which is initiated by IL-6 as secreted by breast cancer cells (Liu et al., 2011). IL-6 induces the production of CXCL7 in MSCs which, in turn, triggers the expression of a number of other cytokines and chemokines, namely IL-6, IL-8, CXCL6 and CXCL5, in both MSCs and breast cancer cells. This mixture of secreted factors then stimulates the expansion of the CSC pool. In line with the observation that MSCs induce the CSC pool to expand is the finding that MSCs stimulated mammosphere formation of normal mammary epithelial cells (Klopp et al., 2010). Evidence has been accumulated suggesting that the generation of mammospheres depends on the presence of mammary stem cells (Dontu et al., 2003). Hence, the number of mammospheres formed is supposed to be a measure of the number of mammary stem cells present (Charafe-Jauffret et al., 2008). A recent study on breast cancer patients support the notion of a link between MSCs and breast cancer stem cells (De Giorgi et al., 2011). It showed that the relative number of disseminated $CD44^{+}/CD24^{low/-}/ALDH\text{-}1^{+}$ breast cancer stem cells correlated with the relative number of MSCs in the bone marrow. Recently, it has been found that epithelial cells after having undergone full EMT display stem cell-like characteristics, including expression of CD44 and ALDH-1 (Mani et al., 2008; May et al., 2011). EMT is linked to E-cadherin loss and the expression of mesenchymal markers. As mentioned above, MSCs have been shown to reduce E-cadherin expression or to induce E-cadherin shedding in luminal A-type, epitheloid breast cancer cells, such as MCF-7 and T47D. In T47D, this downregulation of E-cadherin was accompanied with increased expression of mesenchymal markers suggesting that MSCs may induce at least partial EMT of breast cancer cells. Hence, MSCs may not only be able to trigger the CSC pool to expand, but also to force new CSCs to be generated from the pool of non-CSC breast cancer cells by EMT. These MSC-induced new CSCs may have other features than the CSCs of the existing pool and may further contribute to tumor heterogeneity and progression (Visvader & Lindeman, 2008). Another interesting observation is that the gene expression profile of mesenchymal (basal-type) breast cancer cells show similarities to the expression profile of MSCs (Marchini et al., 2010) suggesting that this type of breast cancer cell and MSCs have also common functions. In support of this notion, a recent study showed that mesenchymal breast cancer cells generated by

transformation of human mammary epithelial cells by SV40 T-antigen and forced expression of EMT-inducing proteins had the potential to undergo adipogenic and chondrogenic differentiation. Also, these mesenchymal breast cancer cells were attracted to wounds and tumors, a feature typical for MSCs. The latter observation may shed a new light on a phenomenon called tumor-self seeding (Leung & Brugge, 2009). Tumor-self seeding describes the ability of metastasized cells to circulate back to the primary tumor. Chemoattraction to the primary tumor was shown to be driven by IL-6 and IL-8. As mentioned above, IL-6 is highly active on MSCs and triggers the production of a number of chemokines (Leung & Brugge, 2009). Based on these data, it is tempting to assume that MSCs are also generated from breast cancer cells (Fig. 1) and that these MSCs containing the mutations (and epigenetic changes) of the breast cancer cells they derived from play a role in breast cancer metastasis and tumor-self seeding. Nestin$^+$ - MSCs have been reported to share with haematopoietic stem cells the same niche in the bone marrow (Mendez-Ferrer et al., 2010). This niche might also be available for breast cancer-derived MSCs and allow these cells to survive in this tissue. An exciting hypothesis would be to assume that, at least in some cases of breast cancer, dormancy (Pantel et al., 2009; Willis et al., 2010) is caused by breast cancer-derived MSCs that are caught in these niches. The niches could fulfill two functions in order to maintain dormancy, preventing the cells from proliferating while, at the same time, protecting them from death-inducing signals. The bone marrow seems to be an attractive tissue for circulating tumor cells to home and form micrometastasis which is an early event in breast cancer development (Pantel et al., 2009). The number of such disseminated breast cancer cells in bone has been linked to prognosis of breast cancer patients. MSCs may also play a role in this early entry of breast cancer cells into bone marrow (Corcoran et al., 2008). MSCs were shown to facilitate the migration of MCF-7 and T47D breast cancer cells across bone marrow endothelial cells *in vitro* and to be in close contact with bone-metastasized breast cancer cells *in vivo*. Evidence was presented that these MSC/breast cancer cell interactions may require the chemokine receptor CXCR4 as well as its ligand SDF-1/CXCL12. Hence, it might be possible that breast cancer-derived MSCs not only would be able to home to the bone marrow and induce tumor dormancy, but also to help other breast cancer cells to enter this tissue and form micrometastasis. The breast cancer cell may not be the only non-stem cell which may be able to convert to an MSC. Vascular endothelial cells have been shown to become MSC-like cells as well as displaying typical MSC features, such as the ability to differentiate to osteoblasts, chondrocytes and adipocytes, upon treatment with ALK2 (activin-like kinase-2), TGFβ2 or BMP4 (bone morphognetic protein-4) (Medici et al., 2010). This suggests that certain non-stem cells under certain conditions can be an additonal source for generating MSCs. These cells may have different features compared to those MSCs that derived from the bone marrow.

4.4 MSCs and carcinoma-associated fibroblasts

Besides differentiating to osteoblast, chondrocytes and adipocytes, MSCs are able to convert to neural cells or to undergo transdifferentiation to different kinds of epithelial cells (Uccelli et al., 2008; Wislet-Gendebien et al., 2005). In tumors, MSCs can also differentiate to the carcinoma-associated fibroblasts (CAFs) (Mishra et al., 2008; Spaeth et al., 2009). These cells are different to normal fibroblasts/myofibroblasts in that they are able to stimulate tumor progression (Olumi et al., 1999) and show higher proliferative and migratory activity (Schor

et al., 1988). Defined as myofibroblasts, CAFs share features with both smooth muscle cells and fibroblasts (Mueller & Fusenig, 2004). CAFs are found in many cancers, including breast cancer (Chauhan et al., 2003), and are linked to tumor invasion and proliferation (De Wever & Mareel, 2003; Tlsty & Coussens, 2006). They are responsible for a phenomenon called desmoplasia and promote angiogenesis and inflammation (Orimo et al., 2005; Tlsty & Coussens, 2006). Interestingly, CAFs secrete factors that are also produced by MSCs. In particular, CAFs and MSCs both secrete IL-6 and SDF-1/CXCL12, cytokines able to induce the proliferation of luminal A-type MCF-7 breast cancer cells (Bhowmick et al., 2004; Fierro et al., 2004; Mishra et al., 2008; Orimo et al., 2005; Sasser et al., 2007b). In addition, both cell types were found to interfere similarly with the response of MCF-7 and MDA-MB-231 breast cancer cells to inhibitors of mTOR and B-RAF (Dittmer et al., 2011). Differentiation of MSCs to CAFs requires the exposure of MSCs to conditioned medium from tumor cells over several weeks (Mishra et al., 2008; Spaeth et al., 2009). Conditioned medium from MDA-MB-231 breast cancer cells and from Skov-3 ovarian cancer cells were similar effective in inducing a MSC/CAF conversion which was accompanied by increased expression of CAF markers, such as tenascin-C, α-smooth muscle actin and IL-6. What are the consequences of this finding? As soon as MSCs enter a tumor, they will be bombarded with a cocktail of cytokines as produced by the tumor cells and may receive additional signals by direct cell-cell contacts. This may then force MSCs to lose their stemness and to undergo differentiation towards CAFs. By converting to CAFs, MSCs may not further be able to act also suppressive on tumor cells and may only keep their potency to promote tumor progression. Hence, the differentiation of MSCs to CAFs may be as much of a benefit for a progressing tumor as is the differentiation of MSCs to particular cells for an injured tissue to be repaired (Dittmer, 2010).

5. Conclusions

MSCs display an astounding plasticity and have shown to differentiate to cells as different as neurons and epithelial cells. The main function of MSCs is likely to promote tissue regeneration after injuries and, since tumors are probably wounds that never heal, also to support repair of tumoral lesions. However, tumors may misguide MSCs and "misuse" them for their "own benefit". Primary tumors may particularly profit from MSCs when they differentiate to tumor-promoting CAFs. MSCs may further facilitate breast cancer to metastasize by helping breast cancer cells to enter the bone marrow as well as by increasing the pool of metastasizing breast cancer stem cells. Most of the interactions between MSCs and tumor cells are mediated by cytokines as secreted by both cell types. Paracrine feedback mechanisms may further increase cytokine concentrations at places where these cells communicate with each other and may attract other cell types, such as macrophages, that are known to support tumor progression. To interfere with the interaction between MSCs and breast cancer cells treatments may be considered involving the inhibition of the activities of key cytokines, such as IL-6 (Liu & Wicha, 2010), which are important for both attraction of MSCs to breast cancer and expansion of the breast cancer stem cell pool by MSCs. On the other hand, there is also evidence that MSCs may have suppressive effects on breast cancer. Different sources from which MSCs were isolated may partially account for these contradictory results. Further studies are necessary to clarify this controversy, before conclusions can be drawn in terms of treatment of breast cancer patients. Certainly, when

engineered to produce anti-tumor factors, MSCs possess anti-tumoral effects and may be used as trojan horses that enter and eradicate tumor cells. Drug-carrying MSCs may have a great advantage over "naked" drugs since they may deliver drugs more selectively and more efficiently at places where they are meant to act.

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Breast Cancer Stem Cells

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1. Introduction

Over 150 years ago, Cohnheim and Durante formalized the concept that cancers might arise from a small subset of cells with stem cell properties [1-3], and in 1961, Till and McCulloch demonstrated for the first time that the existence of hematopoietic stem cells (HSC) in the bone marrow, which was postulated that stem-like cells might be the origin of cancer [4]. However, only recently did an increased interest in cancer stem cells (CSC) occur, thus spurring great advances in cancer stem cell biology. The CSC model was first developed in 1994 when malignant initiating cells were discerned in human acute myeloid leukemia (AML) [5]. Afterwards, similar CSC model was extended to some solid tumors that originated in the breast, brain, lung, prostate, colon, head and neck, and pancreas [6-12]. Most importantly, the development of CSC hypothesis has fundamental implications in terms of understanding the biology of muti-step tumorigenesis, the prevention of cancer, and the creation of novel effective strategies for cancer therapy.

1.1 The definition of cancer stem cells

It is well documented that tumors contain cancer cells with heterogeneous phenotypes reflecting aspects of their apparent state of differentiation. In a tumor, the mutable expression of normal differentiation markers by cancer cells implies that some of the heterogeneity arises as a result of this altered manifestation. Also, cancer is known to be the product of the accumulation of multiple genetic mutations and epigenetic alterations in a single target cell, the occurrences of which can sometimes take place over many decades. Furthermore, chemotherapy and radiation therapy for cancers have limited effectiveness in long-term scenarios, and the possible recurrence of tumors after years of disease-free survival exists in great majority of cancers. All these observations provide persuasive evidence that tumors are not mere monoclonal expansions of cells but might contain a subset of long-lived tumor-initiating cells with the ability to self-renew indefinitely and to regenerate the phenotypic diversity of original tumor [13]. This subpopulation is now widely termed as cancer stem cells (CSCs), also named tumor-initiating cells (T-IC). The exist of CSCs within a tumor was also supported by *in vitro* ''clonogenic assays'' that showed subpopulations of tumor cells (with increased proliferative capacity) using cells isolated from tumor specimens, as well as by *in vivo* self-renewal assays that indicated only a small specific subset of cancer cell population had tumorigenic potential when injected into immunodeficient mice [13, 14].

The definition of CSCs is defined by two main properties: 1) self-renewal that drives tumorigenesis: the ability to form new CSCs with potential for proliferation, expansion, and differentiation; 2) multipotent differentiation, which contributes to the cellular heterogeneity of a tumor: the ability to give rise to a heterogeneous progeny of tumor cells, which diversify in a hierarchical manner.

When distinguished from the majority of differentiated cancer cells, CSCs are resistant to many current cancer treatments, including chemo- and radiation therapy [15-20]. This suggests that lots of cancer treatments, while targeting the majority of tumor cells, may fail in the end due to not eliminating CSCs, which survive by developing new tumors. However, this would open avenues for developing novel effective drugs targeting CSCs. Although CSCs share several properties (i.e. the ability to self-renew and to differentiate, increased membrane transporter activity, the capacity for migration and metastasis, the same intrinsic signaling pathways (Notch, Wnt, Hedgehog etc) for regulation of self-renewal etc) with the normal stem cells [21], they are found to have some particular characteristics. For instance, the proliferation and self-renewal of CSCs are uncontrolled and unlimited (sometimes referred to as "immortality"), and the CSCs always differentiate into abnormal cancer cells, thus they cannot give rise to mature somatic cells [22]. This reveals that therapies targeted at extrinsic signals generated in the microenvironment (such as CXCR1, endothelial cell-initiated signaling, IL-6 and CXCL7) [23-25] or microRNAs (see Part 3 of this chapter) [26-29], which are found to specifically regulate self-renewal and/or differentiation of CSCs, might achieve clinical success with little adverse effects in cancer treatment.

1.2 Leukemia stem cells: The first cancer stem cells identified

In the early 1990s, Dick and his colleagues started a series of groundbreaking investigations to understand whether the functional hierarchy observed in normal hematopoiesis was conserved in leukemia [5, 30]. They used magnetic separation techniques and purified cells from AML patients into several groups according to different surface markers. These groups of cells were then implanted into immunocompromised mice and assessed for the ability to produce leukemic colony forming units. Interestingly, only the $CD34^+$ $CD38^-$ subpopulation of leukemic cells had the ability to generate substantially more leukemic colonies *in vivo*. As well, they found that $CD34^+$ $CD38^-$ leukemic stem cells retained differentiative capacity, giving rise to $CD38^+$ and Lin^+ populations. These observations provided the first compelling evidence that in a human cancer, there was a small population of self-renewing, tumorigenic stem cells.

1.3 Solid tumor stem cells

Subsequent experiments extended the leukemic stem cell model to human solid tumors. In the year 2003, Al-Hajj *et al* reported the identification of CSCs in human breast cancer, the first solid tumor that the existence of a functional hierarchy stem cell system had been demonstrated [7]. In their experiments, human breast cancer specimens obtained from primary or metastatic sites in nine different patients all engrafted in the NOD/SCID (non-obese diabetic/severe combined immune deficiency) mice. They observed that in most human breast cancers, only a minority subset of the tumor clones (defined as $CD44^+$, $CD24^{-/low}$ and representing 11%–35% of total cancer cells) is endowed with the capacity to maintain tumor growth when xenografted in NOD/SCID mice. Importantly, tumors grown from the $CD44^+$, $CD24^{-/low}$ cells were shown to contain mixed populations of epithelial tumor cells, recreating the phenotypic heterogeneity of the parent tumors. The small

subpopulation of cells was further enriched by sorting for those that expressed epithelial surface antigen (ESA). More interestingly, 200 of the enriched $ESA^{+}CD44^{+}CD24^{-/low}$ cells were able to form a tumor following injection into a NOD/SCID mouse, while 20,000 of the $CD44^{+}CD24^{+}$ cells failed to do so [7]. In summery, these results opened a new chapter in the understanding of the biology of CSCs in human solid tumors.

Soon after, Michael F. Clarke's group published similar data about CSCs in brain tumors [8, 31]. They carried out studies to enrich tumorigenic cells in glioblastoma multiforme and medulloblastoma by sorting for those that express positive / high levels of CD133, a neural cell surface stem cell antigen. $CD133^{high}$ cells formed numerous colonies in suspension culture, and injection of as few as 1000 of these cells into an immunocompromised mouse successfully form a tumor. Conversely, $CD133^{low}$ cells showed very limited proliferative potential *in vitro*, and as many as 10,000 of these cells failed to seed tumors in host mice [8]. Furthermore, tumors developing from orthotopic, intracerebral injection of the minority of $CD133^{+/high}$ cells (about 5% - 30% of total tumor cells) reproduced the phenotypic diversity and differentiation pattern of the parent tumors [31].

As mentioned earlier, comparable results have been obtained in other solid tumors, like lung, prostate, colon, head and neck, as well as pancreatic [6, 9-12].

2. Isolation and identification of breast cancer stem cells

In most tumor tissues, including breast cancer, CSCs are rare. As we know, breast cancer is a histologically and molecularly heterogeneous disease, with six different subtypes, including luminal A, luminal B, normal breast-like, basal-like, $claudin^{low}$ and HER2 overexpressing, which are characterized by distinct histology, gene expression patterns, and genetic alterations [32-35]. The molecular heterogeneity between breast cancers has been revealed to issue from different targets of transformation. Recent studies found that basal-like breast cancers with BRCA1 mutations were more likely to arise from luminal progenitors rather than the basal stem cells [36, 37]. However, further studies that focus on breast CSCs and mammary stem/progenitor cells as well as their potential relationship are needed for determining the exact origin of luminal versus basal-like cancers, with the aim of developing targeted therapies for different subtypes of breast cancers. Moreover, CSCs was found to be the main culprit for the failure of chemo- and radiation therapy, as well as the seeds for the distant metastasis and relapse in breast cancers [20, 32, 38-40]. Taken together, in order to better understand the properties and biology of breast CSCs and eventually cure breast carcinoma, it is absolutely necessary and important to identify and separate breast CSCs prospectively.

2.1 Isolation of breast CSCs with cell-surface marker profiles

Since Dick, *et al* isolated a specific subpopulation of leukemia cells (that expressed surface markers similar to normal hematopoietic stem cells) which was consistently enriched for clonogenic activity in NOD/SCID immunocompromised mice from acute myeloid leukemias in the 1990s [5, 30], scientists attempted to see if they could enrich CSCs in human solid tumors by sorting for different cellular markers. CD24, a ligand for P-selectin in both mouse and human cells, was identified as a significant marker for human breast carcinoma invasion and metastasis [41, 42], and another adhesion molecular CD44 was found to correlate with cellular differentiation and lymph node metastasis in human breast cancers [43, 44], whereas B3.8 was described as a breast / ovarian cancer-specific marker [45]. Based on these

observations, in 2003, Al-Hajj *et al* tried to determine whether these surface markers could distinguish tumorigenic from nontumorigenic cells, and flow cytometry was used to isolate cells that were positive or negative for each marker. They demonstrated that a small population of tumorigenic cells, isolated from human breast tumors and characterized by the expression of the cell surface markers $CD44^{+}CD24^{-/low}Lineage^{-}$, was capable of regenerating the phenotypic heterogeneity of the original tumor when injected subcutaneously into NOD/SCID mice [7]. They showed that as few as 100 cells with CD44+CD24−/low phenotype could form tumors in immunodeficient mice, while thousands of cells with fungible phenotypes failed to do so. Since then, CD44 and CD24 are widely accepted as surface markers for breast CSCs, and lots of studies have focused on roles of $CD44^{+}CD24^{-}$ tumor cells in breast cancers. For example, Abraham *et al.* conducted immunohistochemical studies of $CD44^{+}CD24^{-}$ tumor cells in human breast tumors and showed that breast tumors containing a high proportion of $CD44^{+}CD24^{-}$ cells were associated with distant metastases [46].

Nevertheless, besides CD24 and CD44, there are other surface marker candidates for the enrichment of breast CSCs. Ginestier *et al.* reported that they separated breast cancer stem/progenitor cells by sorting for Aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes [47, 48], and they found that fewer ALDH1-positive than $CD44^{+}CD24^{-}$ tumor cells are required to produce tumors in immunodeficient mice [49]. Additionally, recent studies revealed that ALDH1-positive seemed to be a more significantly predictive marker than $CD44^{+}CD24^{-}$ for the identification of breast CSCs, in terms of resistance to chemotherapy and more metastatic [39, 50]. Moreover, it has been reported that the surface marker CD133 could isolate a group of breast CSCs that doesn't overlap with $CD44^{+}CD24^{-}$ cells [51]; and another recent study demonstrated that in a basal breast cancer cell line MDA-MB-231 (known as triple-negative), PROCR and ESA, instead of $CD44^{+}CD24^{-/low}$ and ALDH, could be used to highly enrich breast cancer stem/progenitor cell populations which exhibited the ability to self renew and divide asymmetrically [52].

2.2 Separation of breast CSCs by selecting for side-population (SP) cells

Advances in the separation of breast CSCs was accelerated by the identification of side population (SP) cells, due to lack of dye retention and chemotherapy efflux [53]. The method is based on cells incubated with Hoechst dye 33342 or rhodamine, after which the cells are analyzed by flow cytometry for dye exclusion and size, and SP cells would not retain dye. Isolation of SP cells facilitates purification of adult tissue stem cells comprising human and murine hematopoietic stem cells and a population of putative mammary epithelial stem cells [54-57]. Moreover, because some evidence revealed that breast CSCs and mammary epithelial stem cells represent biologically related entities [58], scientists thought to apply this technique to isolate breast CSCs. In 2005, Patrawala *et al* successfully isolated SP cells from an ER-positive human breast cancer cell line MCF-7, and they demonstrated that these small subset (0.2%) SP cells preferentially express stemness-associated genes (such as Notch1 and β-catenin) and verapamil-sensitive ATP-binding cassette (ABC) transporter ABCG2 mRNA [59]. More interestingly, MCF-7 SP cells were highly tumorigenic, whereas MCF-7 non-SP cells could not give rise to tumors in mice at al[59]. Researchers then took advantage of similar method to separate SP cells with stem cell properties from an ER-negative human breast cancer cell line Cal-51 and an triple-negative human breast cancer cell line MDA-MB-231, respectively, and they both found the SP cells expressed high levels of ABCG2 [60, 61]. Previous

studies showed that SP cells takes advantage of their ability to pump out the fluorescent dye Hoechst 33342 (H33342) through the ABCG2 (also known as breast cancer resistance protein-1), which was regarded as a major mediator of dye efflux in various stem cells [54, 62]. As the ability to efflux substrates is particularly important for the protection of CSCs, and CSCs survive after chemotherapy partially by effluxing cytotoxic drugs, ABCG2 seems to protect stem cells from toxins. This is evident in *ABCG2* knockout mice that are more sensitive to compounds such as vinblastine, ivermectin, topotecan, and mitoxantrone [63-65]. Taken together, SP cells have the capacity to efflux toxic substances out of breast cancer stem like cells via an ABCG2-mediated cytoprotective mechanism and seem to contribute to chemotherapy-resistance. In addition, it is important to consider that identification of cancer stem like cells by selecting for SP cells is not limited to breast carcinomas. Similar observations have been made in other solid tumors (such as glioma, ovarian and pancreatic cancers) where the isolated SP cells proliferated infinitely and could regenerate heterologous NSP cells in culture [59, 66-68].

2.3 Propagation of breast CSCs by isolating "mammospheres" from suspension cultures

Colonial growth in nonadherent culture was used to test for self-renewal capacity in cultures of neural cell in 1996, and in the experiment, suspension culture led to formation of "neurospheres", which consisted of 4% - 20% normal neural stem cells [69]. Based on this approach, Galli *et al.* succeeded in the characterization and isolation from human glioblastoma multiform of "cancer neurospheres", which were highly enriched in long-term self-renewing, multi-lineage-differentiating, and tumor-initiating cells [70]. According to these successful procedures, researchers tried to extend this technology to the identification and propagation of mammary epithelial stem cells and breast CSCs. In 2003, Dontu *et al.* demonstrated that nonadherent mammospheres are enriched in human mammary epithelial progenitor/stem cells and able to differentiate along all three mammary epithelial lineages and to clonally generate complex functional structures in reconstituted 3D culture systems [55]. More encouragingly, two years later (2005), Ponti and colleagues reported the isolation and *in vitro* propagation of spherical clusters of self-replicating cells (''mammospheres'') with stem/progenitor cell properties in suspension cultures from three breast cancer lesions and from an established breast carcinoma cell line MCF-7 [71]. They found that the isolated cells which overexpressed neoangiogenic and cytoprotec-tive factors showed CD44+CD24- and Cx43-, and expressed the stem cell marker OCT-4, and could form tumors *in vivo* when as few as 10^3 cells were implanted. This was the first time showing that breast tumorigenic cells with stem/progenitor cell properties can be propagated *in vitro* as nonadherent mammospheres, and accordingly, this experimental system was then frequently used by researchers for isolating and studying the breast tumor–initiating cells (BT-IC) [72-74].

2.4 Novel strategies for enrichment of breast CSCs

As we mentioned in the first part of this chapter, the cancer stem cell hypothesis suggests that many cancers are maintained in a hierarchical organization of rare, slowly dividing CSCs (or T-IC), rapidly dividing amplifying cells (early precursor cells, EPC) and post-mitotic differentiated tumor cells [22]. Thus, the complex scheme which operates in most tumor tissues seems to be that the slowly dividing CSCs give birth to EPC, which then undertake a program of exponential growth for a limited period of time before the descendant cells differentiate and become post-mitotic (Figure 1). Although the above three

classical methods are widely used for the isolation and identification of breast CSCs, these methods purify both T-IC and some EPC [7 59, 71]. To study the breast CSCs more accurately, our group was trying to search for new strategies to enrich more purified breast CSCs. We found that breast carcinomas from chemo-treated patients were highly enriched for cells with the properties of BT-IC. We then sequentially passaged tumor cells in epirubicin-treated NOD/SCID mice to get a highly malignant breast cancer cell line (SK-3rd) using the chemo-therapeutic resistance of BT-IC. Our SK-3rd cell line showed all the tentatively defined properties of BT-IC, including enhanced mammosphere formation, multipotent differentiation, chemo-therapy resistance, as well as BT- IC phenotype(OCT4+CD44+CD24−lin−)[76] (Figure 2). We assess that about 16% of SK-3rd cells were T-IC, while the rest cells (also CD44+CD24−) were mostly EPC, and mammospheric SK-3rd cells were ∼100-fold more tumorigenic *in vivo* than the parent cell line, metastasize, and can be serial xenotransplanted[26]. Additionally, SK-3rd cells was capable of providing unlimited numbers of cells for BT-IC studies. This method of *in vivo* chemotherapy may provide researchers a novel approach of selecting CSCs from other breast cancer lines or possibly for other cancers.

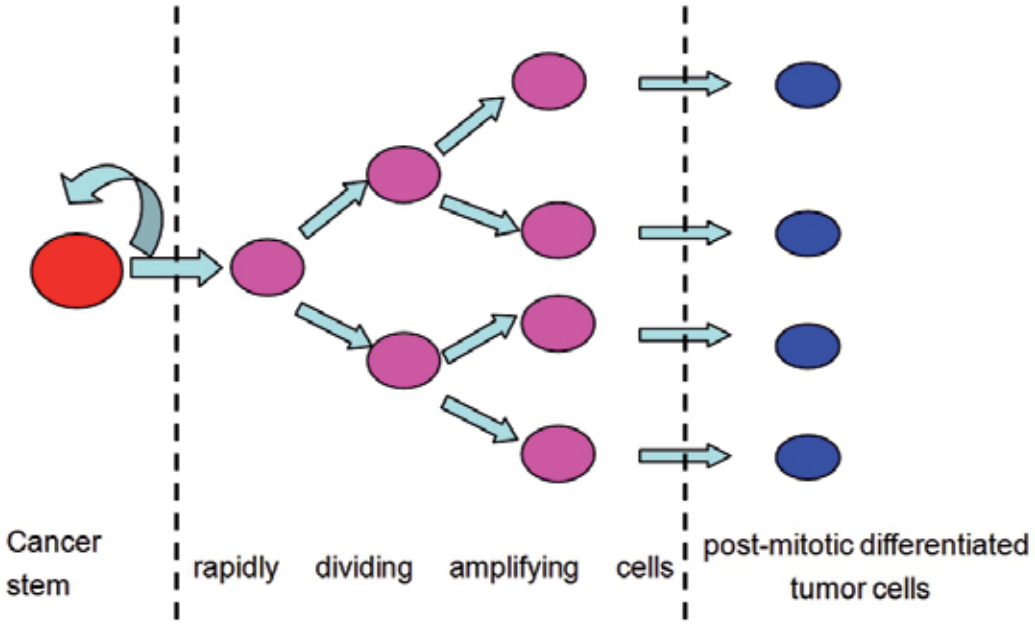

Fig. 1. A Model of the Cellular Hierarchies that May Exist in Human Cancers.

Besides our strategy, there might be other new approaches for generating breast CSCs. The epithelial-mesenchymal transition (EMT) is a key developmental program that is often activated during cancer progression, invasion and metastasis. Associations between the breast CSCs and EMT hypothesis of cancer were established recently as similarities in these two ideas were noted (will be discussed in Part 4 of this chapter). Several very recent studies have found that the EMT could generate mammary epithelial stem cells and breast CSCs [77-79]. This may provide potential novel methods to generate and enrich relatively unlimited numbers of breast CSCs, whose biology may then be studied with far greater facility.

The complex scheme which operates in most tumor tissues seems to be that the slowly dividing CSCs give birth to the rapidly dividing amplifying cells (early precursor cells, EPC), which then differentiate into post-mitotic tumor cells after a small number of cell divisions.

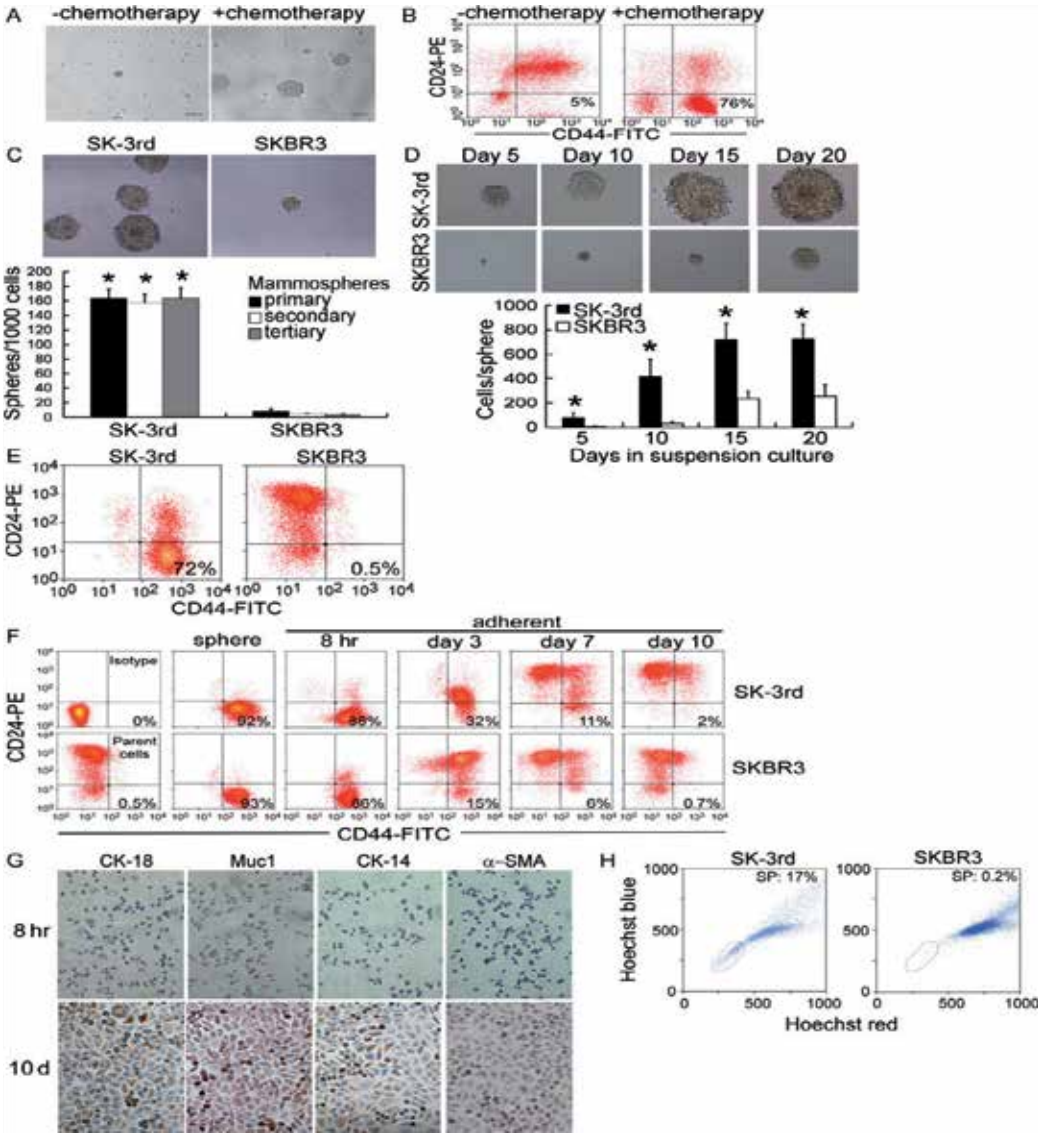

Fig. 2. Breast Cancer Cells under Pressure of Chemotherapy Are Enriched for BT-IC.

(A and B) 1°breast cancers from patients who received neoadjuvant chemotherapy are substantially enriched for self-renewing cells with the expected properties of BT-IC. Representative images show increased numbers of mammospheres after 15 days of culture (A) and a higher percentage of $CD44^{+}CD24^{-}$ cells in freshly isolated tumors (B) from a patient who received chemotherapy. (C) Similarly, passaging the human breast cancer line SKBR3 in epirubicin-treated NOD/SCID mice enriches for cells with BT-IC properties.

Shown are numbers of 1°, 2°and 3°mammospheres on day 15 from 1000 cells. (D) Mammospheres generated from single-cell cultures of SK-3rd and SKBR3, imaged on indicated day of suspension culture. (E) The majority of freshly isolated SK-3rd cells are $CD44^{+}CD24^{-}$, while cells with this phenotype are rare in SKBR3. (F) SK-3rd and SKBR3 cells cultured as spheres are CD44+CD24-. When they differentiate in adherent cultures, they gradually assume the parental SBKR3 phenotype, but somewhat more rapidly for SKBR3 mammospheres. (G) When SK-3rd spheres are removed from growth factors, and plated on collagen for 8 hr (top), they do not express luminal (Muc1 and CK-18) or myoepithelial (CK-14 and a-SMA) differentiation markers, while after further differentiation (bottom), they develop into elongated cells with subpopulations staining for either differentiated subtype. (H) Freshly isolated SK-3rd cells are enriched for $Hoechst^{low}$ SP cells compared with SKBR3 cells[26]. Adapted from Yu F, et al.*Cell*, 2007: 131:1109-23.

3. The dysregulation of MicroRNAs in breast cancer stem cells

MicroRNAs (miRNAs) are endogenously synthesized small non-coding RNAs, 19-25 nucleotides in length that negatively regulate gene expression by repressing translation of target mRNAs or targeting them for degradation[80]. The active miRNA is produced by the RNase III enzyme Dicer in the cytosol from a precursor-miRNA (pre-miRNA) by removing the loop of the pre-miRNA stem-loop. The Dicer-processed miRNA is then taken up by the RNA-induced silencing complex (RISC), which becomes activated when one strand (the antisense or guide strand) is incorporated into the complex and the other strand separates and is discarded. The activated RISC complex can then seek out target mRNAs, which have partially complementary sequences to the guide strand (often in their 3′-UTR), and suppress their translation into protein[81].

MiRNA expression is altered in cancer cells and can be used to predict tumor type and prognosis. Cancer-associated miRNAs are frequently deleted, mutated or associated with satellite DNA expansions in cancers, suggesting that these molecules serve as important regulators of tumor development[82]. Emerging evidence has made it clear that miRNAs also function as important regulators of stemness, collaborating in the maintenance of the pluripotency, control of self-renewal, and differentiation of both normal stem cells and CSCs[83]. Except for certain miRNAs have high level transcripts, the global downregulation of miRNAs are present in CSCs when compared to their differentiated counterparts[82]. Dysregulation of miRNAs may result in excessive self-renewal and survival of CSCs which is a likely cause for the chemo-resistance and relapse in tumor patients.

MiRNAs can serve as either tumor suppressors or oncogenes depend on their expression levels in CSCs. Tumor suppressor miRNAs are supposed to inhibit tumor progression while their expression is downregulated. Oncogenic miRNAs are often called oncomiRs and are upregulated in the cancer cells[84].

3.1 Tumor suppressors

Let-7 is the first human miRNA to be discovered and its expression has been observed to be reduced in a number of tumor cell lines including lung and breast cancer[85]. Recent research indicated let-7 acted as tumor repressor playing an important role in the self-renewal potential of cancer stem cells. Yu and colleagues demonstrated that let-7 family was not expressed by breast CSCs generated from cell lines or 1°patient tumors and increased with differentiation. By expressing of let-7 in breast CSCs or antagonizing let-7 in more

differentiated cells, it was found that let-7 regulated the key features of breast CSCs—self renewal in vitro, multipotent differentiation, and the ability to form tumors. Because the two targets of let-7 RAS and HMGA2 were responsible for the self renewal and multipotent differentiation, respectively, aberrant expression of let-7 in breast CSCs helps to maintain their stemness[26].

Recently, Yu et al. found that similar to let-7, the expression of miR-30 was reduced in breast cancer stem-like cells (BT-ICs), and its target genes, Ubc9, an E2-conjugating enzyme essential for sumoylation, and integrin ß3(ITGB3), were upregulated at protein levels. Overexpression of miR-30 in BT-ICs inhibited their self-renewal ability by repressing Ubc9 and promoted apoptosis by inhibiting Ubc9 and ITGB3. Furthermore, ectopic expression of mir-30 or blocking the expression of Ubc9 in BT-ICs xenografts reduced their tumor-forming capacity and metastasis in NOD/SCID mice, while miR-30 inhibitor enhanced tumorigenesis and metastasis of SKBR3 breast cancer cells with low metastasis potential[86]. These results suggested that miR-30 could be one of the important miRNAs in regulating the stem-like features of breast cancer

MiR-15/ miR-16 are also tumor suppressors. It was first identified in B cell chronic lymphocytic leukaemia (B-CLL) that miR-15/ miR-16 was lower in their expression level while their target protein the anti-apoptosis Bcl-2 was overexpressed[87]. The downregulation or deletion of miR-15/miR-16 was also found in other cancer types, such as prostate cancer[88], pituitary adenomas[89], non-small cell lung cancer (NSCLC)[90], and ovarian cancer[91]. Expression of these miRNAs inhibited cell proliferation, promoted apoptosis, and suppressed tumorigenicity both in vitro and in vivo by targeting multiple oncogenes, including Bcl-2, MCL1, CCND1, Wnt3A and Bmi-1. There has been growing evidence illustrated that the pivotal signaling pathways of the "stem cell genes": Notch, Hedgehog, Wnt, HMGA2, Bcl-2 and Bmi-1 were involved in the self-renewal of CSCs[92]. Since the oncogenic activation of Bmi-1, Bcl-2 and Wnt3A were frequently correlated with the downregulation of miR-15/miR-16, it was strongly suggested miR-15/miR-16 played a key role in the regulation of CSCs.

MiR-34 has been implicated in cell cycle control related to p53[93]. In p53 deficicent human gastric cancer cells, restoration of functional miR-34 inhibited the formation of tumorsphere in vitro and tumor initiation in vivo[94]. In parallel, miR-34 was reported to be involved in pancreatic CSCs self-renewal[95]. The mechanism of miR-34 mediated suppression of self-renewal of CSCs was potentially related to the direct modulation of downstream targets Bcl-2 and Notch, suggesting that miR-34 might play an important role in gastric and pancreatic CSCs' self-renewal and/or cell fate determination. However, reduced expression of miR-34a in prostate cancer stem cells facilitated tumor development and metastasis by directly regulating CD44. Accordingly, CD44 knockdown inhibited prostate cancer growth and metastasis[96]. These results provided a solid experimental basis for developing miR-34a as a promising therapeutic agent against prostate CSCs.

MiR-128 is also a tumor suppressor involved in CSCs. Its expression was dramatically reduced in high grade gliomas, while application of miR-128 inhibited glioma proliferation and self-renewal by targeting Bmi-1 oncogene/stem cell renewal factor[97]. Same result was found in neural tumor medulloblastoma that miR-128a had growth suppressive activity in medulloblastoma and this activity was partially mediated by targeting Bmi-1 and thereby increasing the steady-state levels of superoxide and promoting cellular senescence. This data has implications for the modulation of redox states in CSCs, which are thought to be resistant to therapy due to their low ROS states[98].

miR-200 is an evolutionary conserved family which were found to be strongly suppressed in CD44+/CD24− lineage human breast cancer cells[27] and poorly differentiated pancreatic adenocarcinomas[99]. Recent research conducted in an inducible oncogenesis model showed that inhibition of miR-200b expression resulted in enrichment of the CSC population, and CSC or mammosphere growth was blocked by overexpression of miR-200b. Meanwhile one of its target Suz12 subunit of PRC2 was increased in CSC which in turn repress the transcription of E-cadherin. Thus, miR-200b acts as a tumor suppressor that blocks the formation and maintenance of mammospheres by targetting Suz12-E-cadherin pathway[100]. These results identified miR-200 microRNA family as a critical regulator for CSC growth and function.

3.2 Oncogenes

The miR-17-92 polycistron which is composed of 7 members is found to be overexpressed in multiple tumors, including lung[101], lymphoma[102], myeloid leukemias[103], hepatocellular carcinomas[104], medulloblastoma[105] and colorectal[106]. It's known to function as oncogenes to promotes cell proliferation and tumor progression. Introduction of miR-17-92 into hematopoietic stem cells was shown to significantly accelerated the formation of lymphoid malignancies partly by inhibiting apoptosis[101]. Also Wang et al found members of the miR-17 family were notably more abundant in a mouse model of MLL leukemia stem cells compared with their normal counterpart granulocyte-macrophage progenitors and myeloblast precursors. Forced expression of miR-17-19b in leukemia cells, was consistent with a higher frequency of leukemia stem cell, reduced differentiation and increased proliferation. The oncogenic effects of miR17-92 on leukemia stem cell self-renewal in MLL-associated leukemia in part due to modulating the expression of p21, a known regulator of normal stem cell function[103]. Taken together, these studies implicated the miR-17-92 cluster as a potential human oncogene that played a role in cancer stem cells.

The miR-181 has an oncogenic role within cancers as well. MiR-181 family members were up-regulated in EpCAM(+)AFP(+) hepatocellular carcinoma(HCCs) and in EpCAM(+) HCC cells isolated from AFP(+) tumors which have the cancer stem/progenitor cell features. Downregulation of miR-181 reduced EpCAM(+) HCC cell quantity and tumorigenesis, whereas enforced expression of miR-181 in HCC cells resulted in an enrichment of EpCAM(+) HCC cells. The mechamism underlying the regulation of miR-181 on the stemness of EpCAM(+) HCC cells was partially by negatively regulating two hepatic transcriptional regulators of differentiation and an inhibitor of Wnt/_-catenin signaling (nemo-like kinase [NLK])[107]. Other evidence also showed miR-181 was elevated in breast cancer stem cells. Overexpression of miR-181a/b, or depletion of its target ataxia telangiectasia mutated(ATM), was sufficient to induce sphere formation in breast cancer cells and promote tumorgenesis[108].

3.3 EMT

The epithelial-mesenchymal transition (EMT) is a vital developmental process that is often activated during cancer invasion and metastasis. During EMT, epithelial cells lose its epithelial characteristics including cell polarity and acquire mesenchymal phenotypes. On the molecular level, cells undergoing EMT down-regulated epithelial markers such as E-cadherin and up-regulated mesenchymal markers such as N-cadherin, vimentin, and fibronectin[109]. Mani and colleagues were the first group to demonstrated that the immortalized human mammary epithelial cells (HMLEs) undergoing EMT displayed not

only mesenchymal traits, also cancer stem cell like properties as characterized by their $CD44^{high}/CD24^{low}$ phenotype and increased ability to form mammospheres. On the other hand, HMLE mammospheres expressed markers similar to those of HMLEs that have undergone an EMT[77]. These findings illustrated EMT cells have cancer stem cell features and CSCs exhibit mesenchymal phenotype.

MiR-200 is the most discussed family that involved in the regulation of EMT process. Several studies have demonstrated suppression of endogeneous miR-200 family members was sufficient to induce EMT, whereas their ectopic expression induces MET in normal and cancer cell lines through direct targeting of ZEB1/2[110]. While in CSCs with EMT phenotypes, miR-200 was also detected to be aberrant or absent in breast, pancreas and prostate. Wellner et al showed ZEB1 not only promoted tumor cell dissemination, but also was necessary for the maintaining a stem cell phenotype of pancreatic and colorectal cancer cells by inversely inhibiting the stemness-inhibiting miR-200 family members[111]. Hence, ZEB/miR-200 feedback loop is a driving force for cancer progression towards metastasis by controlling the state of CSCs. MiR-200 and let-7 both were differentiation associated miRNAs, sometimes they work together regulating the EMT status of CSCs. It has been shown in prostate cancer cells the expression of miR-200 and/or let-7 was decreased in EMT phenotypic tumor cells which also expressed stem-like cell features as defined by increased expression of Sox2, Nanog, Oct4, Lin28B and/or Notch1. Restoration of miR-200 in prostate cancer cells inhibited the EMT process, as well as the clonogenic and sphere (prostasphere)-forming ability and tumorigenecity in mice which was consistent with the inhibition of Notch1 and Lin28B expression. Along with the decreased expression of Lin28, let-7 was increased which further repressed self-renewal capability[112].

As discussed above miRNAs are critically involved in the regulation of CSCs and EMT which were considered the "root causes" of chemo-resistant and tumor relapse. Therefore, targeting specific miRNAs could be a very promising therapeutic approach for the treatment optimization aiming at restoring the sensitivity of drug-resistant cells to chemotherapy. If it was possible to introduce miRNA mimics and/or antagonists into CSCs, it could in principle result in reversal of the some of the cells' tumorigenic properties. However, from a clinical/translational research point of view, the critical hurdle to developing this type of approach for cancer therapy is to find an efficient way to selectively deliver miRNAs into CSCs or just cancer cells, but not normal tissues. So far the effective and safe therapeutics are still to be studied.

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Part 4

Breast Cancer Gene Regulation

14

Epigenetics and Breast Cancer

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1. Introduction

The term epigenetic was introduced by Conard Waddington in 1942 as a concept of environmental influence in inducing phenotype modification. His work on developmental plasticity states that the environmental influences during development could induce alternative phenotypes from one genotype, one of the clearest examples is polyphenisms in insects. He showed that exposing the pupae of wild type Drosophila melanogaster to heat shock treatment, results in altered wing vein patterns (Waddington, 1952;Waddington, 1959a). Breeding individuals who have been exposed to these environmentally induced changes led to a stable population exhibiting the phenotype without the environmental stimulus. As a result of Waddington's observations of the dynamic interaction between genes and variation in the environment during the plastic phase of development, he described phenotype induction as genetic canalization. Canalization describes the robustness of phenotypes in response to perturbation (Waddington, 1959b;Waddington, 1961;Waddington & Robertson, 1966).

The epigenome controls the genome in both normal and abnormal cellular processes and events (Szyf et al., 2008;Vaissiere et al., 2008). Epigenetic system includes DNA methylation and histone modification and non-coding RNAs, which work cooperatively to control gene expression. As a result, epigenetic mechanisms are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals. Disruption of epigenetic processes can lead to altered gene function and malignant cellular transformation. Global changes in the epigenetic landscape are a hallmark of cancer (Hanahan & Weinberg, 2000). Methylation of cytosine bases in DNA provides a layer of epigenetic control in many eukaryotes that has important implications for normal biology and disease. DNA methylation is a crucial epigenetic modification of the genome that is involved in regulating many cellular processes. These include embryonic development, transcription, chromatin structure, X-chromosome inactivation, genomic imprinting, and chromosome stability.

Additionally, in 1975, DNA methylation was related to the process of X chromosome inactivation in females (Riggs, 1975). Since then, it has been used as a marker for gene silencing and extensively studied as an important mechanism of epigenetic control (Jaenisch & Bird, 2003). For instance, methylation of CpG islands within the imprinted gene promoters ensures transcriptional silencing of the associated parental allele (Nafee et al., 2008). Consistent with these important roles, a growing number of human diseases

including cancer have been found to be associated with aberrant DNA methylation. Therefore we will summarize the, in this chapter the current knowledge on mechanisms of epigenetic and its potential application in breast cancers.

2. DNA methylation

DNA methylation is a well conserved process that occurs in eukaryotes and prokaryotes (Klose & Bird, 2006). DNA methylation refers to the covalent addition of a methyl group to carbon number five in the nitrogenous base cytosine at the DNA strand (Fuks, 2005;Szyf et al., 2008). However, methylation does not occur in every cytosine, but only those adjacent to guanine are targets for the methylation by the methyltransferases enzymes. The CpG may occur in multiple repeats which are known as CpG islands (Fuks, 2005). These regions are often associated with the promoter regions of genes. Almost half of the genes in our genome have CpG rich promoter regions. In the whole genome, about 80% of the CpG dinucleutides not associated with CpG islands are heavily methylated (Robertson & Jones, 2000). In contrast the CpG islands associated with gene promoters are usually unmethylated (Singal & Ginder, 1999). There are a number of factors that may maintain the undermethylated state of CpG islands, such as sequence feature, SP1 binding sites, specific acting enhancer elements, as well as specific histone methylation mark H3K4me3, which prevents the binding of de novo methylation complexes (Straussman et al., 2009). Methylation of the CpG islands in the promoter region silences gene expression, and the absence of methylation is associated with active transcription. Thus unmethylated CpG islands are associated with the promoters of transcriptionally active genes, such as housekeeping genes and many regulated genes, such as genes showing tissue specific expression (Bird, 1986;Song et al., 2005).

CpG dinucleotides are under-represented in the genome except for small clusters, referred to as CpG islands, located in or near the promoter of greater than 70% of all genes (Balch et al, 2007; Brena et al, 2006; Hellebrekers et al, 2007). Promoter methylation is known to participate in reorganizing chromatin structure and also plays a role in transcriptional inactivation. It is believed that the chromatin surrounding an active promoter containing an unmethylated CpG island is "open" and allows for the access of transcription factors and other coactivators. An inactive promoter containing methylated CpG dinucleotides is associated with a "closed" chromatin configuration and results in transcription factors unable to access the promoter (Dworkin et al., 2009).

2.1 DNA methylation and breast cancer

There are well understood genetic alterations associated with breast carcinogenesis, including specific gene amplifications, deletions, point mutations, chromosome rearrangements, and aneuploidy. In addition to these highly characterized mutations, epigenetic alterations resulting in aberrant gene expression are key contributor to breast tumorigenesis (Campan et al., 2006; Giacinti et al., 2006; Mirza et al., 2007; Sharma et al., 2005; Sui et al., 2007; Vincent-Salomon et al., 2007; Visvanathan et al., 2006; Zhou et al., 2006. Decreased methylation of repetitive sequences in the satellite DNA of the pericentric region of chromosomes is associated with increased chromosomal rearrangements, mitotic recombination, and aneuploidy (Eden et al., 2003,Karpf and Matsui, 2005). Intragenomic endoparasitic DNA, such as L1 (long interspersed nuclear elements) (Schulz, 2006) and Alu (recombinogenic sequence)

repeats, are silenced in somatic cells and become reactivated in human cancer (Berdasco & Esteller, 2010). Furthermore, aberrations in DNA methylation patterns of the CpG islands in the promoter regions of tumor-suppressor genes are accepted as being a common feature of human cancer (Esteller, 2008). CpG island promoter hypermethylation affects genes from a wide range of cellular pathways, such as cell cycle, DNA repair, toxic catabolism, cell adherence, apoptosis, and angiogenesis, among others (Esteller, 2008), and may occur at various stages in the development of cancer. (Berdasco & Esteller, 2010).

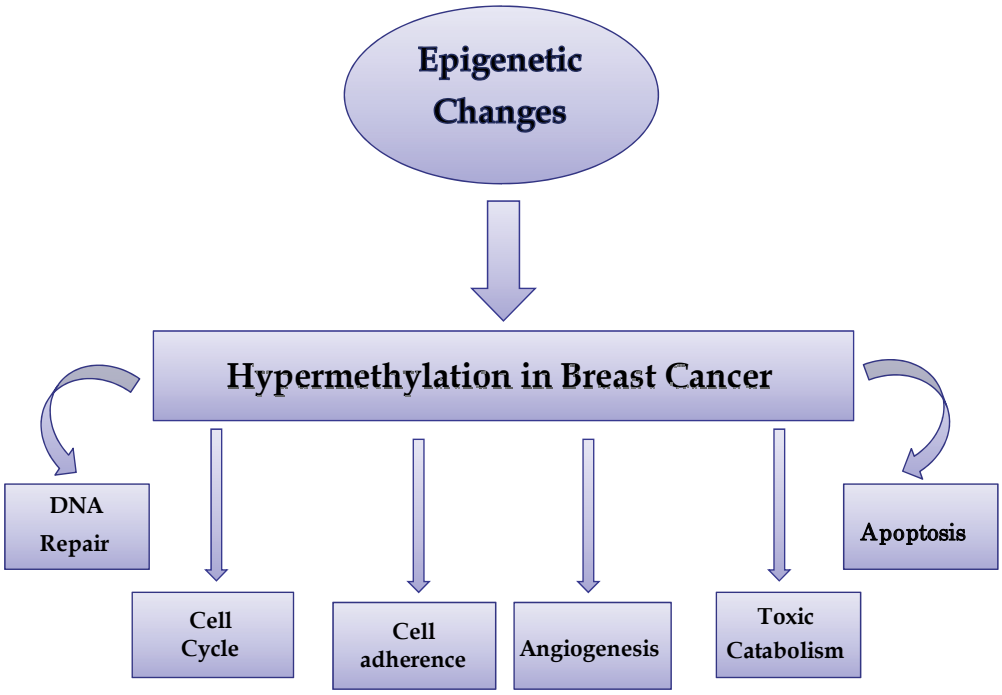

Fig. 1. Hypothetical model that explain how CpG island promoter hypermethylation. Multiple genes are hypermethylated in breast cancer compared to non-cancerous tissue which are affected genes from a wide range of cellular pathways induced by epigenetic changes.

Therefore, DNA methylation not only participates in cancer but has been found to regulate the histone modifications involved in tumor formation. The presence of certain histone modifications such as H4 R3 me2 is a marker of prostate cancer and increased expression of HDAC6 in breast cancer (Kurdistani, 2007). In addition the prognosis of certain malignancies can be affected by epigenetic status (Sakuma et al., 2007) . In normal cells, repetitive genomic sequences (e.g., centromeric satellite α-DNA and juxtacentromeric satellite DNA) are heavily methylated (Esteller, 2007; Jones & Baylin, 2002). The maintenance of methylation in this repetitive DNA could be important for the protection of chromosomal integrity by preventing chromosomal rearrangements, translocations and gene disruption through the reactivation of transposable elements (Eden et al., 2003; Ehrlich, 2002; Jones & Baylin, 2002). Besides hypermethylation of gene-associated CpG islands, hypomethylation of repetitive genomic DNA has also been identified as a specific feature in

human cancers (Feinberg & Vogelstein, 1983; Narayan et al., 1998; Jones & Baylin, 2002). Although less well studied than DNA hypermethylation, several lines of investigation indicate that the global DNA hypomethylation identified in cancer cells might contribute to structural changes in chromosomes, loss of imprinting (LOI), micro satellite and chromosome instability through aberrant DNA recombination, aberrant activation of proto-oncogene expression and increased mutagenesis (Chen et al.,1998; Eden et al., 2003; Kaneda & Feinberg, 2005; Jones & Baylin, 2002). Global genomic hypomethylation in breast cancer has been known to correlate with some clinical features such as disease stage, tumor size and histological grade (Soares et al., 1999). Some proto-oncogenes implicated in proliferation and metastasis (e.g., synuclein γ and urokinase genes) or drug resistance to endocrine therapy (e.g., N-cadherin, ID4, annexin A4, β-catenin and WNT11 genes) have been found to be upregulated in breast cancer through the hypomethylation of their promoters (Fan et al., 2006; Gupta et al., 2003; Pakneshan et al., 2004).

3. DNA methyltransferases

The methylation process is catalyzed by the DNA methyltransferases. There are currently four known DNMTs; DNMT1, 2, 3A and 3B (Okano et al., 1998). DNMT3A and DNMT3B are the de novo methyltransferases while DNMT1 maintains the methylation patterns during DNA replication (mitosis) (Bestor, 2000). The actual function of DNMT2 is not clear. It has been shown that DNMT2 possesses weak methyltransferase activity, and its deletion in the embryonic cells caused no detectible effect on global methylation (Okano et al., 1998). DNMT1 has a 5-30 fold preference for hemimethylated DNA (Goyal et al., 2006;Yoder et al., 1997). As well as to the epigenetic silencing of particular genes, DNMT1 supports the long term silencing of non-coding DNA, including most of the repetitive elements (Brannan & Bartolomei, 1999;Fuks, 2005;Jaenisch & Bird, 2003;Jones & Takai, 2001). DNMT1 exist as a component of the DNA replication complex, and thus methylates the newly synthesized DNA strand in correspondence to the template strand (Vertino et al., 2002). DNMT1 has different isoforms, the somatic tissue isoform DNMT1S, the oocyte specific isoform DNMT1o and the spermatocyte isoform DNMT1p. DNMT1o is responsible for maintaining maternal imprints during cleavage (Howell et al., 2001). In addition to that, over expression of DNMT1 has been reported in human tumours and many contribute to the global methylation abnormalities seen in cancer cells although increased expression of the DNMTs likely to be only partially responsible for the observed methylation abnormalities since not all tumours overexpress these enzymes (Robertson & Jones, 2000).

On the other hand, de novo DNA methylation is catalyzed by DNMT3a, DNMT3b and DNMT3L (Okano et al.,1999; Chedin et al., 2002). DNMT3L lacks the ability to bind to SAM, and is responsible for increasing the binding of DNMT3a to SAM (Chedin et al., 2002; Aapola et al., 2000). DNMT2, a small 391-amino-acid protein, is reported to possess weak DNA methyltransferase activity, but its biological function is not yet elucidated (Dong et al, 2001). Very recent studies have shown that Dicer-mediated microRNA biogenesis is involved in modulation of DNA methylation by indirectly regulating the expression of DNMT3 genes (Sinkkonen et al., 2008; Benetti et al., 2008). Dicer belongs to the RNase III family enzymes and is implicated in processing the biosynthesis of small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Kim et al., 2005). In dicer$^{-/-}$ cells, the microRNAs of the miR-290 cluster are depleted and expression levels of their target Rbl2 protein (retinoblastoma-like protein) are increased, leading to downregulation of DNMT3 gene expression through Rbl2-

mediated transcriptional repression, and in turn causing the DNA methylation defect (global hypomethylation) (Sinkkonen et al., 2008; Benetti et al., 2008). Regarding the role of DNMTs in breast tumorigenesis, it has been reported that DNMT3b mRNA is overexpressed in breast cancer, a finding that correlates well with the hypermethylator phenotype and poor prognosis in breast tumors (Girault et al., 2003; Roll et al.,2008).

4. Histone conformation

Histones are five basic nuclear proteins that form the core of the nucleosome. The histone octamer contains two molecules each of histones H2A, H2B, H3 and H4. Histone H1 the linker histone is located outside the core and involve in the packing of DNA (Kornberg & Lorch, 1999). DNA wraps around the octamer in two turns of 146 base pairs (Luger et al., 1997), and the adjacent nucleosomes are connected and wrapped on each other by H1. Consequently histone modifications play a major role in regulating gene expression and extend the information potential of the DNA which explains the growing interest of the 'Histone Code' (Jenuwein & Allis, 2001;Zhang & Reinberg, 2001a). Modifications to amino acids on the N-terminal tails of histones protruding from the nucleosome core can induce both an open or closed chromatin structure and these affect the ability of transcription factors to access promoter regions to activate transcription. The covalent modification can be acetylation, methylation, phosphorylation and ubiquitination. Methylation of some residues is associated with both transcriptional repression, such as methylation of histone 3 lysine 9 (H3 K9) (Nakayama et al., 2001a)and others with transcriptional activation, such as methylation of histone 3 lysine 4 (H3 K4) (Strahl et al., 1999). Histone methylation is performed by histone methltransferase (HMTs) which can transfer up to three methyl groups to lysine residues within the tails of the histones with different effects on gene activity. Acetylation which occurs at lysine residue is associated with transcriptional activation (Turner, 2000). This modification is performed by histone acetylases (HATs) and removed by the histone deacetylases (HDACs).

Other important regulators of chromatin conformation include the polycomb group (PcG) and trithorax group (trxG) proteins, which have key role in developmental gene regulation (Schuettengruber et al., 2007). They are recruited to response elements near proximal promoters to direct histone modifications, which induce both an active chromatin structure (trxG) and an inactive chromatin structure (PcG). Trithorax group proteins methylate H3 K4 to induce an active chromatin configuration (Schuettengruber et al., 2007), while PcG proteins direct the methylation of H3 K27 to induce a repressive chromatin configuration. The effect of PcG protein are however reversible, as removal of PcG during development leads to gene activation. PcG protein have been found to be implicated in regulation of developmental transcription factors, genomic imprinting and X chromosome inactivation (Heard, 2005).

Acetylation of histones has been extensively studied as one of the key regulatory mechanisms of gene expression (Grant, 2001). Histone acetylation was found to affect RNA transcription as early as the 1960s (Allfrey et al., 1964). The highly conserved lysine residue at the N-terminal of H3 at position 9, 14, 18 and 23, and H4 lysine 5,8,12 and 16, are frequently targeted for modification (Roth et al., 2001). Acetylations of the lysine residues neutralize the positive charge of the histone tails. And therefore, decrease their affinity for DNA which results in open chromatin conformation allowing the transcriptional machinery to reach its target (Hong et al., 1993). Additionally, many histone acetylases (HATs) (Brownell & Allis, 1996; Parthun et al., 1996) and histone deacetylases (HDACs) (Taunton et al., 1996) have been described previously.

The acetyltransferases catalyse the addition of the acetyl group from acetyl coenzyme A (acetyl-CoA) to the epsilon-amino group of specific lysine residues (it-Si-Ali et al., 1998;Kim et al., 2000), where deacetylases reverse the reaction (Kuo & Allis, 1998). There are eighteen HDAC enzymes in mammalian cells which are divided into two families: a) zinc metalloenzymes that catalyses the hydrolysis of acetylated specific residues on histone tails and include class I, II and 1V HDACs, and b) NAD-dependent Sir2 deactylases which are considerd as class III HDACs (Glaser, 2007;Vigushin et al., 2001).
Class I is a group of four enzymes known as HDAC1, 2, 3 and 8 and this class is associated with gene regulation. They are expressed ubiquitously and they function exclusively in the nucleus (Brehm et al., 1998;Glaser, 2007). Class II is subdivided into class IIA, which includes HDAC 4, 5, 7 and 9 and class IIB that includes HDAC 6 and 10. Class II enzymes shuttle between cytoplasm and nucleus, and they involve mainly in cell differentiation and are highly expressed in certain tissues such as heart, skeletal muscle and brain (de Ruijter et al., 2003;Glaser, 2007;Grozinger et al., 1999;Vigushin et al., 2001). Class III includes the NAD-dependent deacetylases which is a group of seven enzymes that are involved in maintaining the chromatin stability. They can remove the acetyl groups from histones as will as other proteins (Kyrylenko et al., 2003). Class IV contains one member which is HDAC 11. It is closely related to class I thus some reviewers consider it as a member of that class. The function of HDAC 11 has not been characterized yet (Crabb et al., 2008;de Ruijter et al., 2003).

5. DNA methylation and histone modification

Besides to the promoter methylation, chromatin modification may also contribute to silencing genes in cancer cells. Post-translational modifications to histone proteins occur after translation primarily in the NH2 terminal tail of histones and include acetylation, methylation, phosphorylation, or ubiquitination (Dworkin et al., 2009). Three decade ago Razin and Cedar (1977) have reported the presence of tight correlation between DNA and chromatin structure (Razin & Cedar, 1977). It was believed the relationship is a unidirectional relationship i.e the state of DNA methylation defines chromatin structure; methylated DNA results in closed chromatin configuration while unmethylated DNA results in open chromatin configuration. This hypothesis was supported by research finding that showed that methylated DNA binding proteins recruits chromatin modification enzymes to methylated genes such as MeCP2 (Meehan et al., 1992;Nan et al., 1997). There is increasing evidence showing that changes in chromatin structure would alter DNA methylation patterns. Furthermore, the targeting of DNA methylation enzymes to genes promoters is guided by chromatin modifying enzymes. The fact that is chromatin configuration is dynamic and that is chromatin modifying enzymes activated by cellular signaling pathways. This provides a link between the extracellular environment and the state of DNA methylation (Szyf, 2007). One of the evidence of the link between chromatin modiling and DNA methylation in humans and mice mutation of the SWI-SNF proteins which are involved in chromatin remidling, result in defect in DNA methylation (Szyf, 2007). A number of histone methyltransferases, such as G9a, SUV39H1 and EZH2, a member of the multiprotein polycomb complex PRC2 can regulate DNA methylation by either recruiting or regulating the stability of DNMTs. DNMTs in turn can recruit HDACs and MBPs to achieve chromatin condensation and gene silencing (Sharma et al., 2010). This relationship between the epigenetic machinery makes the epigenetic mechanisms of genome expression a tightly regulated process.

As a result of that, cancer was thought to be exclusively a consequence of genetic changes in key tumor-suppressor genes and oncogenes that regulate cell proliferation, DNA repair, cell differentiation, and other homeostatic functions. During the last decade, the study of epigenetic mechanisms in cancer, such as DNA methylation, histone modification, nucleosome positioning, and micro RNA expression, has provided extensive information about the mechanisms that contribute to the neoplastic phenotype through the regulation of expression of genes critical to transformation pathways. Regarding DNA methylation, the low level of CpG methylation in tumors compared with that in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer (Feinberg & Vogelstein, 1983;Goelz et al., 1985) this let us to think that the cancer cells have a specific epigenome. hypomethylation in cancer cells is associated with a number of adverse products, including chromosome instability, activation of transposable elements, and loss of genomic imprinting (Berdasco & Esteller, 2010).

6. Micro RNA and epigenetic

As well documented, about 80 % of human transcribed RNA is not translated into protein. This RNA was thought to be either functionless (Mattick, 2001), or transcriptional noise (Dennis, 2002). From this population, micro RNAs (miRNA) have an established epigenetic role with the potential to be implicated in programming. micro RNA (miRNA) are small untranslated RNAs generally 21-25 mucleotides in length (Bartel, 2004), they regulate gene expression by affecting the stability or the translation efficiency of target mRNA. They bind their complementary mRNA and thus dsRNA is formed, this recognized as foreign RNA and cleaved to be degraded. Matching between the miRNAs and mRNA doesn't have to be perfect as even incomplete binding can block translation (Mattick & Makunin, 2005). Nearly 30% of genes expression is probably regulated by miRNA via the interaction between miRNAs and their target mRNA. Individual miRNA may regulate 200 targets by partial base pairing to mRNA, sugessting that one miRNA may control numerous biological or pathological signaling pathway by affecting the expressions and functions of their targets. It has been reported that miRNA has a role in the development process (He & Hannon, 2004), including a role in the process of stem cell differentiation (Houbaviy et al., 2003). Also it has been shown in cancer studies of miRNA that DNA methylation and histone modification control the expression of these small RNAs. This was achieved by studying the effect of DNA demethylating agents and hisdtone deacetylases inhibitors on the expression of miRNA expression particularly the miR-127 which is embedded in CpG island (Saito & Jones, 2006;Saito et al., 2006).

7. Genomic imprinting

Genomic imprinting is a developmental phenomenon that describes a unique form of gene regulation that leads to only one parental allele being expressed depending on its parental origin (Delaval & Feil, 2004;Surani, 1991). Insulin-like growth factor 2 (IGF2) and its receptor IGF2R are two of the first reported genes subjected to imprinting regulation (Barlow et al., 1991;DeChiara et al., 1991). In mouse genome there are 600 predicted imprinted genes (Luedi et al., 2005). These identified imprinted genes have a major common feature in that they are associated with at least one regulatory DNA element, often referred to as imprinted control region (ICR). The ICR region is essential in regulating the parental origin-specific

expression via interaction with specific transcription factors (Kim et al., 2007;Yang et al., 2003). Differential DNA methylation of the parental ICRs is one of the most common features associated with imprinted genes (Kim et al., 2003;Liang et al., 2000;Mancini-Dinardo et al., 2003). Typical disorders associated with imprinted genes include Prader-Willi and Angelman syndromes, Beckwith-Wiedemann syndrome and multiple forms of neoplasia (Weksberg et al., 2003;Zeschnigk et al., 1997). In addition to that, X inactivation is a mechanism that functionally equalizes the difference of X-linked genes between XX females and XY males by silencing one of the two X chromosomes in females. Dosage compensation is a widely known method of silencing the X chromosome in females. This is achieved epigenetically through a cascade of CpG methylation superimposed by global histone deacetylation (Avner & Heard, 2001;Lyon, 1999;Monk, 2002;Pfeifer et al., 1990).

8. PcG and cancer epigenetics

Other epigenetic modifiers have been identified, including the Polycomb group (PcG) proteins and small non-coding RNAs. PcG repressors serve as a docking platform for DNA methyltransferases and target a gene for permanent silencing by methylation of hisone H3 on lysine 27 (H3K27). Reversal of permanent silencing is only overcome by de-differentiation processes in the germline. Small non-coding RNA molecules, such as microRNAs, regulate gene expression by targeting RNA degradation (Luczak & Jagodzinski, 2006). These RNAs have also been found to also target gene promoters and result in transcriptional gene silencing (Balch et al., 2007; Han et al., 2007).

Increasing evidence from cancer epigenomic studies suggests a critical role for PcG factors in abnormal epigenetic silencing of tumor suppressor genes in cancer cells (Baylin & Ohm, 2006;Jones & Baylin, 2007;Lund & van Lohuizen, 2004;Valk-Lingbeek et al., 2004;Ting et al., 2006). There are at least four different PcG complexes identified in mammalian, including the maintenance complex, PRC1, composed of RING, HPC, HPH, and BMI1, and three different initiation complexes, PRC2 through PRC4, which are formed by enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 (SUZ12), and different isoforms of embryonic ectoderm development (EED) (Baylin & Ohm, 2006;Ting et al., 2006;Kuzmichev et al., 2004;Kuzmichev et al., 2005). In particular, PRC4 exists in embryonic, stem, progenitor and cancer cells and associates with a class III HDAC called SIRT1 ((Baylin & Ohm, 2006;Ting et al., 2006). The crucial function of PRC complexes in H3K27 methylation is mediated by EZH2, a histone lysine methyltransferase, that catalyzes this lysine methylation (Cao et al., 2002;Cao & Zhang, 2004;Martin & Zhang, 2005). Methylation of H3K27 possibly stabilizes the binding of PcG complexes to this histone mark to facilitate long-term gene silencing (Fischle et al., 2003;Martin & Zhang, 2005). Importantly, H3K27me is often present at the promoters of the DNA hypermethylated and silenced cancer genes investigated thus far (McGarvey et al., 2006), indicating that PcG proteins play an essential role in aberrant gene silencing in cancer cells. A recent study also showed that PcG-targeted genes in normal cells are closely associated with de novo DNA methylation in cancer cells, suggesting that PcG may preprogram its targeted genes as targets of subsequent DNA methylation in cancer cells (Keshet et al., 2006;Schlesinger et al., 2007).

In addition, several studies have shown that expression of PcG proteins such as EZH2, SUZ12 and BMI1 is aberrantly elevated in breast cancer and other cancers (Dimri et al., 2002;Kleer et al., 2003), suggesting deregulation of components of nucleosomal remodeling complexes can also be a mechanism resulting in gene silencing in cancer cells. In the case of

another repressive histone mark, H3K9me2 (me3), this lysine methylation is catalyzed by several histone lysine methyltransferases, including SUV39H, SETDB1, G9a and GLP among others (Schultz et al., 2002;Lehnertz et al., 2003;Tachibana et al., 2005). Although the defined role of H3K9 methylation in epigenetic gene silencing remains elusive, one possible mechanism is that this mark can serve as a binding site for heterochromatin protein HP1, which has an intrinsic ability to recruit DNA methyltransferases to the silenced genes (Fuks et al., 2003;Lachner et al., 2001).

To establish DNA methylation in a subset of genes, polycomb protein EZH2 must associate with DNMTs (Esteller, 2007). It is thought that polycomb proteins could collaborate with DNMTs by recruiting them to silenced promoters to establish long-term silencing (Matarazzo et al., 2007). Leu et al (2004) investigated whether the removal of ERα signaling could cause changes in DNA methylation and chromatin structure of ERα target promoters. They used RNAi to transiently disable ERα in breast cancer cells and found that polycomb repressors and histone deacetylases assemble in the promoter of an ERα target gene. Accumulation of DNA methylation in these silenced targets like the PR promoter region then occurs and can be stably transmitted to cell progeny for long-term silencing. Both ERα expression and DNA demethylation appear to be required to restore PR expression. They also observed a trend that more ERα negative tumors had more methylated loci than ERα positive tumors (Leu et al., 2004). This indicates that dysregulation of normal signaling in cancer cells may result in stable silencing of downstream targets maintained by epigenetic machinery (Dworkin et al., 2009).

The epigenetic mechanisms for gene silencing involve the interplay between DNA methylation, histone modifications and nucleosomal remodeling. The families of methyl-CpG binding proteins (MBD and Kaiso families) have been identified to play a key role in this interplay. The molecular functions of methyl-CpG binding proteins are dependent on their ability to recognize and bind methylated DNA (Clouaire & Stancheva, 2008;Meehan et al., 1989; ing et al., 2006). Accumulating evidence suggests that methyl-CpG binding proteins can associate directly or indirectly with DNMTs, HDACs and HMTs and cooperate with them to modify chromatin structure and suppress initiation of gene transcription (Fuks et al., 2003;Jones et al., 1998;Kimura & Shiota, 2003;Sarraf & Stancheva, 2004). The associated partners of methyl-CpG binding proteins have also been found to include many nucleosomal remodeling complexes such as NuRD, CoREST, NCoR/SMRT, Sin3A, SUV39H and SWI/SNF (Fujita et al., 2003;Harikrishnan et al., 2005;Le Guezennec et al., 2006;Yoon et al., 2003;Wade et al., 1999;Zhang et al., 1999). The significant role of methyl-CpG binding proteins in cancer epigenetics is supported by the findings that they are localized to DNA hypermethylated and aberrantly silenced cancer genes (Bakker et al., 2002; Lopez-Serra et al., 2006; Nguyen et al., 2001).

Thus, it has been postulated that methyl-CpG binding proteins initially recognize and bind to methylated DNA, and then bring down nucleosomal remodeling complexes to modify chromatin to the repressive compact heterochromatin structure, which causes gene silencing. Inversely, the results from some other studies show that chromatin remodeling activities can further facilitate binding of methyl-CpG binding proteins to methylated DNA sites (Feng & Zhang, 2001;Harikrishnan, et al., 2005), suggesting interaction between methyl-CpG binding proteins and nucleosomal remodeling complexes results in mutual stimulation of each others' activity. Taken together, methyl-CpG binding proteins represent an important class of chromosomal proteins that associate with multiple protein partners to modify surrounding chromatin and silence transcription, providing a functional link between DNA methylation and chromatin modification and remodeling (Lo & Sukumar, 2008).

Again, cancer generally has been viewed as a disease that is driven by progressive genetic abnormalities, involving chromosomal abnormalities, mutations in oncogenes and tumor suppressor genes (Hanahan & Weinberg, 2000;Vogelstein & Kinzler 2004). Nevertheless, it has been shown that breast cancer, similar to other types of cancer, is also a disease that is driven by epigenetic alterations, which do not affect the primary DNA sequence (Widschwendter & Jones, 2002;Polyak, 2007). The result of these alterations is aberrant transcriptional regulation that leads to a modify in expression patterns of genes implicated in survival, differentiation and cellular proliferation (Baylin & Ohm, 2006;Esteller, 2007;Widschwendter & Jones, 2002). In transformed cells, epigenetic alterations occur at the chromosomal level. These involve changes in DNA methylation, histone modifications, altered expression and function of factors implicated in regulating assembly and remodeling of nucleosomes (Baylin & Ohm, 2006;Esteller, 2007;Jones & Baylin, 2002;Jones & Baylin, 2007;Ting et al., 2006). Alterations in DNA methylation include global hypomethyation and focal hypermethylation.

Global hypomethylation has been found to increase with age and is linked to genomic instability and activation of oncogene expression (Eden et al., 2003;Feinberg & Tycko, 2004;Richardson, 2002). Epigenetic inactivation due to aberrant promoter methylation is a key process in breast tumorigenesis. DNA Methylation silencing of tumor suppressor genes, aberrant expression of DNMT1 or demethylation of oncogenes can lead to the conversion of a normal cell to a malignant cell. In addition chromosomal instability and inactivation of the DNA repair system has both the genetic and epigenetic backgrounds (Esteller & Herman, 2002;Szyf, 2008). Epigenetic silencing of tumour suppressor genes is an early event in breast carcinogenesis and reversion of gene silencing by epigenetic reprogramming can provide clues to the mechanisms responsible for tumour initiation and progression. Hypermethylation of the mismatch repair gene MLH1 is associated with tumors exhibiting microsatellite instability, and hypermethylation of the breast cancer gene BRCA1 is found in 10%- 15% of women with non-familial breast cancer (Jones & Baylin, 2002).

9. Epigenetic modifications and breast cancer

Epigenetic modifications are believed to be early events in cancer development (Leu et al., 2004) and breast cancer is a disease characterized by both genetic and epigenetic alterations. It is thought that once epigenetic alterations are established in premalignant tissues, the extent of modifications will accumulate as the disease progresses (Dworkin et al., 2009). Varying theories have been proposed on how this field defect arises. One theory is based on the self-metastasis model and the idea that the primary tumor is composed of multiple self-metastases that form around a seed from the tumor to itself (Norton, 2005). A second theory has been seen in gastric cancers and is based on cell methylation profiles influencing H. pylori infection which leads to additional methylation of promoters in gastric mucosal cells and accompanying increases in risk for gastric cancer (Maekita et al., 2006). Another theory has supportive evidence in breast cancer and is based on the idea that early epigenetic changes are associated with a large area of pre-malignant changes, and the "epicenter" appears to accumulate additional epigenetic changes (Yan et al., 2006).

Allelic losses of 3p, including a critical region at 3p21.3, are frequently detected in many cancers including breast cancer. The Ras-associated domain family member 1 gene (RASSF1) maps to the region of frequent loss. It is comprised of eight exons and through different promoter usage and alternative splicing generates seven unique transcripts, RASSF1A-G.

RASSF1A is transcribed from a CpG island promoter region, and is one of the most frequently hypermethylated genes thus far described in human cancer. The CpG island of RASSF1A is hypermethylated in 60–77% of breast cancers (Lewis et al., 2005;Vincent-Salomon et al., 2007) resulting in gene silencing in cancer cell lines and primary tissues. Its diverse functions include regulation of apoptosis, growth regulation, and microtubule dynamics during mitotic progression. Specifically, RASSF1A is a Ras effector and induces apoptosis through its interactions with pro-apoptotic kinase MST1. When cells lacking RASSF1A expression are treated with a DNA methyltransferase, such as 5-aza-2′-deoxycytidine, expression can be reactivated (Pfeifer & Dammann, 2005). Mouse knockout studies show that RASSF1A$^{-/-}$ mice are prone to spontaneous development of lung adenomas, lymphomas and breast adenocarcinomas. These mice are prone to early spontaneous tumorigenesis and show a severe tumor susceptibility phenotype compared to that of littermate wild-type mice (Pfeifer & Dammann, 2005).

Furthermore, it has been reported that the DNA methylation assay might be used for risk assessment and prognosis of breast cancer. Lewis et al. studied five frequently methylated genes, including RASSF1A, APC, H-cadherin, RARβ, and cyclin D2, and found a higher methylation frequency of both RASSF1A and APC genes in unaffected women at high risk for breast cancer compared with those at low or intermediate risk based on the Gail model analysis. This suggests that promoter hypermethylation of these genes is associated with epidemiologic markers of increased breast cancer risk (Lewis et al., 2005). This finding needs confirmation that such alterations do indeed occur earlier than abnormal histological findings, and by follow-up studies to examine whether these changes are associated with subsequent development of breast cancer (Lo & Sukumar, 2008). The prognostic significance of aberrant DNA methylation has been investigated by Muller et al. (2003) after screening 39 genes in DNA from serum of normal control patients and patients with primary or metastatic breast cancer, they identified two genes, RASSF1A and APC, whose methylation has a statistically significant association with poor outcome. Other methylated genes, such as GSTP1, SFRP1, have also been identified to be associated with poor prognosis (Arai et al., 2006;Veeck et al., 2006).

In breast cancer, multiple genes are hypermethylated compared to non-cancerous tissue (Agrawal & Murphy, 2007). These include genes involved in evasion of apoptosis (RASSF1A, HOXA5, TWIST1), limitless replication potential (CCND2, p16, BRCA1, RARβ), growth (ERα, PGR), and tissue invasion and metastasis (CDH1) (Han et al., 2007; Yan et al., 2001; Widschwendter & Jones, 2002). These genes are not only hypermethylated in tumor cells, but show increased epigenetic silencing in normal epithelium surrounding the tumor site. The first observations of this phenomenon were in oral cancer. Slaughter et al (1953) was the first group to use the term "field cancerization" which refers to the presence of cancer causing changes in apparently normal tissue surrounding a neoplasm. They theorized the existence of (pre-) neoplastic processes at multiple sites, with the unproven assumption that these have developed independently (Slaughter et al., 1953). In subsequent years, the presence of field cancerization has been described in head and neck squamous cell carcinoma, lung, esophagus, vulva, cervix, colon, bladder, skin, and breast cancers (Yan et al., 2006). Studies have demonstrated that normal adjacent cells to tumors frequently harbor loss of heterozygosity, microsatellite and chromosome instability, and gene mutations (Braakhuis et al., 2003). Recently DNA methylation has been added to list as hypermethylated normal tissue immediately adjacent to tumor sites has been found (Ushijima, 2007).

CpG-island-containing gene promoters are usually unmethylated in normal cells to maintain euchromatic structure, which is the transcriptionally active conformation allowing gene expression. Yet, during cancer development, many of these genes are hypermethylated at their CpG-island-containing promoters to inactivate their expression by changing open euchromatic structure to compact heterochromatic structure (Baylin & Ohm, 2006; Esteller, 2007; Jones & Baylin, 2002; Jones & Baylin, 2007). These genes are selectively hypermethylated in tumorigenesis for inactivation owing to their functional involvement in various cellular pathways that prevent cancer formation. Some of the methylated genes identified in human cancers are classic tumor suppressor genes in which one mutationally inactivated allele is inherited. According to Knudson's two-hit model, complete inactivation of a tumor suppressor gene requires loss-of-function of both gene copies (Knudson, 2000). Epigenetic silencing of the remaining wild-type allele of the tumor suppressor gene, thus, can be considered as the second hit in this model. For example, some well-known tumor suppressor genes, such as p16INK4a, APC and BRCA1, are mutationally inactivated in the germline occasionally lose function of the remaining functional allele in breast epithelial cells through DNA hypermethylation (Birgisdottir et al., 2006; Jin et al., 2001; Knudson, 2000). Since the consequence of aberrant DNA methylation is transcriptional silencing, novel tumor suppressor genes can be identified using methylated CpG islands as a marker.

As a result of that, hypermethylated genes identified from breast neoplasms now form a long list. Their biological functions encompass cell cycle regulation (p16INK4a, p14ARF, 14−3−3σ, cyclin D2, p57KIP2), apoptosis (APC, DAPK1, HIC1, HOXA5, TWIST, TMS1), DNA repair (GSTP1, MGMT, BRCA1), hormone regulation (ERα, PR), cell adhesion and invasion (CDH1, APC, TIMP3), angiogenesis (maspin, THBS1), cellular growth-inhibitory signaling (RARβ, RASSF1A, SYK, TGFβRII, HIN1, NES1, SOCS1, SFRP1 and WIF1). In addition to protein-coding genes, recent studies showed that microRNAs with tumor-suppressor function could be silenced in breast cancer cells through DNA methylation (Lehmann et al., 2008). These breast-genome methylation patterns have been developed as biomarkers for early detection and the classification of subtype of breast tumors, as predictors for risk assessment and for monitoring prognosis, and as indicators of susceptibility or response to therapy (Widschwendter & Jones, 2002;Lo & Sukumar, 2008).

These advances in the knowledge of the breast methylome strongly indicate that DNA hypermethylation plays a crucial role in initiation, promotion and maintenance of breast carcinogenesis, which cooperatively and synergistically interact with other genetic alterations to promote the development of breast cancer. For example, human mammary epithelial cells (HMECs) that gained the ability to emerge from the first transient growth plateau lost p16INK4A expression concurrently with hypermethylation of p16INK4A promoter, indicating that loss of tumor-suppressor function of p16INK4A is required for HMECs to gain growth competency by successfully bypassing the stage of cell senescence (Widschwendter & Jones, 2002; Tlsty et al., 2004). This finding is consistent with other studies where the life span of stem cells could be extended by germline loss of this gene (Janzen et al., 2006). Deregulation of cell cycle control by inhibiting the function of the cyclin-dependent kinase inhibitor, p16INK4A, could create a context for facilitating early abnormal clonal expansion of cells at risk for cancer. It is believed that loss of p16INK4A gene is permissive for enabling such expanding cells to develop genomic instability (Kiyono et al, 1998).

In addition to cell-cycle regulatory genes, DNA methylation-mediated silencing of DNA repair genes, such as BRCA1 and MGMT, could result in further inactivation of tumor suppressor genes or activation of oncogenes, which further drive breast tumorigenesis

(Esteller et al., 2000). More recently, the genes that function as inhibitors of WNT oncogenic pathway, such as SFRP1 and WIF1, have been found to be frequently hypermethylated in primary breast tumors (Ai et al., 2006; Lo et al., 2006).). Thus, in addition to the genetic mutation-mediated mechanism, epigenetic gene silencing is another mechanism that fosters malignant transformation of the mammary gland by aberrantly activating oncogenic signaling pathways (Lo & Sukumar, 2008).

10. Breast cancer epigenetic markers

There are two main reasons RASSF1A methylation is a good biomarker for breast cancer. First, RASSF1A methylation is rare in normal tissue providing a marker with high specificity. Second, the frequency of methylation is observed in 60 to 77% of cells from a tumor which provides a high frequency of diagnostic coverage (Campan et al., 2006; Muller et al., 2003). In addition to breast tumors, hypermethylation of RASSF1A can be detected in non-malignant breast cells and patient sera. In one study, hypermethylation of sera in breast cancer patients was detected in six out of 26 cases (Pfeifer & Dammann, 2005). Promoter methylation of RASSF1A was observed in 70% of samples from women at high-risk of developing breast cancer versus only 29% of samples from women at low-risk. Women with a previous history of benign breast growths are statistically more likely to have RASSF1A methylation (Lewis et al., 2005). Thus, hypermethylation of RASSFIA could be used as a form of breast cancer screening to detect breast cancer at its earliest stages (Dworkin et al., 2009).

However, it is well reported that prolonged exposure of undifferentiated (immature) breast cells to estrogen or estrogen-mimetic compounds during early development increases breast cancer risk in adult life. This phenomenon is called estrogen imprinting (Fenton, 2006). These studies can explain why, in addition to genetic factors, the risk of breast cancer is affected by pregnancy, lifestyle in terms of intake of food and drink, and environment. Although the tumorigenic mechanism underlying this phenomenon and its connection with epigenetic regulation are still largely unknown, recently published findings provide insight into this mechanism. One line of evidence is from the study of DNA methylation patterns in several subtypes of breast cells. Bloushtain-Qimron et al. found that several transcription factor genes involved in stem cell function were hypomethylated and highly expressed in breast progenitor/stem (undifferentiated) cells compared with differentiated breast epithelial cells (Bloushtain-Qimron et al., 2008), suggesting the epigenetic programs define mammary epithelial cell phenotypes. Since breast progenitor/stem cells possess self-renewal and proliferating ability and more sensitively respond to estrogenic action, this subtype of cells has been thought to be potent targets of malignant transformation (Shipitsin et al., 2007). The second line of evidence is from the study of the effects of estrogen exposure on breast progenitor/stem cells, using a primary culture system to decipher the phenomenon of estrogen imprinting. Recent study compared the DNA methylation profiles of epithelial progeny of estrogen-exposed breast progenitor cells with those of epithelial progeny of nonestrogen-exposed progenitor cells. They found that estrogen exposure caused epithelial progeny to exhibit a cancer-like methylome, leading to silencing of some tumor suppressor genes (Cheng et al., 2008). Even though the dose of estradiol (E2) used in their study was higher than normal physiological levels, their findings suggest abnormal exposure to estrogen or estrogenic chemicals induces epigenetic alterations in breast progenitor cells, which have been previously implicated in breast cancer (Lo & Sukumar, 2008).

Even though the aberrant activation of estrogen signaling can lead to tumor-associated alterations in the epigenome of breast progenitor cells, approximately 30% of diagnosed breast cancer cases lack estrogen signaling due to loss or downregulation of estrogen receptor (ER)-α, also subject to epigenetic silencing (Lapidus et al., 1998; Ottaviano et al., 1994). ER-negative breast cancers exhibit more aggressive characteristics than ER-positive breast cancers and are resistant to anti-estrogen therapy. How ER-negative breast cancer cells acquire more aggressive properties after loss of estrogen signaling is a very important issue in the field of breast cancer research. Another study provides evidence to link loss of ER signaling to epigenetic silencing of ERα downstream target genes (Leu et al., 2004). Their study showed that abrogation of ERα signaling by small interfering RNA-mediated knockdown of ERα expression resulted in epigenetic inactivation of ERα targets, which began from recruiting PcG repressors and HDACs to their promoters and was then progressively followed by DNA methylation of their promoters (Leu et al., 2004). Their results suggest that epigenetic regulation on ERα target genes is required for establishing ERα-independent growth and other characteristics of ER-negative breast cancer cells (Lo & Sukumar, 2008).

Other post-translational modifications of ERα such as phosphorylation, ubiquitination, glycosylation, and acetylation are believed to play a role in breast cancer promotion. ERα is modified by p300 on two lysine residues (302 and 303) located in the hinge region (between DNA- and ligand binding domains). When these lysine residues are mutated, ERα had increased hormone sensitivity. Thirty-four percent of atypical breast hyperplasia samples have mutations of the lysine at 303 (K303R) of the ERα (Margueron et al., 2004; Popov et al., 2007; Wang et al., 2001) explaining a functional role of these mutations in breast cancer promotion.

Furthermore, BRCA1 is a tumor suppressor gene for both breast and ovarian cancer (Campan et al., 2006). It encodes a multifunctional protein with roles in DNA repair, cell cycle check point control, protein ubiquitization, and chromatin remodeling (Mirza et al., 2007). In vitro experiments showed that decreased BRCA1 expression in cells led to increased levels of tumor growth, while increased expression of BRCA1 led to growth arrest and apoptosis. Recent studies indicate that BRCA1 methylation is an important marker for prognosis. The magnitude of the decrease of functional BRCA1 protein correlates with disease prognosis (Mirza et al., 2007; Vincent-Salomon et al., 2007). Tumors with BRCA1 mutations are usually more likely to be higher-grade, poorly differentiated, highly proliferative, estrogen receptor (ER) negative, and progesterone receptor (PR) negative, and harbor p53 mutations. BRCA1 mutated breast cancers are also associated with poor survival in some studies (Chappuis et al., 2000; Robson et al., 2004; Stoppa-Lyonnet et al., 2000). Phenotypically, BRCA1-methylated tumors are similar to tumors from carriers of germline BRCA1 mutations.

BRCA1 is thought to be a classical tumor suppressor gene for which Knudson's two-hit hypothesis holds true. About 20% of individuals with a strong personal and family history of breast and ovarian cancer carry germline mutations in the BRCA1 gene (Birgisdottir et al., 2006; Tapia et al., 2008). A second hit is thought to be required in the wild-type BRCA1 allele for the development of BRCA-associated cancer (Chenevix-Trench et al., 2006; Osorio et al., 2002; Osorio et al., 2007). However, about 20% of all tumors from BRCA mutation carriers do not show LOH of the wildtype BRCA1 (Chenevix-Trench et al., 2006; Meric-Bernstam, 2007; Osorio et al., 2002; Osorio et al., 2007).). Other studies have looked at the rate of BRCA1 methylation in germline carriers. BRCA1 promoter hypermethylation was observed in one of two tumors from BRCA1 carriers lacking LOH (Esteller et al., 2001). In other study of population-based ovarian tumors, two of eight tumors with germline BRCA1 mutations

showed neither LOH nor promoter methylation (Press et al., 2008). Another study of 47 breast tumors from hereditary breast cancer families identified three BRCA1 carriers of which two showed BRCA1 promoter methylation in their tumors (Birgisdottir et al., 2006). All these investigated studies suggest that methylation of BRCA1 may be serve as a second hit in tumors from a subset of BRCA1 mutation carriers (Dworkin et al., 2009).
Furthermore, BRCA1 promoter methylation was more frequent in invasive than in situ carcinoma and there were no correlation between BRCA1 promoter methylation and ER/PR status in a subset population (Xu et al., 2008). However, they also found a higher prevalence of BRCA1 promoter methylation in cases with at least one node involved and with tumor size greater than 2cm. Based on their findings higher methylation levels may correlate with more advanced tumor stage at diagnosis. They also observed a 45% increase in mortality of individuals with BRCA1 methylation positive tumors compared those who had unmethylated BRCA1 promoters (Xu et al., 2008). Another recent study conducted a familial breast cancer based study and found contradicting results. They found no overall correlation of ER, PR, or grade with hypermethylation of BRCA1 in the tumors from BRCA1 mutation negative families. However, seven individuals had both promoter hypermethylation and LOH; the majority of these tumors had a basal-like phenotype and were triple negative (Honrado et al., 2007).

11. Analysis of DNA methylation in breast cancer

Moreover, much of the research effort to date has concentrated on the identification of silenced genes implicated in breast tumorigenesis. Evron et al. successfully used a three-gene panel (Cyclin D2, RARβ and TWIST) to detect malignant breast cancer cells in ductal fluid from routine operative breast endoscopy (ROBE) and ductal lavage (Evron et al., 2001). Fackler et al. improved this method and tested a four-gene panel (RASSF1A, TWIST, HIN1 and Cyclin D2) using the QM-MSP assay to examine clinical tissue samples (Fackler et al., 2004). The cumulative methylation of these four genes is commonly observed to be higher in primary invasive breast cancers compared with reduction mammoplasty specimens from healthy women (Fackler et al., 2004). Fackler et al. further used the same technique but adopted a nine-gene panel (RASSF1A, TWIST, HIN1, Cyclin D2, RARβ, APC, BRCA1, BRCA2 and p16) to examine ductal lavage samples from women with or without breast cancer. This trial demonstrated that methylation-marker detection was twice as sensitive as cytological diagnosis of ductal lavage cells (Fackler et al., 2006). In addition to biopsied tissue sections and ductal fluid, methylated DNA is also detected in blood since the blood of patients with manifest breast cancer contains detectable amounts of circulating methylated DNA (Widschwendte & Menon, 2006). The blood detection of tumor-specific methylated DNA has been pursued for its potential for prognostic prediction and monitoring relapse of breast cancer after therapy (Widschwendte & Menon, 2006; Muller et al., 2003; Silva et al., 2002).
The analysis of methylation profiles in human cancer indicates that hypermethylation of some of the CpG islands is shared by multiple tumour types, whereas others are methylated in a tumour type-specific manner (Bae et al., 2004; Costello et al., 2000; Esteller et al., 2001; Nass et al., 2000; Parrella et al., 2004; Parrella, 2010). Promoter-aberrant methylation seems to be an early event in tumorigenesis, and an increase in the number of methylated genes during progression has been observed in several tumour types including breast cancer (Lehmann et al., 2002; Subramaniam et al., 2009). Hoque et al (2009) have shown there were differences in the patterns of methylation in pre-invasive breast lesions (atypical ductal hyperplesia and

ductal carcinoma in situ) as compared with invasive breast cancers. They suggested that DNA methylation may represent an interesting target for the development of new molecular markers for the detection of breast cancer cells in tumours and bodily fluids. The most widely used analytical approach for the determination of methylation status is methylation-specific-PCR (MSP). This method is based on bisulphite conversion of unmethylated cytosin to thymidine while methylated cytosines are protected from conversion. PCR primers are designed to specifically amplify the modified methylated sequence (Hoque et al., 2009). Semiquantitative approaches which combine the advantages of MSP which is applicable and highly sensitive to any CpGs and RT-PCR were also developed and used for methylation detection in tumours and bodily fluids (Herman et al., 1996; Lo et al., 1999).

12. Conclusion

Both DNA methylation and histone modifications play a crucial role in the maintenance of normal cell function and cellular identity of cancer cells. In breast cancer cells these epigenetic modification become massively perturbed, leading to significant changes in expression profiles which confer advantage to the development of a malignant phenotype. DNMTs are the enzymes responsible for setting up and maintaining DNA methylation patterns in eukaryotic cells. Intriguingly, DNMTs were found to be overexpressed in cancerous cells, which is believed to partly explain the hypermethylation phenomenon commonly observed in tumors. Thus, epigenetic modifications are clearly involved in breast cancer initiation and progression. Early studies focused on single genes important in prognosis and prediction, but newer genome-wide methods are identifying many genes whose regulation is epigenetically altered during breast cancer progression. Detection of hypermethylation in specific genes like RASSF1A could be used as a form of surveillance to detect early stage breast cancer, however future studies may find that the addition of multiple genes and the inclusion of histone alterations to predictive panels may improve sensitivity and specificity. In addition to the use of epigenetic alterations as a means of screening, epigenetic alterations in a tumor or adjacent tissues may also help clinicians in determining prognosis and treatment in breast cancer patients. As we understand specific epigenetic alterations contributing to breast tumorigenesis and prognosis, these discoveries will lead in future to significant advances for breast cancer treatment.

13. References

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Histone Modification and Breast Cancer

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1. Introduction

In eukaryotic cells, DNA is maintained in a highly ordered and condensed form via its association with small, basic histone proteins. The fundamental subunit of chromatin, the nucleosome, is composed of an octamer of four core histones, an H3/H4 tetramer and two H2A/H2B dimers, around which 146 bp of DNA are wrapped. Dynamic modulation of chromatin structure, that is, chromatin remodeling, is a key component in the regulation of gene expression, apoptosis, DNA replication and repair and chromosome condensation and segregation. Enzymes that eovalently modify histones control many cellular processes by affecting gene expression. These modifications of core histones mainly include of methylation, acetylation, phosphorylation, ubiquitination/sumoylation, ADP-ribosylation, deamination, and proline isomerisation (Ito, 2007; Bartova et al., 2008). The abnormal regulation of these processes is intimately associated with human diseases, including cancer.

Breast cancer, the leading cause of death from cancer in women, is a heterogeneous disease ranging from premalignant hyperproliferation to invasive and metastatic carcinomas (Jemal et al., 2011). The disease progression is poorly understood but is likely due to the accumulation of genetic mutations leading to widespread changes in gene expression. Accumulating evidence has suggested that abnormal alteration of histone modification plays roles in the process of breast cancer. This chapter will summarize the relationship between histone modification and the molecular mechanism of breast cancer, and the therapy strategies focused on histone modification for breast cancer will also be discussed.

2. Histone modification and breast cancer

2.1 Chromatin structure and histone modifications

Chromatin is the physiological template of eukaryotic genome. Its fundamental unit, the nucleosome core particle, contains ~200 bp of DNA, organized by an octamer of small, basic proteins. The protein components are histones (two copies of each highly conserved core histone protein – H2A, H2B, H3 and H4). They form an interior core; the DNA lies on the surface of the particle. Nucleosomes are an invariant component of euchromatin and heterochromatin in the interphase nucleus, and of mitotic chromosomes. The nucleosome core particle represents the first level of organization, with a packing ratio of ~6. The second level of organization is the coiling of the series of nucleosomes into a helical array

to form the fiber with ~30 nm diameter, which is found in both interphase chromatin and mitotic chromosomes. This brings the packing ratio of DNA to ~40 in chromatin. The fiber-like structure requires additional proteins, which has not been well defined. The final packing ratio is determined by the third level of organization, the packaging of the 30 nm fiber itself. This gives a total packing ratio of ~ 1000 in euchromatin, cyclically interchangeable with packing into mitotic chromosomes to reach an overall ratio of ~10,000. Heterochromatin generally has a packing ratio -10,000 in both interphase and mitosis (Fig 1) (Lewin, 2004).

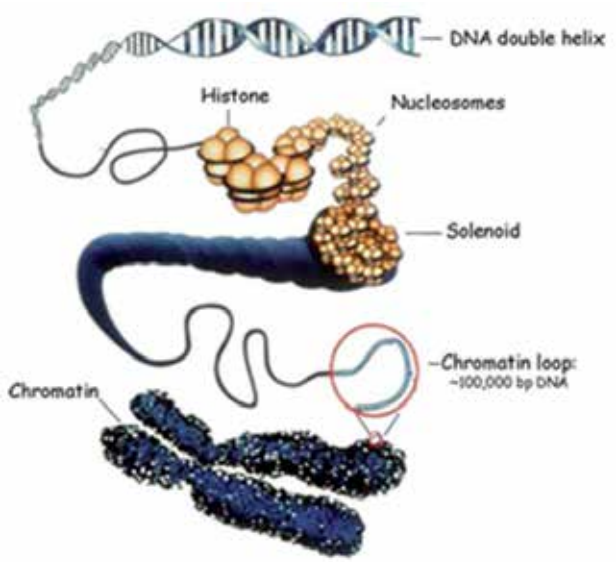

Fig. 1. Chromatin structure in eukaryotic cells

Local chromatin architecture is now generally recognized as an important factor in the regulation of gene expression. This architecture of chromatin is strongly regulated by post-translational modifications of the N-terminal tails of the histones. Core histones are subjected to a wide range of covalent modifications including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deamination, prolineisomerization (Fig 2) (Jovanovic et al., 2010). These modifications lead to a combinatorial histone code that demarcates chromatin regions for transcription activation or repression. Although the histone code is not fully investigated, specific marks such as lysine acetylation (H3K9ac, H3K18ac, and H4K12ac), lysine trimethylation (H3K4me3), and arginine dimethylation (H4R3me2) are generally associated with transcriptionally active gene promoters, whereas some other modifications such as lysine methylation (H3K9me2, H3K9me3 and H4K20me3) are associated with transcriptional repression. Global loss of acetylation (K16) and trimethylation (K20) of histone H4 have been shown to be characteristic of human cancer (Elsheikh et al., 2009).

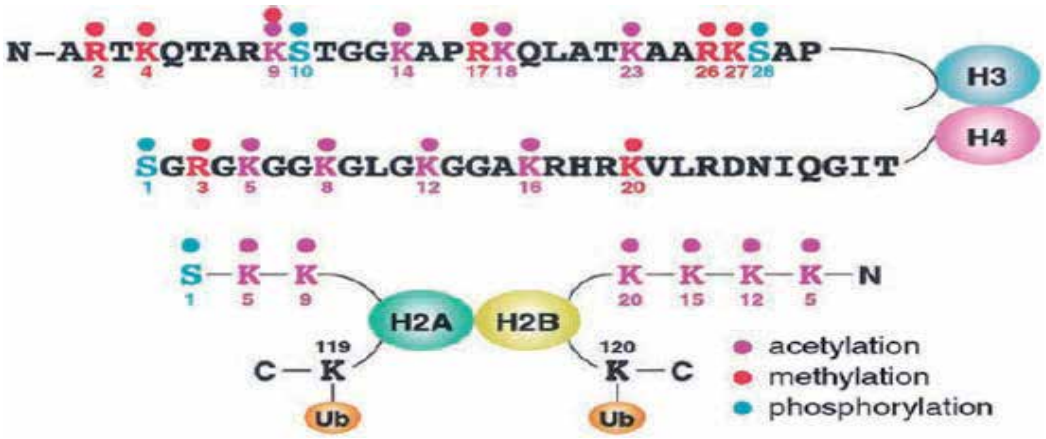

Fig. 2. Major sites of histone modifications

2.2 Histone modifications in breast cancer

2.2.1 Histone acetylation in breast cancer

Histone acetylation is a dynamic process directed by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Normally, Transcription factors recruit coactivators with HAT activity to regulatory DNA sites, whereas transcriptional repressors recruit corepressors with HDAC activity (Sun et al., 2001). A summary of known HAT proteins is presented in Table 1 (Sterner et al., 2000; Yang, 2004; Kimura et al., 2005).

Many HATs have also be showed to be involved in breast cancer. Among of them, p300/CBP and NCOAs are the most important and well-characterised HAT proteins associated with breast cancer.

2.2.1.1 p300/CBP

p300 and its close homolog CBP (CREB-binding protein) are often referred to as a single entity. p300 and CBP share several conserved domains: (1) the bromodomain (Br), which is frequently found in mammalian HATs; (2) three cysteine-histidine (CH)-rich domains (CH1, CH2 and CH3); (3) a KIX domain; and (4) an ADA2-homology domain, which shows extensive similarity to Ada2p, a yeast transcriptional co-activator. The N- and C-terminal domains of p300/CBP can act as transactivation domains, and the CH1, CH3 and the KIX domains are likely to be important in mediating protein-protein interactions, and a number of cellular and viral proteins bind to these regions. The acetyl-transferase domain is located in the central region of the protein, and the Br domain could function in recognising different acetylated motifs (Fig 3A, B) (Chan et al., 2001). p300/CBP contribute to acetylation of H3-K56 and promotes the subsequent assembly of newly-synthesized DNA into chromatin (Das et al., 2009). It is a non-DNA-binding transcriptional coactivator which stimulates transcription of target genes by interacting, either directly or through cofactors, with numerous promoter-binding transcription factors such as CREB, nuclear hormone receptors, and oncoprotein-related activators such as c-Fos, c-Jun, c-Myb and AML1 (Fig 3C) (Kitabayashi et al., 1998; Sterner et al., 2000).

Family	Members	Histone specificity	Basic functions
P300/CBP		H2A/H2B/H 3/H4	Global transcriptional coactivator
Nuclear receptor coactivators (p160, SRC)		H3/H4	Nuclear receptor coactivators (transcriptional response to hormone signals)
	NCOA1 (SRC-1)		
	NCOA2 (SRC-2)		
	NCOA3 (SRC-3)		
GNAT			
	Hat1	H4	Histone deposition, chromatin assembly and gene silencing
	Gcn5	H3/H4	Transcriptional coactivator
	PCAF	H3/H4	Transcriptional coactivator
MYST			
	Tip60	H2A/H3/ H4	Transcriptional co-regulator, DNA repair and apoptosis
	MOZ	H3	Transcriptional coactivator
	MORF	H2A/H3/ H4	Transcriptional coactivator (strong homology to MOZ)
	HBO1	H3/H4	DNA replication, transcriptional corepressor
$TAF_{II}250$		H3/H4	TBP-associated factor, transcription initiation, kinase and ubiquitin ligase
TFIIIC		H3/H4	RNA polymerase III transcription initiation
	TFIIIC220		
	TFIIIC110		
	TFIIIC90		
ATF-2		H4/H2B	Transcriptional activator
CIITA		H4	Transcriptional coactivator
CDY		H4	Histone-to-protamine transition during spermatogenesis

Table 1. Summary of major human HATs

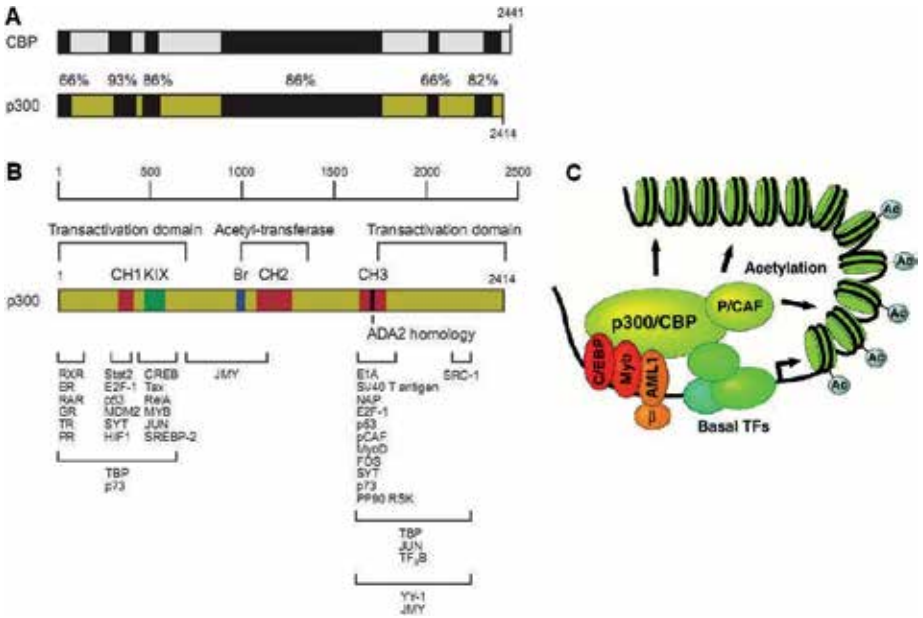

Fig. 3. Organisation of p300/CBP proteins. (A) Comparison of p300 and CBP. The dark regions indicate the areas of highest homology; (B) The functional domains in p300; (C) One of the potential model for the action of p300/CBP in the transcriptional regulation (Kitabayashi et al., 1998; Sterner et al., 2000).

p300/CBP is a ubiquitously expressed, global transcriptional coactivator that is involved in most important cellular programs, such as cell cycle control, differentiation, and apoptosis. Mice nullizygous for p300 or double heterozygous for p300 and CBP showed defects in neurulation and heart development, and then exhibited embryonic lethality, and mutations in p300 and CBP are associated with certain human disease processes (Giles et al., 1998; Yao et al., 1998; Giordano et al., 1999). A role for p300 in tumor suppression has been proposed by the fact that disturbance of p300 function by viral oncoproteins is essential for the transformation of rodent primary cells and, consistent with this hypothesis, mutations of p300 have been identified in certain types of human cancers, including breast carcinomas (Gayther et al., 2000).

It showed that both the localization of p300 and the recruitment to aggresomes differ between breast cancers and normal mammary glands. The expression level of p300 in breast cancer epithelia is higher than that in normal mammary gland. Cytoplasmic localization of p300 was also observed in tumor epithelia whereas nuclear localization was found in normal mammary glands in both animal models and in non-malignant adjacent areas of human breast cancer specimens. Proteasomal inhibition induced p300 redistribution to aggresomes in tumor but not in normal mammary gland-derived cells (Fermento et al., 2010).

The regulation of gene expression by nuclear receptors (NRs) controls the phenotypic properties and diverse biologies of target cells. In breast cancer cells, estrogen receptor alpha (ERα) is a master regulator of transcriptional stimulation and repression (Frasor et al., 2003). Upon E2 treatment, gene transcription is widely impacted, creating highly complex regulatory networks whose ultimate goal is the stimulation or suppression of specific biological processes. p300/CBP can function as a transcriptional cofactor of ERs and other

nuclear hormone receptors (Hanstein et al., 1996). Compared to CBP, NRIP1 and NCOAs, which play more gene-specific roles in the ER-dependent transcription, p300 seemed to be the only cofactor that appeared to be recruited at all the target genes of ER and plays a central role in both transcriptional activation and repression. After E2 treatment, ERα recruits coactivator complexes including of p300 and initiates transient stimulation of transcription via binds to ERα binding sites of target genes. If it could offer a more stable nucleation site for coactivator proteins (i.e. SRC-3), leading to histone acetylation and engagement of RNA polymerase II (Pol II), the transcriptional activation status would be maintained. Alternatively, ERα can cause transcriptional repression by recruiting, via p300, CtBP1-containing repressor complexes which lead to RNA polymerase II dismissal and histone deacetylation (Fig 4) (Stossi et al., 2009). In addition, the breast cancer susceptibility gene BRCA1 can strongly inhibits the transcriptional activity of ERα in human breast and prostate cancer cell lines, and this event is correlates with its down-regulation of p300 (but not CBP) (Fan et al., 2002). p300 also plays roles in the regulation of CYP19 I.3/II (aromatase), the key enzyme in estrogen biosynthesis and an important target in breast cancer (Subbaramaiah et al., 2008).

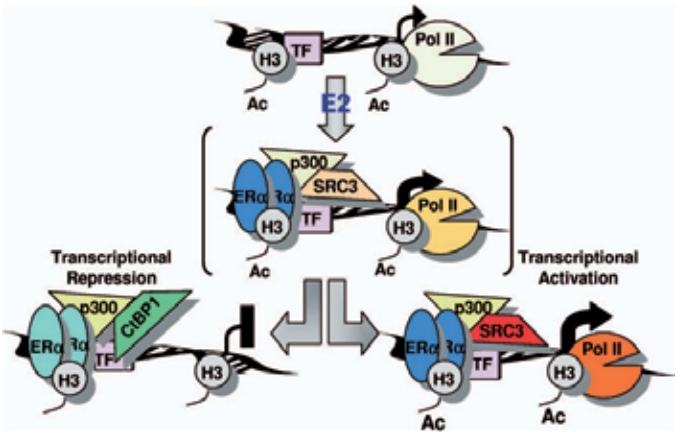

Fig. 4. Proposed model for ERα-mediated activation or repression of target genes via p300 (Stossi et al., 2009).

Another important role of p300 in breast cancer is the regulation of p53, a famous tumor suppressor. p53 can be acetylated by p300 in response to DNA damage to regulate its DNA-binding and transcriptional functions (Yuan et al., 1999). What's more, the N terminus of p300/CBP exhibits the ubiquitin ligase E3/E4 activity and is required for physiologic p53 polyubiquitination and degradation. Depletion of CBP or p300 could enhance the stabilization of p53 (Grossman et al., 2003; Shi et al., 2009).

Furthermore, p300/CBP has also been identified as a coactivator of HIF1α (hypoxia-inducible factor 1 alpha), and thus plays a role in the stimulation of hypoxia-induced genes (such as VEGF, GLUT1, etc) and development of glycolysis, which is the most important metabolic marker of cancer (Ruas et al., 2005).

2.2.1.2 Nuclear receptor coactivators

The Nuclear receptor coactivator family (NCOA), also named as p160 or steroid receptor coactivator, contains three homologous members: NCOA1 (SRC-1), NCOA2 (SRC-2, GRIP1

or TIF2) and NOCA3 (SRC-3, p/CIP, RAC3, ACTR, AIB1 or TRAM-1). These three members have an overall sequence similarity of 50–55% and sequence identity of 43–48%. They contain three structural domains. The N-terminal basic helix-loop-helix-Per/ARNT/ Sim (bHLH-PAS) domain is the most conserved region and is required for interact with several transcription factors (such as myogenin, MEF-2C and TEF, but not be obligator for NRs) and then enhance the transcription (Onate et al., 1995; Belandia et al., 2000). The central region contains three LXXLL (L, leucine; X, any amino acid) motifs, which form an amphipathic α-helix and are responsible for interacting with NRs (Heery et al., 1997; Darimont et al., 1998). The C-terminus contains two intrinsic transcriptional activation domains (AD1 and AD2). The AD1 region binds p300/CBP (but not interact with NRs), and this recruitment of p300/CBP to the chromatin is essential for NCOA-mediated transcriptional activation (Yao et al., 1996). The AD2 domain interacts with histone methyltransferases, coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferases (PRMT1) (Koh et al., 2001). Based on such molecular features, NCOAs interact with ligand-bound nuclear receptors and recruit histone acetyltransferases and methyltransferases to specific enhancer/promotor regions, which in turn results in chromatin remodeling, assembly of general transcription factors and recruitment of RNA Polymerase II for transcriptional activation (Fig 5) (Zhang et al., 2004; Xu et al., 2009). Furthermore, The C-termini of NCOAs itself also contain HAT activity domains (Chen et al., 1997; Spencer et al., 1997), and the poly Q encoding sequence in the C-terminal of NCOA3 gene is genetically unstable and is an easy target for somatic mutations in cancer cells (Wong et al., 2006).

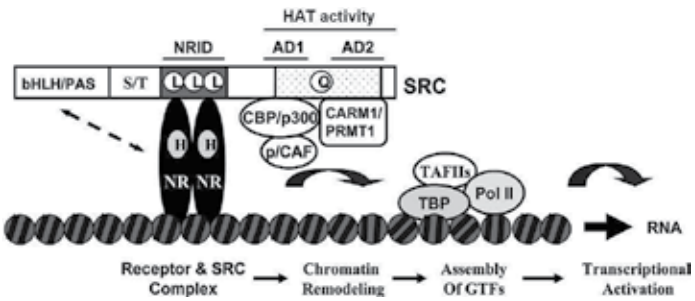

Fig. 5. Molecular structure of NCOAs and their functional mechanisms in steroid hormone-induced gene expression. Abbreviations: H, hormone; NRID, NR interaction domain; TBP, the TATA binding protein; TAFIIs, TBP-associated general transcription factors (GTFs).

Except of NRs, NCOAs also serve as coactivators for many other transcription factors associated with breast cancer, such as HIF1, NF-κB, E2F1, p53, RB and MRTFs (Zhang et al., 2004; Xu et al., 2009). By regulating a broad range of gene expression controlled by NRs and non-NR transcription factors, NCOAs regulate diverse events in the development of breast cancer. Either NCOA1 or NCOA2 deficiency can reduce ductal side branching and alveologenesis in the mammary gland (Xu et al., 1998; Mukherjee et al., 2006), and NCOA3$^{-/-}$ mice show growth retardation, delayed puberty, reduced female reproductive function and blunted mammary gland development (Xu et al., 2000).

In normal human breast, the levels of the three NCOA proteins in epithelial cells are usually low or undetectable (Hudelist et al., 2003). NCOA1 is overexpressed in 19% to 29% of breast cancers and plays important roles in cell proliferation, lymph node metastasis, disease recurrence and poor disease-free survival (DFS) (Fleming et al., 2004). Therefore, elevated

NCOA1 has been regarded as an independent predictor of breast cancer recurrence following therapy (Redmond et al., 2009). Although the evidence were not very sufficient, NCOA2 overexpression might also promote proliferation and invasion of breast cancer cells (Kishimoto et al., 2005). The amplification (in less than 10%) and elevated expression (in over 30%) of NCOA3 were be detected in breast cancer, and its overexpression in breast cancer usually correlates with the expression of ERBB2 , matrix metalloproteinase 2 (MMP2), MMP9 and PEA3 and with larger tumor size, higher tumor grade, and/or poor DFS (Anzick et al., 1997; Hudelist et al., 2003; Harigopal et al., 2009; Xu et al., 2009). What's more, elevated NCOA3 is able to promote estrogen-independent cell proliferation depends on the function of E2F1 and the association between NCOA3 and E2F1, but not ER (Louie et al., 2004).

In addition, NCOAs play important roles in the chemotherapy resistance of breast cancer. Increased expression levels of the ER-NCOA3 complex were found in tamoxifen-resistant cells, and such overexpression of NCOA3 could enhance the agonist activity of tamoxifen and therefore, reduces its antitumor activity in patients with breast cancer (Smith et al., 1997; Zhao et al., 2009).

2.2.1.3 HDACs

The 18 HDACs identified so far can be categorized into four classes: class I (HDAC1–3, HDAC8), class II (HDAC4–7, 9–10), class III (Sirtuin1–7) and class IV (HDAC11). Class I, II, and IV HDACs share homology in both sequence and structure and all require a zinc ion for catalytic activity. In contrast, class III HDACs shares no similarities in their sequence or structure with class I, II, or IV HDACs and requires nicotinamide adenine dinucleotide (NAD+) for catalytic activity (Ellis et al., 2009; Mottet et al., 2010). HDACs remove the acetyl groups from histone lysine tails and are thought to facilitate transcriptional repression by decreasing the level of histone acetylation. Like HATs, HDACs also have non-histone targets (Bolden et al., 2006; Wang et al., 2007).

Several HDACs have been found to be involved in breast cancer. In ER-positive breast cancer MCF-7 cells, expression of HDAC6 was increased after being treated by estradiol, and the elevated HDAC6 could deacetylate alpha-tubulin and increase cell motility. While the ER antagonist tamoxifen (TAM) or ICI 182,780 could prevent estradiol-induced HDAC6 upregulation, and then reduce cell motility. The *in vivo* assays showed that the patients with high levels of HDAC6 mRNA tended to be more responsive to endocrine treatment than those with low levels, indicating that the levels of HDAC6 expression might be used as both as a marker of endocrine responsiveness and also as a prognostic indicator in breast cancer (Zhang et al., 2004; Saji et al., 2005). Besides, HDAC1, Sirtuin3 (SIRT3), SIRT7 are all overexpressed in breast cancer (Zhang et al., 2005; Michan et al., 2007; Saunders et al., 2007). HDAC4 overexpression and mutations have also been found in breast cancer samples (Sjoblom et al., 2006).

2.2.2 Histone methylation in breast cancer

Histones can be mono-, di-, or tri-methylated at lysine or arginine residues by histone methyltransferases (HMTs). Many HMTs, including both lysine-specific HMTs (eg. SMYD3) and arginine-specific HMTs (eg. PRMT1 and CARM1), have been shown to act as ER coactivators and be involved in breast cancer.

2.2.2.1 Histone lysine methyltransferase (HKMTs)

Histone lysine methylation occurs on histone H3 at ε-amino group of lysines 4, 9, 14, 27, 36, and 79 and on histone H4 at lysines 20 and 59 (Strahl et al., 2000; Lee et al., 2005). In general,

methylation at H3K4 or H3K36, mono- methylations of H3K27, H3K9, H4K20, H3K79, and H2BK5 is associated with transcriptional activation, whereas trimethylations of H3K27, H3K9 H3K79, and H4K20 are linked to transcriptional repression (Rea et al., 2000; Kouzarides, 2007; Wang et al., 2007). Many HKMTs have been isolated and characterized (Tab 2). Up to now, except of Dot1, all the HKMTs contains a conserved SET [Su(var), Enhancer of zeste, trithorax] domain that is responsible for catalysis and binding of cofactor S-adenosyl-l- methionine (AdoMet), and many of them has been shown to play roles in the breast cancer.

NSD3 is amplified in human breast cancer cell lines and primary tumors and identified at the breakpoint of t(8;11)(p11.2;p15), resulting in a fusion of the NUP98 and NSD genes (Angrand et al., 2001; Rosati et al., 2002).

SMYD3 is a novel SET-domain-containing lysine histone methyltransferase which has been regarded as an important factor in carcinogenesis. Formed a complex with RNA polymerase II through an interaction with the RNA helicase HELZ, SMYD3 specifically methylates H3K4 and activates the transcription of a set of downstream genes (including of Nkx2.8, hTERT, WNT10B, VEGFR1, c-Met, etc) containing a ‘‘5′ - CCCTCC - 3′’’ or “5′ - GGAGGG - 3” sequence in the promoter region (Fig 6) (Hamamoto et al., 2004; Hamamoto et al., 2006; Kunizaki et al., 2007; Zou et al., 2009). It seems that the N-terminal region of SMYD3 plays an important role for the regulation of its methyltransferase activity, and the cleavage of 34 amino acids in the N-terminal region or interaction with heat shock protein 90 alpha (HSP90α) may enhance the histone methyltransferase (HMTase) activity compared to the full-length protein (Silva et al., 2008). Enhanced expression of SMYD3 is essential for the growth of many cancer cells (such as breast cancer, colorectal carcinoma, hepatocellular carcinoma, etc), and it also could stimulate cell adhesion and migration, whereas suppression of SMYD3 by RNAi or other reagents induces apoptosis and inhibits cell proliferation and migration (Hamamoto et al., 2004; Hamamoto et al., 2006; Luo et al., 2007; Wang et al., 2008; Luo et al., 2009; Zou et al., 2009; Luo et al., 2010). SMYD3 may be an important coactivator of estrogen receptor (ER) in the estrogen signal pathway. It can directly interact with the ligand binding domain of ER, in turn augments ER target gene expression via histone H3-K4 methylation (Kim 2009).

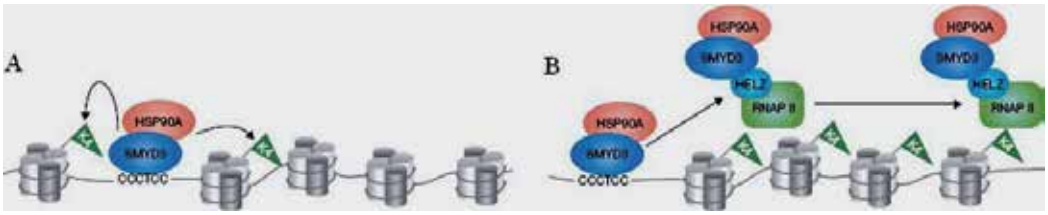

Fig. 6. SMYD3-mediated histone H3-K4 methylation and transcriptional regulation. (Sims et al., 2004)

EZH2 overexpression has been found in breast cancer, its elevation is associated with poor prognosis. It seems that EZH2 might be associated with the regulation of pRB–E2F pathway and genes involved in homologous recombination pathway of DNA repair (Zeidler et al., 2005). However, the detailed mechanism of EZH2 in cancer is not yet clear. Another study has shown that EZH2 is also overexpressed in preneoplastic breast lesions and morphologically normal breast epithelium adjacent to the pre-invasive and invasive lesions, indicating that it might be a marker of epithelium at higher risk for neoplastic transformation (Ding et al., 2006).

Family	Members	Histone specificity	Basic functions
SET domain-containing proteins			
SUV39	SUV39H1, SUV39H2, SULT1E1, G9A, CLLL8	H3K9	Transcriptional repression
SET1	MLL1, MLL2, MLL3	H3K4	Transcriptional activation
SET2	NSD1	H3K36, H4K20	Transcriptional activation
	NDS2	H4K20	Transcriptional activation
	NSD3	H3K4, H3K27	Mainly be transcriptional repression
	SETD2	H3K36	Transcriptional activation
SMYD	SMYD1	H3K4	Transcriptional repression
	SMYD2	H3K36	Transcriptional activation
	SMYD3	H3K4	Mainly be transcriptional activation
	SMYD4	Unclear	Transcriptional repression
	SMYD5	Unclear	Unclear
EZ	EZH2	H3K27	Transcriptional repression
SUV4~20	SUV4~20H1, SUV4~20H2	H4K20	Heterochromatin
PRDM2		H3K9	Transcriptional activation
Others	SET7/9	H3K4	Transcriptional activation
	SETD8	H4K20	Transcriptional repression
	SETDB1	H3K9	Transcriptional repression
	EHMT1	H3K9, H3K27	Transcriptional repression
Non-SET domain-containing proteins			
Dot1	Dot1L	H3K79	Transcriptional repression

Table 2. Summary of major human HKMTs (Pan et al., 2010)

PRDM2 (RIZ1) was originally identified as a pRb-binding protein, and its inactivation and underexpression via mutations or promoter hypermethylation had been found in a number of tumors including breast, colon, liver and lung cancers, as well as neuroblastoma, melanoma and osteosarcomas (Kim et al., 2003; Wang et al., 2007). Overexpression of PRDM2 induces G2/M cell-cycle arrest and apoptosis in tumor cell lines, while PRDM2$^{-/-}$ mice are prone to developing B cell lymphoma and stomach cancer (Steele-Perkins et al., 2001; Gibbons, 2005).

2.2.2.2 Histone arginine methyltransferase (HRMTs)

The protein arginine methyltransferase (PRMT) family is the major HRMTs up to now. The PRMTs are classified into four groups depending on the type of methylarginine they generate: Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8) catalyze the formation of ω-NG, monomethylarginines (MMA) and ω-NG, NG-asymmetric dimethylarginines (aDMA); Type II PRMTs (PRMT5, PRMT7 and PRMT9) catalyze the formation of MMA and ω-NG, N′G-symmetric dimethylarginines (sDMA); Type III PRMTs (remained unclear) catalyze only the monomethylation of arginine residues in proteins; Type IV PRMTs (only be found in *Saccharomyces cerevisiae* up to date) catalyze the methylation at delta (Δ) nitrogen atom of arginine residues (Niewmierzycka et al., 1999; Boisvert et al., 2005; Bachand, 2007).

Compared to HKMTs, The evidence for the involvement of HRMTs in human cancers is not as solid. However, underexpression of PRMT1 has been observed in breast cancer (Scorilas et al., 2000). PRMT4, also known as coactivator-associated arginine methyltransferase-1 (CARM1), is a coactivator for nuclear receptors and is oversexpressed in prostate and breast cancers (El et al., 2006). PRMT4 plays an important role in estrogen-induced cell cycle progression in the MCF-7 breast cancer cell line. Upon estrogen stimulation, the E2F1 promoter is subject to PRMT4-dependent dimethylation on H3R17, and this recruitment of PRMT4 by ERα are dependent on the presence of the NCOA3 (Frietze et al., 2008).

2.2.2.3 Histone demethylase

It used to be considered that histone methylation was a permanent and irreversible histone modification. However, in recent decade, many enzymes have been identified with the ability to demethylate methylated histone lysine/arginine residues via amine oxidation, hydroxylation or deimination (Cloos et al., 2008). The histone demethylases could be divided into three distinct classes. The first class (petidylarginine deiminase 4, PADI4) converts a methyl-lysine to citrulline. The second class (lysine-specific demethylase 1, LSD1) reverses histone H3K4 and H3K9 modifications by an oxidative demethylation reaction. The third class of demethylases is the family of Jumonji C (JmjC)-domain containing histone demethylases (JHDMs). Contrast to LSD1, JHDMs can demethylate all three methylated states (mono- di- and tri-methylated lysine). Up to now, JHDMs have been found to demethylate H3K36 (JHDM1), H3K9 (JHDM2A) and H3K9/K27 (JHDM3 and JMJD2A-D) (Klose et al., 2006; Miremadi et al., 2007).

Histone demethylase JARID1B (PLU-1) is shown to be overexpressed in breast cancers but low expressed in normal adult tissues, and it is essential for the proliferation of the MCF-7 breast cancer cell line and for the tumor growth of mammary carcinoma cells in nude mice. Several target genes of JARID1B have also been identified to be associated with breast cancer proliferation, such as 14–3–3σ, BRCA1, CAV1, and HOXA5 (Lu et al., 1999; Yamane

et al., 2007). LSD1 might be a coactivator in the ER signalling (Garcia-Bassets et al., 2007). JMJD1C expression is decreased in breast cancer tissues compared with normal breast tissues, indicating that it might be a tumor suppressor (Wolf et al., 2007).

2.2.3 Histone phosphorylation in breast cancer

Phosphorylation is also thought to have a role in chromatin remodeling and in the initiation of gene transcription, and therefore be associated with the development of human cancer (Espino et al., 2006; Wang et al., 2007). Phosphorylation of H3 on S10 and S28 is important not only during mitotic chromosome condensation but also in transcriptional activation of immediate early genes. The number of H3 pS10 foci was increased, and these TPA-induced foci were positioned next to actively transcribed regions in the nucleus after TPA stimulating of MCF-7 breast cancer cells. Presumably, these nuclear sites represent the nuclear location of genes that are induced or in a competent state. Thus, growth factors stimulating the Ras/MAPK and increasing H3 pS10 at transcriptionally active loci may contribute to aberrant gene expression and breast cancer progression (Espino et al., 2006).

2.2.4 The other histone modifications in breast cancer

Besides the acetylation, methylation and phosphorylation, there are some other modification occurred in the histone. These epigenetic changes include ubiquitination/sumoylation, ADP-ribosylation, deamination, and proline isomerisation. Although the knowledge of their functions and mechanisms is still little, some studies have showed that they are also associated with breast cancer and other human cancers.

The regulation of gene expression by phosphorylated and undersumoylated PRs is a novel form of hormone independent PR action that is predicted to contribute to breast cancer cell growth and survival (Daniel et al., 2009). Recent studies revealed that E3 ubiquitin ligases play important roles in breast carcinogenesis. ubiquitin-mediated protein degradation plays an important role in many cancer-related cellular processes. E3s play critical roles because they control the substrate specificity. Accumulating evidence suggests that genetic and expression alteration of E3s contributes to breast carcinogenesis (Chen et al., 2006).

histone sumoylation as a component of the group of modifications that appear to govern chromatin structure and function to mediate transcriptional repression and gene silencing (Shiio et al., 2003). A better understanding of the epigenetic mechanisms that cause transcriptional repression has allowed researchers to find new agents that are very effective in inducing apoptosis , differentiation, and/or cell growth arrest in human breast cancer, lung cancer, thoracic cancer, leukemia, and colon cancer cell lines (Giacinti et al., 2006).

2.3 Histone modification inhibitors and breast cancer

As discussed above, histone modification could be used as a novel target for the research of anticancer drugs. So far, several histone modification inhibitors have been developed. HDAC inhibitors are the most studied type of histone modification inhibitor up to now (Tab 3).

It showed that combination of the HDAC inhibitor vorinostat with paclitaxel and bevacizumab could induce a partial or complete response in more than 50% of patients with metastatic breast cancer (Wong, 2009; Jovanovic et al., 2010). In addition, the HDAC inhibitors have different role in ER+ and ER- breast cancer cells. In ER+ cells, HDAC inhibitors reduce the transcriptional level of ER and its response genes, while they

reestablish ER expression in ER- cell lines. But the HDAC inhibitor could potentiate and restore the efficacy of anti-estrogen therapy in preclinical models in either ER+ or ER- breast cancer cells. This has led to the initiation of several clinical trials combining HDAC inhibitors with anti-estrogen therapy (Thomas et al., 2009). LAQ824 is a novel inhibitor of HDAC that shows antineoplastic activity and can activate genes that produce cell cycle arrest. Combination of the LAQ824 and a DNMT inhibitor (decitabine) showed a synergistic (re-)activation of silenced tumor-suppressor genes in human MDA-MB-231 and MCF-7 breast carcinoma cells (Hurtubise et al., 2006).

Class	In vivo preclinical activity	Clinical phase
Carboxylates (short-chain fatty acids)		
PA	Leukemia, glioblastoma	I/II
PB	Prostate, endometrial	I/II
VA	Brain, melanoma	I/II
AN-9	NSCLC, leukemia	I/II
Hydroxamic acids		
SAHA	Lung, prostate, melanoma	I/II
m-Carboxycinnamic acid bishydroxamic acid	Neuroblastoma	
Suberic bishydroxamic acid	Melanoma, sarcoma	
Pyroxamide		I
TSA	Cervical, hepatoma,	
Oxamflatin	Melanoma	
NVP-LAQ824	Colon, multiple myeloma	I
Electrophillic ketones (epoxides)		
TPX		
AOE		

Class	In vivo preclinical activity	Clinical phase
Depudecin		
Cyclic peptides		
Apicidin	Melanoma, leukemia	
FK-228, FR901228	Melanoma, colon, sarcoma, fibrosarcoma, lung, gastric	I/II
Benzamides		
MS-275	Leukemia, colorectal, gastric, pancreatic, lung, ovarian	I/II
CI-994	Colorectal, pancreatic, mammary, prostate, sarcoma, leukemia	I
Other hybrid compounds		
CHAPs	Melanoma, lung, stomach, breast	
Scriptaid		
Tubacin		
JNJ16241199		
A-161906		
6-(3-Chlorophenylureido)caproic hydroxamic acid		
PXD101	Breast, prostate, ovarian, colon, NSCLC	

Table 3. Summary of major HDAC inhibitors (Acharya et al., 2005; Laird, 2005).

3. Conclusion

In summary, Histone modifications provide crucial regulatory functions in the process of gene transcription, and they play very important roles in the proliferation, metastasis, chemotherapy and other aspects of breast cancer, as well as many other human cancers. The reversibility of histone modification makes it could be regarded as one valuable target for

the development of novel anticancer strategies. The understanding of all these epigenetics changes and their contribution to breast cancer might take great progress in the field of diagnosis, prognosis and therapy of breast cancer.

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MCF-7 Breast Cancer Cell Line, a Model for the Study of the Association Between Inflammation and ABCG2-Mediated Multi Drug Resistance

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1. Introduction

Breast cancer is one of the most common and serious malignancies worldwide. Despite intensive cancer control efforts, it remains the second-leading cause of cancer death among women (Harris et al., 2000). While the overall response rate can be high, the duration of response is relatively short, and most patients with initially responsive tumors will experience a drug-resistance phenotype. Therefore, a lot of studies have centered on the field of drug resistance to improve cancer chemotherapy and management of cancers Gottesman, 2002).

The development of intrinsic or acquired resistance to a wide variety of anticancer drugs is a major obstacle to successful cancer chemotherapy. Some cancers show primary resistance or natural resistance in which they do not respond to standard chemotherapy drugs from the beginning. On the other hand, many types of sensitive tumors respond well to chemotherapy drugs in the beginning but show acquired resistance later (Choi, 2005). Multidrug resistance (MDR) can be defined as the intrinsic or acquired resistance of cancer cells to multiple classes of structurally and mechanistically unrelated antitumor drugs (Teodori et al., 2002). To date, the most widely studied cellular mechanisms of MDR are those associated with drug efflux involving members of the adenosine triphosohate-binding cassette (ABC) membrane transporter family (Mao et al., 2005).

Recently, several human ABC transporters with a potential role in drug resistance have been discovered. Among them, a novel known protein is ATP-binding cassette sub-family G member 2 (ABCG2). Human ABCG2 (also known as MXR, BCRP, and ABCP) was first cloned by Doyle et al. (Doyle et al., 1998) in the drug-resistant breast cancer cell line (MCF-7). ABCG2 is an efflux pump, which transports a variety of xenobiotics and endogenous compounds across cellular membranes. Tissue localization of ABCG2 in the mammary glands, intestine, kidney, liver, ovary, testis, placenta, endothelium and in hematopoietic stem cells indicates that ABCG2 plays an important role in absorption, distribution, and elimination of its substrates (Krishnamurthy et al., 2004; Mao & Unadkat, 2005). The expression of ABCG2 protein and/or mRNA has been detected in numerous types of human cancers (Diestra et al., 2002; Ross et al., 2000), and a large spectrum of anticancer drugs are effluxed by ABCG2

(Doyle et al., 2003). It has also been shown that ABCG2 expression may be associated with poor response to chemotherapy (van den Heuvel-Eibrink et al., 2002, Steinbach, 2002 #216) . Alteration in ABCG2 expression and function can significantly affect the disposition of the transporter drug substrates, it is possible that its overexpression in cancer cells is responsible for decreasing in drug concentration within the cell and a reduced cancer-chemotherapy efficacy (Glavinas et al., 2004; Mao & Unadkat, 2005).

Inflammation is a state consisting of complex cytological and chemical reactions that occur in affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation caused by physical, chemical or biological agents (Ho et al., 2006; Philip et al., 2004). Although inflammation is essential, it can be harmful to the host and therefore it is subject to multiple levels of biochemical, pharmacological, and molecular controls involving a diverse and potentially huge array of cell types and soluble mediators including cytokines (Haddad, 2002). In fact tumors are similar to healing or desmoplastic tissue in many ways and the micro-environment of the tumor highly resembles an inflammation site (Caruso et al., 2004). Breast cancer is a prototype of these kinds of cancer. Indeed, proinflammatory cytokines have been found to be present within the microenvironment of breast carcinomas and secreted by infiltrating host leukocytes, malignant and/or stromal cells of the breast cancer (Basolo et al., 1996; Jin et al., 1997; Lithgow et al., 2005; Miles et al., 1994).

In recent years, it has been demonstrated that the expression and function of the MDR transporters is altered in numerous tissues during an inflammatory response. The current review focuses on the elucidation of the effects of inflammation on the ABCG2 expression and function, using MCF-7 human breast carcinoma cell line.

2. The role of inflammation on the ABCG2 expression and function

In an overview, the results of several studies on the effect of inflammation on the levels of ABCG2 protein expression and function in MCF-7 cells will be reviewed in this paper. In the first part, the observed effects of the proinflammatory cytokines on the ABCG2 protein expression and function will be expressed. In the next section, the effects of cyclooxygenase 2 on drug resistance due to ABCG2 will be reviewed and eventually the influence of treatment with anti-inflammatory drugs indomethacin and dexamethasone on the incidence of drug resistance phenotype will be expressed.

2.1 Proinflammatory cytokines and ABCG2 expression and function

Proinflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are well-known regulators of inflammatory response. Inflammatory components including host leukocytes, chemokines and cytokines are also present in the microenvironment of most probably all tumors including those not casually related to an obvious inflammatory process (Germano et al., 2008).

Numerous in vitro and in vivo investigations reported that inflammation and proinflammatory cytokines are able to modulate the expression or function of different drug transporters including Multi-Drug Resistance transporter 1 (MDR1/ABCB1), Multidrug Resistance-associated Proteins (MRPs/ABCCs) and Lung-resistance Related Protein / Major Vault Protein (LRP/MVP). These modulations appeared to happen at various levels of expression including transcriptional, posttranscriptional, translational, and/or post-translational levels (Bertilsson et al., 2001; Hartmann et al., 2002; Hirsch-Ernst et al., 1998;

Piquette-Miller et al., 1998; Stein et al., 1997; Sukhai et al., 2001; Theron et al., 2003; Vos et al., 1998; Walther et al., 1994; Walther et al., 1995).

The influence of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) on ABCG2 expression and function in human MCF-7 breast cancer cell line were studied using real-time PCR and flow cytometry, respectively. The results showed that, the levels of ABCG2 mRNA, protein expression and function in MCF-7 cells increased significantly after treatment with either IL-1β or TNF-α (Fig. 1).

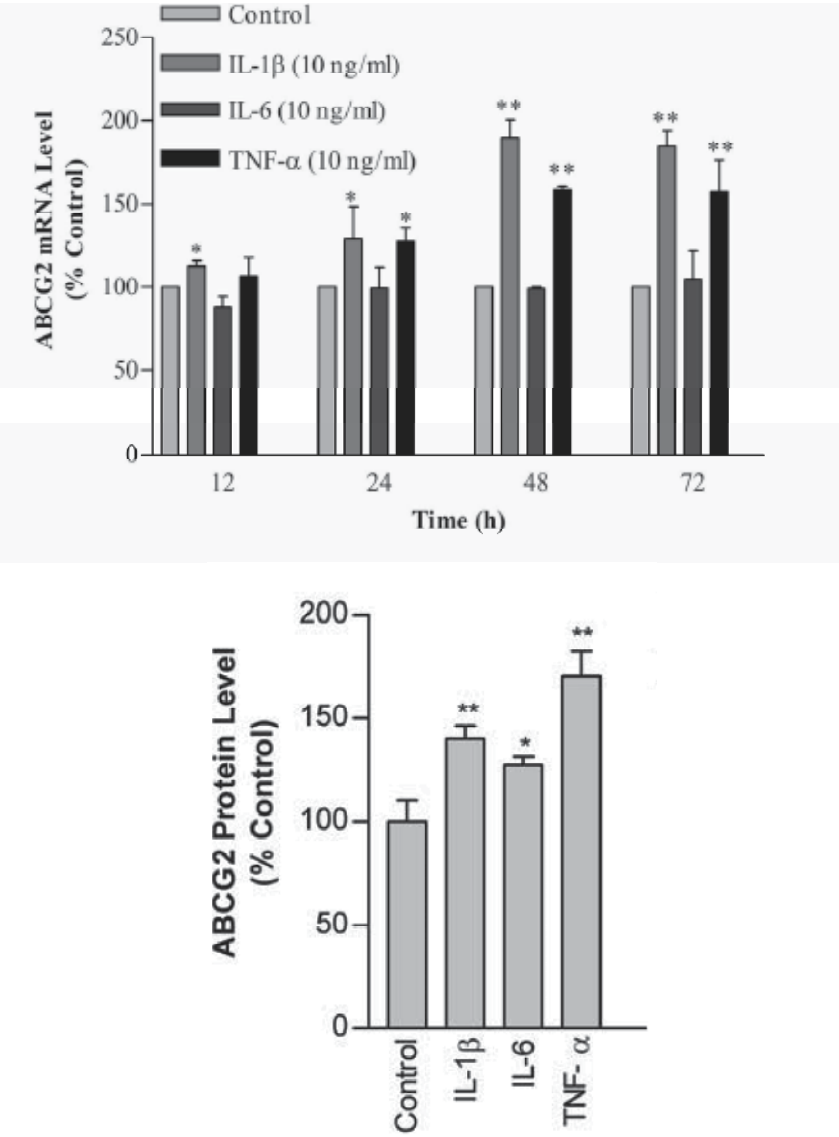


Fig. 1. The effects of proinflammatory cytokines on ABCG2 mRNA expression (A) and protein levels (B) in MCF-7 cells.

A;Cells were treated with 10 ng/ml of each cytokine for 12–72 h and real-time RT-PCR analysis was performed on total RNA extracted from control and treated cells. Values were normalized to the β-actin content of samples and expressed as mean (% control) ±SD (n = 3); *P<0.05; **P<0.01 versus control (0 ng/ml). B; After 72-h incubations with IL-1β (50 ng/ml), IL-6 (50 ng/ml) or TNF-α (50 ng/ml), expression of ABCG2 protein was measured by flow cytometry. Each value represents MFI mean (% control) of at least three independent experiments ± SD; *P<0.05, **P<0.01 versus controls.

Pradhan and colleagues also found that under proinflammatory conditions two transcription factors, estrogen receptor (ER) and NF-κB are cooperatively recruited to the promoter region of the ABCG2 gene at adjacent sites. ER allows the NF-κB family member p65 to access a latent NF-κB response element located near the estrogen response element (ERE) in the gene promoter; in turn, this p65 recruitment is required to stabilize ER occupancy at the functional ERE. Once present together on the ABCG2 promoter, ER and p65 act synergistically to potentiate mRNA and subsequent protein expression. This study has important implications for patients with ER-positive breast tumors, as it reveals a mechanism whereby inflammation enhances the expression of an ER target gene, which in turn can exacerbate breast tumor progression by promoting drug resistance mechanism whereby inflammation enhances the expression of an ER target gene, which in turn can exacerbate breast tumor progression by promoting drug resistance (Pradhan et al., 2010).
On the other hand, while IL-6 had no significant effects on ABCG2 mRNA expression and function in MCF-7 cells, it could slightly increase ABCG2 protein expression in these cells. This shows that IL-6 probably modulates ABCG2 expression by affecting ABCG2 protein translation and/or stability, but not ABCG2 transcription. For unknown reasons, this modulation did not result in the increased activity of the protein (Mosaffa et al., 2009).
In contrast to the results obtained for MCF-7 cells, in its mitoxantrone-resistance derivative, MCF-7/MX cells, none of the cytokines (even at high concentrations and long incubation times) exerted significant effects on ABCG2 mRNA levels. Because MCF-7/MX cells overexpress ABCG2 mRNA, it is likely that although modulation of the signaling pathway(s) responsible for increased transcription of ABCG2 in IL-1β and TNF-α-treated MCF-7 cells, has already happened in MCF-7/MX cells, but treatment with these cytokines could not cause further induction in ABCG2 mRNA levels (Mosaffa et al., 2009).
The results showed that IL-1β increased ABCG2 function and TNF-α enhanced both ABCG2 protein expression and function in MCF-7/MX cells. This lack of correspondence between mRNA and expression/function data suggests that perhaps in addition to the transcriptional regulatory effects of IL-1β and TNF-α, these two cytokines can also mediate ABCG2 expression and function via translational and/or post-translational effects (Mosaffa et al., 2009).

2.2 Cyclooxygenase-2 and ABCG2 expression and function

Cyclooxygenases (COX), also known as prostaglandin endoperoxide synthases or prostaglandin H synthases, comprise a group of enzymes that participate in the conversion of arachidonic acid to prostaglandins (PGs) that affect a number of physiological and pathological states in neoplastic and inflamed tissues (Smith et al., 1996). There are two isoforms of the enzyme that have been identified, COX-1 and COX-2. Constitutively expressed COX-1 supplies normal tissues with prostaglandins required to maintain physiological organ functions (O'Neill et al., 1993), such as cytoprotection of the gastric

mucosa (Chan et al., 1995) and regulation of renal blood flow (Tanioka et al., 2003). On the other hand, COX-2 is highly induced by growth factors (epidermal growth factor (EGF)), cytokines (IL-1β, IL6, TNF-α (Davies et al., 2002; Zhang et al., 2006)), and carcinogens (phorbol esters (Liu et al., 1996; Rigas et al., 2005)) via protein kinase C (PKC) and RAS-mediated signaling at sites of inflammation. Therefore, it is assumed that COX-2 plays an important role in the prostaglandin E2 (PGE2) production involved in pathophysiological processes (Trebino et al., 2003). COX-2 may be implicated in tumor promotion through modulating cell proliferation, inhibiting apoptosis, control of cell migration, cell adhesion, tumor invasion and suppression of immune response (Cao et al., 2002). In recent years, it has been reported that COX-2 modulates ABC transporter expression and is involved in the development of the MDR phenotype (Ratnasinghe et al., 2001, Fantappiè O, 2002 #78, Puhlmann, 2005 #103).

Kalalinia et al. studies had aimed to explore the potential link between COX-2 expression and development of multidrug resistance phenotype due to ABCG2 expression in MCF-7 cell line. In one study they used of 12-O-tetradecanoylphorbol-13-acetate (TPA) for induction of COX-2 expression in MCF-7 cells. TPA often employed in biomedical research to activate the signal transduction enzyme protein kinase C (PKC). The effects of TPA on PKC result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol (DAG).

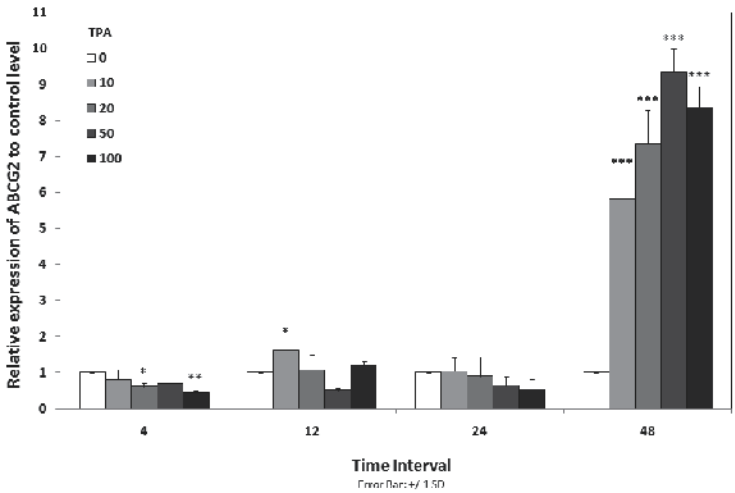
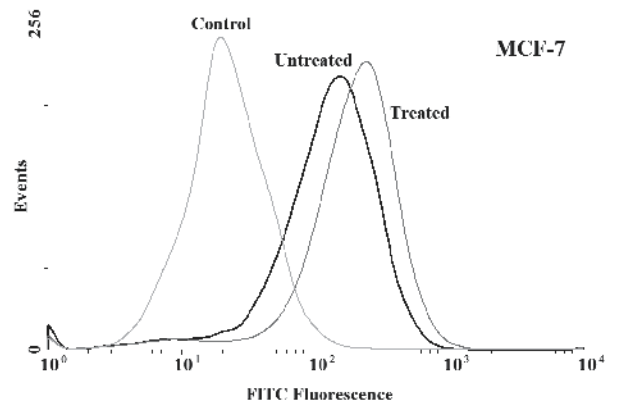


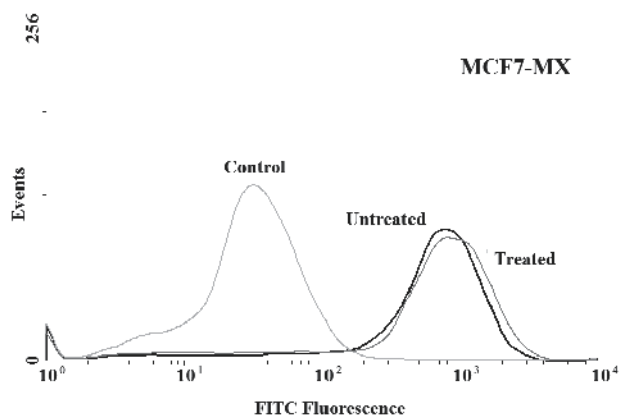
Fig. 2. Effects of TPA on the levels of ABCG2 mRNA in MCF-7 cells.
Cells were treated with TPA (0-100 nM for 4-48 h) and ABCG2 mRNA expression was measured by real-time RT-PCR using total RNA extracted from control and treated cells. Relative expression levels for each gene were normalized to that of the β-actin. The results were expressed as: (target/reference ratio) treated samples / (target/reference ratio) untreated control sample. Values were expressed as mean ± SD (n = 3); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The real-time PCR analysis showed that COX-2 inducer TPA caused a considerable increase up to 9-fold in ABCG2 mRNA expression in parental MCF-7 cells (Fig. 2). While a slight increase in ABCG2 expression was observed in the resistant cell line MCF-7/MX. The results

of flow cytometry showed a slight increase of ABCG2 expression at protein level in MCF-7, while no significant changes in the level of ABCG2 protein expression was observed in MCF-7/MX (Fig. 3). As we mentioned earlier, in the drug resistant MCF-7/MX cells, ABCG2 is already overexpressed, and its expression may be at a threshold maximum level, so an induction with TPA treatment may not be causing any detectable increase in ABCG2 mRNA level. Likewise, a close association between MDR and COX-2 has been reported in non-Hodgkin's lymphomas (Szczuraszek et al., 2009), non-small cell lung cancer (Surowiak et al., 2008) and breast cancer cases (Surowiak et al., 2005). Adenovirus-mediated transfer of rat COX-2 cDNA into renal rat mesangial cells increased P-glycoprotein (P-gp/MDR1) expression, and this was blocked by COX-2 inhibitor NS398, suggesting that COX-2 products may be implicated in this response (Miller et al., 2006; Patel et al., 2002). All of these studies strongly suggest that COX-2 could be involved in the development of the MDR phenotype (Sorokin, 2004).



(A)



(B)

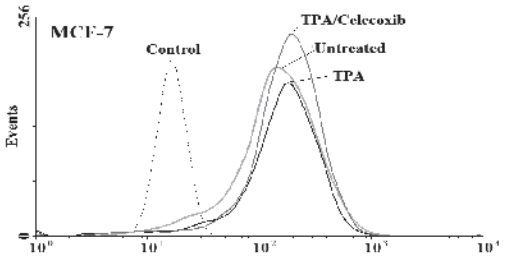
Fig. 3. Effect of TPA on ABCG2 protein levels in MCF-7 (A) and MCF-7/MX (B) cells. After 48 h incubation with TPA (10 nM), expression of ABCG2 protein was measured by flow cytometry. Each histogram shows the overlay of the treated sample (dark gray), untreated sample (black) and secondary antibody as negative control (light gray).

Different studies showed that incubation of MDR cells with PKC activator TPA stimulate P-gp phosphorylation, reduce drug accumulation, and enhance drug resistance (Ramachandran et al., 1998). Fine et al. demonstrated that phorbol 12,13-dibutyrate [P(BtO)2] led to an increase in protein kinase C activity and induced a drug-resistance phenotype as a result of increased phosphorylation of an unknown 20-kDa particulate protein (Fine et al., 1988). Similar to TPA treatment, diacylglycerol (DAG), a physiological stimulant of PKC, also increased the expression of MDR1 mRNA and protein. Whereas, protein kinase inhibitor staurosporine suppressed the induction of MDR1 expression by TPA and DAG (Chaudhary et al., 1992). These reports suggest that MDR gene expression in different cell types is regulated by a PKC-mediated pathway.
ABCG2 function was measured by flow cytometric mitoxantrone efflux assay. In long term exposure TPA enhanced the ABCG2 function, which was more considerable in MCF-7/MX than parental MCF-7 cells (Kalalinia et al., 2010). There is considerable precedent that PKC activation is associated with increased transport processes. Fine et al showed that, protein kinase C activity was 7-fold higher in the drug-resistant mutant MCF-7 cells compared with the control MCF-7 cells, sensitive parent cells (Fine et al., 1988). Fine et al reported that exposure of drug-sensitive cells to the phorbol 12, 13-dibutyrate [P (BtO)2] caused an enhanced PKC activity and induced drug-resistance phenotype, whereas drug-resistant cells in the same exposure to P(BtO)2 showed further increased in drug resistance. So phorbol ester might be the reason of decreased drug accumulation by inducing phosphorylation of a drug efflux pump or carrier protein (Fine et al., 1988).

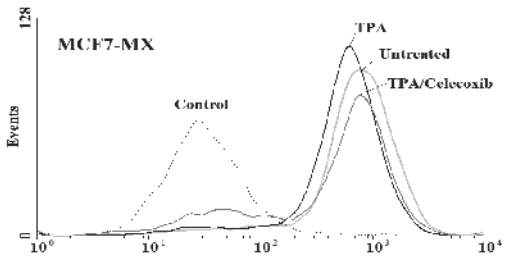
2.3 Celecoxib (a selective inhibitor of COX-2) and ABCG2 expression and function

Numerous studies showed that COX-2 inhibitors (coxibs) enhance the efficacy of different anticancer therapy methods. Different mechanisms have been suggested to contribute to the antitumor activity of coxibs such as the inhibition of cell cycle progression, induction of apoptosis, inhibition of angiogenesis and decreased invasive potential of tumor cells (Fife et al., 2004; Gasparini et al., 2003; Hashitani et al., 2003; Masferrer et al., 2000). Another mechanism by which COX-inhibitors could sensitize cells to chemotherapeutic drugs is functional blockade of membrane transporter proteins of the ABC-transporter family (Patel et al., 2002; Zatelli et al., 2005).
Kalalinia et. al. investigated the relationship between the inhibition of COX-2 and expression of ABCG2 in parental and resistance breast cancer cell lines. They reported that treatment of MCF-7 and MCF-7/MX cells with celecoxib up-regulates ABCG2 expression at mRNA levels. The results also indicated that, celecoxib reversed the inhibitory effects of TPA on ABCG2 protein expression and increased its expression to the basal level in MCF-7/MX, while co-treatment of MCF-7 cells with TPA and celecoxib caused increased ABCG2 protein expression to a small amount more than TPA lonely (Fig. 4). In the same way , Zrieki et al. showed that treatment of human colorectal Caco-2 cell line with COX-1/ COX-2 inhibitor naproxen led to an stimulation of ABCG2 expression which corresponded to the significant decrease of Rho123 retention achieved in activity study. In contrast, treatment with selective COX-2 inhibitors nimesulide did not influence the expression of ABCG2 at protein level (Zrieki et al., 2008). Several studies have shown that specific COX-2 inhibitors could prevent or reduce the development of chemoresistance phenotype by downregulation of the expression and function of P-glycoprotein (MDR1) (Huang et al., 2007; Kim et al., 2004; Roy et al., 2010; Zatelli et al., 2005; Zatelli et al., 2007). Xia et al. found that celecoxib significantly inhibited MDR1 expression without any effects on pump function of P-gp.

They demonstrated that the inhibitory effect of celecoxib on MDR1 was COX-2-independent but directly correlated to hypermethylation of MDR1 gene promoter (Xia et al., 2009). In addition, it is shown that COX-2 inhibitors induced PGH2 generation and NF-κB activation, which result in inhibition of P-gp expression and function in breast cancer cells (Zatelli et al., 2009).



(A)



(B)

Fig. 4. Effects of celecoxib on the expression of ABCG2 at protein levels in MCF-7 (A) and MCF-7/MX (B) cell lines were studied by flow cytometry.
Cells were fixed and permeabilized by formaldehyde and methanol, blocked with BSA and then incubated with primary monoclonal antibody BXP-21. After washing, cells were incubated with a FITC-conjugated goat anti-mouse antibody. Each histogram shows the overlay of the TPA treated sample (black), TPA and celecoxib treated sample (dark gray), untreated sample (ligh gray) and secondary antibody as negative control (broken light gray).

In MCF-7 cell line, celecoxib in presence of TPA 10 nM caused reduction of ABCG2 function in a dose- and time-dependent manner (Kalalinia et al., 2010). Another study provides evidence that NS-398, selective COX-2 inhibitor, sensitizes chemoresistant breast cancer cells to the cytotoxic effects of doxorubicin and notably enhances intracellular DOX accumulation and retention in vitro. It was shown that these effects depended on the inhibition of P-gp expression and function in both native and chemoresistant MCF-7 cells (Zatelli et al., 2007).

2.4 The influence of indomethacin on ABCG2 expression and function

Several preclinical and clinical trials have shown that nonsteroidal anti-inflammatory drugs (NSAIDs), used as classical COX inhibitors, could reduce the incidence of cancers (Cha et al., 2007; Kang et al., 2005; Lin et al., 2005). Although the exact anticancer mechanisms of NSAIDs are not fully understood, it seems to be related closely to their suppression of COX

enzyme and subsequent reduction in prostaglandin production (Kismet et al., 2004; Zatelli et al., 2005). Modulation of the efficacy of cancer chemotherapy by NSAIDs has not been examined in detail.

Elahian et. al. investigated the pharmacological silencing of ABCG2 in MCF-7 cells through the use of indomethacin, in the hopes of opening a novel way in management of breast cancer. MTT assay showed that indomethacin did not significantly change the survival of MCF-7 and MCF-7/MX cells, but cotreatment of mitoxantrone with indomethacin increased the mitoxantrone cytotoxicity and reduced the IC50 of mitoxantrone in these cells. Altough indomethacin sensitized MCF-7 cells to mitoxantrone, but it did not alter mitoxantrone accumulation in MCF-7 cells, compared to the control (Elahian et al., 2010). It might suggest that indomethacin exerts the sensitising effects through a mechanism not involving the inhibition of ABCG2, but possibly reducing the synthesis of COX and its end-products (Spugnini et al., 2006; Verdina et al., 2008). Indeed, further studies would be necessary to clarify the molecular mechanisms involved in the potentiation of mitoxantrone cytotoxicity by indomethacin in MCF-7 and MCF-7/MX cells. Real-time PCR results showed that indomethacin-treated MCF-7 cells indicated no significant change in the amount of ABCG2 mRNA expression. This observation has been also confirmed on the level of ABCG2 protein expression (Elahian et al., 2009). As a result, expression of a MDR phenotype in human malignant cells may not always be sensitive to potentiation of drug cytotoxicity by NSAIDs (Roller et al., 1999). The present results also confirmed other studies that show NSAIDs' effects are cell and efflux transporter specific (Nozaki et al., 2007).

2.5 The influence of dexamethasone on ABCG2 expression and function

Glucocorticoides are efficacious in the reducing of chemotherapy adverse side effects and show their intrinsic anticancer activity (Vee et al., 2009) (Pavek et al., 2005). Some glucocorticoids, such as beclomethasone, 6α-methylprednisolone, dexamethasone, and triamcinolone, at micromolar concentrations, are shown to efficiently decrease the transport of ABCG2 substrates (Pavek et al., 2005).

Glucocorticoide receptor agonists regulate gene expression in various ways, at the transcriptional (Adcock, 2001), posttranscriptional (Korhonen et al., 2002), and posttranslational levels (Kritsch et al., 2002). Direct interaction of ligand-activated GR with control elements of target genes could regulate gene transcription in a positive or negative way. However, there are different mechanisms for the negative regulation of gene transcription by glucocorticoides. They could interfere with general transcription factors such as activator protein-1 (AP-1) (Herrlich, 2001) and nuclear factor-κB (NF-κB) (Almawi et al., 2002), resulting in decreased transcription of AP-1- and NF-κB -responsive genes. Genomic organization of the ABCG2 gene revealed the presence of several AP-1 sites in the ABCG2 promoter (Bailey-Dell et al., 2001). So it could be a direct target of transcriptional repression in a similar way. On the other hand, it has been reported that dexamethasone mediates negative regulation of gene expression by destabilizing the mRNA of some target genes (Garcia-Gras et al., 2000; Lasa et al., 2002).

Investigating the effects of dexamethasone on ABCG2 expression in MCF-7 cells showed that dexamethasone decreased the mRNA level of ABCG2 gene in comparison with control in MCF-7 and MCF-7/MX cell lines. Flow cytometry analysis indicated that a decrease in the level of ABCG2 protein was observed in dexamethasone treated MCF-7/MX cells. While the level of ABCG2 protein expressed as a ratio of the corresponding control was unchanged in

MCF-7 treated cells (Elahian et al., 2009). Cotreatment with different concentrations of mitoxantrone and dexamethasone increased the sensitivity of MCF-7 and MCF-7/MX cells to the toxic effects of mitoxantrone. In addition, the flow cytometry results showed that dexamethasone could inhibit the efflux and consequently caused increase in the accumulation of mitoxantrone in MCF-7/MX cells. However, ABCG2 inhibition by dexamethasone was not significant in MCF-7 cells (Elahian et al., 2010).
These studies also confirmed that suppression role of dexamethasone on ABCG2 expression in MCF-7/MX cells was more significant than MCF-7 cells. It could be a confirmation for higher level of ABCG2 in MCF-7/MX cells compared with their parental cells and also confirmed other studies that show hormonal regulation of MDR gene expression is cell type specific (Demeule et al., 1999; Imai et al., 2005).

3. Conclusion

In this review we aimed to focuse on the explanation the role of inflammation on the ABCG2 expression and function, using MCF-7 human breast carcinoma cell line. Pro-inflammatory cytokines have been found to be present within the micro-environment of tumors and inflammation. They are able to modulate the expression and function of different drug transporters. Mosaffa et al. showed that that proinflammatory cytokines IL-1β and TNF-α induce ABCG2 mRNA and protein expression and increase its function in MCF-7 cells. In MCF-7/MX, these cytokines increased ABCG2 protein expression and function, but they have no influence on the transporter mRNA levels.
Cyclooxygenase-2 (COX-2) is induced by mitogenic and inflammatory stimuli such as growth factors and cytokines, which results in enhanced synthesis of PGs in neoplastic and inflamed tissues. Kalalinia et al. studies had aimed to explore the potential link between COX-2 expression and development of multidrug resistance phenotype in MCF-7 cell line. They reported that COX-2 inducer TPA (12-O-tetradecanoylphorbol-13-acetate) caused a considerable increase up to 9-fold in ABCG2 mRNA expression in parental MCF-7 cells, while a slight increase in ABCG2 expression was observed in the resistant cell line MCF-7/MX. They also showed a positive corrolation between ABCG2 protein expression and COX-2 protein level in each cell line. On the other hand, celecoxib (a selective inhibitor of COX-2) up-regulated the expression of ABCG2 mRNA in MCF-7 and MCF-7/MX cells, which was accompanied by increased ABCG2 protein expression. Furthermore, TPA could increase ABCG2 function in all cell lines with the greatest stimulatory effects in MCF-7/MX (more than 6 times the control level). In addition, celecoxib inverted the effects of TPA on ABCG2 function. This effect was more obvious in MCF-7/MX.
Several studies have demonstrated that anti-inflammatory drugs like NSAIDs and some glucocorticoids could be effective in chemosensitizing of the many carcinoma cell lines to cytotoxic agents. The pharmacological modulation of ABCG2 in MCF-7 cells by dexamethasone and indomethacin was investigated by elahian et al. . They showed that dexamethasone induced downregulation of ABCG2 mRNA compared to controls in both MCF-7 and MCF-7/MX cell lines, whereas no changes were noted in the presence of indomethacin. The level of ABCG2 protein was decreased in dexamethasone treated MCF-7/MX cells. Cotreatment of mitoxantrone with different concentrations of dexamethasone and indomethacin sensitized parental and resistant MCF-7 cells to mitoxantrone cytotoxicity. Dexamethasone also increased the accumulation of mitoxantrone in the MCF-7/MX cell line, indicating an inhibitiory effect on the ABCG2 protein.

In this review, we describe the effects of proinflammatory cytokines (IL-1β and TNF-α), inflammatory mediator (COX-2) and anti-inflammatory drugs (celecoxib and dexametashone) on the expression of ABCG2 which addressed concerning to finding a new adjutant therapy for patients with cancer experiencing resistance to cancer treatment.

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Interaction of Alkylphospholipid Formulations with Breast Cancer Cells in the Context of Anticancer Drug Development

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1. Introduction

Alkylphospholipids have shown promising results in several clinical studies (Mollinedo 2007) and among them Perifosine (octadecyl(1,1-di-methyl-4-piperidinium-4-yl)phosphate, OPP), and miltefosine (hexadecylphosphatidylcholine (HPC)) seems to be most promising for breast cancer therapy (Fichtner, Zeisig et al. 1994). For this type of tumor, an antitumor effect was found only for hormone receptor negative tumors *in vivo*, while no effect was found for receptor positive tumors. The reason for this difference is not yet understood and requires further studies. The exact mechanism of action of alkylphospholipids on the molecular level is still not well known in detail. It is clear that they do not target DNA, but they insert into the plasma membrane and subsequently induce a broad range of biological effects, ultimately leading to cell death.

Unfortunately, administration of free (micellar) alkylphospholipids results in unwanted side effects, reflected in gastrointestinal toxicity and hemolytic activity, which limits the application of higher doses of alkylphospholipids. To achieve better therapeutic effects of alkylphospholipids *in vivo* with less side effects, different liposomal formulations of alkylphospholipids have been tested and showed diminished hemolytic activity. On the other hand, in most cases, cytotoxic activity of liposomes was also lower as compared to free alkylphospholipids (Zeisig et al., 1998).

For efficient application of liposomes as nanocarriers in breast cancer therapy it is not only necessary to investigate the properties of the nanocarrier, which has to transport the drug to the (target) cell, but also the properties of the target cell. The main difference between Perifosine (OPP) resistent MCF7 cells and OPP sensitive MT-3 cells is in the uptake of OPP liposomes by cells and the transport of OPP across plasma membrane. At physiological temperatures the rate of transfer of OPP across plasma membrane increases to greater extent in OPP resistant MCF7 cells, while the uptake of liposomal OPP formulations is lower for

OPP resistant MCF7 cells as compared to OPP sensitive MT3 breast cancer cells. On the other hand the properties of an efficient OPP formulation are mainly determined by cholesterol concentration, which should be below 50 mol%.

2. Alkylphospholipids in clinical trials

In the late 1970's and early 1980's systemic investigations of structure – activity relationship were performed to screen lysophospholipids, alkylphospholipids and etherlipids to identify new candidates for cancer treatment. Among them, especially Miltefosine (Fig. 1), basically a simple phosphorus acid diester, displayed high inhibitory activity against chemically induced mammary carcinomas in rats (Eibl & Unger, 1990). It became the first drug based on a phospholipid structure demonstrating the high potential of this simple structured molecule class. The main advantage of this class of drugs is the target. In contrast to most anti-cancer drugs, which interfere at the DNA level with cell proliferation, alkylphospholipids act at the cell membrane, where they disturb the PI3K/Akt/mTOR signal transduction pathway (Fig. 2). Initial preclinical tests were promising, indicating a good anti-cancer activity against several human tumour xenograft models in the mouse (Arndt et al., 1997; Fichtner et al., 1994), including different breast cancer cell lines like: MT-3 (Zeisig et al., 1998), MDA-MB 435 and MDA-MB 231 (Sobottka & Berger, 1992), MaTu (Arndt et al., 1999), MT-1 (Naundorf et al., 1992), C3H, Ca 755 (Zeisig et al., 1991) and also syngeneic models like murine P388 leukemia, and B 16 melanoma (Zeisig et al., 1991). Preclinical experiments further demonstrated that alkylphospholipids, if used in liposomal form, are able to abolish multi drug resistance in human breast cancer xenografts (Zeisig et al., 2004) and inhibit metastasis if combined with an aggregation inhibitor inside liposomes in murine syngene (Wenzel et al., 2010) and human xenograft breast cancer models (Wenzel et al., 2009). Perifosine in combination with dioleylphosphoethanolamine, as a component of the liposome bilayer, also enhances transport of drugs across the blood brain barrier and in this way improves the treatment of intracerebral tumours and metastases (Orthmann et al., 2010). Miltefosine was also tested as an alternative approach for the treatment of patients with progressive cutaneous lesions from breast cancer in Phase I and II studies, which indicated that Miltefosine (either used alone or in conjunction with other therapies for distant metastases) is an effective and tolerable local treatment for cutaneous breast cancer (Clive et al., 1999; Unger & Eibl, 1991).

Only small changes in the molecular structure (slightly longer alkyl chain, a modified head group) while maintaining molecular size and shape resulted in Perifosine (OPP). Gills et al. (Gills & Dennis, 2009) summarised the clinical trials with Perifosine as single agent until 2009. Seven Phase 1 single agent studies of Perifosine have been completed. The trials demonstrated that Perifosine can be safely given to humans with a manageable toxicity profile. The dose limiting toxicity in the Phase I studies was, similar to Miltefosine, gastrointestinal: nausea, vomiting and diarrhea. Perifosine as single agent has been further evaluated in Phase II studies for the treatment of most common cancers, including breast, prostate, head and neck, pancreatic cancers, melanoma, renal cell carcinoma, advanced brain tumours, soft-tissue sarcomas, hepatocellular carcinoma, as well as in haematological malignancies including multiple myeloma and Waldenstrom's macroglobulinemia (WM). Potent activity with Perifosine, given as single-agent, has been observed so far in sarcoma and WM patients.

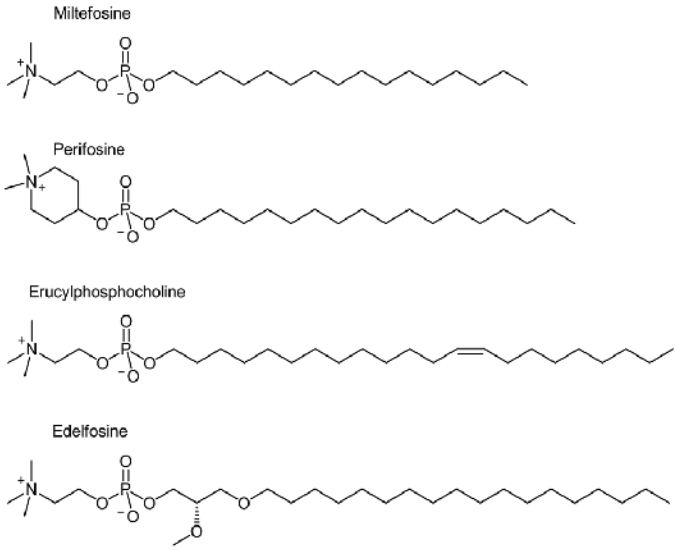

Fig. 1. Structural formula of pharmaceutically tested alkylphospholipids.

Name	Abbreviation(s) CAS-number	IUPAC-name	Formula, molecular weight (g/mol), reference
Miltefosine	HPC, HePC, 58066-85-6	Hexadecyl-2-(trimethylazaniumyl) ethylphosphat	$C_{21}H_{46}NO_4P$, 407.57, (Eibl & Unger, 1990; Unger et al., 1988)
Perifosine	OPP, D21266 57716-52-4	(1,1-dimethylpiperidin-1-ium-4-yl) octadecyl phosphate	$C_{25}H_{52}NO_4P$, 461.66, (Hilgard et al., 1997)
Erucyl phosphocholine	EuPC; C22:1-PC 143317-74-2	[(Z)-docos-13-enyl] 2-(trimethylazaniumyl) ethyl phosphate	$C_{27}H_{56}NO_4P$, 489.71, (Erdlenbruch et al., 1998)
Edelfosine	ET-18-OCH3 77286-66-9	(2-methoxy-3-octadecyloxypropyl) 2-(trimethylazaniumyl) ethyl phosphate	$C_{27}H_{58}NO_6P$, 523.73, (Heesbeen et al., 1991)

Table 1. Names, abbreviation, IUPAC names, formula, molecular weights and references of most common alkylphospholipids

Erucylphosphocholine is an alkylphospholipids derivative with a 22 carbon atom chain and a cis-13,14 double bond. Although it differs from miltefosine only in alkyl chain length and the presence of a double bond (Fig. 1), significant differences were found in pharmacological properties. This structural modification increases hydrophobicity resulting in the formation of lamellar supramolecular structures, which abolished hemolytic side effects and allows Erucylphosphocholine to be administrated intravenously (Erdlenbruch et al., 1999; Kaufmann-Kolle et al., 1996; van Blitterswijk & Verheij, 2008). It is a potent inducer of apoptosis (Jendrossek et al., 2003) that exerts more potent antineoplastic effects *in vitro* and *in vivo* than Miltefosine.

3. Mode of action of APL

Anti-cancer mechanisms of alkylphospholipids have been described and extensively discussed in some recent reviews (Danker et al., 2010; Gajate & Mollinedo, 2002; Gills & Dennis, 2009; van Blitterswijk & Verheij, 2008). Early interest focussed on immune stimulating activity of alkylphospholipids. It could be demonstrated that Miltefosine and other lipids of this class are able to activate T-cells and macrophages to express and release chemokines like GM-CSF (Vehmeyer et al., 1992), IFgamma (Hochhuth et al., 1992) and/or nitric oxide (NO) (Zeisig et al., 1995). This effect could be improved if the alkylphospholipids were used in liposomal form. Because of their amphiphilic structure, alkylphospholipids are able to form lamellar bilayers, if combined with lipids of opposite molecular shape. Liposomes were taken up by macrophages much better than the free, micellar lipids and induced, after cellular uptake, the release of IF gamma and NO (Eue et al., 1995). But their potency as immune stimulator was limited and not sufficient enough amount of chemokines was released for a complete inhibition of tumor cell proliferation.

3.1 Uptake and absorption of alkylphospholipids

Due to their amphiphilic nature alkylphospholipids are easily incorporated into cell membranes in substantial amounts and then spread among intracellular membrane compartments, where they accumulate and interfere with a wide variety of key enzymes (Unger et al., 1992; van Blitterswijk et al., 1987). At lower, clinically relevant concentrations alkylphospholipids interfere with phospholipid turnover and lipid-based signal transduction pathways. In mouse S49 lymphoma cells alkylphospholipids accumulate in detergent-resistant, sphingolipid- and cholesterol-enriched lipid raft domains and are rapidly internalized by clathrin-independent, raft-mediated endocytosis (van der Luit et al., 2007). Alkylphospholipid uptake in KB carcinoma cells, however, appears to be raft-independent and mediated by a yet unidentified ATP-dependent lipid transporter (Vink et al., 2007). In leukemic cells treatment with alkylphospholipids induces the formation of membrane raft aggregates containing Fas/CD95 death receptor and the adaptor molecule Fas-associated death domain-containing protein (FADD), which are critical in the triggering of apoptosis (Gajate et al., 2009). Miltefosine and other alkylphospholipids also alter intracellular cholesterol traffic and metabolism leading to an increased uptake, synthesis and accumulation of cholesterol in the cell (Carrasco et al., 2008; Jimenez-Lopez et al., 2006; Marco et al., 2009). As cholesterol and sphingomyelin content are critical for the integrity and functionality of membrane lipid rafts, the disturbance of the cholesterol/sphingomyelin ratio could alter signaling pathways associated with these membrane domains.

3.2 Inhibition of phosphatidylcholine biosinthesis

Inhibition of phosphatidylcholine (PC) biosynthesis is a major alkylphospholipid target (Fig. 2). Inhibition of the biosynthesis of PC causes stress on cells sufficient to trigger apoptosis. In the endoplasmic reticulum, alkylphospholipids inhibit CTP (phosphocholine cytidyltransferase, CT), which chatalyses the rate-limiting step of the *de novo* PC synthesis. Alkylphospholipids inhibit CT in all exponentially growing tumor and normal cells, including leukemic and endothelial cells (Zerp et al., 2008). Synthesis of PC is essential for cell proliferation and is upregulated in tumor cells. PC is not only the most abundant membrane lipid and crucial for new membrane formation, but also the precursor for the second messengers diacylglycerol (DAG) and phosphatidic acid (PA) and for

sphingomyelin (SM) in membrane lipid rafts. Inhibition of PC biosynthesis blocks the downstream sphingomyeline synthase (SMS) that catalyzes synthesis of sphingomyelin and diacylglycerol in the trans-Golgi (van Blitterswijk et al., 2003). Possible consequence is the accumulation of the ceramide, which is a second SMS substrate and can trigger apoptosis (Wieder et al., 1998). Another consequence of the PC shortage is the oxidative stress with reactive oxygen species (ROS) formation (Smets et al., 1999; Vrablic et al., 2001; Wagner et al., 1993).

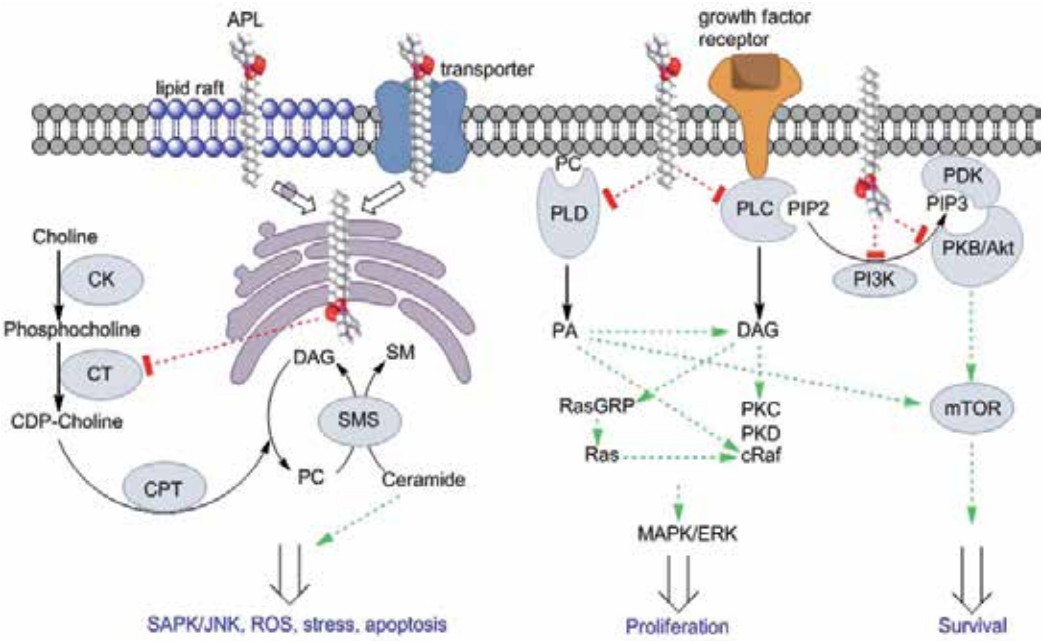

Fig. 2. Alkylphospholipid targets in lipid metabolism and signalling pathways summarized after van Blitterswijk et al. (van Blitterswijk & Verheij, 2008).

3.3 Influence of alkylphospholipids on major signaling pathways

Alkylphospholipids interfere with phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB)/Akt survival pathway, which is important for proliferation, differentiation, survival and intracellular trafficking (Fig. 2). They inhibit phosphorylation and recruitment of PKB/Akt to the membrane, which is essential for its activation (Elrod et al., 2007; Kondapaka et al., 2003; Rahmani et al., 2005; Tazzari et al., 2008) probably by decreased production of PIP_3 (Gills & Dennis, 2009; van Blitterswijk & Verheij, 2008).

Alkylphospholipids inhibit PC hydrolysis to phosphatidic acid (PA) by phospholipase D and further to diacylgycerol (DAG) (Kiss & Crilly, 1997; Lucas et al., 2001). PA and DAG are second messengers, essential for the mitogen-activated protein kinase (MAPK) pathways, which regulate mitosis, metabolism, survival, apoptosis and differentiation (Chen et al., 2001; Kyriakis & Avruch, 2001; Pearson et al., 2001). PA is also involved in the activation of protein kinase C-ζ (Limatola et al., 1994), mTOR (Fang et al., 2001) and c-Raf (Rizzo et al., 2000). DAG activates proteins with the C1 domain, such as protein kinases C and D, Ras guanine-releasing protein (RasGRP) and indirectly MAPK/ERK pathway (Carrasco &

Merida, 2007; van Dijk et al., 1997; Yang & Kazanietz, 2003). Alkylphospholipids also inhibit DAG formation by phospholipase C (Maly et al., 1995; Ruiter et al., 2001; Strassheim et al., 2000). Alkylphospholipids also activate the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) pathway responsible for apoptosis in tumor cells (Gajate et al., 1998; Nieto-Miguel et al., 2007; Nieto-Miguel et al., 2008; Nieto-Miguel et al., 2006; Ruiter et al., 2001; Ruiter et al., 1999). Apoptosis can be triggered by imbalance between apoptotic and survival signals (Ruiter et al., 2001; Ruiter et al., 1999), which can be influenced by alkylphospholipids, since they have an influence on cross-talk between several membrane dependent signaling pathways.

4. Interaction of free Perifosine (OPP) with breast cancer cells

In this chapter the investigation of the interaction of Perifosine (octadecyl(1,1-di-methyl-4-piperidinium-4-yl)phosphate – OPP), with ER positive (ER+) and ER negative (ER-) breast cancer cell lines is emphasized. Perifosine was chosen since it is one of the most cancerostatically active lipids, with strong antitumor effect on xenotransplanted human breast cancer. We summarize results obtained mostly by the electron paramagnetic resonance (EPR) method in order to measure the influence of Perifosine on cell membrane fluidity and to measure transport of free and liposome incorporated Perifosine into breast cancer cells.

4.1 Influence of Perifosine (OPP) on cell membrane fluidity as studied by EPR

To get the information about the influence of a biologically active substance on cell membrane by EPR it is necessary to introduce a lipophilic paramagnetic probe into the membrane bilayer. This so called spin probe serves as a marker, which reflects the motion of the alkyl chains in the vicinity of the nitroxide group of the spin probe and in this way gives information about its surrounding. Motional characteristics that determine membrane fluidity are reflected in the EPR spectra line-shape. Main parameters obtained directly from the line-shape of the EPR spectra are order parameter (S) and correlation time (τ_c). Order parameter describes the orientational order of the phospholipids alkyl chains with S = 1 for perfectly ordered chains and S = 0 for isotropic alignment, and rotational correlation time (τ_c) describes the dynamics of the spin probe motion; more fluid membranes are characterized by a small τ_c. The changes in the EPR spectra line-shape give direct information about the external influences (temperature, interactions, damages) on cell membrane fluidity. More exact information about the membrane alterations can be obtained by computer simulation of the EPR spectra taking into account that the membrane is heterogeneous, composed of several coexisting domains with different fluidity characteristics. Therefore the EPR spectrum is composed of several spectral components reflecting different modes of restricted rotational motion of the spin probe molecules in different membrane environments (Pabst et al., 2007; Stopar et al., 2006; Strancar et al., 2003; Strancar et al., 2005).

In order to see how Perifosine (OPP) influences the plasma membrane fluidity of ER+ MCF7 and ER- MT-3 breast cancer cells, the cells were labeled with the spin probe 5P. This is a spin labeled OPP (5P), containing the nitroxide group at the 5th C atom (counting from the polar head group), (Mravljak et al., 2005). Structural formula of 5P is shown in Fig. 3.

It enters the plasma membrane easily but due to its charge, it only slowly crosses from outer to inner side of cell membrane, therefore it is suitable spin probe for detecting changes in the properties of the outer layer of plasma membrane. For spin labeling of cell membranes, MCF7 and MT-3 cells were mixed with 5P (2 μM) as a spin probe and with different amounts of Perifosine (OPP) to achieve final concentrations of OPP in extracellular medium: 0 μM, 25 μM, 50 μM or 150 μM.

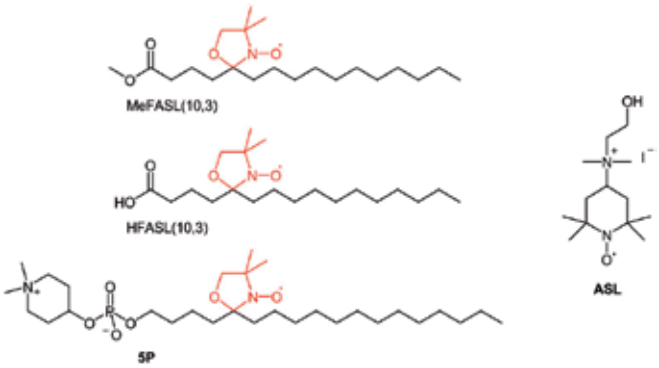

Fig. 3. Structural formulas of spin probes. MeFASL(10,3) – 5-doxylpalmitoyl methylester; HFASL(10,3) – 5-doxyl-palmitic acid, ASL - spin labeled tempocholine; 5P – spin labeled OPP, containing the doxyl group at the 5th C atom (counting from the polar head group).

From the spectra the order parameter was calculated. In the absence of OPP, S = 0.66 for MT-3 and S = 0.68 for MCF7 cells were obtained. After addition of 150 μM OPP, the order parameter decreased to S = 0.57 for MT-3 and to 0.60 for MCF7 cells. At lower concentrations of Perifosine no significant differences in order parameter were observed. This result indicates that OPP increases membrane fluidity of both cell lines at concentrations higher than 50 μM. The influence of OPP is less pronounced for MCF7 as for MT-3 cells. This indicates that OPP either doesn't incorporate into the alkylphospholipid resistant, ER+ MCF7 cell membranes as well as into alkylphospholipid sensitive, ER- MT-3 cells, or it doesn't concentrate in plasma membrane of MCF7 cells at such high concentrations as it does in MT-3 cells.

4.2 Transport of Perifosine (OPP) into the cell

To get information about the transport of Perifosine (OPP) into breast cancer cells by EPR, several spin labeled OPPs were synthesized in our group (Mravljak et al., 2005), which have an EPR detectable nitroxide group at various positions along the alkyl chain. We have chosen spin labeled OPP (5P) (structural formula in Fig. 3) with the lowest critical micellar concentration (CMC) of all synthesized spin labeled OPPs (Mravljak et al., 2005). Its CMC is around 10 μM, while 14P (spin labeled OPP, containing the nitroxide group at the 14th C atom) exhibited the CMC of around 200 μM (Mravljak et al., 2005). Edelfosine and OPP disperse in water in the form of micelles, due to their inverted-cone shape (Busto et al., 2007), displaying a CMC at 2.5 - 3 μM (Rakotomanga et al., 2004). It appears that addition of the doxyl group to OPP distorts the inverted-cone shape of molecule to greater extent when it is placed further away from the polar head group, which results in increased CMC. Since the CMC of 5P is similar to CMC of other similar alkylphospholipids one can assume that

the disturbance caused by attaching doxyl group to OPP is small. Therefore we asumed that from all of synthesized spin labeled OPPs, 5P is the best candidate as a model molecule for studying behavior of Perifosine (OPP).

When 5P is transported into the cell, the EPR spectra intensity decreases due to the reduction of the nitroxide group to the corresponding EPR non-visible hydroxylamine by oxy-redoxy systems inside the cells (Chen et al., 1988; Swartz et al., 1986; Ueda et al., 2003) and can be detected by measuring the amplitude of the middle line of EPR spectra with time. From the kinetics of EPR spectra intensity decrease, information about the transport and/or interaction of spin probe with cells can be obtained. Reduction kinetics of 5P was found to be much slower as for spin probes usually used in EPR investigations of cell membranes MeFASL(10,3) and HFASL(10,3) (Chen et al., 1988; Yonar et al., 2010) indicating that its transport into the cell cytoplasm and organelles is slower as for the other probes. This is not surprising due to the charge at the head group of 5P (Fig. 3), which prevents passive transport of OPP across the membrane. For human KB carcinoma cells it has been demonstrated that OPP is internalized by an ATP-dependent translocase activity across the plasma membrane (Munoz-Martinez et al., 2008).

In order to investigate whether there is a difference in the uptake of OPP by alkylphospholipid (APL) resistant MCF7 cells (estrogen receptor positive, ER+) and APL sensitive MT-3 cells (estrogen receptor negative, ER-), both cell lines were incubated with 5P and EPR spectra intensity decrease was measured with time after incubation (Fig. 4).

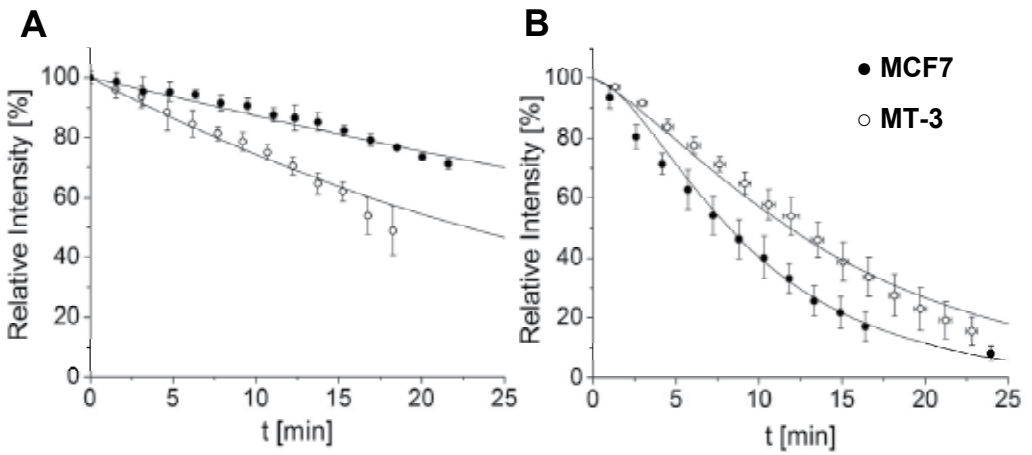

Fig. 4. EPR spectra intensity decrease with time after incubation of MCF7 cells (●), and MT-3 (○) cells with 5P at A) room temperature and B) 37 °C MCF7 (estrogen receptor positive) and MT-3 (estrogen receptor negative) breast cancer cells (5-7 x 10^6 MCF7 cells and 12-20 x 10^6 MT-3 cells) were incubated with 5P (2-3 μM concentration, depending on the estimated number of total cell membrane bilayer lipids), which was adsorbed to the wall of a glass tube in order to achieve gradual accumulation of OPP in cells during 10 min incubation at room temperature and EPR spectra intensity decrease was measured with time after incubation.

From the kinetics of EPR spectra intensity decrease (Fig. 4) the rate of transfer of spin labeled OPP (5P) across the cell membrane was calculated using a similar model as

described previously (Koklic et al., 2008). The rate of nitroxide reduction inside the cells does not differ significantly between the two cell lines and is faster at physiological temperature as at room temperature, while by increasing the temperature from room to physiological temperature the rate of transfer remains in the range of error for MT-3 cells (estrogen receptor negative), but increases significantly for estrogen receptor positive MCF7 cells (Podlipec et al., manuscript in preparation).

5. Interaction of liposomal Perifosine (OPP) with breast cancer cells

5.1 Effect of supramolecular organization of liposomal OPP formulations on their interaction with breast cancer cells

Alkylphospholipids are amphiphilic molecules and usually form micelles under physiological conditions. Unfortunately, administration of free (micellar) alkylphospholipids results in unwanted side effects, reflected in gastrointestinal toxicity and hemolytic activity, which limits the application of higher doses of alkylphospholipids. To achieve better therapeutic effects of alkylphospholipids *in vivo* with less side effects, different liposomal formulations of alkylphospholipids were prepared. This is possible only in the presence of lipids or other amphiphiles with a complimentary molecular shape. Usually cholesterol fulfills this role and enables the preparation of stable liposomal formulations from alkylphospholipids and lipids of different chain length and head groups. Among different alkylphospholipids, most investigations with liposomal formulations were performed with Perifosine (OPP). *In vivo* data show that the hemolytic effect of OPP is significantly diminished in liposomal formulations, but unfortunately in most cases, cytotoxic activity of OPP liposomes was also lower than of free OPP (Zeisig et al., 1998).

In an early study (Zeisig et al., 2001) we investigated the influence of cholesterol in liposomes consisting of Perifosine (OPP), dicetylphosphate and cholesterol (CH) on liposome stability and in vitro cytotoxicity. It was found that the ratio between the alkylphospholipid and cholesterol affects the cytotoxicity of the liposomes (Table 2). An increase in the OPP/CH ratio correlated directly with an increase in cytotoxicity against breast cancer cells. In the same time it was shown that a portion of 10 – 30% of OPP was present as micelles in liposomal formulations with OPP/CH ratio between 10:10 and 10:5, while the remaining OPP was stabilised by CH and forms liposomes. This was concluded, using ^{1}H-NMR spectroscopy, by the analysis of lipid composition after centrifugation of liposomal formulations, where micelles remain in supernatant in comparison to the initial sample. This micellar part of OPP molecules can easily be exchanged with the external environment and is able to become incorporated into other (bi)layers, as monolayer incorporation experiments demonstrated. It was assumed that this part of OPP is also mainly responsible for the cytotoxicity against tumor cells, which are not able to internalize the vesicles very well (Zeisig et al., 2001). A similar composition dependent effect was found *in vivo*, when the hemolytic effect of differently composed liposomes was followed. Again, liposomes with higher OPP/CH ratio, and thus containing a higher proportion of micellar OPP, were more hemolytically active than liposomal OPP formulations with a lower CH content (Zeisig et al., 1998).

Recently we developed a new method achieving more accurate estimates of the relative proportion of micelles, in comparison to the previously used methods (Koklic et al., 2010). The method is based on the spectral decomposition of EPR spectra. We confirmed findings of previous studies, which showed that the amount of micelles in liposomal OPP formulations increases with decreasing amount of cholesterol (Table 2).

According to the results presented in Table 2 we concluded that the amount of micelles in OPP liposome formulations is too small to be the main reason for better efficiency of liposomes with low amount of cholesterol in experimental breast cancer therapy (Koklic et al., 2010). Therefore we proposed that better efficiency of liposomes with lower amount of cholesterol depends also on the physical and chemical characteristics of liposome membranes and their interaction with cells.

Code	Molar ratio OPP:CH:X:PEG	OPP*	CH*	X*	Micelles** (%)	Hemolysis increase# (%)	Cytotoxicity $IC_{50\ MT\text{-}3}$#
N5 PEG	10:5:2:1	55.6	27.8	11.1	18 ± 7	179 ±23	23 ± 1
N5	10:5:2:0	58.8	29.4	11.8	20 ± 9	126 ± 30	18 ± 3
N7.5	10:7.5:2:0	51.3	38.5	10.2	11 ± 4	147 ± 32	18 ± 3
N10	10:10:2:0	45.5	45.5	9	5 ± 2	127 ± 29	28 ± 5
P10	10:10:2:0	45.5	45.5	9	0.5 ± 0.6	nd	19 ±2
N15	10:15:2:0	37.0	55.6	7.4	0 ± 1	nd	n.d.
Micelles	100:0:0:0	100	0	0	100	248 ± 11	17 ±9

N and P denote charge of the formulation (- or +, respectively)
X is a charged compound (DCP (dicetylphosphate) for N formulations and DDAB (dimethylioctadecylammonium bromide) for P formulation)
PEG are stearically stabilized liposomes with PEG_{2000}DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[cyanur(polyethylene glycol)-2000])
* mol% of total lipids
** reference (Koklic et al., 2010)
\# IC_{50} concentration (μM) required for 50% inhibition of cell growth for MT-3 breast cancer cells, (Zeisig et al., 1998)

Table 2. Composition of OPP liposomes, relative portion of micelles obtained by EPR and their hemolytic and cytotoxic activity.

5.2 Membrane domain structure of liposomal OPP formulations

For a better understanding of the interaction between liposomal OPP formulations and tumor cells, a deeper understanding of liposomal bilayer organization is necessary. Therefore, EPR with spin labels was used to study the influence of cholesterol, charge and sterical stabilization by PEG_{2000} DSPE on physical and chemical characteristics of liposomal OPP formulations. For this purpose liposomes with the composition presented in Table 2 were spin labeled with 5-doxylpalmitoyl methyl ester (MeFASL(10,3), Fig. 3) and EPR spectra were measured. By computer simulation of the EPR spectra line-shape, information about membrane fluidity and membrane domain structure was obtained, taking into account that the membrane is heterogeneous, composed of regions with different fluidity characteristics (Koklic et al., 2002; Koklic et al., 2008). Typical spectra are presented in Fig. 5A.

It was found that in general the experimental spectra are composed of at least three spectral components. Each spectral component corresponds to a mode of motion of a portion of spin probes partitioned in different parts of the membrane with the same physical properties and characterizes a certain type of lateral membrane domains with different fluidity characteristics. EPR parameters (order parameter S, rotational correlation time τ_c , polarity correction factor p_A), which describe the motional modes of nitroxide in a certain domain

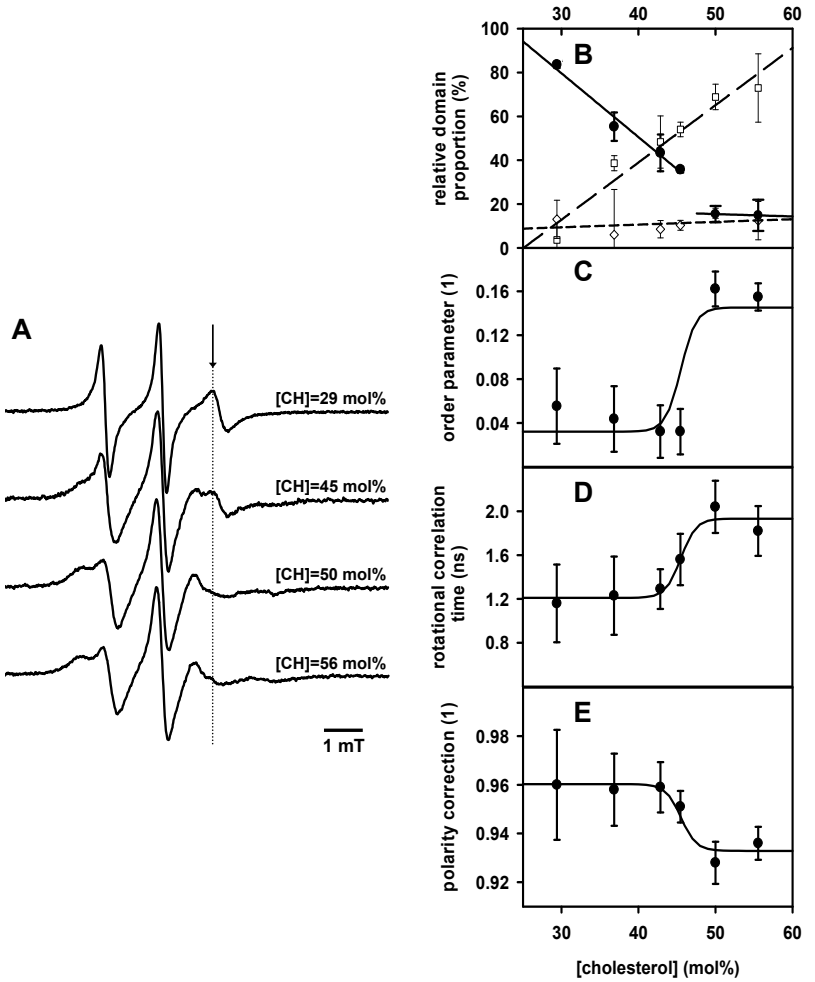

Fig. 5. A) EPR spectra of lipophilic spin-probe methyl ester of 5-doxylpalmitate (MeFASL(10,3)) in the membrane of OPP liposomes with different concentrations of cholesterol (the amount of cholesterol is indicated in mol%) in PBS buffer at 39 °C. The arrow points to a peak, which vanishes at around 50 mol% of cholesterol in the liposome membrane. B) - E) Dependence of EPR spectral parameters of spectral components on cholesterol concentration ([cholesterol]). Spectral parameters of EPR spectra of spin-probe MeFASL(10,3) in membranes of liposomal OPP formulations were derived by fitting of the calculated to the experimental spectra. B) Relative proportions of spectral components with: the lowest order parameter – domain type 1 (●); middle order parameter – domain type 2 (◊); and the highest order parameter – domain type 3 (□). Solid black line is a linear fit to the relative proportion of domain type 1, C) Order parameter, D) rotational correlation time, and E) polarity correction factor of the less ordered domain type (domain type 1). (republished with permission from (Koklic et al., 2008)).

type and reflect the fluidity characteristics of the domains as well as the proportion of spin probes in each domain type were determined. They were found to depend mainly on the amount of cholesterol, and only to a minor part on charge and sterical stabilization (Koklic

et al., 2002). Dependance of EPR parameters, reflecting the properties of the least ordered domain type on cholesterol concentration is presented in Fig. 5 B, C, and D. A sudden increase in order parameter and rotational correlation time was observed when cholesterol concentration increases from 45 mol% to 50 mol%, while at the same time the polarity correction factor decreased, indicating that the spin probes in the domain type with lowest order parameter are less accessible to water. Relative proportion of this domain type (Fig. 5B) decreases at higher cholesterol concentrations, whereas the relative proportion of the domain type with the highest order parameter increases. It seems that above 50 mol% cholesterol the least ordered domains are transformed into a new type of domains with higher order parameter (S = 0.15) and with proportion of 15 %.

5.3 Release of liposome encapsulated material during the interaction of Perifosine (OPP) liposomal formulations with breast cancer cells

In order to better understand the factors that determine the therapeutic activity of liposomal OPP formulations, the interaction of liposomal OPP formulations at different cholesterol/Perifosine (CH/OPP) ratios with MT-3 and MCF7 breast cancer cells was measured and correlated with the membrane domain structure of liposomal OPP formulations (Koklic et al., 2008). For this purpose, spin labeled tempocholine (ASL) (Fig. 3), which cannot penetrate an intact liposome membrane easily, was entrapped into the liposomes. Labeled liposomal formulations were mixed with the cells and the kinetics of ASL reduction in the presence of human breast cancer cells was measured by EPR. ASL gets reduced to EPR non-visible hydroxylamine when it is released from liposomes and exposed to the oxy-redoxy systems inside the cells (Chen et al., 1988; Swartz et al., 1986; Ueda et al., 2003), which is reflected in an EPR spectra intensity decrease. Therefore, from the kinetics of EPR spectra intensity decrease information about the interaction of liposomes with cells can be obtained. Results are presented in Fig. 6.

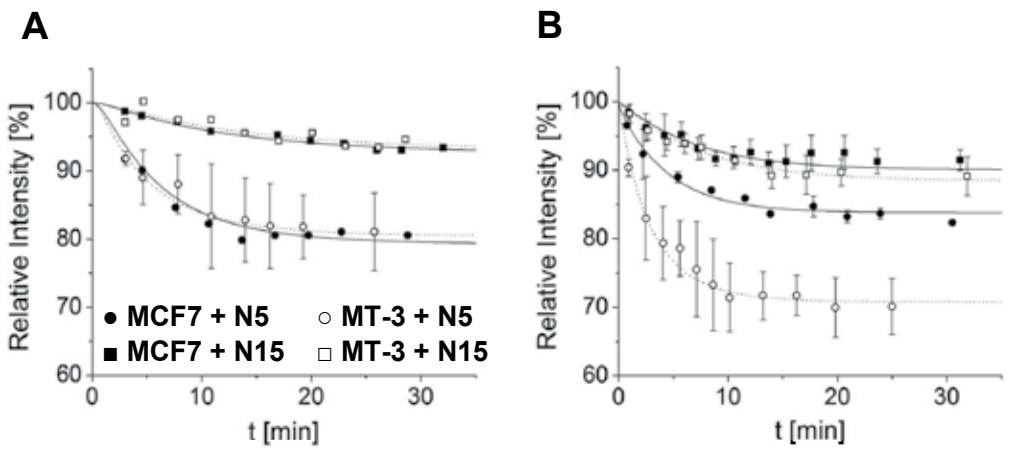

Fig. 6. EPR spectra intensity decrease after mixing of MCF7 cells (closed signs) or MT-3 cells (open signs) with Perifosine (OPP) liposomal formulations with two concentrations of cholesterol: 29 mol% (N5) (circles) and 56 mol% (N15) (squares) at A) room temperature and B) 37 °C. Symbols represent mean values of two to three measurements with error bars representing standard deviations.

For liposomal OPP formulation with low cholesterol content N5 (circles) a fast decrease of the EPR signal was observed in first 10 minutes after mixing liposomes with cells (Fig. 6), indicating that about 30% of spin-probes were released fast from the liposome interior into the cell cytoplasm. On the other hand, for liposomal OPP formulation with high cholesterol content N15 (Fig. 6 sguares), only a very small amount of liposome entrapped ASL was released into the cells, since the intensity decrease was less than 10% at room and physiological temperature. This indicates that the liposomes remained intact either in the extracellular space or entered the cells by endocythosis, but remained intact at least for the time of measurement. It is important to note that at room temperature both cell lines behave similarly, while at physiological temperature significantly higher amount of liposomes with low CH (N5) interact with alkylphospholipid sensitive, estrogen receptor negative, MT-3 cells (open circles in Fig. 6B) than with alkylphospholipid resistant, estrogen receptor positive, MCF7 cells (Podlipec et al., manuscript in preparation). These results, obtained on trypsinated cells, which are presented here, agree well with the results published by Koklic et al. (Koklic et al., 2008), which were obtained on scraped MT-3 cells, although small differences could originate from different procedures of removal of cells from the culture flasks (Batista et al., 2010).

In order to investigate interaction of OPP liposomes with breast cancer cells in more detail, we have added 0.5 mol% of a phospholipid fluorescent probe C6-NBD-PC, where 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) is attached to the phosphatidylcholine phospholipid (16:0-06:0 NBD PC, Avanti polar lipids, Alabaster, AL, USA) to OPP liposomes as described previously (Arsov et al., 2011). Results with the lipophilic fluorescence probe C6-NBD-PC (Fig. 7) confirm the EPR measurements, indicating that liposomes with high amount of

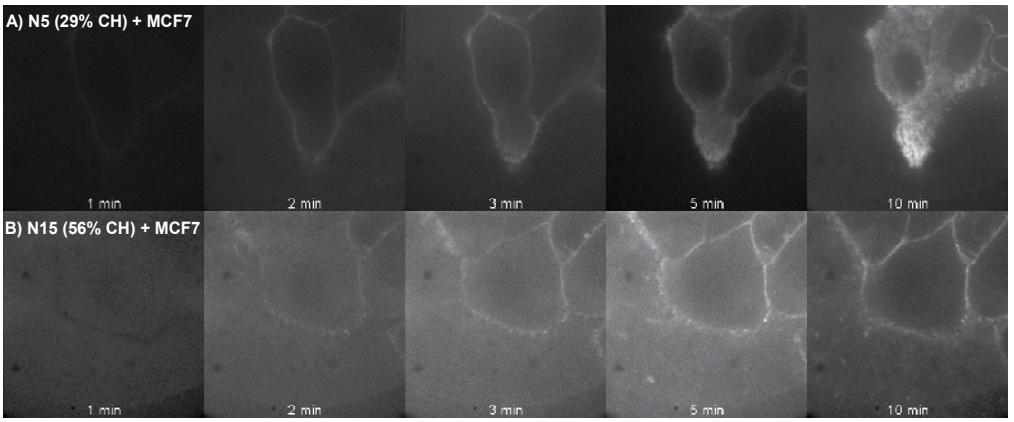

Fig. 7. Interaction of fluorescently labeled Perifosine (OPP) liposomal formulations with MCF7 breast cancer cells. Fluorescence microscopy was performed to localize C6-NBD-PC labeled liposomal formulation immediately after addition to cells, at room temperature.
A) Negatively charged liposomal OPP formulation (N5) with 29 mol % of cholesterol (1 mM final total lipid concentration) were added to MCF7 cells attached to the bottom of a well and the distribution of the lipophilic fluorescent probe C6-NBD-PC was followed with time as indicated at the bottom of each image.
B) Negatively charged liposomal OPP formulation (N15) with 56 mol % cholesterol were added to MCF7 cells and measured under same conditions as in experiment A.

cholesterol do not interact with cells. Fluorescence microscopy clearly shows that OPP liposomes with high amount of CH (N15) remain outside the cells, while low cholesterol – containing, N5 liposomes interact with cell membranes because the fluorescent probe distributes in the cell interior. In addition for liposomes with low amount of cholesterol (N5), for which C6-NBD-PC distributes inside the cells, maximum of fluorescence emission spectrum shifts for a few nanometers, indicating that either micelles or liposomes interacted with cells and delivered C6-NBD-PC into lipophilic compartments of MCF7 cells, where the environment of the fluorescent probe changed (Arsov et al., 2011).

Comparing liposome membrane characteristics, derived from EPR spectra (Fig. 5) and summarized in Table 2, with liposome cell interaction experiments (Fig. 6 and Fig. 7) we can see that the propensity of Perifosine (OPP) liposomal formulations for interaction with tumor cells and for delivery of OPP into cells coincides with the existence of disordered domains as well as with the existence of micelles. We have shown that by increasing the concentration of cholesterol above 50 mol% the domains with the lowest order parameter (between 0.06 and 0.03, Fig. 5) are transformed into a new type of domains with higher order parameter (S = 0.15). This suggests that disordered motion of lipid alkyl chains within the liquid-disordered domains, which coexist with liquid-ordered domains, is necessary for fast delivery of liposome encapsulated probe into the cells. On the other hand, the presence of micelles in liposome formulations with concentration of cholesterol below approximately 50 mol% suggests that micelles are necessary for efficient delivery of liposome encapsulated probe into cells.

5.4 Transport of spin labeled Perifosine (OPP) from liposomes to cell membrane does not depend on the liposome cell interaction

In order to get information about the rate of transfer of OPP from liposomes containing two different concentrations of cholesterol to cells, a portion of OPP molecules in liposomal OPP formulations was replaced with spin labeled OPP (5P), so that the final concentration of 5P in OPP liposomes was 17 mol%. Because of such high amount of P5 in the liposomal formulations, the EPR lines are highly broadened (Fig. 8A) due to the spin exchange interaction between paramagnetic probes.

EPR spectra in Fig. 8B are similar as obtained for MCF7 cells labeled with 5P, indicating that high amount of 5P from liposomal OPP formulation was transferred to cell membranes in a time shorter than 2 minutes. It was not possible to resolve any difference in the rate of transfer from N5 or N15 liposomes after 2 minutes of mixing liposomes with cells. Very fast transfer was also observed when giant liposomes (composition: POPC:POPE:POPS:CH molar ratios 40:20:10:40), which represent a model for cell membrane, were incubated with N5 or N15 liposomes (Mravljak et al., 2010), proving that spin labeled OPP molecules are transferred from one type of membrane to other membranes within several minutes, and the rate of transport does not depend significantly on the membrane composition. We can conclude from the above experiment that analkylphospholipid-like molecule can easily exchange between membranes and can accumulate in cells when they are in contact with liposomal OPP formulations. This is in agreement with lipid monolayer experiments, which showed that alkylphospholipids, below the critical micellar concentration (CMC), insert progressively into lipid monolayers as monomers from the aqueous medium, while above CMC, not only monomers but also groups of monomers (micelles) are transferred into the monolayers (Rakotomanga et al., 2004). It was also shown that, while the alkylphospholipid HePC is miscible with POPC, there is high affinity between HePC and sterols (ergosterol, and cholesterol) and that maximum

condensation is reached at a ratio of HePC/sterol around 50:50 (mol/mol) (Rakotomanga et al., 2004). This kind of behavior is generally known as the condensing effect of cholesterol towards phospholipids (Chapman et al., 1969; Ghosh & Tinoco, 1972). Micelles constituted a reservoir of monomers both for monomer insertion between condensed phospholipids and for insertion of groups of monomers between fluid phospholipids. Since biological membranes are composed of dynamically condensed domains surrounded by fluid domains, it has been suggested that, above the CMC, alkylphospholipids can insert into both kinds of phases: as monomers into the condensed phase and as a group of monomers into the fluid phase. (Rakotomanga et al., 2005). The presence of albumin in the medium has the effect of increasing the CMC value by binding molecules of lipids and, hence, reducing the concentration of free monomers in the medium (Kim et al., 2007). Like albumin acts as an alkylphospholipid reservoir - it binds reversibly to the cell surface and may release the drug gradually - the role of liposomes seems to be similar.

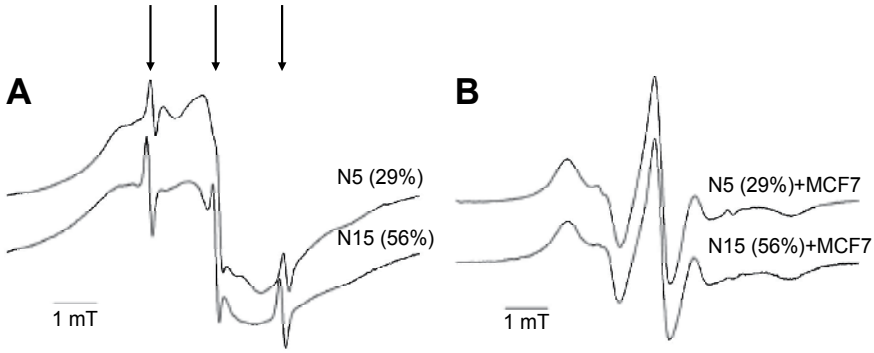

Fig. 8. EPR spectra of lipophilic spin-probe – spin labeled OPP (5P)
A) in the membrane of liposomal OPP formulation with different concentrations of cholesterol (the amount of cholesterol is indicated in mol%) in PBS buffer at room temperature. The arrows point to peaks corresponding to free 5P, which is neither incorporated in liposomes, neither in micelles. A broad spectrum corresponds to supramolecular structures of OPP (liposomes and micelles);
B) in the membrane of MCF7 cells. Spectra were recorded 2 minutes after mixing of spin labeled OPP liposomes (1.5 μL) with pellet of MCF7 cells (1.5 μL with 5 -10 x 10^6 cells).

At first glance these results are in stark contrast with the experiments with fluorescently labeled liposomal OPP formulations (Fig. 7) and with OPP liposomes with the entrapped hydrophilic probe (Fig. 6), since those experiments suggest that OPP liposomes with high amount of cholesterol almost do not interact with breast cancer cells. Fast transfer of OPP from liposomes to other membranes would lead to destabilization of liposomes, which was not the case for N15 liposomes with high amount of CH. It seems that OPP differs from spin labeled OPP (5P), which is not surprising with respect to the doxyl group attached to the alkyl chain, which probably prevents the condensing effect of cholesterol.

6. Summary of differences between MT-3 and MCF7 breast cancer cell lines

Main differences between OPP sensitive MT-3 and OPP resistant MCF7 cells are:

1. Plasma membrane fluidity is slightly larger for estrogen receptor negative (ER-) MT-3 as for estrogen receptor positive (ER+) MCF7.

2. OPP increases plasma membrane fluidity of both cell lines at concentrations higher than 50 μM. The influence of Perifosine (OPP) is less pronounced for MCF7 as for MT-3 cells. This indicates that OPP either doesn't incorporate into alkylphospholipid resistant, ER+ MCF7 cells as well as it incorporates into alkylphospholipid sensitive, ER- MT-3 cells, or it doesn't concentrate in plasma membrane of MCF7 cells at such high concentrations as it does in MT-3 cells.
3. Transport of alkylphospholipids across plasma membrane and subsequent reduction in breast cancer cells (Fig. 4) showed that the transport and the reduction of spin labeled OPP is faster for MT-3 than for MCF7 cells at room temperature, whereas it is just the opposite at physiological temperature. The main difference between MCF7 and MT-3 cells is the transport of OPP across the plasma membrane, which increases significantly for MCF7 cells at physiologic temperature, but remains almost unchanged for MT-3 cells. Because of this we suspect that OPP uptake by OPP resistant MCF7 cells might be mediated, similarly as in the case of KB carcinoma cells (Vink et al., 2007), by a lipid transporter. This observation could explain lower influence of OPP on cell membrane fluidity of MCF7 cells and support the hypothesis that OPP doesn't concentrate in the plasma membrane of MCF7 cells.
4. Liposomal OPP formulations with low CH concentration (N5) quickly release a portion of their content when mixed with breast cancer cells. At room temperature the release is comparable for MT-3 and MCF7 cells (Fig. 6). However, at physiological temperature the amount of released content increases for OPP sensitive MT-3 cells, but remain in the same range for MCF7 cells.

7. Conclusions with respect to experimental breast cancer therapy with alkylphospholipids

For efficient application of liposomes as nanocarriers in breast cancer therapy it is not only necessary to investigate in detail the physical properties of the nanocarrier, which has to transport the drug to the (target) cell, but also the properties of the target cell. In the case of application of alkylphospholipids, where plasma membrane is the specific target, one has to know the properties of the plasma membrane and differences among membranes of different breast cancer cell lines. We have shown that plasma membrane of MT-3 cells is more fluid (lower order parameter) as the membrane of MCF7 cells and was influenced more by OPP. Besides, it should be taken into account also that the properties of plasma membrane depend on external factors. For example, it has been shown that confluent MT-3 breast cancer cells have significantly higher membrane fluidity and higher relative proportion of disordered membrane domains as compared to cells harvested during exponential growth (Koklic et al., 2005). The fluidity of plasma membrane of MT-3 breast cancer cells also has an important role in metastasis development. The increase in membrane fluidity of MT-3 breast cancer cells was correlated with 2-fold increase in sialyl Lewis X and/or A ligand-mediated adhesion of these cells and a higher motility of ligands in the membrane of confluent cells, together with an accumulation of these ligands in distinct areas on a cell membrane (Zeisig et al., 2007).

In order to better understand the interaction of OPP micelles or liposomes with breast cancer cells, one has to take into account the following main characteristics of alkylphospholipids and of liposomal formulations:

1. In mouse S49 lymphoma cells, alkylphospholipids accumulate in detergent-resistant, sphingolipid- and cholesterol-enriched lipid raft domains and are rapidly internalized by clathrin-independent, raft-mediated endocytosis (van der Luit et al., 2007).
2. Alkylphospholipid uptake in KB carcinoma cells appears to be raft-independent and mediated by a yet unidentified ATP-dependent lipid transporter (Vink et al., 2007).
3. Lipid monolayer experiments showed that alkylphospholipids, below the critical micellar concentration (CMC), insert progressively into lipid monolayers as monomers from the aqueous medium, while above CMC, not only monomers but also groups of monomers (micelles) are transferred into the monolayers (Rakotomanga et al., 2004).
4. While alkylphospholipid HePC is miscible with POPC, there is high affinity between HePC and sterols (ergosterol, and cholesterol) and that maximum condensation is reached at a ratio of HePC/sterol around 50:50 (mol/mol) (Rakotomanga et al., 2004). This kind of behavior is generally known as the condensing effect of cholesterol towards phospholipids (Chapman et al., 1969; Ghosh & Tinoco, 1972).
5. Alkylphospholipid OPP increases membrane fluidity of both MCF7 and MT-3 cell line at concentrations higher than 50 μM, indicating that OPP incorporates into the membrane of breast cancer cells and is slowly transferred into the cell interior as it was detected by reduction kinetics of spin labeled OPP (Fig. 4). OPP transfer increases at physiologic temperature for OPP resistant MCF7 cells, but remains almost the same for OPP sensitive MT-3 cells. We believe that OPP uptake by OPP resistant MCF7 cells might be mediated, similarly as in the case of KB carcinoma cells (Vink et al., 2007), by a lipid transporter.
6. Liposomal OPP formulations efficient in experimental breast cancer therapy should have cholesterol concentration below 50 mol%.
7. *In vivo* data show that hemolytic effects of liposomal OPP formulations is diminished as compared to free OPP, but cytotoxic activity of liposomal formulations is also lower (Zeisig et al., 1998).
8. Liposomal formulations with lower cholesterol/OPP ratio containing higher proportion of micellar OPP, are hemolytically more active than liposomal formulations with lower cholesterol concentration (Zeisig et al., 1998). At approximately 55 mol% cholesterol liposomal formulations do not contain any OPP micelles (Koklic et al., 2010). While there is almost no release of content from liposomal OPP formulations with 56 mol% cholesterol for both cell lines, liposomal formulations with low cholesterol concentration quickly release a portion of their content when mixed with breast cancer cells (Fig. 6). Similarly lipid phase of liposomal OPP formulations with low cholesterol concentration quickly enters and crosses plasma membrane of OPP resistant MCF7 cells, but remains outside of cells in the case of liposomal formulations with 56 mol% cholesterol, as measured by C6-NBD-PC labeled liposomal formulations.
9. Fluidity characteristics of liposomal OPP formulations depend mainly on the amount of cholesterol, and only to a minor part on charge and sterical stabilization (Koklic et al., 2002). A sudden increase in order parameter of most disordered domain type occurs at cholesterol concentration around 50 mol%, while at the same time the polarity correction factor decreases, indicating that the spin probes are located in an environment less accessible to water.
10. Surface activity of an alkylphospholipid Edelfosine is decreased by other lipids, especially sterols, which indicates that Edelfosine is slowly released from the lipid mixture to the aqueous environment (Busto et al., 2008).

Based on all of the above mentioned properties, there are two competing hypothesis of OPP liposome formulation – breast cancer cell interaction, which still need further experimental validation. Either OPP liposome formulations with low cholesterol concentration are able to deliver OPP into breast cancer cells by fusing with plasma membrane of breast cancer cells, due to liposome membrane properties, or alkylphospholipids whether in free or micellar form insert with high affinity into cholesterol containing target membranes (cells) as long as liposomal carriers contain low amounts of cholesterol. Once the remaining liposomal carriers have cholesterol concentration above around 50 mol% the remaining alkylphospholipids are stabilized in liposomes and do not interact with cells anymore. In this way liposomes serve as a reservoir capable of releasing alkylphospholipids and preventing side effect associated with high alkylphospholipid concentrations.

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Part 5

Breast Cancer Cell Interaction, Invasion and Metastasis

18

The Mesenchymal-Like Phenotype of the MDA-MB-231 Cell Line

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1. Introduction

Mesenchymal stem cells (MSCs) are progenitor cells that can be isolated from all connective tissues such as bone, adipose, cartilage, blood and muscle (Wang *et al*., 2009). MSCs have recently been described to localise within breast carcinomas where the stem cells integrate into tumour-associated stromal tissues whereby the MSCs promote breast cancer cell invasion and metastasis (Karnoub *et al*., 2007). Previous studies have demonstrated that when combine with weakly metastatic human breast carcinoma cells, bone marrow-derived mesenchymal stem cells (BMSCs) increase the metastatic potency of the cancer cells greatly (Hombauer & Minguell, 2000). This phenomenon was significantly observed in MCF-7 cells where increase in cancer cell proliferation was observed when the cancer cells were co-cultured on the BMSCs feeder layer. Furthermore, light and epifluorescence microscopy studies revealed that the MCF-7 cluster grew in a dispersed fashion on the BMSCs feeder layer due to the decrease expression of adhesive molecules, such as E-cadherin and epithelial-specific antigen (ESA), in the cancer cells. The interaction between the MCF-7 cells and the BMSCs likely causes the loss of the adhesive molecules in the cancer cells. A phenomenon similar to this interaction was also observed in our recent study. Indeed, the study found that the growth of the MCF-7 cells was enhanced not only when the cancer cells were adhesively co-cultured with the BMSCs but also when they were co-cultured non-adhesively.

In the adhesive cell interaction, the growth or proliferation rate of the MCF-7 cells, which was measured by colony size, was observed to increase when the cancer cells were co-cultured on the BMSCs feeder layer (Fig. 1A and Fig. 1B). The non-adhesive interaction of the MCF-7 cells with BMSCs was also found to increase the growth of the cancer cells. When the cancer cells were incubated with the conditioned medium (culture supernatant) of the BMSCs, the proliferation rate of the MCF-7 cells increased approximately 16.6% when compared to the proliferation rate of the cancer cells incubated with growth medium only (Fig. 1C). This phenomenon indicates that the increase in the proliferation rate of the cancer cells due to the presence of the BMSCs must not be related to a direct physical cell–cell interaction, as similar findings are observed in both the adhesive and non-adhesive co-culture conditions.

Note: In this chapter, adhesive co-culture is defined as the growth of cancer cells on a non-tumorigenic cell monolayer where direct physical cell–cell interaction occurs. Non-adhesive

co-culture is defined as the incubation of cancer cells with a conditioned medium that is withdrawn from the non-tumorigenic cells; here, the cells interact with one another *via* the culture medium.

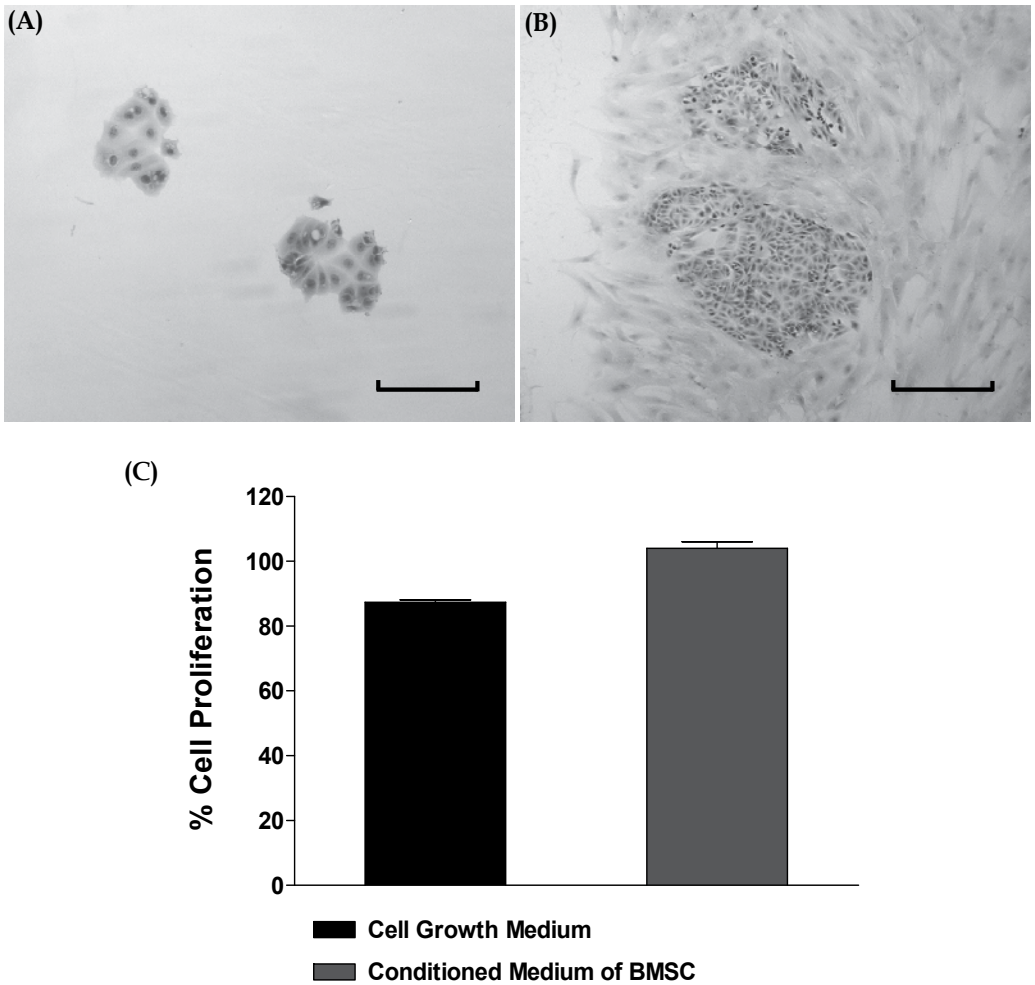

Fig. 1. Colony formation of the MCF-7 cells cultured with or without BMSCs. The pictures show Oil-Red-O staining of **(A)** the MCF-7 colonies alone and **(B)** the MCF-7 colonies grown on the BMSCs feeder layer for one week. The pictures were visualised under an inverted light microscope using same magnification. **(C)** The proliferation rate of the MCF-7 cells incubated with cell growth medium (control) and conditioned medium of BMSCs for one week. One hundred cells were used as the input prior to incubation. The values were expressed as mean±SD from three replicates, and the determination was carried out from three replicates each of three independent experiments.

The BMSCs likely secreted or influenced the MCF-7 cells to secrete certain soluble growth factors into the conditioned medium whereby the growth factors stimulated the MCF-7 cells in cluster and grow into single cell layer, after which the cells dispersed without any

evidence of direct cell–cell contact (Fig. 2). Thus, the influence of the BMSCs on the changed of cell morphology and increased proliferation rate of the MCF-7 cells may be achieved *via* the culture medium without the need for any direct physical cell–cell interaction between the two cell lines. However, this phenomenon, in which the BMSCs increased the proliferation rate of the breast cancer cells, was not observed when the BMSCs were co-cultured with highly invasive and metastatic human breast cancer cells, such as MDA-MB-231 cells. The MDA-MB-231 cell line likely contains its own source that similar to the MSCs as progenitor factor in the cell population that is able to secrete a standard level of the soluble growth factors into the conditioned medium of the MDA-MB-231 cells. Therefore, the activity of the MDA-MB-231 cells was not influenced by their exposure to the MSCs-conditioned medium and few effects were observed when the cells were co-cultured with the MSCs (Sasser *et al*., 2007a).

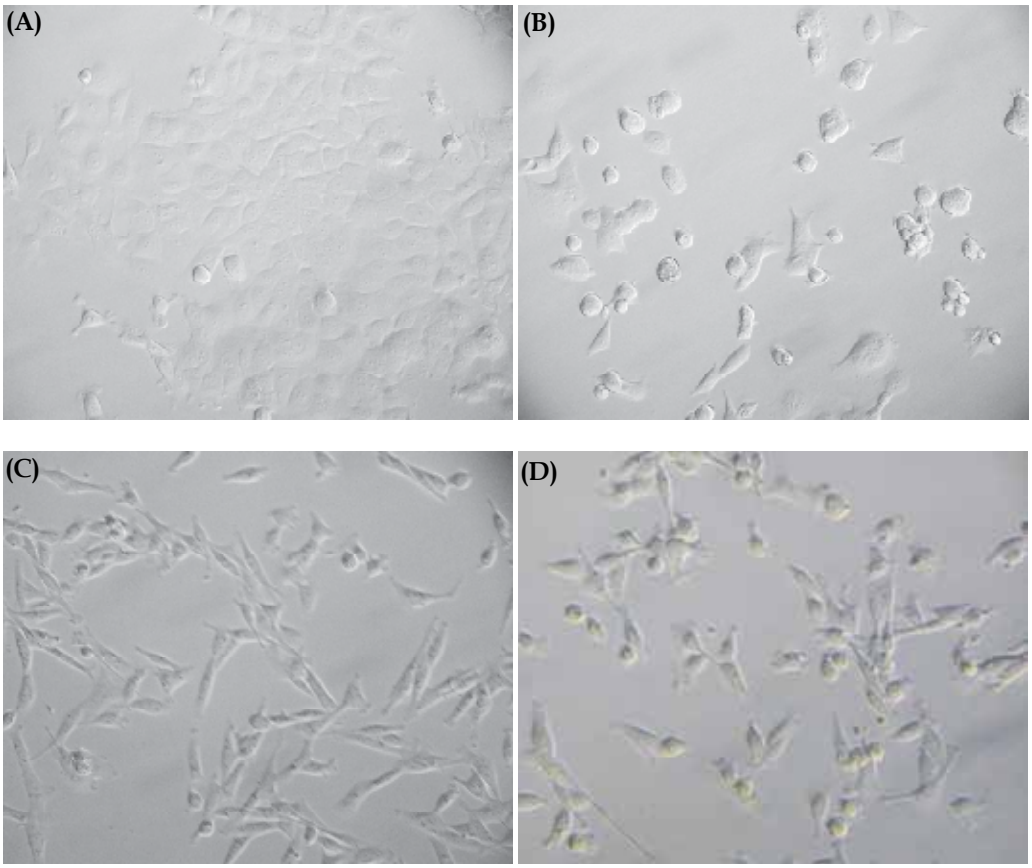

Fig. 2. Unstained MCF-7 and MDA-MB-231 cells as visualised under an inverted light microscope. Panels A and B show clustered and single cell layer of MCF-7 cells that were incubated with cell growth medium and BMSCs-conditioned medium, respectively, whereas panels **C** and **D** show few effects on cell morphology were observed when the MDA-MB-231 cells were incubated with cell growth medium and BMSCs-conditioned medium, respectively. The pictures were taken after one week of cell incubations.

2. Soluble growth factors in the conditioned medium of the MDA-MB-231 cells

2.1 Expression of MMPs in the conditioned medium

The MDA-MB-231 cell line is an estrogen receptor alpha (ERα)-negative human breast cancer cell line (Liu *et al.*, 2003). It was derived from a metastatic adenocarcinoma of the mammary gland of a 51-year-old Caucasian woman, according to the data sheet of the American Type Culture Collection (ATCC). This adherent epithelial cell line that likely contains more than one cell populations is a highly aggressive, invasive and poorly-differentiated human breast cancer cell line. Similar to other invasive cancer cell lines, the MDA-MB-231 cells display the invasiveness by mediating the proteolytic degradation of the extracellular matrix (ECM), including basement membrane and several mechanical barriers to the ECM, through the increased expression of matrix metalloproteinases (MMPs), including gelatinases, en route to their destinations (Fig. 3).

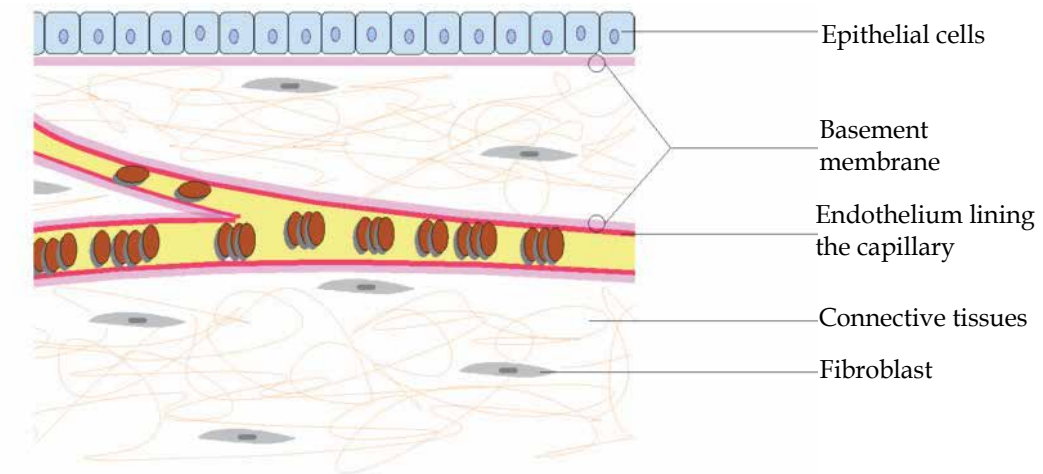

Fig. 3. Illustration depicts the ECM in relationship to the epithelium, endothelium and connective tissues. To reach their destination, the invasive cancer cells must penetrate the mechanical barriers of the ECM and basement membrane through proteolytic degradation. The figure was modified from the Wikimedia.

Type IV collagen, which is the main component of the basement membrane, is the first component that must be degraded to allow the invasion process (Boutaud *et al.*, 2000). The ability to degrade and penetrate the basement membrane is related with an increased potential of the cells for invasion and metastasis (Castro-Sanchez *et al.*, 2011). Tumour cells are able to produce MMPs that degrade the matrix barriers surrounding the tumour, including basement membrane, permitting invasion into connective tissues, entry and exit from blood and lymphatic vessels, and metastasis to distant organs. MMPs are family of zinc-dependent endopeptidases that collectively are capable of degrading all components of the ECM, including the basement membrane. Binding of breast cancer cells to type IV collagen in the basement membrane induces Discoidin domain receptor 1 (DDR1) activation and then it triggers signal transduction pathways and cellular processes that promotes secretion of MMPs which contributes to basement membrane degradation and cancer cell

invasion. A previous study demonstrated that gelatinase B or MMP-9, which degrades the type IV collagen in the basement membrane, plays a crucial role in the invasion process of the MDA-MB-231 cells (Liu *et al.*, 2003). This phenomenon can be observed by determining the metastatic potential of the MDA-MB-231 cells in an experimental model that is closely correlated with the expression of the MMP-9 and the activities of the gelatinases in the conditioned medium of the MDA-MB-231 cells. According to the study, the invasion of the MDA-MB-231 cells was blocked by MMP-9-neutralising antibodies that reduced the gelatinolytic activities in the conditioned medium, as detected using Enzyme-linked immunosorbent assay (ELISA). This phenomenon also led to the significant inhibition of the invasive capacities of the MDA-MB-231 cells. This inhibition was induced by specific drugs e.g., peroxisome proliferator-activated receptor gamma ligands and all-trans-retinoic acid that were administered on a reconstituted basement membrane in a Matrigel® chamber *in vitro*. Therefore, MMP-9 was shown to play a crucial role in the invasion process of the MDA-MB-231 cells and it was shown to be absolutely required for the transmigration of this cell line.

Note: In this chapter, conditioned medium is denoted as culture supernatant that is withdrawn from feeder layer. To accomplish this, a culture of feeder layer e.g., BMSCs is maintained with fresh growth medium. After certain duration, the growth medium is withdrawn from the feeder layer as conditioned medium. The conditioned medium is believed to contain growth factors released by the feeder layer.

2.2 Activation of STAT3 and soluble IL-6 in the conditioned medium

In addition to MMP-9 in the conditioned medium, the MDA-MB-231 cells are also demonstrated to contain elevated level of signal transducer and activator of transcription 3 (STAT3) in the cells (Sasser *et al.*, 2007b). STAT3 is typically maintained in the cytoplasm as an inactive monomer. Once it is phosphorylated, the STAT3 forms homodimers and enters into nucleus where it activates the transcription of multiple genes associated with cell proliferation and survival (Heinrich *et al.*, 1998; Zinzalla *et al.*, 2010). The activation of STAT3 has been correlated with enhanced breast cancer cell growth, survival and immune evasion (Selander *et al.*, 2004; Ling *et al.*, 2005; Yu *et al.*, 2007). According to a previous study, exposure of MSCs-conditioned medium to MCF-7 and T-47D activated the levels of $pTyr^{705}$ STAT3 in the cells (Sasser *et al.*, 2007a). Correlatively, the enhancement of the cancer cell growth rates was observed in ERα-positive human breast cancer cell lines, including MCF-7 and T-47D, in the presence of the MSCs-conditioned medium. The growth rates of BT474 and ZR-75-1 cells were also observed to increase after the cancer cells were co-cultured with the MSCs-conditioned medium. All cancer cell growth rates were enhanced by approximately 2-3 fold, after the exposure to the conditioned medium (Fig. 4). The growth rate of an ERα-negative breast cancer cell line, MDA-MB-468, was also elevated in the presence of the MSCs-conditioned medium, albeit to a lesser extent than the other ERα-positive cell lines that were tested (Sasser *et al.*, 2007a; Sasser *et al.*, 2007b). However, this induction was not observed when the MDA-MB-231 cell line was exposed to the MSCs-conditioned medium.

Few effects were observed when the MDA-MB-231 cells were co-cultured with the MSCs-conditioned medium because the cell line likely contained a subpopulation in the cell population that secreted a standard level of soluble growth factors in the conditioned medium (Sasser *et al.*, 2007b). In this non-adhesive co-culture study, paracrine interleukin-6

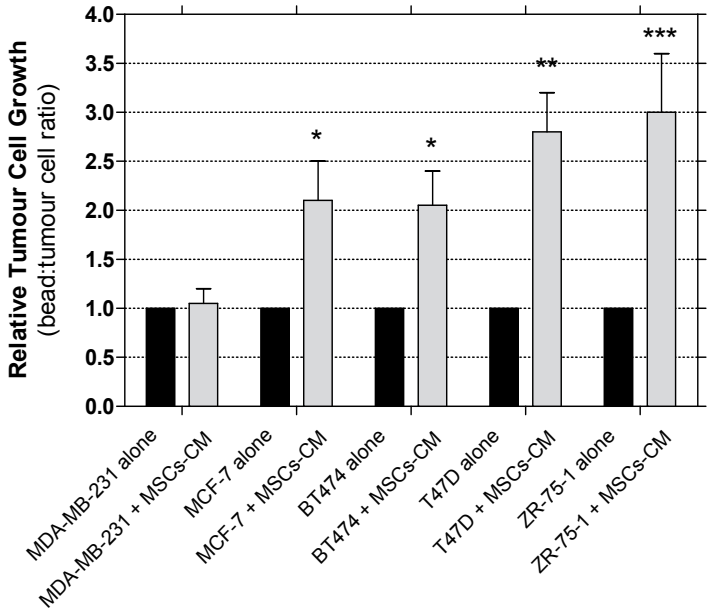

Fig. 4. Breast cancer cell growth in the presence or absence of MSCs-conditioned medium was assessed for MDA-MB-231, MCF-7, BT474, T47D and ZR-75-1 cells. The MDA-MB-231 cell growth was unaltered by MSCs-conditioned medium, whereas the growth of the four remaining ERα-positive cell lines in the presence of MSCs-conditioned medium was significantly elevated when compared to the cell lines growing alone for eight days (Sasser *et al.*, 2007b).

(IL-6) was found to be the principal mediator of the STAT3 phosphorylation in the cells. MSCs-induced STAT3 phosphorylation was lost when the IL-6 was depleted from the MSCs-conditioned medium. A similar phenomenon was observed when the IL-6 receptor in the cancer cells was blocked. This secretion of IL-6 from the MDA-MB-231 cells allowed for the activation and maintenance of the level of STAT3 as well as the growth in the MDA-MB-231 cells. Therefore, the conditioned medium of MDA-MB-231 cells has similar effect as the conditioned medium withdrawal from the MSCs, as evidenced by previous study, where the conditioned medium from the MDA-MB-231 cells with constitutively active STAT3 is sufficient to induce p-STAT3 levels in various recipients that do not possess elevated p-STAT3 levels, such as MCF-10A cells, a non-tumorigenic cell line (Lieblein *et al.*, 2008). This signalling occurs through the JAK/STAT3 pathway, leading to STAT3 phosphorylation as early as 30 minutes and was persistent for at least 24 hours, indicating that a correlation between elevated levels of IL-6 production and p-STAT3 in the cells, as confirmed by ELISA analysis. Neutralisation of the IL-6 ligand or gp130 was sufficient to block the increased levels of p-STAT3 (Y705) in the treated cells. These results demonstrate that the STAT3 phosphorylation in breast epithelial cells can be stimulated by paracrine signalling through soluble growth factors from both breast cancer cells and breast cancer associated fibroblasts with elevated STAT3 phosphorylation. The finding of growth factors within the MDA-MB-231 conditioned media was also sufficient to stimulate an increase in IL-6 production from

MCF-10A cells, as indicated in the previous study, may not correct as both MDA-MB-231 cells and MCF-10A cells secret IL-6 in the conditioned medium. Indeed, our study demonstrated that the conditioned media of the MDA-MB-231 cells and MCF-10A cells contained a high level of IL-6, although the level was not as high as in the MSCs-conditioned medium (Fig. 5).

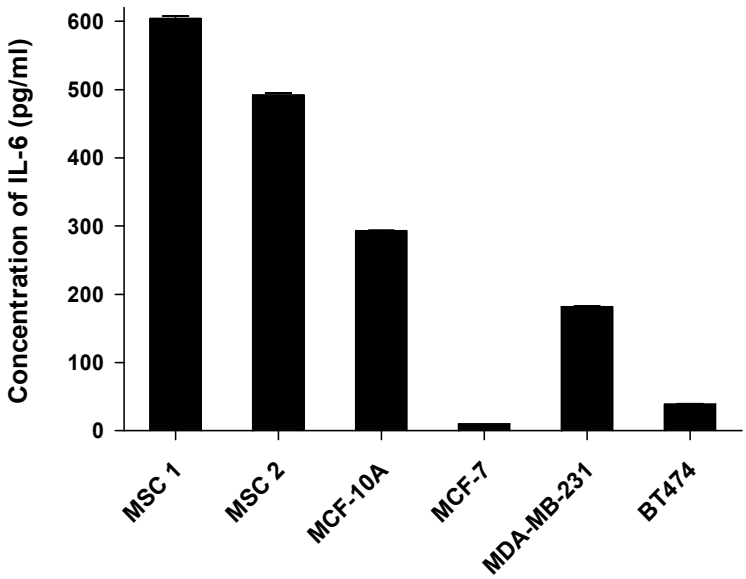

Fig. 5. The activity of IL-6 in the conditioned media of MSCs, non-tumorigenic cells and human breast cancer cells. The culture supernatants were withdrawn from one-week-old feeder layer of above cultures. The level of IL-6 in the conditioned medium was assayed using ELISA. The values were expressed as the mean±SD from three replicates, and the determination was carried out from three replicates each of three independent experiments.

Therefore, the finding indicates that the soluble growth factors within the MDA-MB-231 conditioned medium to stimulate an increase in IL-6 production from the MCF-10A cells might be due to combine of both conditioned media. Anyhow, this result indicates that the MDA-MB-231 cells may contain similar progenitor factor as MSCs in the cell population. This factor likely expresses the high level of IL-6 where it contributes to the induction of STAT3 phosphorylation and appears to be associated with cell proliferation of the MDA-MB-231 cells. Although the secretion of IL-6 allows for the activation and maintenance of the level of STAT3 as well as the growth in the MDA-MB-231 cells have been demonstrated, its role in invasiveness of the MDA-MB-231 remains unclear. Nevertheless, targeting the IL-6 in the conditioned medium can be an idea to diagnose patients with tumour that are ERα negative or express lower level of ERα.

2.3 CCL2 and CCL5 in the conditioned medium

Chemokines or chemotactic cytokines are small proteins that are classified into four conserved groups, CXC, CC, C and CX3C, based on the position of the first two cysteines that are adjacent to the amino acid (Balkwill, 2004; Lu & Kang, 2009). Among more than 50

identified human chemokines, chemokine (C-C motif) ligand 2 (CCL2 or MCP-1) and chemokine (C-C motif) ligand 5 (CCL5 or RANTES) are of particularly important. CCL2 is a potent chemoattractant for monocytes, memory T lymphocytes and natural killer cells whereas CCL5 is a potent inducer of leukocyte motility (Lu & Kang, 2009; Melgarejo *et al.*, 2009; Yaal-Hahoshen *et al.*, 2006). Both chemokines stimulate migration of leukocytes in response to inflammatory signals. The roles of CCL2 and CCL5 in breast malignancy have been extensively addressed in breast cancer studies (Goldberg-Bittman *et al.*, 2004; Soria *et al.*, 2008; Soria & Ben-Baruch, 2008; Wu *et al.*, 2008; Fujimoto *et al.*, 2009). Overexpression of the CCL2 and CCL5 are stimulated during breast cancer development and progression. They are also frequently associated with advanced tumour stage and metastatic relapse in breast cancer. Both chemokines act directly on the tumour cells to promote their pro-malignancy phenotype by increasing their migratory and invasion-related properties (Soria & Ben-Baruch, 2008). The chemokines are expressed by the cells of the tumour microenvironment osteoblasts and MSCs. In breast cells, the chemokines are highly expressed by breast tumour cells at primary tumour sites and minimally expressed by normal breast epithelial duct cells (Soria *et al.*, 2008; Soria & Ben-Baruch, 2008). The chemokines are soluble growth factors that can be easily detected in serum and conditioned culture medium. Consistently, our recent study demonstrated that high levels of CCL2 and CCL5 were detected in the conditioned medium of the MDA-MB-231 cells, as determined by ELISA (Fig. 6). The results indicated that the CCL2 and CCL5 were present in the conditioned media of all of the tested cell lines. As expected, elevated levels of CCL2 and CCL5 were observed in the MSCs and MCF-10A cells. CCL2 was additionally more stably expressed in the non-tumorigenic cells, such as MCF-10A, than in the MDA-MB-231 cells. However, an opposite event was observed for CCL5 in the MCF-10A and MDA-MB-231 cells. Both CCL2 and CCL5 displayed relatively higher expression levels in the MDA-MB-231 cells than that in the weakly metastatic cells, such as MCF-7 and BT-474. However, the CCL2 level in the MDA-MB-231 cells was only slightly higher than in the MCF-7 and BT-474 cells.

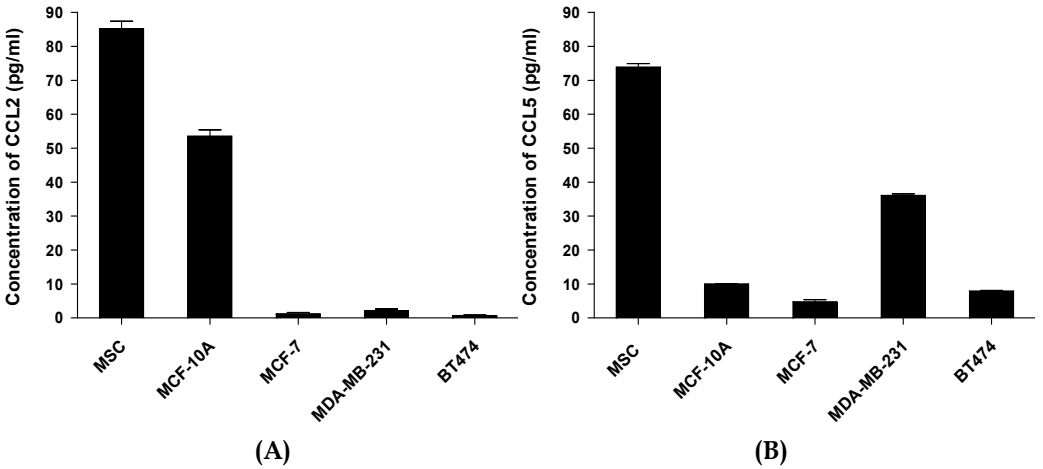

Fig. 6. The activities of **(A)** CCL2 and **(B)** CCL5 in the conditioned media of MSCs, human breast tumorigenic and non-tumorigenic cells. The levels of the soluble growth factors were assayed using ELISA. The values were expressed as mean±SD from three replicates, and the determination was carried out from three replicates each of three independent experiments.

The overexpression of chemokine decoy receptor proteins, such as D6 and Duffy antigen receptor for chemokines (DARC), have been demonstrated to inhibit the proliferation and invasion of the human breast cancer *in vitro*, tumorigenesis and lung metastasis *in vivo* (Wu *et al*., 2008). This inhibition is associated with decrease in chemokines, such as CCL2 and CCL5, vessel density and tumour-associated macrophage infiltration. The inhibition of CCL5 expression by short interfering RNA (siRNA) or by the use of neutralising antibodies against CCL5 impaired the tumour-supporting roles that were mediated by the CCL5-CCR5 loop; this significantly inhibited the metastatic potential of the MDA-MB-231 cells (Karnoub *et al*., 2007; Soria & Ben-Baruch, 2008). Moreover, CCL2-neutralizing antibodies inhibited bone resorption *in vitro* and bone metastasis *in vivo* as well as the tumour conditioned media-induced osteoclast formation *in vitro* and bone metastasis *in vivo*, indicating a role of the CCL2 and CCL5 in metastasis (Lu & Kang, 2009). The MDA-MB-231 cells are obviously having its own progenitor factor, which is in the cell population that produce the soluble growth factors in order to maintain the invasive and progressive phenotypes in the cells. Further identification and functional characterisation of CCL2 and CCL5, as well as MMP-9 and IL-6, would provide an effective treatment for systemic metastasis. Perhaps, the effects of certain inhibitors or drugs on the inhibition of proliferation and the reduction of invasion of breast cancer cell growth can be easily determined using these growth factors as they can be detected *via* serum and conditioned culture medium. Thus, all four molecules mentioned in this chapter could be considered as potential therapeutic targets for the development of a detection assay for human breast cancer.

3. The subpopulation in the MDA-MB-231 cells

Most of the cancer cell lines have recently been demonstrated by flow cytometry to contain a subpopulation of $CD44^+/CD24^-$ where the MDA-MB-231 cells are found to contain a high percentage of the $CD44^+/CD24^-$ subpopulation (85±5%) in the cells (Sheridan *et al*., 2006). Other cell lines that contain a high level of this subpopulation are MDA-MB-436 (72±5%), Hs578T (86±5%) and SUM1315 (97±3%) (Table 1). The subpopulation is shown to possess the capacity for self-renewal and the generation of heterogeneous progeny in the cells. Moreover, the subpopulation of the breast cancer cells has been reported to have stem/progenitor cell properties that contribute a unique ability to allow these cells to invade. Similar to the ability of the MSCs that was described above, the inherent properties of this subpopulation may impart their transformed counterparts with the ability to evade traditional antitumour therapies and to establish breast cancer metastasis (Reya *et al*., 2001; Behbod *et al*., 2005; Dean *et al*., 2005). Several studies suggested that this subpopulation of cells, as a subset of human breast cancer cells, possessed an enhanced ability to form tumour in immunocompromised mice (Al-Hajj *et al*., 2003; Ponti *et al*., 2005). However, the potential of this subpopulation to establish breast cancer metastasis in the cell line remains unclear.

The expression levels of pro-invasive genes, such as interleukin-1-alpha (IL-1α), IL-6, interleukin-8 (IL-8) and urokinase plasminogen activator (UPA), are higher in the cell lines that contained a significant $CD44^+/CD24^-$ subpopulation (Sheridan *et al*., 2006). The results indicate that the cell lines with a significant number of $CD44^+/CD24^-$ subpopulation are more invasive is consistent with the studies that demonstrate the metastatic process in breast cancer cells requires the following: (1) ECM degradation-associated proteins, including the

UPA/UPA receptor system and MMPs; (2) cytokines, including interleukin-1 (IL-1), IL-6, IL-8, interleukin-11 (IL-11), tumour necrosis factor (TNF) and transforming growth factor-beta 1 (TGF-β1); and (3) chemokines and their receptors, including stromal cell-derived factor-1-alpha (SDF-1α) and CXC chemokine receptor (CXCR4) (Edwards and Murphy, 1998; Dumont and Arteaga, 2003; Kang *et al*., 2003; Yodkeeree *et al*., 2010). In addition, a recent study described the role of nuclear factor-kappa B (NF-κB) and its ligand in the metastasis of breast cancer cells to the bone matrix (Jones *et al*., 2006). All of these factors may be directly related to breast cancer metastasis. However, the contribution of the subpopulation of $CD44^+/CD24^-$ to the pro-invasive factors in breast cancer cells remains unclear.

No.	Cell Line	$CD44^+/CD24^-$	Cell Type Classification
a	MDA-MB-231	85±5	Mesenchymal
b	MDA-MB-436	72±5	Myoepithelial
c	Hs578T	86±5	Mesenchymal
d	MDA-MB-468	3±1	Basal
e	MCF-7	0	Luminal
f	T47-D	0	Luminal
g	ZR-75-1	0	Luminal
h	BT-474	0	Luminal/$ErbB2^+$
i	SK-BR-3	0	Luminal/$ErbB2^+$
j	MCF-10A	17±4	Basal

Table 1. Subpopulation of $CD44^+/CD24^-$ in commonly used breast cancer cell lines. The CD44 and CD24 expression patterns in the subpopulation $CD44^+/CD24^-$ were determined by flow cytometry. CD44 and CD24 were detected by a combination of fluorochrome-conjugated monoclonal antibodies against human CD44 (FITC) and CD24 (PE), respectively (Sheridan *et al*., 2006).

Demethoxycurcumin (DMC) is recently demonstrated to inhibit the adhesion, migration and invasion of the MDA-MB-231 cells (Yodkeeree *et al*., 2010). According to the study, the DMC-treated MDA-MB-231 cells contained decreasing levels of ECM degradation-associated proteins, which included MMP-9, membrane type-1 MMP (MT1-MMP), UPA and UPAR. DMC reduced also the expression of intercellular adhesion molecule-1 (ICAM-1) and CXCR4 in the MDA-MB-231 cells. These molecules are involved in the modulation of the tumour metastasis process. In addition, the study showed that treatment of the MDA-MB-231 cells with DMC inhibited the DNA binding activity of NF-κB, which is known to mediate the expression of MMPs, UPA, UPAR, ICAM-1 and CXCR4 in breast cancer cells. These results indicated also that NF-κB may play a role in the invasion process of the MDA-MB-231 cells. All of these findings suggest the presence of a correlation between the above molecules and the invasiveness of the MDA-MB-231 cells. However, the specific correlation between the above molecules and the subpopulation in the MDA-MB-231 cells has yet to be fully elucidated. The inhibition or depletion of the progenitor factor from the subpopulation is hypothesised to reduce the expression of the above molecules, thereby reducing the invasiveness of the MDA-MB-231 cells. Therefore, it is essential to identify more surface markers that can specifically be used to isolate the subpopulation. By targeting this subpopulation in the cells, the expression of the above molecules and the invasiveness of the MDA-MB-231 cells can be further elucidated.

4. Mesenchymal-like phenotype of the MDA-MB-231 cells

Before the subpopulation and invasiveness of the MDA-MB-231 cells can be further elucidated, hierarchy of the breast cancer stem cells in the breast cancer cell compartment should be understood. A breast cancer stem cell, as described in the cancer stem cell compartment hierarchy, is capable of undergoing an asymmetric cell division to generate one cell that is identical to itself (orange colour) and one that it is more committed towards a certain differentiation pattern (a breast cancer cell, grey colour) (Cariati & Purushotham, 2008; Fig. 7).

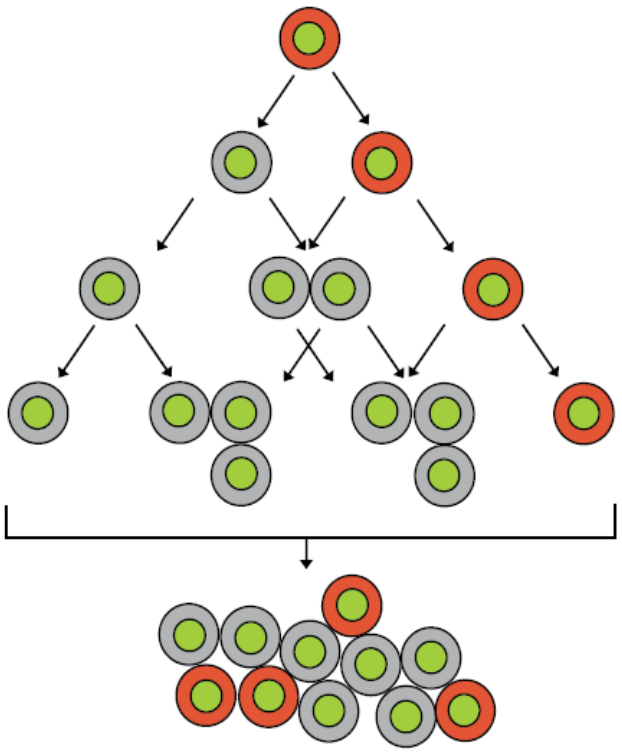

Fig. 7. A breast cancer stem cells-breast cancer cells compartment hierarchy. A breast cancer stem cell is capable of going through an asymmetric cell division to generate one cell that is identical to itself (orange colour) and one that tends toward a certain differentiation pattern (breast cancer cells, grey colour) (Modified from Cariati & Rurushotham, 2008).

The formation of the identical cell ensures that the cancer stem cell compartment is maintained throughout its time in the subpopulation. These distinct cells undergo series of divisions and differentiation steps that result in the generation of a terminally differentiated

population of breast cancer stem cells and breast cancer cells. The existence of these cancer stem cells explains why only a small minority of cancer cells are capable of extensive proliferate and transfer to the tumour. Chemotherapy can remove breast cancer cells, but it fails to eliminate the cancer stem cells that can revive the breast cancer cells. This allows the regrowth of the breast cancer after the treatment has ended (Sanchez-Garcia *et al.*, 2008). This shortcoming explains the high recurrence of the disease. Therefore, the current strategy for the development of anti-breast cancer agents is to target both breast cancer stem cells and breast cancer cells. Moreover, the healthy breast cells transform into cancer cells *via* the formation of breast cancer stem cells is also a possibility, but, the precise mechanism of the transformation for this disease remains unclear. Therefore, study of the transformation remains warranted. By understanding the precise mechanism that transforms normal stem cells into cancer cells *via* the formation of the cancer stem cells, it would be possible to develop more effective tools for the ERα-negative human breast cancer prevention, detection and treatment.

The MDA-MB-231 cell line is a good example of a breast cancer cell line that consists of the above mentioned populations of breast cancer stem cells and breast cancer cells. As described above, the MDA-MB-231 cells have been demonstrated to contain a subpopulation of $CD44^+/CD24^-$ that provides stem/progenitor cell properties to enhance the invasiveness of the cancer cells. Surprisingly, in our present study, we found that the MDA-MB-231 cells were positive for CD105 staining, while the weakly metastatic breast cancer cell lines, such as MCF-7 and T47D, were negative for the CD105 staining; the staining was visualised by fluorescent microscopy (Fig. 8A). CD105, also known as Endoglin, is a type I integral trans-membrane glycoprotein and is an accessory receptor for transforming growth factor-alpha (TGFβ) superfamily ligands (Barbara *et al.*, 1999). CD105 is found on activated monocytes, mesenchymal stromal cells and leukemic cells of lymphoid and myeloid lineages. The BMSCs, as well as other non-hematopoietic MSCs, are positive for the CD105 antibody staining, as visualised by fluorescent microscopy (Miao *et al.*, 2006; Bernacki *et al.*, 2008). Therefore, it is hypothesised that the MDA-MB-231 cells may have similar mesenchymal phenotypes as the progenitor factors that contribute to the metastatic potency of the cancer cells. Therefore, the cell line contains not only the stem/progenitor cell properties but also the mesenchymal-like stem/progenitor cell properties. This property likely contributes to the invasiveness of the cell line and causes the cell line to express high levels of MMP-9, IL-6, CCL2 and CCL5 in the conditioned medium. The Oil-Red-O-stained MDA-MB-231 cells were also observed to contain a mixture of epithelial cells and a mesenchymal-like subpopulation, as visualised by light microscopy (Fig. 8B).

These results focused our attention and research on the mesenchymal-like phenotype in the MDA-MB-231 cells. MDA-MB-231 and MCF-7 are cell lines that originate from pleural effusion metastatic cells in ductal invasive breast carcinomas (Burdall *et al.*, 2003; Lacroix & Leclercq, 2004). These cells are among the most commonly used breast cancer cell lines in medical research laboratories. MDA-MB-231 is a mesenchymal-like cell line that is highly aggressive and invasive, whereas MCF-7 is classified as a luminal (epithelial)-like cell line with a relatively low invasive phenotype and potential (Lacroix & Leclercq, 2004; Charafe-Jauffret *et al.*, 2006). A comparison of the two cell lines in terms of DNA copy number variation and gene expression profiles has been performed, and the expression levels of 2157 transcripts were shown to be significantly increased in the MDAMB-231

cells compared with the MCF-7 cells; the expression levels of 2345 transcripts were significantly increased in the MCF-7 cells compared with the MDA-MB-231 cells (Forozon *et al*., 2000; Charafe-Jauffret *et al*., 2006). Moreover, 387 of the above transcripts have been defined by the gene expression profile to be mesenchymal-like cellular subtypes (Charafe-Jauffret *et al*., 2006). Recently, 31 mesenchymal-like and luminal-like subtype features of breast cancer cell lines were revealed in Charafe-Jauffret's study, which was based on the gene expression profiles. The study found that 680 transcripts were preferentially expressed in the group of mesenchymal-like cell lines, and 629 transcripts were expressed preferentially in the group of luminal-like breast cancer cell lines. In a recent expression study, 387 transcripts, which are also identified in the mesenchymal-like subtype gene list in Charafe-Jauffret's study, showed significantly higher expression levels in the MDA-MB-231 cells; and 328 transcripts, which were present on the luminal subtype gene list from Charafe-Jauffret's study, showed significantly higher expression levels in the MCF-7 cells (Li *et al*., 2009). These data revealed the differential expression profiles of mesenchymal-like and luminal-like subtypes of the breast cancer cell lines. These expression profiles can be utilised to effectively overcome the invasion and metastasis of human breast cancer.

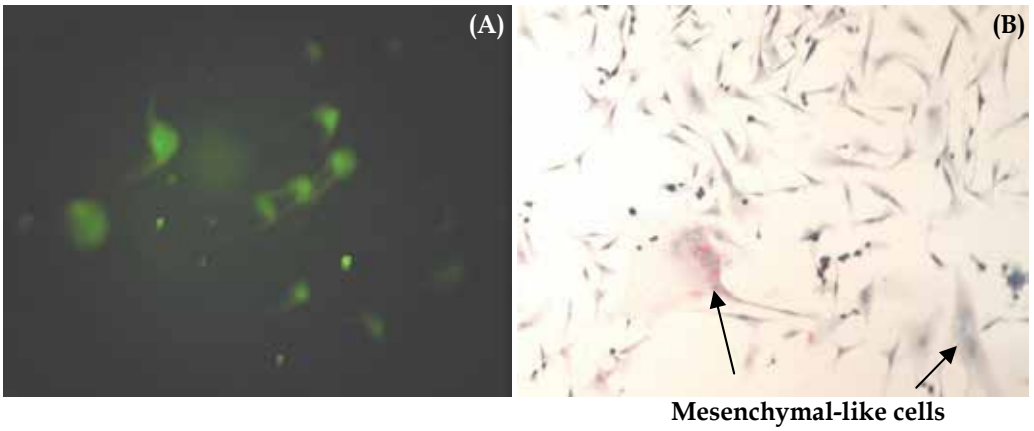

Fig. 8. **A:** The MDA-MB-231 cells, which were predicted to have a mesenchymal-like phenotype subpopulation, existed in the epithelial cell population. The cells were stained with Oil-red O and visualised using an inverted light microscope. **B:** The MDA-MB-231 cells were positive for the CD105 antibody staining as visualised using fluorescent microscope. These images were captured using a digital camera.

5. Future prospects

Our recent study proposes to isolate or withdraw the mesenchymal-like stem cells from the MDA-MB-231 population using CD105 and other known antibody-conjugated microbeads, thus allowing for a clearer understanding of the subpopulation of the cancer cells. Potential drugs will then be applied to the isolated $CD105^+$ (mesenchymal-like stem

cells) and CD105$^-$ (epithelial cells) MDA-MB-231 cells. The invasion rate of the drug-treated CD105$^+$ and CD105$^-$ MDA-MB-231 will then be determined using the Matrigel invasion assay. The mRNA and protein expression levels of the ECM degradation-associated molecules in the drug-treated CD105$^+$ and CD105$^-$ MDA-MB-231 cells will also be assessed using real-time PCR and Western Blotting, respectively, and the gelatinase activities in the conditioned medium of drug-treated CD105$^+$ and CD105$^-$ MDA-MB-231 cells will be investigated using ELISA. The proposed project that will utilise cell separation and isolation techniques to study the breast cancer cell invasion is a new area of cancer research in the institute of my home country. The previous research projects regarding the MDA-MB-231 cell invasion and metastasis were related to drug treatments, the effects of herbal and plant extracts, and the understanding of a gene or protein activity in cancer cells and animal models. However, the approaches to study the cancer cell invasion and metastasis by isolating or withdrawing the mesenchymal-like breast cancer cells (CD105$^+$) from the MDA-MB-231 cell population using cell separation or isolation have not been demonstrated. Therefore, this project may establish a new fundamental cancer research and new research topic in my institute. This study may also lay a research foundation that is focused on the inhibition of invasion for the ERα-negative human breast cancer cells. I also believe that, by targeting the mesenchymal-like phenotype in the MDA-MB-231 subpopulation, the invasion rate of the ERα-negative human breast cancer cells can be easily monitored and controlled.

6. Conclusion

All of the results mentioned above show that the MDA-MB-231 cells likely display a mesenchymal-like phenotype that facilitates the cells to be a highly metastatic breast cancer cell line. However, a deeper understanding of the cell morphology, gene expression and intracellular mechanisms and pathways of the cancer cells that can explain the interaction between mesenchymal-like and epithelial cells in the MDA-MB-231 cells is warranted. By targeting this phenotype, the metastatic potency and the growth of the cancer cells may be controlled or effectively reduced. A potential anticancer drug can also be identified to treat both human breast cancers and other malignancies. Perhaps, withdrawing the progenitor factor from a tumour may serve as a potential machinery target in cell-mediated therapy for human breast cancer.

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p130Cas and p140Cap as the Bad and Good Guys in Breast Cancer Cell Progression to an Invasive Phenotype

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1. Introduction

Breast cancer is an aggressive malignancy affecting a large woman population. Even though important progress have been made in providing new therapies to treat this neoplasia, our knowledge on the mechanisms underlying the transformation of breast epithelial cells in tumor cells is still superficial. The neoplastic phenotype results from the alteration of multiple cellular signaling mechanisms controlling proliferation, survival and invasiveness. Moreover, the prognosis of breast cancer patients is tightly correlated with the degree of spread beyond the primary tumor. However the mechanisms by which epithelial tumor cells escape from the primary tumor and colonize a distant site are not entirely understood. In this chapter we will discuss recent data on the relevance of p130Cas and p140Cap adaptor molecules in breast cancer signalling related to the acquirement on invasive properties. Due to the presence of adaptor modules, these proteins create signalling platforms proximal to plasma membrane cell surface receptors, such as integrins and growth factor receptors. p130Cas and p140Cap exert opposite regulation on cell signalling. Indeed p130Cas has been shown to increase survival, proliferation and migration of normal and transformed cells either in response to cell matrix adhesion or to hormones and growth factors. Moreover, p130Cas has been recently linked to resistance to breast cancer treatments, revealing its potential use as a novel therapeutic target. Instead, p140Cap behaves as a potent negative regulator of signalling pathways leading to cancer cell proliferation and migration. In this chapter, we will discuss the increasing evidence that highlight the importance of these adaptor proteins in breast cancer.

It is well established that to migrate and to invade a cell needs to detach from its neighbors, i.e. adjacent cells in an epithelium, to extend lamellipodia and filopodia from the leading edge and to create new dynamic adhesions, which form and rapidly disassemble at the base of protrusions (Mitra *et al.*, 2005; Ridley *et al.*, 2003). Cell invasion also requires the release or activation of proteases that degrade the extracellular matrix (ECM) and allows cells to sort out from the basal lamina invading surrounding tissues (Eliceiri *et al.*, 2002). Under physiological conditions cell motility and invasion are tightly controlled by a complex interplay among cell-cell, cell matrix and growth factors receptors resulting in the maintenance of the architectural integrity of human tissues. This subtle regulation is lost in

human tumours leading to uncontrolled dissemination of cancer cells into the body (Berx *et al.*, 2007; Cavallaro and Christofori, 2004; Giancotti, 2003; Guo and Giancotti, 2004),
At least three major classes of membrane proteins are involved in these events, namely, the E-cadherin, the Receptor tyrosine kinases (RPTKs), and the integrin receptors. The cell-cell adhesion receptor E-cadherin is the major membrane protein involved in binding between neighbouring cells in adherens junctions. As a practical consequence of its adhesive functions, E-cadherin has also been shown to prevent EGFR activation and downstream signalling, leading to negative regulation of proliferation (Berx and Van Roy, 2001; Gutkind, 2000; Perrais *et al.*, 2007; Qian *et al.*, 2004). E-cadherin is frequently down-regulated or lost in epithelial tumours, and its loss correlates with increased cancer cell invasiveness ((Peinado *et al.*, 2007; Reynolds and Carnahan, 2004);.
Integrins are cell surface heterodimeric receptors for the ECM formed by the non covalent association of alpha and beta subunits (Hynes, 2004). Integrins specifically localize to focal adhesions, which are sites of close apposition with the ECM where actin filaments are anchored to the plasma membrane. Integrins are catalytically inactive and translate positional cues into biochemical signals by direct and/or functional association with intracellular adaptors or growth factor and cytokine receptors, thus regulating integrin ability to transduce signals inside the cells, the so called "outside-in signalling" (Cabodi *et al.*, 2010). A growing body of evidence shows that integrins, RPTKs and cytokine receptors have no longer to be considered as individual receptors, but rather as joint modules in which attachment to the matrix confers positional control to respond to soluble growth factors (Cabodi *et al.*, 2010b; Cabodi *et al.*, 2008; Desgrosellier *et al.*, 2009; Streuli, 2009; Uberti *et al.*). In the case of the EGF receptor (EGFR), beta1 integrin is both sufficient to partially activate the receptor itself and required for the full activation of the EGFR in response to EGF (Morello *et al.*; Moro *et al.*, 1998)). Integrin-dependent EGFR trans-activation accounts for a specific repertoire of mechanisms, namely cell survival and actin cytoskeleton organization involved in cell migration.
In this chapter we will focus on p140Cap and p130Cas adaptors as major regulators of cell migration and invasion (Cabodi *et al.*). Owing to their modular structure, both proteins can undergo tyrosine phosphorylation and association with effector proteins, leading to the assembly of molecular platforms that regulate the variety of signalling events originating from the complex cross-talk among integrins, E-cadherin and RPTKs.

2. p130Cas adaptor protein

2.1 p130Cas adaptor features

p130Cas is coded in human by the BCAR-1 (Breast Cancer Anti-oestrogen Resistance 1) gene. This gene is conserved through many species and in humans is localized on Chromosome 16q23.1. Knock-out of the mouse gene results in embryonic lethality at 9.5 days, indicating that any other protein cannot fill in for its role during development. p130Cas is an ubiquitously expressed multi-site docking protein that consists of i) an N-terminal Src homology 3 (SH3) domain, ii) a substrate domain, which contains 15 repeats of a YXXP sequence (tyrosine-any two aminoacids-proline), iii) a serine rich region, and iv) a C-terminal domain (Figure 1A). The presence of these multiple conserved sequence motifs and extensive post-translational modification, mainly consisting of tyrosine and serine phosphorylation, allow the assembly of specific multi-protein complexes. In particular, the SH3 domain interacts with polyproline-rich sequences present in several proteins including

Fak, PYK2/RAFTK, phosphatases like PTP-PEST, PTP1B, and effectors as C3G and CIZ (Sakai *et al.*, 1994; Tikhmyanova *et al.*). The substrate domain, upon Src family kinases activation, is tyrosine phosphorylated and exposes additional binding sites for SH2 containing proteins such as the Crk adaptors (Salgia *et al.*, 1996), while the serine rich region represents a docking site for other partners such as 14-3-3 and Grb2. Lastly, the C-terminus contains a polyproline-rich region responsible for the binding of the Src family kinase, PI3K, Bcar3/AND-34, Chat-H and ubiquitin ligases such as AIP4, APC/C and CDH1, as well as a binding site for the adaptor protein p140Cap (Bouton *et al.*, 2001; Cabodi *et al.*, 2004; O'Neill *et al.*, 2000).

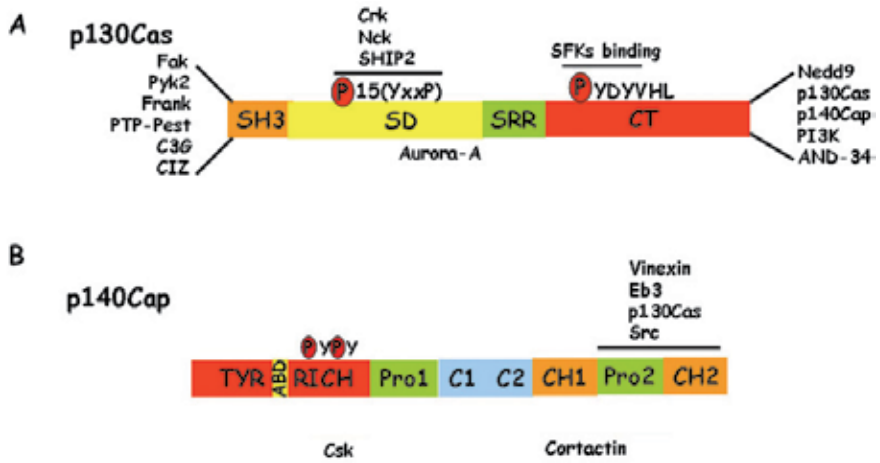


Fig. 1. p130Cas and p140Cap structure.
A) p130Cas consists of an N-terminal SH3 domain, a substrate domain (SD), a serine rich region (SRR), and a C-terminal domain (CT). The main interactors are indicated. In particular, many proteins associate to the N-terminal domain and the Src family kinases (SFKs) bind the CT domain. The 15 YxxP motifs are phosphorylated by Src family kinases to mediate Crk binding.
B) p140Cap consists of an N-terminal tyrosine–rich region (Tyr-rich), an actin binding domain (ABD), a proline rich domain (Pro1), a coil-coiled region (C1-C2), two domains rich in charged amino acids (CH1, CH2) and a C-terminal proline rich domain (Pro2). Src, p130Cas, EB3 and Vinexin bind to the Pro2 domain of p140Cap. The binding regions of Cortactin and Csk have yet to be defined.

2.2 p130Cas in human breast cancer

Although several reports highlight the relevance of p130Cas in tumour cell lines and animal models, investigation of its expression in biopsies of different human malignancies using immunohistochemistry, is still limited. However, it is noteworthy that a significant subset of human breast cancers where both ErbB2 and p130Cas are over-expressed are associated with increased proliferation and low prognosis (Cabodi *et al.*, 2006). In estrogen receptor (ER)-positive human breast tumours, p130Cas over-expression correlates with intrinsic resistance to tamoxifen treatment, high risk of relapse and loss of oestrogen-receptor in a large subset of human breast cancer samples, indicating that elevated BCAR1 might be a prognostic marker for breast tumours (Dorssers *et al.*, 2001; van der Flier *et al.*, 2001).

Therefore, at least in two classes of breast cancer that account for more than 90% of breast tumors, p130Cas over-expression is revealing its potential as prognostic factor in terms of therapy and disease progression.

2.3 p130Cas tyrosine phosphorylation in cell migration and invasion

p130Cas represents a nodal signalling platform on which integrin and RPTKs signalling convey. Integrins, RPTKs and oestrogen receptor (ER) are major upstream regulators of p130Cas, mainly through the activation of Src and Fak kinases, leading to p130Cas tyrosine phosphorylation on the C-terminal binding site YDYVHL (Figure 1) (Cabodi *et al.*). Moreover, physical stretching of p130Cas induces a conformational change that enables Src-family kinase-dependent p130Cas tyrosine phosphorylation. These findings point out a function for p130Cas as a sensor that integrates mechanical forces coming from the extracellular environment into intracellular signals leading to actin cytoskeleton reorganization (Kostic and Sheetz, 2006; Sawada *et al.*, 2006). The role of p130Cas in cell migration was initially inferred by studies performed on mouse embryo fibroblasts (MEFs) derived from p130Cas knock-out mice. p130Cas null MEFs show defects in stress fibre formation and cell spreading, impaired actin bundling and cell migration (Honda *et al.*, 1998), that were restored by full-length p130Cas expression. The tyrosine phosphorylation of the substrate domain of p130Cas provides binding sites for Crk proteins that in turn associates with DOCK180, a guanine nucleotide exchange factor that switches the small GTPase Rac1 from a GDP-bound inactive to a GTP-bound active state at lamellipodia and filopodia adhesion sites (Figure 2) (Kiyokawa *et al.*, 1998; Klemke *et al.*, 1998). This drives localized Rac activation, membrane ruffling and actin cytoskeleton remodelling, focal adhesion turnover, pseudopodia formation and extension. In addition, ARP2/3 and PAK kinase activation enhance cell migration (Heasman and Ridley, 2008). Uncoupling of p130Cas/Crk negatively regulates cell migration. Indeed, the non-receptor tyrosine kinase Abl phosphorylates Crk-II on tyrosine 221, inducing intramolecular folding that prevents binding of the C-terminal Crk-II SH2 domain to the phosphorylated p130Cas substrate domain, leading to decreased cell movement (Holcomb *et al.*, 2006; Kobashigawa *et al.*, 2007). Additional molecules that play important roles in modulating tyrosine phosphorylation of p130Cas leading to cell migration are the zyxin/Ajuba family of LIM proteins. These proteins bind to actin cytoskeleton and are implicated in cell motility. Ajuba allows p130Cas localization to nascent adhesive sites in migrating cells thereby leading to the activation of the small GTPase Rac, whereas Zyxin interacts with the SH3 domain of p130Cas and with a nucleocytoplasmic transcription factor, CIZ/NMP4/ZNF384 (Janssen and Marynen, 2006). Recent data also show that p130Cas activates several GTPases other than Rac. The association between p130Cas and And-34, an NSP family member, which acts as a GTP exchange factor for Ral, Rap1 and R-Ras enhances Src activation and cell migration, likely through a Rap1-dependent mechanism (Figure 2)(Riggins *et al.*, 2003). p130Cas tyrosine phosphorylation upon integrin or growth factor receptor activation has also been linked to cell invasion and it has been reported that the SH3 domain of p130Cas is also required for this process. Indeed, Focal adhesion kinase (Fak)-null cells are not invasive when transformed by v-Src, but they acquire invasive properties upon over-expression of p130Cas SH3 domain, indicating that this domain is required for rescue of v-Src cell invasion. In this context, the formation of Src/p130Cas/Crk/DOCK180 complex increases Rac1 and JNK activities and MMP-9 expression, leading to an invasive cell phenotype (Hsia *et al.*, 2003).

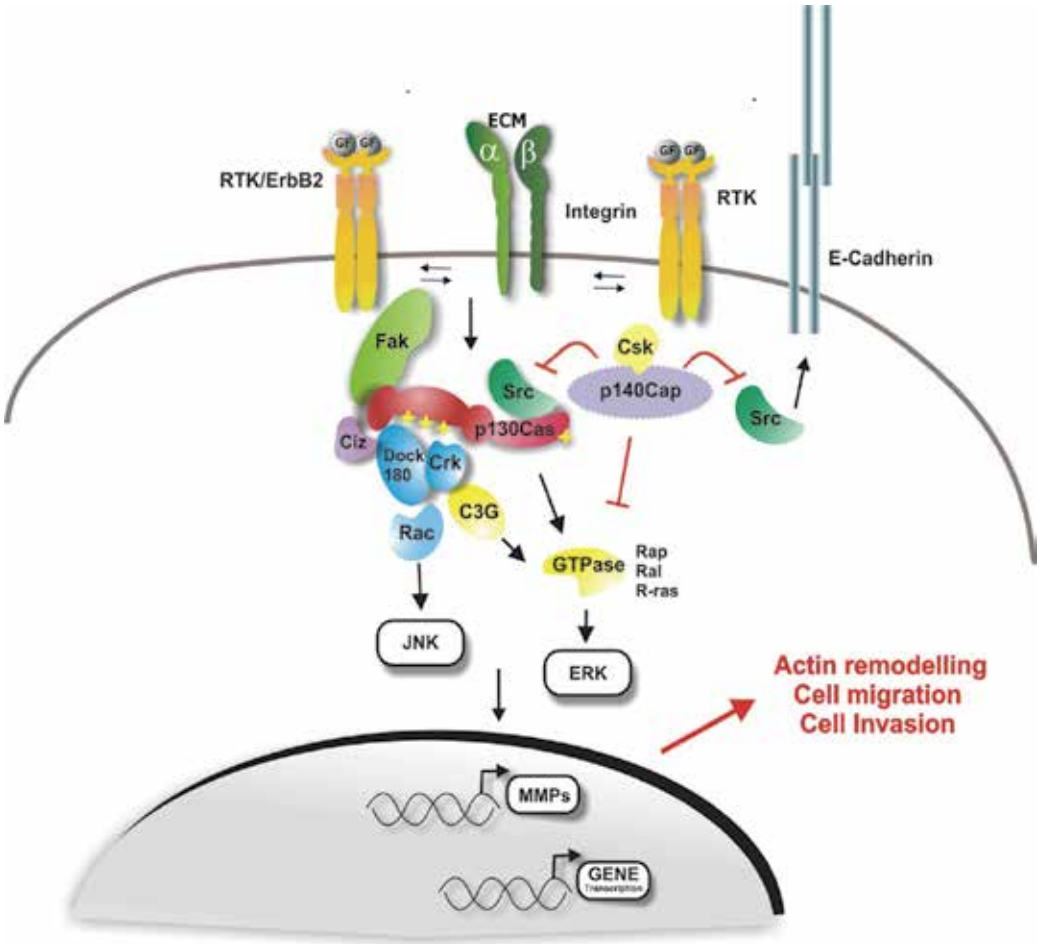


Fig. 2. p130Cas and p140Cap signalling involved in migration and invasion of breast cancer cells.

Upon extracellular matrix binding or growth factors stimulation, integrins and Receptor Protein Tyrosine Kinases (RPTK) represent the major upstream regulators of p130Cas and p140Cap, mainly through the regulation of Src kinase activity. Once tyrosine phosphorylated by Src, p130Cas recruits proteins that activate downstream pathways, resulting in actin cytoskeleton re-organization, increased cell motility and migration. p130Cas by acting on metalloproteinases (MMPs) promoter is also required for the invasive program. Upon cell matrix adhesion or mitogen stimulus, p140Cap inhibits Src kinase activity and p130Cas tyrosine phosphorylation and p130Cas/Crk complex formation. As a consequence, the effect of p130Cas on actin cytoskeleton re-organization is impaired and cell migration and invasion are inhibited. (Di Stefano et al., 2007) Moreover, by inactivating Src, p140Cap also regulates the epidermal growth factor receptor (EGFR) pathway through E-cadherin-dependent inactivation of EGFR signalling. p140Cap by interacting with E-cadherin and EGFR at the cell membrane, immobilizes E-Cadherin at the cell membrane thus preventing cell migration and invasion. (Damiano et al., 2010)

2.4 Role of p130Cas in c-Src dependent cell transformation

Hyper-phosphorylation or over-expression of p130Cas has been implicated in transformation induced by several oncogenes. For example, p130Cas involvement in c-Src-mediated tumourigenesis has been demonstrated by the inability of c-Src to transform p130Cas-null MEFs (Honda *et al.*, 1998). The C-terminal region of p130Cas containing the Src binding domain is sufficient to recover the ability of Src to promote anchorage-independent growth. In breast carcinoma cells p130Cas over-expression accelerates and up-regulates Src activity (Cabodi *et al.*, 2004) as well as increases tyrosine phosphorylation of multiple endogenous cellular proteins (Brabek *et al.*, 2004; Burnham *et al.*, 1996; Cabodi *et al.*, 2004). It was recently reported that bosutinib, a novel Src inhibitor, derived from breast cancer patients, inhibits cell spreading, migration, and invasion of human cancer cells, derived from breast cancer patients by stabilizing cell-to-cell adhesions and membrane localization of beta-catenin. These effects are dependent on the inhibition of the Src/Fak/p130Cas signaling pathway (Buettner *et al.*, 2008). It has been recently reported that Fak promotes mammary tumorigenesis by enabling Src-mediated phosphorylation of p130Cas. Consistently, knock-down of p130Cas causes proliferative arrest in breast cancer cell lines harbouring oncogenic mutations in K-Ras, B-Raf, PTEN and PIK3CA (Pylayeva *et al.*, 2009), underlying a role for p130Cas as a general regulator of breast cancer cell growth induced by different oncogenes.

2.5 Role of p130Cas in TGF-beta signalling in breast cancer cells

Transforming growth factor-beta (TGF-beta) is a powerful suppressor of mammary tumorigenesis because of its ability to repress mammary epithelial cell proliferation, as well as through its creation of cell microenvironments that inhibit mammary epithelial cells (MECs) motility, invasion, and metastasis. Yet, paradoxically, cancer cells elicit mechanisms that subvert the tumour suppressing functions of TGF-beta, and in doing so, confer oncogenic and metastatic activities upon this multifunctional cytokine (Massague, 2008). In epithelial cells, integrin beta1 suppresses apoptosis and growth inhibition induced by TGF-beta (Zhang *et al.*, 2003). In this context p130Cas has been shown to be a crucial player by binding to Smad3, and preventing its phosphorylation by TGF-beta receptor. As a consequence, the transcription of the cyclin-dependent kinase inhibitors p15 and p21 is inhibited, resulting in cell cycle progression (Kim *et al.*, 2008). Recently, it has been reported that p130Cas over-expression in MECs shifts TGF-beta signalling from Smad2/SMAD3 phosphorylation to p38 MAPK activation, rendering MECs resistant to TGF-beta -induced growth arrest and enhancing their metastatic potential (Wendt *et al.*, 2009). Overall, p130Cas can act as a molecular rheostat that switches the tumour suppressor function of TGF-beta to a pro-metastatic role during breast cancer progression.

3. The ErbB2 oncogene in breast cancer

The ErbB2 oncogene is a member of the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases (RTKs). This family comprises four related members: EGFR, ErbB2 (also known as Neu, HER-2), ErbB3 (HER-3), and ErbB4 (HER-4) (Holbro *et al.*, 2003). Over-expressed and mutated ErbB2 has been found in human tumors and cancer cell lines (Mukohara; Yarden *et al.*, 2004). In addition, several studies have shown a strong correlation of ErbB2 over-expression with a negative clinical prognosis in breast cancer (Choi *et al.*, 2009; Mukohara). Significantly, ErbB-2 may be useful not only as a prognostic marker but

also as a predictive marker, given that its elevated expression predicts tamoxifen resistance of the primary tumor and the response to anti-HER2 targeted therapy such as the monoclonal antibody Herceptin.

Further understanding of the mechanisms by which ErbB2 leads to tumorigenesis in the mammary gland comes from studies of ErbB2 mouse models. Expression of Neu mutation that promotes spontaneous receptor dimerization (NeuT), under the MMTV promoter, or more recently under the ErbB2 endogenous promoter (ErbB2/KI model), leads to the formation of mammary adenocarcinomas (Andrechek *et al.*, 2000; Muller *et al.*, 1998). Interestingly, the expression of the ErbB2 protooncogene in a MMTV-transgenic mice show late tumor latency with a low penetrance of lung metastasis, suggesting that gene amplification of the wild type receptor may be the main mechanism implicated in ErbB2-mediated tumorigenesis. Indeed, elevated protein and mRNA ErbB2 levels in the ErbB2/KI model also correlated with selective genomic amplification of the activated ErbB2 allele (Andrechek and Muller, 2000; Hodgson *et al.*, 2005; Montagna *et al.*, 2002). One of the most significant effects associated with ErbB2 activation is enhanced and sustained signal transduction cascades leading to the regulation a variety of cellular processes, including proliferation, apoptosis, cell polarity, migration and invasion (Feigin and Muthuswamy, 2009). Activation of specific ErbB homo- or heterodimer pairs leads to initiation of the mitogen activated protein kinase (MAPK) cascade, activation of phospholipase C gamma (PLCγ) and phosphatidylinositol 3 kinase (PI3K), as well as induction of the small GTPases Rho, Rac and Cdc42, among many other effectors (Hynes and MacDonald, 2009; Kurebayashi, 2001). Several reports have demonstrated a role for these pathways in ErbB-induced cell migration.

3.1 p130Cas in ErbB2 dependent transformation

In the context of ErbB2 positive breast cancer, previous studies generated by our group placed p130Cas as an important regulator of ErbB2-dependent tumorigenesis. To investigate the mechanisms through which p130Cas is linked to tumorigenesis, we generated mouse mammary tumor virus (MMTV)-p130Cas mice overexpressing p130Cas in the mammary gland. MMTVp130Cas transgenic mice are characterized by extensive mammary epithelial hyperplasia during development and pregnancy and by delayed involution at the end of lactation. These phenotypes are associated with activation of Src kinase, Erk1/2 MAPK, and Akt pathways, leading to an increased rate of proliferation and a decreased apoptosis. A double-transgenic line derived from crossing MMTV-p130Cas with MMTV-HER2-Neu mice expressing the activated form of the HER2-Neu oncogene develops multifocal mammary tumors with a significantly shorter latency than the HER2-Neu parental strain alone (Figure 3). MECs isolated from tumors of double-transgenic mice display increased tyrosine phosphorylation, c-Src, and Akt activation compared with cells derived from HER2-Neu tumors. In addition, p130Cas down-regulation by RNA interference increases apoptosis in HER2-Neu-expressing cells, indicating that p130Cas regulates cell survival. These findings provide evidences for a role of p130Cas as a positive regulator of both proliferation and survival in normal and transformed mammary epithelial cells. Its overexpression contributes to HER2-Neu-induced breast tumorigenesis, thus identifying this protein as a putative target for clinical therapy (Cabodi *et al.*, 2006).

More recent studies further assessed the functional role of p130Cas in ErbB2-dependent breast tumorigenesis by its silencing in breast cancer cells derived from mouse mammary tumours over-expressing ErbB2 (N202-1A cells), and by its re-expression in ErbB2-

transformed p130Cas-null mouse embryonic fibroblasts. We demonstrate that p130Cas is necessary for ErbB2-dependent foci formation, anchorage-independent growth and *in vivo* growth of orthotopic N202-1A tumours. Moreover intra-nipple injection of p130Cas-stabilized siRNAs in the mammary gland of MMTV-HER2-Neu mice decreases the growth of spontaneous tumours (Figure 4) (Cabodi *et al.*, 2010c).

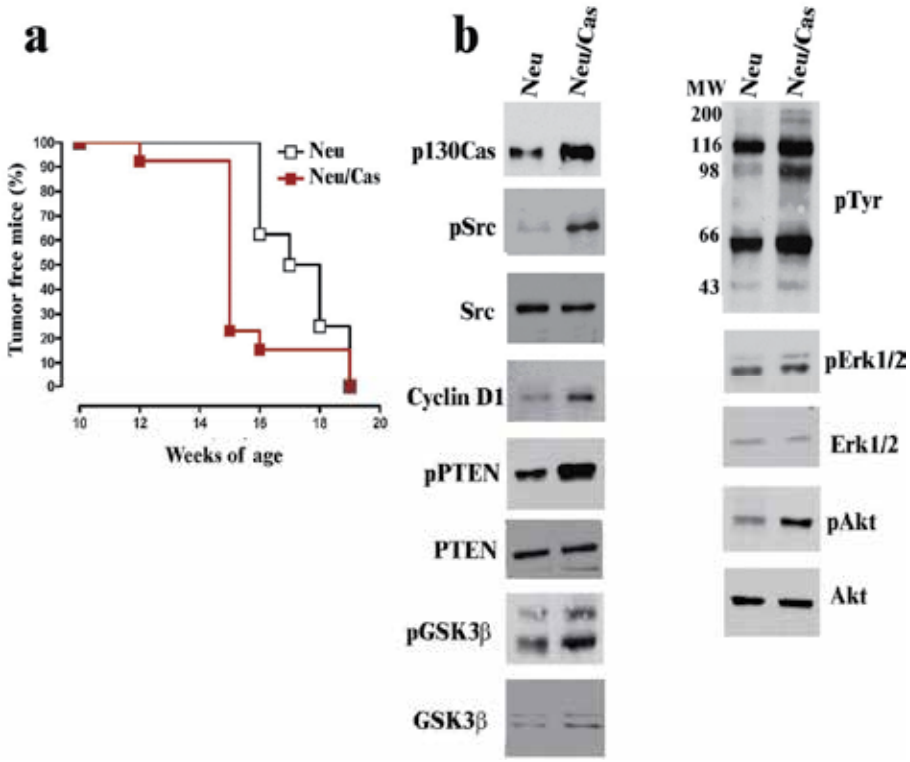


Fig. 3. Kinetics of tumor occurrence in p130Cas/HER2-Neu and HER2-Neu mice.
A) Tumor formation in p130Cas/HER2-Neu (gray line and black circles) and HER2-Neu (black line and empty squares) mice. Twenty mice were analyzed for each group. The difference of occurrence between the two groups is statistically significant, $P < 0.001$.
B) Independent epithelial cell culture were derived from four distinct tumors excised from p130Cas/HER2-Neu and HER2-Neu mice. Western blot analysis of protein extracts was done with the indicated antibodies and representative results are shown. MW, molecular weight markers. The figure is modified from Cabodi *et al.*, 2006.

To precisely underline the mechanism implicated in p130Cas/ErbB2-mediated transformation, cultures of MECs grown on three dimensional matrix, that share several properties with breast epithelial acini were evaluated. These in vitro three-dimensional acini-like structures provide a developmental context and serve as an important tool to study the biological effects of oncogenic signals. Most oncogenic signals that promote proliferative signals have the ability disrupt acini organization with oncogene-specific features. For instance, activation of ErbB2 induces formation of abnormal non invasive structures consisting of individual units (Muthuswamy *et al.*, 2001). Interestingly, in human

mammary cells MCF10A.B2, the concomitant activation of ErbB2 and p130Cas over-expression provides invasive properties (Figure 5). Consistently, p130Cas drives N202-1A cells *in vivo* lung metastases formation. These results demonstrate that p130Cas is an essential transducer in ErbB2 transformation and highlight its potential use as a novel therapeutic target in ErbB2 positive human breast cancers (Cabodi *et al.*, 2010c).

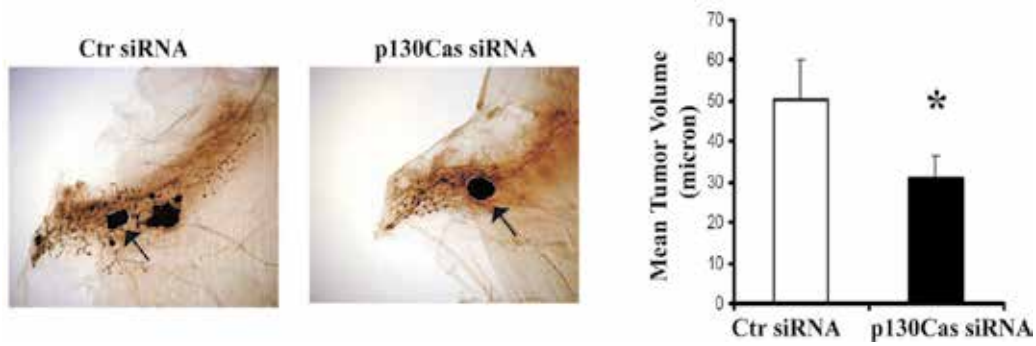


Fig. 4. p130Cas is required for *in vivo* ErbB2 tumorigenesis. Intra-nipple injection was performed in BalbC-NeuT female mice. Control (Ctr siRNA) or p130Cas stabilised siRNA (p130Cas siRNA) were injected once a week for 5 weeks starting from week 12. Left: Whole mount analyses of fixed mammary gland at week 18. The gland is composed of a tree-like structure of branching ducts. Small lesions that have histologic aspects of a solid carcinoma are visible. Black arrows indicate the lymph node. Ctr siRNA picture shows larger lesions on the right of the lymph node. Right: The histogram shows the mean tumour volume measured from two independent experiments with 8 mice per group. *p<0.0329 (two-tailed P value). The figure is modified from Cabodi *et al.*, 2010c.

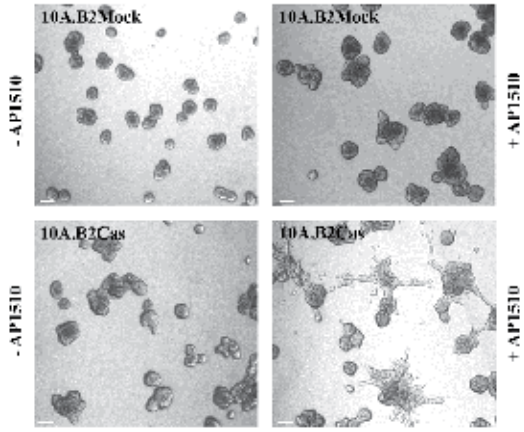


Fig. 5. p130Cas triggers acina invasion of ErbB2 transformed MCF10 cells. p130Cas over-expressing or Mock ErbB2 transformed MCF10 cells were plated on a Matrigel/collagen 1:1 matrix and left un-stimulated or activated for ErbB2 by treating with the small molecule AP1510. 3D invasive protrusions are present only in p130Cas over-expressing and ErbB2 activated acinar structures. The figure is modified from Tornillo *et* al., 2010.

We further analysed the molecular mechanisms through which p130Cas controls ErbB2-dependent invasion in three-dimensional cultures of mammary epithelial cells. Concomitant p130Cas over-expression and ErbB2 activation enhance PI3K/Akt and Erk1/2 MAPK signalling pathways and promote invasion of mammary acini. By using pharmacological inhibitors, we demonstrate that both signaling cascades are required for the invasive behaviour of p130Cas over-expressing and ErbB2 activated acini. Erk1/2 MAPK and PI3K/Akt signaling triggers invasion involving mTOR/p70S6K and Rac1 activation, respectively (Figure 6). Moreover, in silico analyses indicate that p130Cas expression in ErbB2 positive human breast cancers significantly correlates with higher risk to develop distant metastasis, thus underlying the value of the p130Cas/ErbB2 synergism in regulating breast cancer invasion. In conclusion, high levels of p130Cas favour progression of ErbB2-transformed cells towards an invasive phenotype (Tornillo *et al.,* 2010).

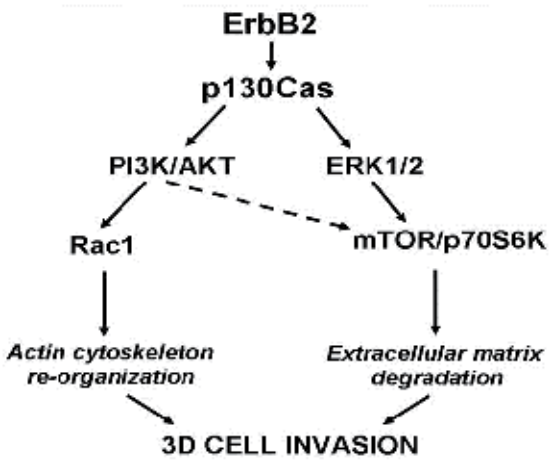


Fig. 6. Scheme illustrating the signaling pathways leading to 3D invasion of ErbB2 transformed MCF10 over-expressing p130Cas.

Both PI3K/Akt and Erk1/2 pathways are activated during invasion triggered by ErbB2 transformation of p130Cas over-expressing MEC. ErbB2/p130Cas/Erk1/2 MAPK signalling pathway preferentially targets mTOR/p70S6K, whereas the ErbB2/p130Cas/PI3K/Akt cascade triggers Rac1 activation. Both signaling pathways are required for mammary epithelia invasion in 3D suggesting that they cooperate in the regulation of different processes that ultimately lead to cell invasion. The figure is modified from Tornillo *et* al., 2010.

4. p140Cap adaptor protein

4.1 p140Cap structure and phosphorylation

The human p140Cap (Cas associated protein) is codified by the gene Srcin1, previously known as SNIP, P140 or p140Cap. The Srcin1 gene is conserved in human, mouse, rat, dog, cow, and zebrafish and in human is localized on Chromosome 17 q21.1.

The p140Cap protein was originally identified in rat brain as SNIP, a Synaptosome-associated protein SNAP-25b-interacting protein implicated in regulated exocytosis (Chin *et al.,* 2000). The name p140Cap derives from its identification as a protein associated to

p130Cas by affinity cromatography and MALDI-Mass spectrometry in epithelial cells (Di Stefano, 2004). p140Cap is a multisite docking protein, composed by a putative N-terminal mirystilation site, a tyrosine-rich domain, two prolin-rich regions, a coil-coiled domain, two regions rich in charged amino acids and a putative actin binding site (Figure 1)(Chin *et al.*, 2000; Di Stefano *et al.*, 2004).

p140Cap is mainly expressed in brain, testis and epithelial rich tissues such as mammary gland, lung, colon and kidney (Chin *et al.*, 2000; Di Stefano *et al.*, 2004; Ito *et al.*, 2008). The protein is present at least in two N-terminal alternative and two C-terminal different isoforms. The presence of many conserved sequence motifs that could undergo extensive post-translational modification, mostly tyrosine and serine phosphorylation, led to predict that p140Cap could promotes protein–protein interactions, leading to the formation of multiprotein complexes. Indeed p140Cap is tyrosine phosphorylated in epithelial cells upon integrin-mediated adhesion and EGF receptor activation (Di Stefano *et al.*, 2004). In addition, global phospho-proteomic analysis of human brain extracts revealed that p140Cap is phosphorylated on serine 859 in the context of the sequence 857RGS*DELTVPR866 (DeGiorgis *et al.*, 2005). The same sequence has also been found phosphorylated in mouse brain (Collins *et al.*, 2005).

4.2 p140Cap interacting proteins

Since its discovery, many proteins have been shown to bind directly or to associate in molecular complexes with p140Cap. In normal epithelial cells, p140Cap was found associated to the adaptor protein p130Cas. Although *in vitro* binding studies indicate that p140Cap and p130Cas are not directly linked, their association is mediated by the last 217 amino acids of the p140Cap C-terminal region and the p130Cas region encompassing amino acids 544-678. Through the same C-terminal region, p140Cap binds directly to the SH3 domain of the Src kinase. Moreover in MCF7 cells p140Cap has been shown by Far Western Blotting to bind directly the kinase C-terminal Src kinase (Csk), a potent negative regulator of Src (Di Stefano *et al.*, 2007). The physiological significance of p140Cap interaction with Src and Csk relates to p140Cap ability to regulate Src activation and downstream signaling (see below).

By two hybrid screen in human brain, the C-terminal motif of p140Cap has also been found to associate with the SH3 domain of Vinexin (Ito *et al.*, 2008), belonging to a family composed of vinexin, c-Cbl associated protein/ponsin, and Arg-binding protein 2 (Kioka *et al.*, 2002; Matsuyama *et al.*, 2005). In non-neuronal cells, Vinexin is localized at focal adhesions and shown to be involved in growth factor- and integrin-mediated signal transduction, actin cytoskeletal organization, cell spreading, motility, and growth (Kioka *et al.*, 2002). Always in brain, p140Cap directly associates with all the members of the microtubule plus-end tracking protein EB family through a short 92 amino acid C-terminal region, likely through a positively charged S/P-rich region (Jaworski *et al.*, 2009). The p140Cap interaction with Vinexin and EB family proteins in tumour cells remains to be established.

Finally, in breast cancer cells, p140Cap has also been shown to bind with Cortactin (Damiano *et al.*, 2011). Cortactin is a major substrate of Src kinase and localizes to cortical actin structures where it regulates early cell migration and invasion by controlling actin assembly (Weed *et al.*, 2000; Wu and Parsons, 1993; Wu *et al.*, 1991). p140Cap/Cortactin association requires the second proline-rich domain of p140Cap and the Cortactin SH3 domain, suggesting a direct interaction between the two proteins. p140Cap binding to Cortactin controls invasion properties of breast cancer cells (Damiano *et al.*, 2011).

In conclusion, p140Cap is involved in direct interactions with several proteins (Figure 1). The p140Cap binding partners are mainly implicated in membrane fusion and actin cytoskeleton remodelling. p140Cap association to p130Cas, Src, Cortactin and the presence of a putative actin binding domain in the p140Cap sequence, suggest that p140Cap could be an actin binding protein. Indeed, p140Cap has been described to co-localize with actin stress fibers and cortical actin both in epithelial and in neuroectodermal cells (Chin *et al.*, 2000; Di Stefano *et al.*, 2004; Jaworski *et al.*, 2009).

4.3 140Cap in human breast cancer

So far, few data are available on p140Cap in human tumors. Immunohistochemistry analysis of normal mammary tissue show that p140Cap expression is confined to the luminal cells of alveoli, suggesting that in normal conditions p140Cap might play a role in mammary cell differentiation. In contrast, in human breast tumours p140Cap is not expressed in 70% of tumour specimens, showing an inverse correlation with the state of malignancy.

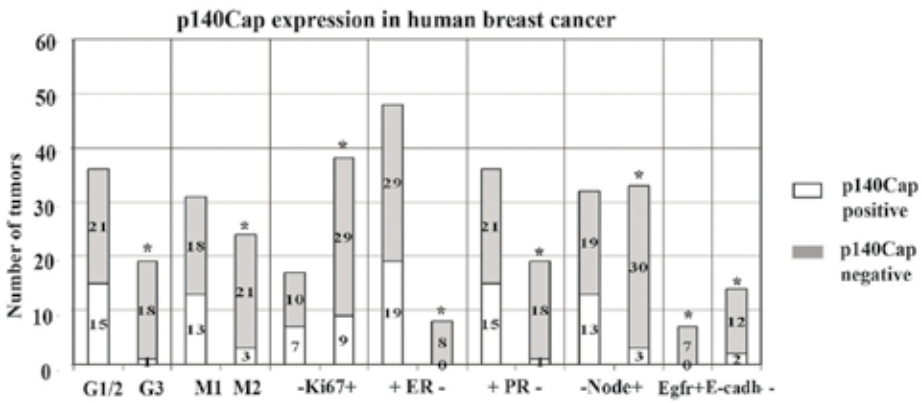


Fig. 7. p140Cap expression is lost in aggressive human breast cancers. In the histogram we reported the number of tumours positive (white) or negative (grey) for p140Cap expression according to tumour grade (low grade G1/G2, high grade G3), number of mitosis 10/10 HPS (M1 Mitosis < 10, M2 Mitosis>10), Ki67 proliferation index (Ki67+>24% , Ki67- <24%), Estrogen Receptor staining (ER-, ER+), Progesterone Receptor staining (PR-, PR+), infiltration in lymph nodes (Node+, Node-), EGFR staining (EGFR+), E-Cadherin staining (E-Cad-). The figure is modified from Damiano *et al.*, 2010.

Interestingly, 94.8% of aggressive G3 tumours, 87% of the Node +, 86.5% of tumours with a mitosis major number of 10/10HPF, and 76% of highly proliferative tumours (revealed by Ki67 staining), lose p140Cap expression. Moreover, none of the E-cadherin negative and EGFR positive tumours express p140Cap, suggesting mutually exclusive correlation between EGFR and p140Cap expression (Figure 7) (Damiano *et al.*, 2010). Therefore, although limited, these data point out that only low grade breast tumors express p140Cap. Further analysis is required to draw a general picture of the relevance of p140Cap in human breast cancers, and to delineate a potential use of p140Cap as a diagnostic and prognostic factor.

4.4 p140Cap modulates Src activity and EGFR signalling in breast cancer cells

The major function of the p140Cap adaptor is its ability to regulate Src kinase activation. In particular, in breast cancer cells, upon cell-matrix adhesion or EGF stimulation, p140Cap activates the Csk kinase, that phosphorylates the negative regulatory tyrosine 530 on the C-terminal domain of Src (Latour and Veillette, 2001) , resulting in inhibition of Src kinase. Consistently p140Cap silencing increases Src activation, leading to a fine tuning of integrin and growth factor receptor signalling (Figure 2) (Damiano *et al.,* 2010; Di Stefano *et al.*, 2007)
As a consequence, in breast cancer cells expressing high levels of p140Cap, upon integrin-mediated adhesion, the association between Src and Fak is impaired as well as integrin-dependent p130Cas phosphorylation (Figure 2). As described above p130Cas phosphorylation leads to the assembly of a p130Cas-Crk signalling complex that drives for cell migration and invasion through activation of Rac. Therefore elevated levels of p140Cap severely impair integrin-dependent Rac activity, while its down-regulation induces a sustained Rac activation (Di Stefano *et al.*, 2007).
In MCF7 breast cancer cells, p140Cap functionally interacts with E-cadherin and EGFR at the cell membrane, behaving as a new player in E-cadherin-dependent down-regulation of EGFR signalling. Indeed p140Cap-dependent inhibition of Src kinase activity results in E-cadherin immobilization at the cell membrane (Damiano *et al.,* 2010). E-cadherin is known to inhibit EGFR, either by interaction through the extracellular domains or by a beta catenin-dependent mechanism (Perrais *et al.*, 2007; Qian *et al.*, 2004; Takahashi and Suzuki, 1996). Consistently, EGFR activation, association and phosphorylation of Grb2 and Shc and Ras/Erk1/2 MAPK activities are profoundly impaired by p140Cap over-expression and enhanced by its silencing (Damiano *et al.,* 2010). Interestingly, rescue of Src activity and of E-cadherin mobility is sufficient to recover EGFR phosphorylation, but not Ras and Erk1/2 activation, that require an active RasV12, suggesting that p140Cap might regulate the Ras pathway through an additional mechanism. Therefore, in MCF7 cancer cells, p140Cap regulates EGFR signalling with dual mechanisms, involving both an E-cadherin-dependent inactivation of EGFR and a Ras-dependent inhibition of Erk1/2 activity (Damiano *et al.,* 2010).
Moreover, p140Cap expression also inhibits EGFR, Src and Erk phosphorylation in the highly aggressive MTLn3-EGFR breast cancer cells. Interestingly, in these cells, p140Cap affects also Cortactin phosphorylation in response to EGF (Damiano *et al.*, 2011).

4.5 p140Cap affects cell proliferation and in vivo tumour growth of breast cancer cells

The ability of p140Cap to regulate Src and Ras pathways profoundly affects cell proliferation. Elevated expression of p140Cap in both breast and colon cancer cells inhibits in vitro proliferation, but does not affect cell survival (Damiano *et al.*; Di Stefano *et al.*, 2007). Interestingly, p140Cap over-expression impairs colony formation in soft agar, while its silencing leads to a significantly increased number of colonies, demonstrating that p140Cap, likely through the regulation of integrin signalling, controls anchorage-independent growth (Di Stefano *et al.*, 2007). *In vivo* xenografts of breast and colon cancer cells show that cells expressing high levels of p140Cap are impaired in tumour formation. Consistently, p140Cap silencing in carcinoma cells dramatically increases *in vivo* tumour formation. Strikingly, p140Cap knock-down is sufficient for *in vivo* growth of MCF7 cells even in the absence of estrogen pellets, a condition in which control cells are unable to grow. These last findings also rise the possibility that p140Cap may regulate estrogen receptor signalling, contributing

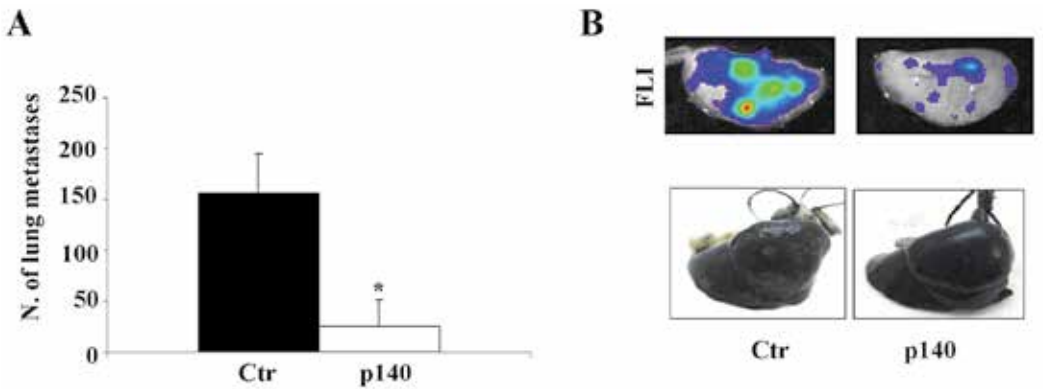
to breast cancer resistance to hormonal therapies. Thus these data provide evidence that p140Cap behaves mechanistically as a tumour suppressor molecule in breast and colon cancer cells, with a broad effect on cell proliferation and tumorigenesis.

4.6 p140Cap affects in vitro motility and invasion of breast cancer cells

As expected for the major role of Src in actin cytoskeleton dynamics and cell migration, high levels of p140Cap impair spreading and extension of lamellipodia and filopodia on extracellular matrix proteins of breast cancer cells. In addition, p140Cap over-expression also inhibits migration on fibronectin-coated transwells and invasion in Matrigel. Consistently, p140Cap silencing induces an increase in cell spreading in the early phases of cell adhesion, a fibroblastic-like shape and increased motility and invasion. Cells expressing a truncated form of p140Cap, lacking the Src-binding domain, restores integrin-dependent Src and Rac activation and are capable of migrating and invading properly (Di Stefano *et al.*, 2007).

In addition, p140Cap specifically interferes with invasive and migratory properties of cancer cells blocking E-cadherin/EGFR cross-talk in both breast and colon cancer cells. The ability of p140Cap to immobilize E-cadherin at the cell surface strengthenes cell-cell adhesion and inhibition of cell scatter in response to EGF. Rescue of Src activity by the expression of a kinase-defective Csk mutant or by Csk silencing, recover E-cadherin mobility at the cell surface and the ability to scatter in response to EGF (Damiano *et al.*, 2010)

Moreover, we recently identified p140Cap as a critical regulator of *in vitro* cell motility and invasion and *in vivo* metastasis formation of highly metastatic MTLn3-EGFR breast cancer cells. Our data show that increasing p140Cap expression in the highly aggressive MTLn3-EGFR cells results in an 80% decrease in *in vivo* lung metastasis formation (Figure 8).



The figure is modified from Damiano *et al.*, 2011.

Fig. 8. p140Cap over-expression inhibits spontaneous lung metastasis formation.
A) $5x10^5$ Ctr and p140 cells were injected subcutaneously in $Rag2^{-/-}\gamma c^{-/-}$ mice. Right panels: after sacrificing the mice, lungs were coloured with ink, metastasis were counted and the number of metastasis reported in the y axis of the histogram. Statistical significances were evaluated by Student's t-test: Ctr EGF vs p140 EGF (*$p<0.05$).
B) Upper panels: two representative pictures of lung metastases visualized with the FLI (GFP detection) after spontaneous metastasis assay with the MTLn3-EGFR Ctr and p140 cells. Lower panels: two representative pictures of the lungs coloured with ink are shown.

Consistently, p140Cap over-expressing MTLn3-EGFR cells show also reduced anchorage-independent cell growth, which is an *in vitro* characteristic that predicts the *in vivo* metastatic potential of many tumour cells. Furthermore, detailed *in vitro* analysis of cell migratory and invasive abilities showed that p140Cap over-expressing cells have an impaired capacity to migrate in response to EGF. Remarkably, p140Cap over-expressing cells display an increased number and area of focal adhesions, which correlate with the presence of actin stress fibers consistent with a less dynamic turnover of adhesive structures. Cortactin tyrosine phosphorylation has been shown to regulate MTLn3 cells invadopodia assembly and maturation (Oser *et al.*, 2009). Our results show that in p140Cap over-expressing cells cortactin phosphorylation in response to EGF is decreased. Indeed, the expression of the phosphomimetic cortactin mutant is sufficient to completely rescue the defects in migration and invasion of MTLn3-EGFR p140Cap over-expressing cells. Taken together, these data demonstrate that p140Cap suppresses the invasive properties of highly metastatic breast carcinoma cells by inhibiting cortactin-dependent cell motility (Damiano *et al.*, 2011).

5. Conclusions

As outlined in this chapter p130Cas and p140Cap adaptor proteins represent key elements in the control of cell migration and invasion in breast cancer cells. Interestingly, in breast cancers, p130Cas results frequently over-expressed, while p140Cap is not expressed in the more aggressive human breast cancers. Interestingly, Src kinase is a common target of these two proteins. However, even though both p130Cas and p140Cap have been described to bind to Src, they exert opposite roles on Src activity. Indeed p130Cas enhances and sustains Src activity, while p140Cap is a negative regulator of Src kinase. Therefore, it is likely that Src activity is finely tuned by p130Cas and p140Cap relative expression in cells in which they are co-expressed. As a consequence in breast tumors their reciprocal levels of expression might profoundly influence the ability of cancer cells to acquire invasive properties. Although still limited, the analysis of human breast tumors suggests that an overbalance towards p130Cas over-expression might represent a negative prognostic marker in human breast cancer specimens, indicating progression to a more aggressive phenotype.

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20

Fibrillar Human Serum Albumin Suppresses Breast Cancer Cell Growth and Metastasis

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1. Introduction

1.1 Breast cancer classification

Breast cancer is one of the most common cancers among women worldwide and approximately one-third of women diagnosed will eventually develop metastases and die (Jemal et al, 2010). Breast cancer is heterogeneous at the molecular, histopathologic and clinical levels and is commonly classified into several categories according to multiple schemes, each based on different criteria. A typical description of breast cancer can be comprised of tumor grade, histologic type, tumor stage, and the expression of proteins and genes etc. (McSherry et al, 2007). Normal non-cancerous cells are differentiated and have specific cell shapes and functions; whereas, cancer cells lose differentiation (de-differentiate), have less uniform nuclei, and exhibit uncontrolled cell division. Pathologists, therefore, determine breast cancer by grade according to the degree of differentiation of cells compared to normal breast cells: highly differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade). Cancers classified as high grade generally have a worse prognosis (McSherry et al, 2007). The majority of breast cancers are derived from the epithelium lining the ducts or lobules of the breast. They can be classified histologically according to characteristics seen upon light microscopy of biopsy specimens. Histologic classification is divided into: ductal carcinoma in situ (DCIS), invasive ductal carcinoma, and invasive lobular carcinoma (McSherry et al, 2007). Breast cancer can further be classified using the TMN Classification of Malignant Tumors, TMN stage is based on tumor size, lymph node micrometastasis, and macrometastasis, where 'T' describes tumor size; 'N' indicates whether or not the tumor has spread to the lymph nodes; and 'M' indicates whether or not distant metastasis has occurred. Larger tumor size with lymph nodal spread and distal metastasis has a worse prognosis (Gonzalez-Angulo et al, 2007). Expression of certain proteins and genes can also be used to classify breast cancer (McGrogan et al, 2008; Stickeler et al, 2009). Whole-genome analysis using expression microarray and immunohistochemical analysis has revolutionized the understanding of breast carcinomas in recent years, and led to the discovery of five distinct subtypes of breast carcinomas (luminal A, luminal B, HER-2 overexpression, basal-like, and normal-like), each with unique recognizable phenotypes and clinical outcomes (McGrogan et al, 2008; Stickeler et al, 2009). By using classification to characterize each cancer patient, it may help select the suitable treatment strategies to achieve an optimal outcome and increase therapeutic efficacy.

2. Breast cancer metastasis

In the United States, about 178,480 new cases of invasive breast cancer were diagnosed in 2007 and approximately 40,460 women died (Jemal et al, 2007). In 2010, about 207,090 new breast cancer cases were diagnosed and 39,840 died (Jemal et al, 2010). The breast cancer incidence rate has been decreasing in the USA since 1999 and the majority of 40,000 women died each year were due to breast cancer metastasis (Giordano & Hortobagyi, 2003; Jemal et al, 2010). Cancer metastasis is a complex process that includes intercellular and intracellular signaling, activation, adhesion, migration and invasion (Im et al, 2004; Lee et al, 2006). Epithelial-to-mesenchymal transition (EMT) is also thought to be involved in cancer metastasis. EMT may promote cancer-cell progression and invasion into the surrounding microenvironment. Historically, epithelial and mesenchymal cells are distinct in their unique cellular appearance and the morphology of the multicellular structures they create (Shook & Keller, 2003). A typical morphology of epithelium is sheeted and thick with individual epithelial cells abutting each other in a uniform array. Cell-to-cell junctions and adhesions between neighboring epithelial cells hold cells tightly together and inhibit the movement of individual cells away from the epithelial monolayer. Mesenchymal cells, on the other hand, possess usually a more extended and elongated shape and do not exhibit either a regimented structure or tight intracellular adhesion. Mesenchymal cells are irregular in shape and not uniform in composition or density. Adhesions between mesenchymal cells are not as strong as those of their epithelial counterparts, allowing for increased migratory capacity. The transformation of an epithelial cell into a mesenchymal cell not only alters cellular morphology, architecture, adhesion capacity, and migration capacity but also enhances capability of the cell to metastasize (Shook & Keller, 2003). Conversely, the transformation of a mesenchymal cell into an epithelial cell (MET) may prevent cell invasion and suppress cell metastatic ability.

3. Breast cancer therapy

To date, adjuvant and neo-adjuvant therapies are commonly used in cancer metastasis therapy (McGrogan et al, 2008). Currently, there are three main groups of medications used for adjuvant breast cancer treatment: (1) hormone blocking therapy; (2) chemotherapy; and (3) monoclonal antibody therapy (McGrogan et al, 2008). The cell surfaces of some breast cancers are estrogen receptors positive (ER^+) and/or progesterone receptors positive (PR^+) and the cells require estrogen to continue growing. These cancers can be treated with drugs that block either the hormone receptors, such as tamoxifen or the production of estrogen, such as anastrozole (Arimidex) or letrozole (Femara). The drugs that inhibit estrogen production are only suitable for post-menopausal patients (Gonzalez-Angulo et al, 2007). Combination chemotherapy is predominately used for patients at stages 2-4, being particularly beneficial in ER-breast cancer. One of the most common treatments is cyclophosphamide plus doxorubicin (Adriamycin) which destroys rapidly growing or replicating cancer cells by causing DNA damage; however, these drugs also damage normal cells causing serious adverse effects. Damage to heart muscle is the most dangerous complication associated with doxorubicin. Taxane drugs such as paclitaxel, a microtubule-stabilizing agent that interferes with spindle microtubule dynamics causing cell-cycle arrest and apoptosis through interaction with β-tubulin (Bergstralh & Ting, 2006), is also used in the breast cancer metastasis therapy. However, resistance to paclitaxel is common and there

is a need to identify patients most likely to respond to treatment (McGrogan et al, 2008). Other treatments like methotrexate and fluorouracil are also used in chemotherapy. Approximately 15-20% of breast cancers have an amplification of the HER-2/neu gene or overexpression of its protein product. This receptor is a marker for poor prognosis that is associated with increased disease recurrence during the period of cancer therapy (Brown et al, 2008). Trastuzumab (Herceptin), a humanized monoclonal antibody that specifically binds to the extracellular domain of the HER-2 receptor, has improved the 5-year disease free survival of stage 1-3 HER-2^+ breast cancers to about 87%. However, about 2% of patients suffer significant heart damage after Herceptin treatment (Brown et al, 2008). Trastuzumab has also been used in combination with doxorubicin and proven to be highly effective for metastatic breast cancer patients with HER-2 over-expressing tumors. However, this regimen causes severe cardiac toxicity in 27% of treated patients when the two substances are given concurrently (Stickeler et al, 2009). Lapatinib (Tykerb, GlaxoSmithKline) is an orally active small molecule that inhibits the tyrosine kinases of HER-2 and epidermal growth factor receptor type 1 (EGFR). In preclinical studies, lapatinib showed no cross-resistance with trastuzumab (Jahanzeb, 2008).

Conventional radiotherapy is usually given after surgery to destroy remaining tumor cells that may have escaped surgery. Recently, radiotherapy has also been given at the time of surgery and found to reduce the risk of recurrence by 50-66% (Belletti et al, 2008). Despite such improvements in treatment modalities, there is still a high rate of failure among adjuvant interventions mainly due to tumor invasion and metastasis. Therefore, the search for new therapeutic targets and the development of new inhibitors of tumor cell resettlement and metastatic growth continues.

4. Surface membrane integrins as potential drug-discovery targets

It is well known that cell activation, migration, proliferation, and differentiation require direct contact between cells and the extracellular matrix (ECM). Cell-to-cell and cell-to-matrix interactions are mediated by the integrin, selectin, cadherin and/or immunoglobulin families and several studies have focused on investigating cancer therapies based on the integrin superfamily. Integrin expression on cancer cells is frequently associated with cancer progression and metastasis; therefore, targeting small-molecule antagonists of the integrin superfamily provides an opportunity to suppress cancer development and metastasis (Mullamitha et al, 2007). β1 integrin, which frequently aberrantly expressed in human breast carcinomas, has been verified to play a central role in metastasis and contribute to growth factor receptor signaling. Inhibition of the β1 integrin signaling pathway has been shown to abolish the formation of metastasis in breast and gastric cancer models. Additionally, the β1 integrin signaling pathway also plays a significant role in mediating resistance to cytotoxic chemotherapies by enhancing cell survival in hematologic malignancies, lung, and breast cancers (Lu et al, 2008). Recent studies have shown that α1β1, α2β1, and α3β1 integrins regulate hepatocarcinoma cell invasion, angiogenesis of human squamous cell carcinoma, and increase migration and invasion of malignant glioma, melanoma and mammary adenocarcinoma cells, respectively. Expression of α5β1 integrin in colon cancer cells decreases HER-2-mediated proliferation (Kuwada et al, 2005). Loss of the α7β1 integrin in melanoma increases highly tumorigenic and metastatic phenotypes (Ziober et al, 1999). Several preclinical and clinical trials have shown that some integrin targeting antibodies can effectively block tumor growth and metastasis. These antibodies include MEDI-522 (vitaxin)

against αvβ3 integrin (Brooks et al, 1994), CNTO 95 against both αvβ3 and αvβ5 integrins (Mullamitha et al, 2007), 17E6 against αvβ3, αvβ5, and αvβ1 integrins (Mitjans et al, 2000), LM609 against αvβ3 integrin, and Tysabri (natalizumab) against α4 integrins (O'Connor, 2007). In addition, β1 integrins possess a RGD-binding region, therefore, based mainly on their RGD containing peptides and RGD peptidomimetics, some small molecule integrin antagonists have also shown potent inhibition of angiogenesis (Kumar et al, 2001). Both fibronectin and its receptor integrin α5β1 directly regulate angiogenesis (Kim et al., 2000). Thus, antagonist(s) of α5β1 integrin might be useful targets for the inhibition of angiogenesis associated with human tumor growth, and neovascular-related ocular and inflammatory diseases (Pasterkamp et al, 2003; Suzuki et al, 2007). Further, our own studies recently found that fibrillar bovine serum albumin (F-BSA) induced apoptosis in human breast duct carcinoma cell line T47D, and fibrillar fibronectin (F-FN) induced apoptosis in human breast cancer cell line MCF-7. F-BSA and F-FN induced BHK-21 cell (baby hamster kidney cell) apoptosis through negatively regulating the integrin/FAK/Akt/GSK-3β signaling pathway and activating SHP-2 and RhoA/ROCK (Huang et al, 2009; Huang et al, 2010). Together these results suggest that inhibition of the β1 integrin signaling pathway may provide a promising therapeutic approach to breast cancer metastasis.

5. Formation and purification of fibrillar human serum albumin

Some diseases like Alzheimer's disease, transmissible spongiform encephalopathies, pancreatic islet amyloidosis, and familial amyloidosis are caused primarily by amyloid-like fibrils aggregation in organs and in the circulation (Jackson & Clarke, 2000). Recently, it has been documented that amyloid-like fibrils are cytotoxic to neuronal cells, BHK-21 cells, SKOV-3, and MCF-7 cancer cells (Gharibyan et al, 2007; Su & Chang, 2001; Zamotin et al, 2006). Whether the fibrillar proteins may be used as anti-cancer drugs in the cancer therapies is largely unclear. We have developed a novel process to convert globular proteins, bovine serum albumin and fibronectin, to fibrillar forms using detergent assisted refolding chromatography (Huang et al, 2009; Huang et al, 2010). This procedure is easier to perform than other methods reported to convert proteins to fibrillar structures such as glycation, sonication, or high temperature incubation (Azakami et al, 2005; Taboada et al, 2006). Fibrillar protein F-BSA induced apoptosis in human breast duct carcinoma cell line T47D, and F-FN induced apoptosis in human breast cancer cell line MCF-7 suggesting that fibrillar proteins may have therapeutic effect in human breast cancer cells. We thus further investigated the effects of the fibrillar form of human serum albumin (F-HSA) on the malignant breast cancer cell lines, TS/A and MDA-MB-231. We chose F-HSA for further study for two reasons: first, because F-HSA is less likely to provoke an immune response in the human body; and second, because HSA is easier to obtain and less costly than FN. We produced F-HSA using the same process as was used to produce F-BSA. In brief, 20 mg of HSA from human serum was dissolved in 10 ml of PBS with 1% SDS (w/v). The HSA solution was sonicated for 5 minutes and subsequently applied to a Superdex-200 column previously equilibrated with the eluting buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl, and 0.05% SDS). The fractions that contained HSA were pooled and dialysed against PBS to remove SDS. The yield of the F-HSA was about 67% (Fig. 1). The F-HSA produced was then tested for fibrillar structure by transmission electron microscopy (TEM). For TEM analysis of F-HSA, 2 mg/ml of protein was applied to a 300-mesh carbon-coated copper

grid. Excess samples were removed, and the grid was air dried. The protein-bearing grid was negatively stained with 1% (w/v) phosphotungstic acid for 1 minute. Transmission electron micrographs were observed at 20,000–150,000× magnification at 75 kV on a Hitachi H-7000 electron microscope. TEM analysis showed that F-HSA did indeed have a fibril structure (Fig. 2).

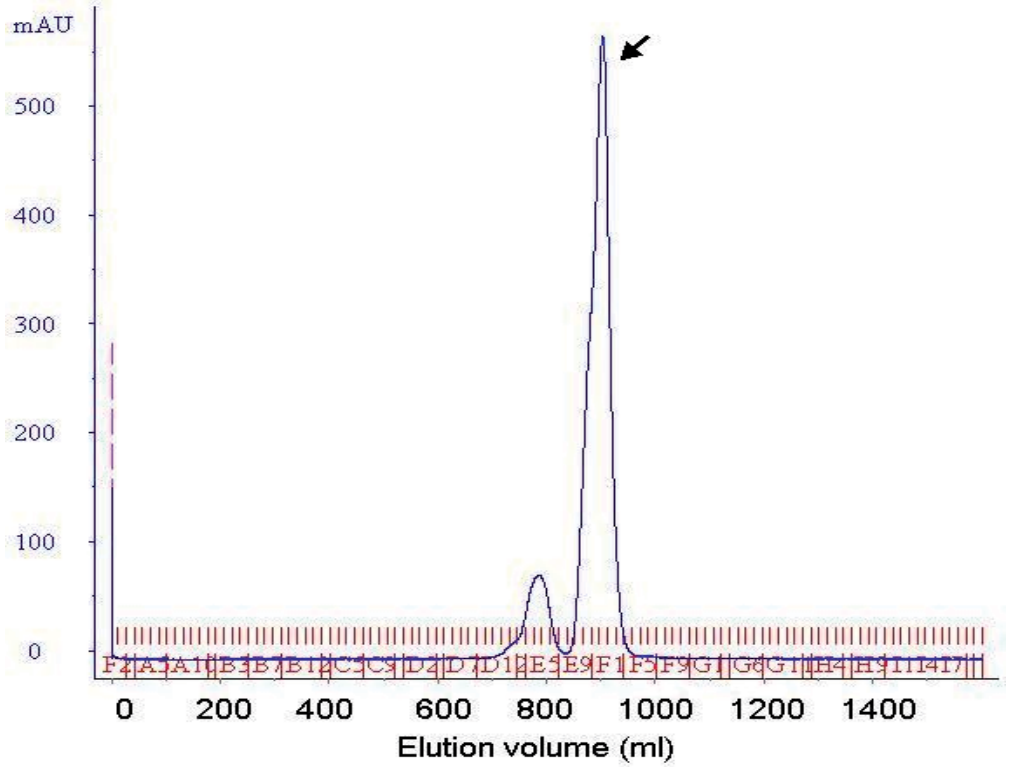


Fig. 1. Elution profile of F-HSA from a Superdex-200 column. HSA (2 mg/ml dissolved in PBS containing 1% SDS) was applied to a Superdex-200 column and eluted at a rate of 1 ml/min with a buffer solution containing 0.05% SDS. Arrow shows F-HSA.

Specific binding to Thioflavin T (ThT) is one of the characteristics of amyloid-like proteins. ThT fluorescence assay was, thus, used to identify amyloid-like fibrils (LeVine, 1999). Like Aβ (1-42), which is known to have fibrillar structure and was used as a positive control, F-HSA obtained from the Superdex-200 column exhibited a gradual dose-dependent increase in ThT fluorescence level (Fig. 3).

6. Effects of F-HSA on cell viability

Previously, we demonstrated that F-BSA and F-FN induced apoptosis in the less malignant T47D and MCF-7 breast cancer cell lines, respectively (Huang et al, 2009; Huang et al, 2010). In this study, we examine whether F-HSA induced cytotoxicity in the more malignant breast

cancer cell lines, TS/A and MDA-MB-231, using a 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-colorimetry assay to measure the cell viability (MERCK, Darmstadt, Germany). TS/A, a murine mammary adenocarcinoma cell line that is estrogen dependent, was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO®); and MDA-MB-231 (ATCC **HTB-26™**), a metastatic human breast cancer cell line that is estrogen independent, was cultured in DMEM/F12 medium (GIBCO®). In brief, 2×10^4 breast cancer cells were incubated in serum-free medium and treated with serial dilutions of F-HSA. After incubation for 24 hours to allow the drug to take effect, 10 µl MTT solution was added to each well. After incubation at 37°C in 5% CO_2 for another 2 hours to allow the MTT solution to be metabolized, formazan (MTT metabolic product) was resuspended in 200 ul DMSO. Finally, the proportions of surviving cells were determined by optical density (570 nm test wavelength, 630 nm reference wavelength). The percentage of surviving cells was calculated as $(O.D._{treatment}/O.D._{control}) \times 100\%$, and the percentage of growth inhibition was calculated as $[1 - (O.D._{treatment}/O.D._{control})] \times 100\%$. IC_{50} value is the concentration at which the reagent produces 50% inhibition of cellular viability. F-HSA inhibited growth of the breast cancer cell lines TS/A and MDA-MB-231 in a dose dependent manner with IC_{50} values of 0.15 and 0.48 µM, respectively (Fig. 4). F-HSA at concentrations over 0.4 µM induced dose-dependent cytotoxicity in both TS/A cells and MDA-MB-231 cells, whereas concentrations of 0.1-0.2 µM did not affect cell viability significantly.

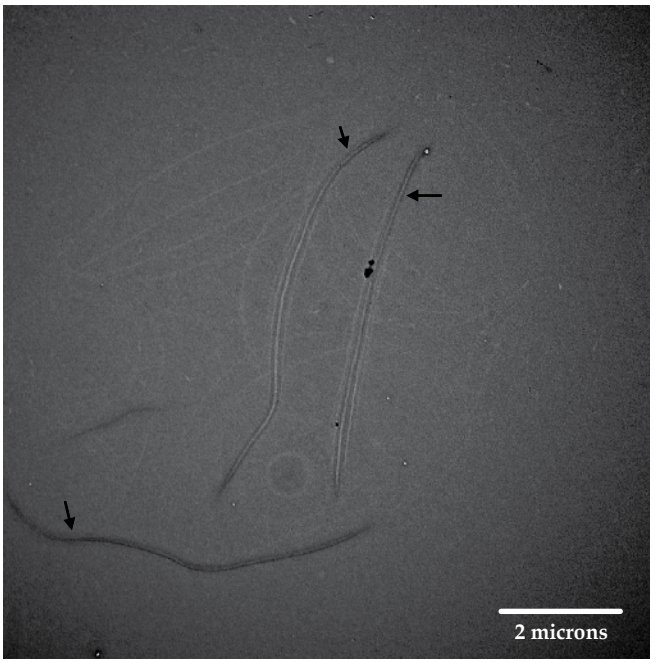


Fig. 2. Ultra-structures of F-HSA were observed by TEM. F-HSA was applied to a 300-mesh carbon-coated copper grid then the grid was air-dried. The F-HSA-bearing grid was negatively stained with 1% (w/v) phosphotungstic acid. Finally, transmission electron micrographs were observed at 20,000–150,000× magnification at 75 kV on a Hitachi H-7000 electron microscope. Arrows show F-HSA.

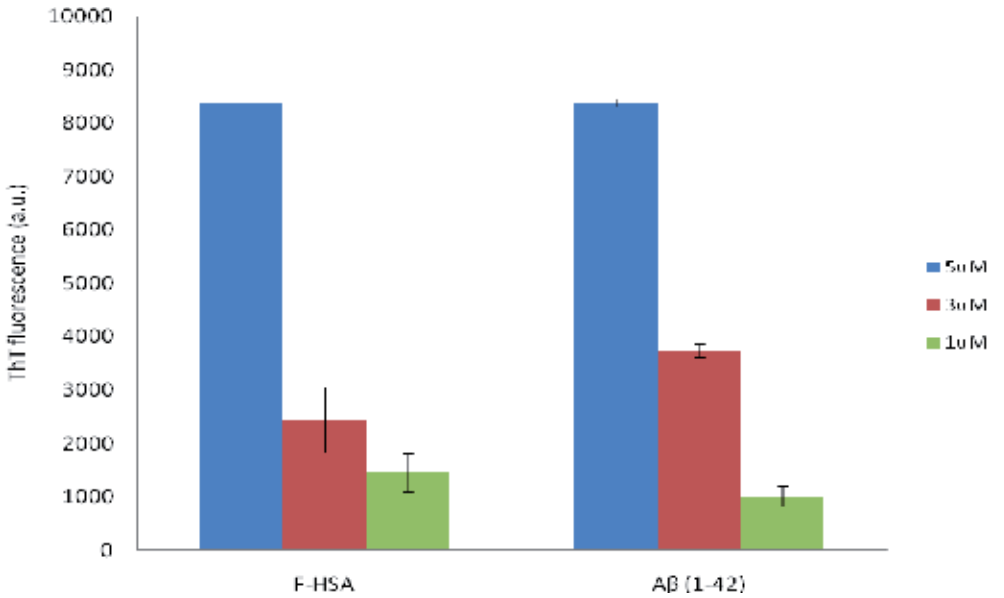
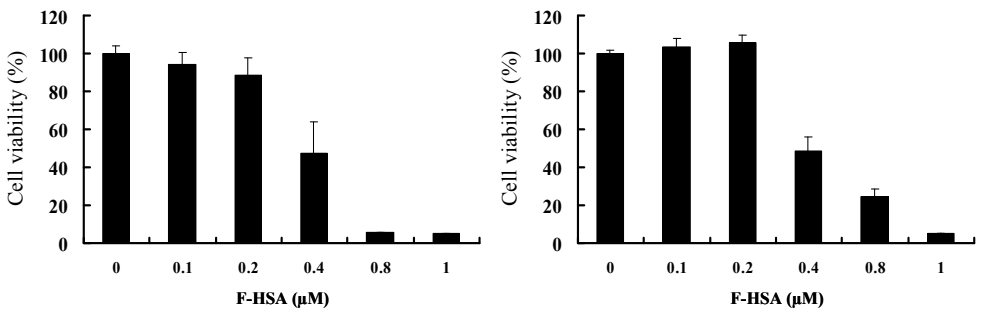


Fig. 3. ThT fluorescence assay of F-HSA. For fluorescence measurements, increasing concentrations of proteins were incubated with 20 μM ThT for 1 h at room temperature, and fluorescence was measured in triplicate on a Wallac Victor² 1420 Multilabel Counter (Perkin Elmer Life Science, Waltham, MA, USA). Excitation and emission wavelengths were 430 nm and 486 nm, respectively. ThT background signal from buffer solution was subtracted from the corresponding measurements. Aβ (1-42) was used as a positive control.



(a) (b)

Fig. 4. Effect of F-HSA on viability of TS/A (A) and MDA-MB-231 cells (B).

To understand the effects of F-HSA on cell morphology and MET in TS/A cells and MDA-MB-231 cells, breast cancer cells were treated low concentrations of F-HSA and cell morphology was observed under light microscopy. F-HSA induced a morphological alteration in cells, from a fibroblast-like shape to a round shape (Fig. 5). We also examined whether F-HSA suppressed breast cancer-cell migration at non-cytotoxic concentrations by

wound-healing assay. TS/A and MDA-MB-231 cells were plated onto six-well tissue culture dishes in complete tissue culture medium until they formed a confluent monolayer. The cell monolayer was scratched with a sterile pipette tip to generate a wound (width 2 mm). The remaining cells were washed three times with culture medium to remove cell debris. The medium was immediately replaced with serum-free medium with 0.1 or 0.2 μM of F-HSA, and cultured at 37°C for 24 hours. Spontaneous cellular migration was then monitored at 0 hours (immediately after wounding) and 24 hours (the end of F-HSA treatment) using and inverted microscope (Axiovert 200M; Zeiss) at 100× original magnification. The extent of wound healing was determined by the distance (migrating distance) traversed by cells migrating into the denuded area. F-HSA at concentrations of 0.1 to 0.2 μM suppressed cell migration of both TS/A and MDA-MB-231 cells (Figs. 6-7).

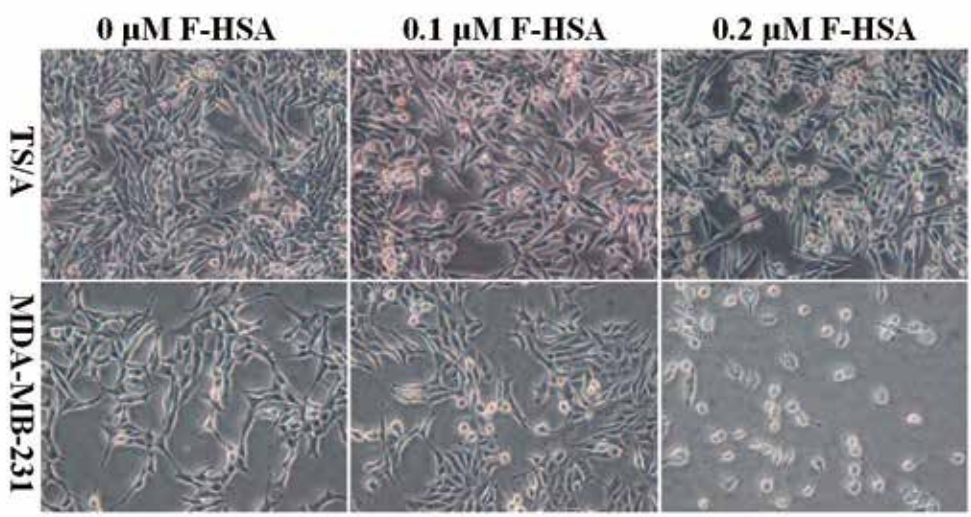


Fig. 5. F-HSA induced morphological alterations and mesenchymal-to-epithelial transition in breast cancer cells. After 0.1 μM and 0.2 μM of F-HSA treatment at 37°C for 24 h, cell morphology was observed under light microscopy. Scale bar, 5 μm

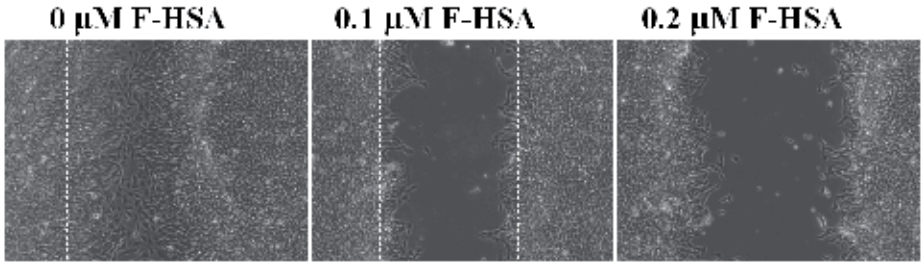


Fig. 6. F-HSA suppressed TS/A cell migration in a breast cancer cell wound-healing assay. After 0.1 μM and 0.2 μM of F-HSA treatment at 37°C for 24 h, cell migration was observed under light microscopy.

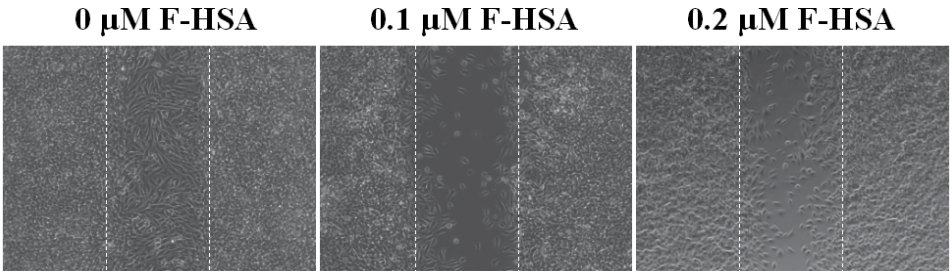


Fig. 7. F-HSA suppressed MDA-MB-231 cell migration in a wound-healing assay. After 0.1 μM and 0.2 μM of F-HSA treatment at 37°C for 24 h, cell migration was observed under light microscopy.

7. F-HSA suppresses breast cancer cell migration via β1 integrin signaling pathway

Cell surface receptors mediate cell-to-matrix and cell-to-cell interactions. Integrins are a large family of heterodimeric transmembrane receptors that mediate cell-ECM interactions. In eukayotic cells, integrins consist of 18 α subunits and 8 β subunits that form 24 different αβ integrins. The particular combination of α and β subunits in integrin dimers determines their specificity for ligands, which include most of the ECM proteins such as FN and collagen (Plow et al, 2000). Upon activation by ECM proteins, integrins mediate cellular adhesion, migration, survival, and proliferation (Ginsberg et al, 2005). Integrin signaling is activated by ECM proteins or growth factors through focal adhesion kinase (FAK), PI3K, and Akt, a major downstream target of PI3K signaling, known to be involved in various cellular processes such as cell survival, cell cycle, metabolism, protein synthesis, and transcriptional regulation (Mitra & Schlaepfer, 2006). We showed that fibrillar proteins induced cellular apoptosis (Huang et al, 2009; Huang et al, 2010). The mechanism of the cytotoxic effects of F-BSA in BHK-21 cells (baby hamster kidney cell) was due to modulation of the α5β1 integrin/FAK/Akt/GSK-3β/caspase-3 signaling pathway. Furthermore, F-FN induced cytotoxicity via activating SHP-2 and RhoA/ROCK, and deactivation of Akt/GSK-3β. Taken together these findings suggested that β1 integrin may play a critical role in mediating cancer growth and metastasis. Therefore, we measured the proportion of α5 integrin$^+$ cells or β1 integrin$^+$ cells in TS/A and MDA-MB-231 cells by flow cytometry. First, TS/A or MDA-MB-231 cells were collected and washed with 1× PBS three times. Then, specific monoclonal antibodies for α5 integrin-FITC and β1 integrin-FITC were added and co-incubated with cells (1×10^5/ml) at 4°C for 30 minutes. Cells were then washed three times using 1× PBS and finally stained with 5 μg/ml propidium iodide (PI) at 4°C for 10 minutes to exclude dead cells. Cell viability was determined using a flow cytometer (FACSCalibur; BD Bioscience) and CellQuest software. Data showed that 58.67% and 66.19% of TS/A cells were α5 integrin$^+$ and β1 integrin$^+$, respectively. 42.99% and 97.65% of MDA-MB-231 cells were α5 integrin$^+$ and β1 integrin$^+$, respectively (Table 1). Blocking β1 integrin signaling pathway with a specific mAb (mouse anti-human integrin beta1 monoclonal antibody; Millipore) could reverse F-HSA's effect on TS/A and MDA-MB-231 breast cancer cell migration (Fig. 8). Taken together, these results indicated that the suppression of breast cancer migration by F-HSA may be mediated by binding of β1 integrin.

	α5 integrin	β1 integrin
TS/A	58.67 (%)	66.19 (%)
MDA-MB-231	42.99 (%)	97.65 (%)

Table 1. Percentages of α5 integrin$^+$ cells and β1 integrin$^+$ cells in TS/A and MDA-MB-231 cells.

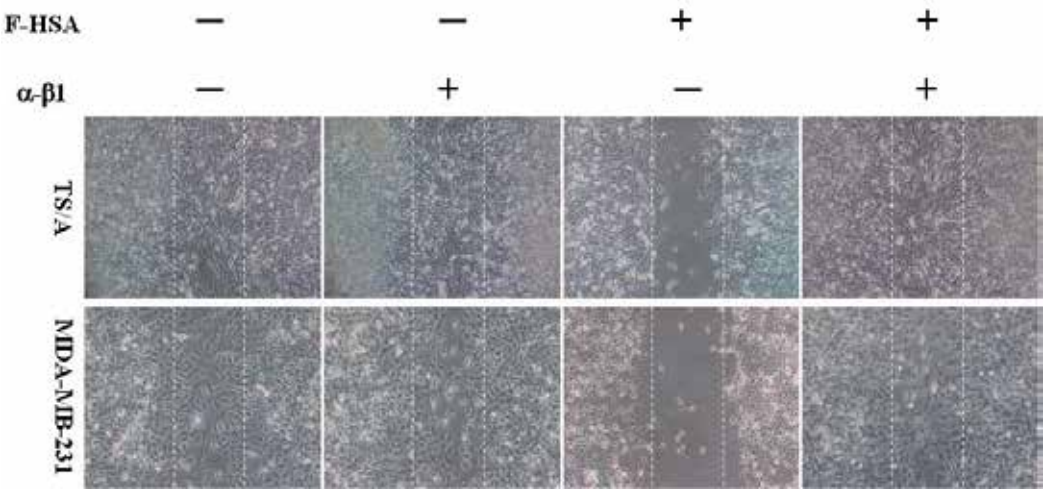


Fig. 8. Blocking the β1 integrin signaling pathway with a specific mAb (mouse anti-human integrin beta1 monoclonal antibody) reversed the effect of 0.2 μM F-HSA on TS/A and MDA-MB-231 breast cancer cell migration.

8. Conclusion

The search for novel therapeutic targets and the development of inhibitors of cancer metastasis is an ongoing challenge. Herein, we used a detergent assisted refolding chromatography process to convert globular HSA into fibrillar F-HSA. Unlike globular HSA, this novel F-HSA caused cell death, reversed EMT, and suppressed breast cancer cell migration through targeting β1 integrin signaling pathway. These important findings may be useful for the development of better therapeutics for the intervention of breast cancer metastasis.

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On the Role of Cell Surface Chondroitin Sulfates and Their Core Proteins in Breast Cancer Metastasis

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1. Introduction

Breast cancer is the most common cancer diagnosis among women worldwide (Jemal et al., 2011). Significant numbers of women present with advanced metastatic breast cancer despite major improvements in population screening and health awareness (Breast Cancer Facts & Figures 2009-2010, 2009; Autier et al., 2011). Metastatic spread leads to the poor prognosis and incurring low survival rates of patients presenting with advanced stage breast cancer or tumor recurrence. Therefore, effective therapies targeting metastatic spread should be designed to prevent the devastating consequences of breast cancer progression. In this regard, novel pro-metastatic molecules must be identified and their functional roles in the progression of the disease need to be addressed.

Cell–cell and cell–matrix adhesions have a profound role in the hematogenous phase of cancer metastasis. Tumor-associated glycans participate in these cell–cell and cell–matrix adhesions and their expression is associated with the metastatic potential of tumor cells and the prognosis of cancer patients (Hakomori, 1996; Couldrey and Green, 2000; Gorelik et al., 2001; Kawaguchi, 2005; Korourian et al., 2008).

We have been studying the role carbohydrates play in breast cancer metastasis (Monzavi-Karbassi et al., 2005; Carcel-Trullols et al., 2006; Monzavi-Karbassi et al., 2007). A large body of evidence indicates that P-selectin expressed on endothelial cells and platelets plays a crucial role during hematogenous metastasis (Borsig et al., 2001; Kohler et al., 2010). In a murine model of breast cancer we observed that the expression of carbohydrates that react with the P-selectin receptor plays a major role in metastasis (Monzavi-Karbassi et al., 2005). This evidence indicates that P-selectin-mediated interaction of breast cancer cells with platelets is a relevant cellular adhesion mechanism that participates in establishing distant metastases. A novel finding in our work is the observation that chondroitin sulfate glycosaminoglycans (CS-GAGs) can serve as P-selectin ligands on breast cancer cells. This observation links CS-GAGs to P-selectin binding in defining the metastatic phenotype dependent on the interaction of cancer cells with platelets (Monzavi-Karbassi et al., 2007). Therefore, CS-GAGs can be targeted for development of novel anti-metastatic therapies.

Large variation exists in CS-GAG sequences and in proteoglycans (PGs) presenting them. The prevalence of a presenting core protein may predict the functional outcomes of P-selectin-mediated adhesion of tumor cells. To use these molecules as targets for diagnostic or therapeutic purposes, a thorough understanding of their presentation and expression is necessary. This chapter reviews the biological roles of chondroitin sulfates (CS) in tumor development and metastasis and the role of different types of CS and the core protein carrying these polysaccharides.

2. Chondroitin sulfate biosynthesis and presentation

A relative variation in the composition of CS/DS has been reported in neoplastic tissues (Chiarugi and Dietrich, 1979; Bumol et al., 1982; Reisfeld and Cheresh, 1987; Olsen et al., 1988; Alini and Losa, 1991; Vijayagopal et al., 1998; Vynios et al., 2008).

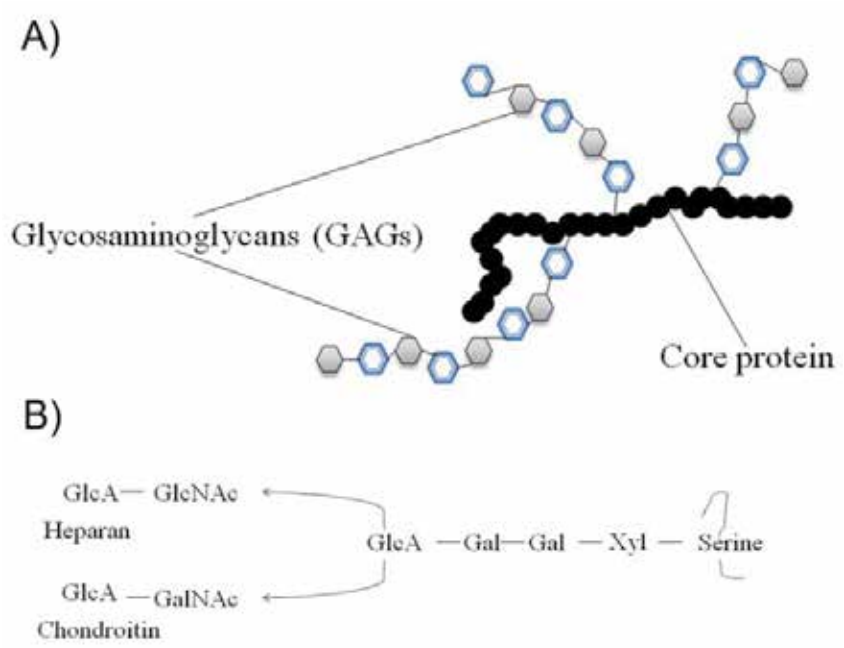

Fig. 1. **A)** Proteoglycans consist of a core protein and covalently attached GAG chains. **B)** Biosynthesis of chondroitin and heparan sulfate building blocks initiates by the formation of a linkage tetrasaccharide attached to serine residue on the core protein. GlcA: Glucuronic acid; GlcNAc: N-acetyl-D-glucosamine; GalNAc: N-acetyl-D-galactosamine; Gal: Galactose; Xyl: xylose.

Chondroitin sulfate (CS)/ dermatan sulfate (DS) polysaccharides are widely distributed in extracellular matrices and at cell surfaces as PGs, in which glycosaminoglycan (GAG) chains are covalently attached to a variety of core proteins (**Figure 1A**) (Esko et al., 1999). Chondroitin or heparan backbone is synthesized on the common GAG-protein linkage region tetrasaccharide (GlcUA-Galactose-Galactose-Xlylose) (**Figure 1B**), which is attached to specific serine residues in the respective core protein (Silbert and Sugumaran, 2002; Sugahara et al., 2003).

The Chondroitin chain backbone consists of repetitive disaccharide units containing D-glucuronic acid (GlcUA) and *N*-acetyl-D-galactosamine (GalNAc) residues. They further differentiate into variable chains with distinct structures and functions after various modifications. Sulfation and epimerization will further generate CS/DS isomers (**Table 1**). DS or CS-B is a stereoisomeric variant of CS with varying proportions of L-iduronic acid (IdoUA) in place of GlcUA, which forms by epimerization of GlcUA to IdoUA (**Table 1**).

Chondroitin type	Disaccharide repeat	Modifying enzymes	
		Sulfotransferase	Epimerase
A	[GlcUAβ1-3GalNAc(4S)]	Carbohydrate (chondroitin-4) sulfotransferase 11, 12 and 13 (CHST11, CHST12 and CHST13)	-
B	[IdoUA(2s)α1-3GalNAc(4S)]	Uronyl-2-O-sulfotransferase (UST) and CHST11, CHST12 and Carbohydrate (N-acetylgalactosamine 4-O) Sulfotransferase 14 (CHST14)	Dermatan-sulfate 5-epimerase
C	[GlcUAβ1-3GalNAc(6S)]	Carbohydrate (chondroitin 6) sulfotransferase 3 (CHST3) and Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7 (CHST7)	-
D	[GlcUA(2S)β1-3GalNAc(6S)]	UST, CHST3 and CHST7	-
E	[GlcUAβ1-3GalNAc(4S,6S)]	CHST11, CHST12, CHST13 and CHST15 (N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase)	-
iE	[IdoUAα1-3GalNAc(4S,6S)]	CHST11, CHST12, CHST14 and CHST15	Dermatan-sulfate 5-epimerase

Table 1. Chondroitin sulfate types

The monosulfated disaccharide A-unit [GlcUA-GalNAc(4S)] and C-unit [GlcUA-GalNAc(6S)] are common and major components of mammalian CS chains. Disulfated disaccharide D-unit [GlcUA(2S)-GalNAc(6S)] and E-unit [GlcUA-GalNAc(4S,6S)] also exist that are based on further sulfation of monosulfated C and A units, respectively.

CS/DS chains that often found as CS/DS hybrid structures have the potential to display an enormous structural diversity by embedding multiple overlapping sequences constructed with distinct disaccharide blocks modified by different patterns of sulfation (Kusche-Gullberg and Kjellen, 2003; Sugahara et al., 2003). Given the complexity of these structures, the expression of modifying enzymes may correlate better with an aggressive tumor phenotype. Therefore, linking the expression of these enzymes with a functional role of cell surface CS glycans is highly significant

2.1 Biological functions of CS/DS chains

CS/DS chains specifically interact with heparin binding proteins. The interaction of DS chains with fibroblast growth factor (FGF) activates FGF-2 to signal cell proliferation (Penc et al., 1998). DS also acts as a cofactor for FGF-7 (Trowbridge et al., 2002). In addition, DS has been shown to bind and activate hepatocyte growth factor/scatter factor (HGF/SF), a

paracrine growth factor whose receptor, c-met (previously characterized as a proto-oncogene), is also a transmembrane tyrosine kinase.

The CS/DS chains of the PG versican, which is expressed in many tissues including kidney, skin, aorta, and brain, bind the adhesion molecules L- and P-selectin (Kawashima et al., 2002), molecules that have been implicated in leukocyte trafficking, inflammatory disease, and tumor dissemination. Interestingly, these interactions are specifically inhibited by CS or DS containing the 'E' disaccharide unit GlcUA-GalNAc (4S, 6S) or the 'iE' unit IdoUA-GalNAc (4S, 6S), respectively.

In previous studies we found that CS/DS-GAGs are expressed on the cell surface of murine and human breast cancer cell lines with high metastatic capacity. This suggests that CS/DS-GAGs can mediate P-selectin binding and P-selectin-mediated adhesion of cancer cells to platelets and endothelial cells (Monzavi-Karbassi et al., 2007). In inhibition assays performed in vitro, we showed that among the CS types only CS-B (DS), and CS-E can efficiently block P-selectin binding to tumor cells (Monzavi-Karbassi et al., 2007). Other studies have also suggested important interactions mediated by CS-A and CS-E in tumor progression and metastasis (Iida et al., 2007; Li et al., 2008; Basappa et al., 2009). Therefore, enzymes involved in sulfation (sulfotransferases) or epimerization (DS epimerase) of CS chains may play a fundamental role in defining the malignant phenotype of breast tumors.

The expression of several sulfotransferases including CHST11 and CHST15 appears to be greater in human breast carcinoma compared to normal breast tissue (Potapenko et al., 2010). An increase in CHST11 expression is observed in malignant plasma cells from myeloma patients compared to normal bone-marrow plasma cells (Bret et al., 2009). In searches for genes involved in the transition of DCIS to IDC, Schuetz et al. (Schuetz et al., 2006) found a significant increase in DS epimerase (Maccarana et al., 2006).

Collectively, the evidence implicates CS/DS GAGs in a wide array of molecular and cellular interactions resulting in tumorigenesis and metastasis.

3. Potential cell membrane CS/DS-carrying PGs of breast carcinoma

Malignant neoplasms exhibit changes in production of PGs (Bumol and Reisfeld, 1982; Iozzo, 1985; Iozzo, 1988; Stylianou et al., 2008). The variation, abundance and function of CS/DS-GAGs are also affected by the expression of the PG core protein presenting them. Therefore, it is imperative to study these polysaccharides in the context of their carrying PG. PG are involved in signaling and tumorigenicity and their attached GAG contributes to their functions. There is a growing list of PGs that have been implicated as possessing CS/DS side chains (Esko et al., 1999; Taylor and Gallo, 2006). PGs that may be modified by CS/DS chains include aggrecan, neurocan, brevican, bamacan, a CD44 isoform, chondroitin sulfate proteoglycan 4 (CSPG4), syndecans, betaglycan, serglycin, versican, decorin, biglycan, and endocan, most of which are extracellular matrix PGs. Our focus is on the cell membrane PGs that are able to bind to P-selectin (Monzavi-Karbassi et al., 2007). CD44 variants (CD44v), CSPG4, syndecan-1 (SDC-1) and syndecan-4 (SDC-4) are among the cell surface candidates (Faassen et al., 1992; Jackson et al., 1995; Barbareschi et al., 2003; Burbach et al., 2003; Baba et al., 2006; Gotte et al., 2007; Wang et al., 2010). Recently, It has been demonstrated that substantial fraction of neuropilin-1 (NRP-1), a membrane glycoprotein, is a PG modified with either HS or CS-GAG chains (Shintani et al., 2006).

Many articles are now devoted to CD44 in cancer stem cells and its role in cancer progression and metastasis (Lesley et al., 1997; Naor et al., 1997; Lesley and Hyman, 1998; Kalish et al., 1999; Toole, 2009). Here we focus on SDC-1, SDC-4, CSPG4 and NRP-1 as potential CS-carrying PGs on the surface of breast tumor cells.

3.1 Role of CS-carrying PGs in tumor progression and metastasis

Alteration in the production and structure of GAG chains and the functional consequences of such alterations is dependent on the PG carrying the GAG chain. PGs isolated from carcinomas contained 32.2% more CS, 18% less DS, and 30% less HS than PGs of normal breast tissue (Vijayagopal et al., 1998). Chondroitin sulfate proteoglycans (CSPGs) were expressed significantly more often in metastases than in primary tumors of uveal melanoma (Kiewe et al., 2006). We have recently found that CSPGs on breast cancer cells also bind to P-selectin receptors, and interruption of this interaction leads to significant reduction in hematogenous metastasis (Monzavi-Karbassi et al., 2007).

Selectin-mediated binding of tumor cells to platelets, leukocytes, and vascular endothelium may regulate their hematogenous spread in the microvasculature (Krause and Turner, 1999). Among selectin molecules, evidence strongly supports P-selectin involvement in tumor metastasis (Kim et al., 1998; Stevenson et al., 2005). Our data suggest that inhibition of P-selectin interaction with CS-GAGs significantly attenuates hematogenous lung metastasis (Monzavi-Karbassi et al., 2007). We have demonstrated that P-selectin binding to the surface of the aggressive breast cancer cell line MDA-MB-231 and MDA-MET is also CS-dependent, suggesting a role for CSPGs in metastatic behavior of human cancer cells. Because of the role of some of these PGs in signaling and tumor phenotype, we speculate that P-selectin interaction with a particular PG may lead to an exclusive tumor cell activation, and consequently survival in circulation. Here, we review the role of the surface PGs able to present CS-GAGs in malignancy.

3.1.1 CSPG4

CSPG4 is a human homolog of Rat neuroglycan 2 (NG2), which is also known as High Molecular Weight Melanoma Associated Antigen and Melanoma Chondroitin Sulfate Proteoglycan (Stallcup, 1981; Bumol and Reisfeld, 1982; Pluschke et al., 1996) and exclusively carries CS chains (Bumol and Reisfeld, 1982; Nishiyama et al., 1991). This tumor-associated cell surface PG potentiates cell motility, promotes invasiveness and the metastatic potential of tumor cells in melanoma (Burg et al., 1998; Campoli et al., 2004; Iida et al., 2007; Wang et al., 2010), and modulates responses to growth factors (Grako and Stallcup, 1995; Yang et al., 2009), processes that are critical for the proliferation and migration of tumor cells. It is suggested that CSPG4 facilitates the invasion of aggressive primary tumors within the dermis by enhancing the local concentration and/or activation of specific matrix metalloproteinases (MMPs) at sites of contact between melanoma cells and the underlying ECM (Iida et al., 2001). The authors demonstrated that CSPG4 on WM1341D cells, interacts with membrane-type matrix metalloproteinase (MT3-MMP), facilitating invasion, and that the interaction is CS-dependent. Inhibiting CS presentation by treating cells with p-nitrophenyl beta-D-xylopyranoside (beta-D-xyloside or βDX), a compound that uncouples the CS chain from the PG, led to a decrease in melanoma cell invasion into type I collagen (Faassen et al., 1992). CSPG4 is highly expressed on aggressive breast cancer cell lines **(Figure 2)** and is considered as a major CS-carrying PG.

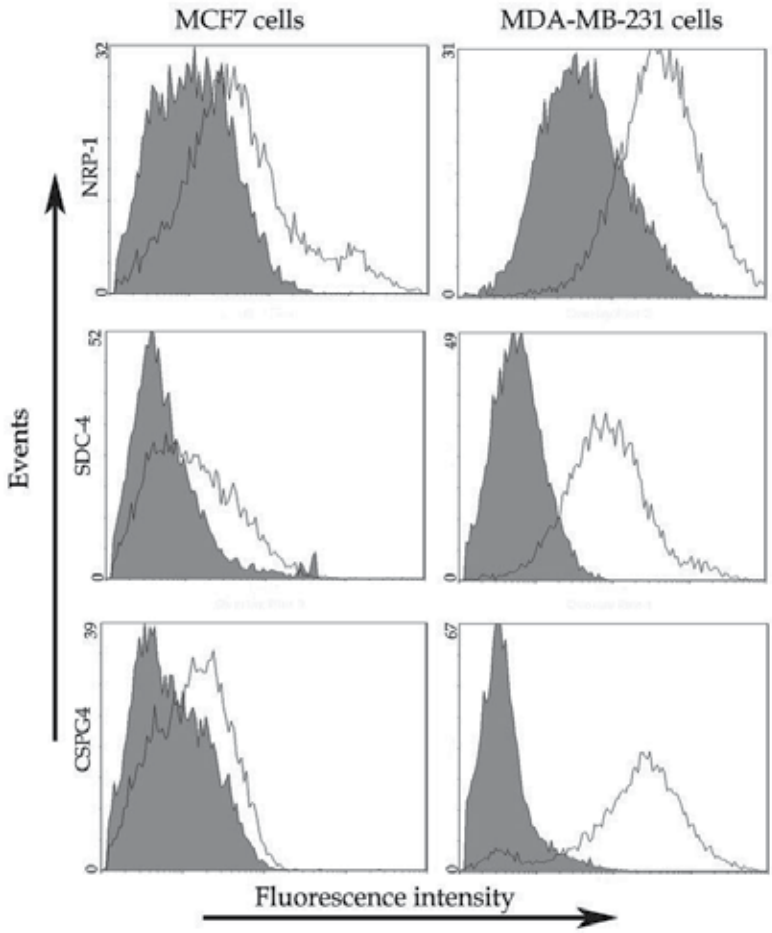

Fig. 2. Expression of NRP-1, SDC-4 and CSPG4 in breast cancer cells. Cells were grown in standard medium, harvested and then stained with monoclonal antibodies against the indicated targets. Stained cells were then analyzed by flow cytometry.

3.1.2 NRP-1

NRP-1 is a 120-130 kDa transmembrane glycoprotein, initially characterized as a neuronal receptor for specific secreted members of the semaphorin family involved in exon repulsation (Kolodkin et al., 1997). A substantial fraction of NRP-1 is a PG with a GAG chain attached (Shintani et al., 2006). In addition to being a receptor for a number of class 3 semaphorins, NRP-1 also serves as a receptor for some members of vascular endothelial growth factor (VEGF), and placental growth factor (PlGF) (Migdal et al., 1998; Soker et al., 1998; Makinen et al., 1999; Wise et al., 1999; Klagsbrun et al., 2002).

Considerable data support a functional role for NRP-1 in regulating VEGF activities in endothelium. It has been shown that semaphorin-3A competes with $VEGF_{165}$ binding to NRP-1 and inhibits angiogenesis *in vitro* (Miao et al., 1999). NRP-1 knock-out mice, in addition to neural defects, exhibit transposition of large vessels, disorganized and insufficient capillary

formation, and defects in heart development (Kawasaki et al., 1999). In contrast, over-expression of NRP-1 leads to over-stimulation of blood vessel formation (Kitsukawa et al., 1995). Studies have shown that NRP-1interacts with a subset of heparin binding proteins like FGF-1, FGF-2, FGF-4, FGF-7, FGF receptor-1, and HGF/SF (West et al., 2005). Investigation of the role of NRP-1 in human glioma progression, Hu et al. (Hu et al., 2007) have shown that NRP-1 expression correlates with tumor progression in clinical setting, and that NRP-1 expression promotes tumor growth and survival through an autocrine HGF/SF/c-met signaling pathway. We observed an overexpression of NRP-1 in aggressive human breast cancer cell line MDA-MB-231 compare to MCF-7 cells (**Figure 2**). This PG is also considered as a potential CS-carrying PG that can present CS-GAGs to P-selectin.

3.1.3 SDC-1 and SDC-4

SDC-1 is mainly expressed by epithelia and plasma cells. Although there are inconsistent reports (Barbareschi et al., 2003; Tsanou et al., 2004), the expression of SDC-1 is generally down-regulated in malignant tumors, and lower levels of expression have been associated with high metastatic/aggressive potential in many tumors (Nackaerts et al., 1997; Kumar-Singh et al., 1998; Mikami et al., 2001; Numa et al., 2002). SDC-1 has also been shown to act as a tumor suppressor molecule by inhibiting cell growth and inducing apoptosis (Mali et al., 1994; Dhodapkar et al., 1998). Therefore, during tumor development the decrease of SDC-1 expression may be an important step from tumorigenesis to a metastatic phenotype. However, there are conflicting data on the role of SDC-1; both its loss and over-expression in carcinoma cells have been associated with malignant progression (Baba et al., 2006).

SDC-4 is more ubiquitously expressed by most cell types, and little is known about its role in malignancy. Among the four members of the syndecan family, SDC-4 is the only one involved in the formation of fibronectin-induced focal adhesions, in cooperation with β1-integrin receptors (Woods and Couchman, 1994; Woods et al., 2000). SDC-4 has been implicated in cytoskeletal organization and regulation of cell adhesiveness. The migratory capacity of lymphocytes and dendritic cells has been reported to be mediated by SDC-4 (Kaneider et al., 2002; Greene et al., 2003; Feistritzer et al., 2004; Averbeck et al., 2007). Our data suggest a role for relative expression of SDC-1 and SDC-4, low SDC-1 and high SDC-4 expression, in metastatic breast cancer cells (**Figure 3**).

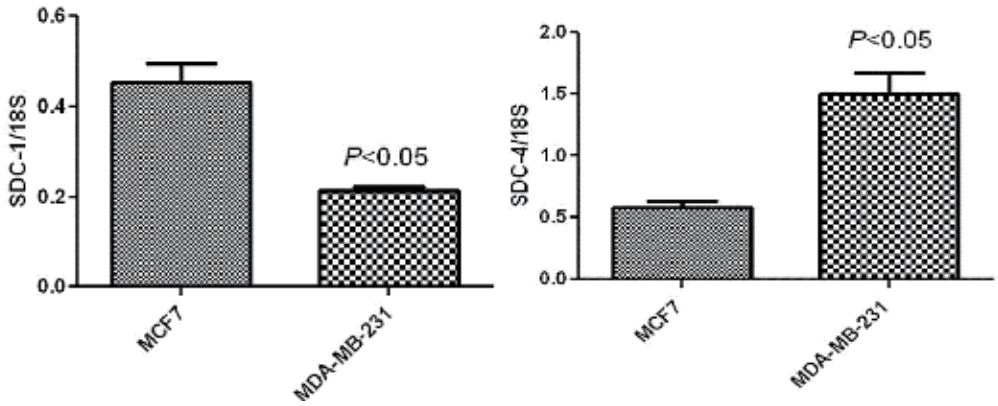

Fig. 3. Relative expression of SDC-1 and SDC-4 in human breast cancer cells using quantitative real-time PCR. Means of three independent experiments (±SD) are shown.

Therefore, relative expression of certain PGs or modification in their GAG chains may affect tumor aggressive phenotype through promoting survival, growth, and the metastatic capability of tumor cells. P-selectin can bind to CS-GAGs of these PGs and binding to each PG can have different functional consequences. These molecules has been linked to motility, invasion, angiogenesis, and cancer stem cell properties. Therefore, depending on the setting and expression of other molecules, P-selectin interaction may lead to various tumor promoting outcomes.

In studying the role of P-selectin in tumor growth and metastasis in a P-selectin-deficient Rag2-/- background, it was demonstrated that growth of subcutaneously challenged tumor cells were reduced significantly in the absence of P-selectin (Kim et al., 1998). This significantly slower growth rate in P-selectin deficient mice was unexpected because P-selectin is assumed to play a role in leukocytic infiltrates within tumors, which are generally inversely associated with tumor growth (Kreider et al., 1984). These findings, consistent with our hypothesis, demonstrate that the presence of P-selectin ligands on tumor cells and P-selectin-mediated interactions with stroma leads to tumorigenesis and tumor growth promotion.

4. Diagnostic and therapeutic values

Overexpression of particular CS chains can be used to develop diagnostic tests predicting tumor behavior or for prognostic purposes. In this regard, further attempts should be made to link the expression of a combination of genes that define GAG remodeling to the initiation and outcome of the disease in clinical setting.

Expression of CS can also be used for drug delivery purposes. Polyethylene glycol coated liposomes, containing a cationic lipid with CS specificity were used to deliver cisplatin to metastatic tumor cells (Lee et al., 2002). The cisplatin loaded CS-reactive liposomes suppressed metastatic spread of the murine osteosarcoma cells to the liver.

We have shown CS interactions with P-selectin and the significance of P-selectin binding in metastasis of a murine mammary cell line (Monzavi-Karbassi et al., 2007). Our findings support the concept that CS chains promote survival in the circulation, and tumor cell extravasation via P-selectin-mediated binding to platelets and endothelial cells. Using heparin to block P-selectin binding to tumor cells as anti-metastatic therapy has been the subject of many studies (Borsig et al., 2001; Stevenson et al., 2005). However, blocking P-selectin action through the inhibition of binding to its many ligands may affect cellular immunity that could be a tumor friendly side effect of a potential treatment. To avoid unfavorable impact of such a treatment on lymphocyte trafficking and infiltration, targeting relevant tumor-specific P-selectin ligands should be prioritized as an alternative long-term therapeutic strategy for aggressive breast cancer.

To develop therapeutics targeting CS entity we envision three major strategies. 1) Targeting particular CS types through blocking the expression of particular CS structures or the usage of small molecules, is supposed to attenuate metastasis efficiency. In this category, blocking the expression of a key sulfotransferase with siRNA may be considered a potential therapeutic approach at this point. Development of small molecules with fine specificity can also be proposed for blocking particular isomers of CS with reactive molecules. 2) Specific targeting of a prominent CS-carrying PG with definite impact on tumor progression and metastasis. CSPG4 is considered a prominent CSPG with a tumor promoting role. MAb targeting CSPG4 have been developed in melanoma and testing them for treatment of

patients with aggressive breast cancer falls in line with our data (Wang et al., 2010a; Wang et al., 2010b). However, targeting a core protein may bring in specificity issues as these PGs are also expressed in stroma. Additionally, tumor cells can escape treatment by immune editing and replacing a PG with another one. 3) Targeting a combination of sugar and PG that can be accomplished by simultaneous targeting of the core protein and the polysaccharide, or by developing reagents like mAb specific for the whole entity (polysaccharide and the core protein).

5. Conclusion

Breast cancer cell surface CS-GAGs and their interaction with P-selectin should be considered as viable targets for the development of novel diagnostic or therapeutic strategies. Our studies suggest that CS-GAGs, their biosynthetic pathway, or the core protein carrying them, can be potential targets in dealing with aggressive breast tumors. However, in order to efficiently block tumor cell dissemination by interrupting P-selectin/CS interaction, targeting any single PG does not seem to be enough, as other PGs can probably compensate and support metastatic processes. In this regard, global targeting of specific CS isomers, or combined targeting of the glycan and the PG, may be effective approaches.

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22

Endocrine Resistance and Epithelial Mesenchymal Transition in Breast Cancer

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1. Introduction

Estrogen plays a major part in the regulation of cell proliferation and survival, controlling female physiology, reproduction and behaviour (Musgrove and Sutherland, 2009). It however assumes a more malevolent role in its association with breast cancer pathogenesis. Consequently, therapies have been designed to block the actions of estrogen mediated through its receptors (ERα and ERβ), or to simply reduce its levels in the body (Zilli et al., 2009). Since Beatson (1896) first introduced ovariectomy over a century ago as the first therapeutic modality to reduce the adverse effects of estrogen, endocrine therapy has developed into the cornerstone of breast cancer treatment for those 60-70% of patients whose tumours over-express ER and/or progesterone receptor (PR) (Massarweh and Schiff, 2007; Zilli et al., 2009). For three decades, selective estrogen receptor modulators (SERMS), predominantly tamoxifen, have proved to be effective agents for the suppression of breast cancer growth in both early and advanced disease (Normanno et al., 2005). Tamoxifen has significantly improved the quality of life and survival of many patients with metastatic disease, as well as displaying prophylactic benefit, particularly in women with ductal carcinoma-*in situ* (Fisher et al., 1999).

However, about half of ER+ patients with advanced disease and nearly all patients with metastatic disease fail to respond to first-line tamoxifen therapy. About 40% of patients receiving tamoxifen as adjuvant therapy experience tumour relapse and die from their disease, and a third of women treated with tamoxifen for 5 years develop recurrent disease within 15 years (Normanno et al., 2005). The introduction of pure estrogen antagonists such as fulvestrant, to overcome the apparent disadvantage of tamoxifen with its partial agonist properties, did not resolve the resistance problem (Osborne and Schiff, 2011). Second line therapy with other endocrine agents designed to inhibit peripheral extra-gonadal synthesis of estrogen in postmenopausal women produces some beneficial effects but for the most part serves merely to delay onset of endocrine resistance (Massarweh and Schiff, 2007). This refractiveness to continued administration of anti-estrogens and aromatase inhibitors poses a significant therapeutic problem that has been addressed by a large number of studies. Several theories have been proposed to explain this phenomenon, based on observations made with a variety of *in vitro* cellular models (Normanno et al., 2005). The consensus opinion seems to be that whereas *de novo* resistance is most likely due to low levels of ER expression, *acquired* resistance is predominantly the consequence of an attenuated response to other peptide growth factors that normally play a subsidiary role in cell proliferation.

These molecules exert their action through a variety of trans-membrane receptors that possess intrinsic tyrosine kinase activity. Fig 1 depicts the various potential influences that govern the behaviour of breast cancer cells.

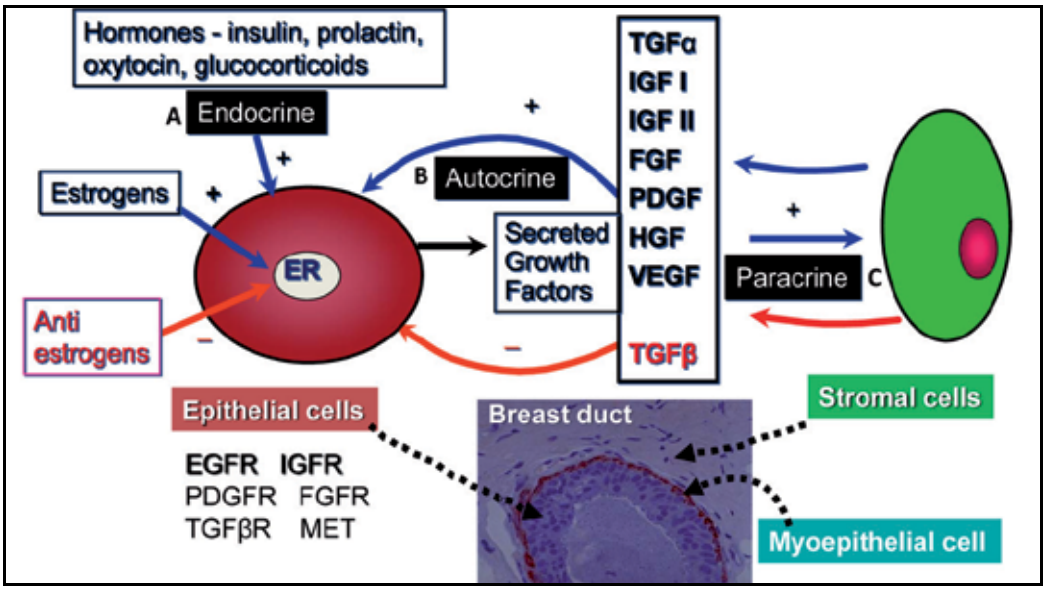

Fig. 1. Factors affecting growth and proliferation of breast cancer cells. Breast epithelial cells are subject to various influences that can either promote or inhibit cellular activity. (A) Endocrine stimulation by a variety of hormones, most significantly estrogen, promotes long term effects. (B) Autocrine stimulation involves, under various conditions, the production and secretion of a number of peptides that act back on the producer cell to modify its activity through membrane bound receptors that frequently possess intrinsic tyrosine kinase activity which initiates a signalling cascade that terminates in the action of transcriptional regulators to modify gene expression. (C) Paracrine stimulation is effected by the action of mediators which include the listed peptide growth factors as well as others originating from myoepithelia (in the normal breast) and stromal elements that include fibroblasts and macrophages in tumours. All of these pathways have been found to operate both *in vitro* (in tumour-derived cell lines) and *in vivo*, but their relative contributions vary considerably in both cases and may be influenced not only by biological heterogeneity but also by therapeutic interventions.

It is also a general experience that endocrine resistance is associated with increased aggressiveness and frequent metastasis (Hiscox et al., 2007), characteristics that more often typify ER-ve tumours. Identification of ligands, receptors and downstream signaling molecules with increased activity in the resistant phenotype, both in cell culture and in tumour biopsies, has highlighted a bewildering collection of molecules that may play a direct causative role, be a consequence or simply innocent bystanders in the progressive cellular change towards endocrine independence. For the purposes of therapeutic discrimination, attempts have been made to reduce this plethora, generated principally by microarray analyses (eg Charafe-Jauffret et al., 2006 ; Luqmani et al., 2009; Al Saleh, 2010) to

a manageable number, and given the designation of 'gene signature' by virtue of selectively circumscribing a particular sub-group of patients.

In a separate scenario, new insights have been gained into our understanding of cell differentiation from studies that have demonstrated that epithelial cells have the potential to trans-differentiate into mesenchymal cells (epithelial to mesenchymal transition: EMT) and vice versa (mesenchymal to epithelial transition: MET). Many recent reports have indicated that this process, which was previously observed during transition between developmental stages, is synonymous with the process of tumour metastasis. Both processes share similar pathways of activation. Our recent data (Luqmani et al., 2009; Al Saleh, 2010; Al Saleh et al., 2011a) suggests that there may also be causal links between the development of endocrine resistance and the onset of EMT. In this report we summarise the molecular pathways of ER activity, the mechanisms proposed to account for resistance and finally review the evidence for the above hypothesis.

2. Mechanisms of estrogen receptor induced cell proliferation

ERα and ERβ are transcribed from distinct genes located on separate chromosomes (6 and 14, respectively) (Green et al., 1986; Kuiper et al., 1996). These receptors differ in their tissue distribution, with ERα being highly expressed in the pituitary gland, ovaries (thecal and interstitial cells), uterus, liver, kidneys, adrenals and the mammary glands while ERβ is found mainly in the prostate, bone, ovaries (granulosa cells), lungs and in various parts of the central and peripheral nervous system (Emmen et al., 2005; Kuiper et al., 1997). Nevertheless, ERα and ERβ do overlap in their expression in some tissues (Zilli et al., 2009). More importantly, the two receptors have different roles in breast development. Only ERα appears to be essential for ductal growth although both receptors are present in the breast. ERα-knockout mice show very little growth of mammary ducts, while ERβ-knockout mice develop a normal mammary gland with regular ductal branching (Förster et al., 2002; Lubahn et al., 1993). This suggests that ERβ might be exerting pro-differentiative and anti-proliferative functions. In addition, increased ERα/ERβ ratio in breast cancer as compared with benign tumours and normal tissues suggest that ERα is most closely associated with breast cancer pathogenesis, while ERβ can protect against the mitogenic activity of estrogens in pre-malignant lesions (Roger et al., 2001; Shaw et al., 2002). It has even been suggested that the estrogen-induced proliferation of ER+ breast cancer cells can be inhibited by ERβ over-expression (Ström et al., 2004; Williams et al., 2008). Thus ERα remains the main focus of attention in studies on breast cancer. Unless otherwise specified, 'ER' in this review will refer to ERα.

In what is now referred to as the nuclear or genomic action of ER, binding of estrogen induces activation of the receptor by initiating its dissociation from cognate heat shock proteins, and leads to conformational changes, dimerisation and autophosphorylation (Osborne & Schiff, 2005). The activated ER binds to estrogen response elements (EREs) located in the promoter regions upstream of estrogen-regulated genes. Frasor et al., (2003) observed from microarray analysis of gene expression in MCF-7 cells that about 70% of such estrogen-regulated genes were actually down-regulated following treatment with estradiol. Many of these genes are transcriptional repressors, or genes with anti-proliferative or pro-apoptotic function. On the other hand, there is increased expression of genes inducing cell proliferation and survival. Up-regulation of gene expression is mediated through two domains; activating function-1 (AF-1) and activating function-2 (AF-2). AF-1 is a hormone

independent domain located at the N-terminus of the receptor with its function regulated by phosphorylation. AF-2 is the site where ligand-binding actually occurs and is therefore hormone dependant. Almost all gene promoters are activated through both AF-1 and AF-2, though some are activated independently by AF-1 or AF-2 (Gronemeyer 1991; Osborne et al., 2001). Subsequent to formation of the ER-ligand complex, binding of co-regulatory molecules such as nuclear-receptor co-activator 1 (NCOA1 or SRC1), NCOA2 (TIF2) and NCOA3 (AIB1, TRAM1, RAC3 or ACTR) (Leo & Chen 2000; McKenna et al., 1999) enhance the transcriptional activity of ER accompanied by increased activity of histone-acetyltransferase (HAT) at the promoter site. Other co-regulatory molecules can also partly suppress the transcriptional activity of ER by recruitment of histone-deacetylase complexes such as nuclear-receptor co-repressor 1 (NCOR1) and NCOR2 that influence ER-induced transcription (Chen & Evans, 1995; Horlein et al., 1995). Several of these groups of molecules have been reported to have prominent roles in cancer. AIB1 (SRC-3) is over-expressed in almost two thirds of all breast cancers and associated with a shorter disease-free survival in patients receiving tamoxifen as adjuvant treatment (Osborne et al., 2003). In untreated patients, high levels of AIB1 were associated with improved outcome, consistent with studies that suggest the possibility of an association between an enhanced agonistic effect of tamoxifen and the high levels of co-activators. However, ER can also co-operate with FOS/JUN and bind with other transcription factors such as AP-1 (activator protein-1) and SP-1 (specificity protein-1) at their specific sites on DNA (Kushner et al., 2000; Ray et al., 1997; Safe 2001) commonly designated as serum response elements (SRE).

In addition to its classical mode of action through a nuclear-located receptor, estrogen has also been reported to interact with membrane associated receptors, leading to a more rapid reaction than would be expected from a transcriptionally mediated response, such as initiation of cAMP production (Rosner et al., 1999; Zivadinovic et al., 2005) and activation of intrinsic kinases present in other plasma membrane receptors such as insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and ERBB2 (Bunone et al., 1996; Campbell et al., 2001; Font de Mora & Brown 2000) as well as receptors for fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). It has been suggested that interaction of SERMs including tamoxifen with such membrane associated receptors may be responsible for their agonist behaviour. There is however much controversy over this issue, with other studies discounting the involvement of such postulated receptors as G protein-coupled receptor as targets of estrogen action (Otto et al., 2008). However this may be, any non-genomic interactions of estrogen would depend on the levels of the above-mentioned kinases, and they will likely be modest in ER+ breast cancer cells that express low levels of tyrosine kinase receptors such as EGFR and ERBB2 (Normanno et al., 2005).

Ligand independent activation of ER can occur via the downstream signaling cascades transmitted through membrane receptor tyrosine kinases such as EGFR, ERBB2, and IGF1R In particular. MAPK/ERK, PI3K/AKT, p90RSK and p38 MAPK pathways can specifically activate ER at key positions (serine 118 and 167 and threonine 311) in the AF-1 domain and in other domains (Bunone et al., 1996; Campbell et al., 2001; Joel et al., 1998; Kato et al., 1995). Expression of ligands and receptors such as transforming growth factor-α (TGFα), IGF1 and IGF1R can be increased by estrogen and those can then initiate signalling while expression of other receptors such as EGFR and ERBB2 is decreased by estrogen signaling (Kushner et al., 2000; Massarweh et al., 2008; Umayahara et al., 1994; Vyhlidal et al., 2000; Yarden et al., 2001). In addition, activation of the PI3K/AKT and the p42/44 MAPK

pathways by these receptors down-regulates the expression of ER and PR causing reduction in estrogen dependency while activating the transcriptional function of ER, which suggests a contribution of this cross talk to the relative resistance to endocrine therapies in tumours with amplified ERBB2 expression (Bayliss et al., 2007; Brinkman and El-Ashry, 2009; Creighton et al., 2010; Lopez-Tarruella and Schiff, 2007).

The two types of ER actions, genomic and non-genomic, are not mutually exclusive and do overlap. For example, ER induces the expression of transcripts for both TGFα and amphiregulin (Normanno et al., 1993; Saeki et al., 1991) which can both bind and activate EGFR resulting in activation of MAPK and AKT signaling which are also activated by direct interaction with ER (Salomon et al., 1995). ER binding to membrane caveolin-1 leads to the activation of specific G proteins resulting in the activation of SRC and in turn of matrix metalloproteinases that cleave transmembrane precursors of the EGFR ligand, heparin binding-EGF (HB-EGF) (Levin, 2003; Razandi et al., 2003). Fig 2 illustrates the major identified downstream events involving ER activation.

3. Mechanisms of endocrine resistance

It should be noted that most tumours are heterogeneously composed and a biphasic response to treatment could reflect the survival and eventual clonal outgrowth of an intrinsically resistant minor sub-population.

3.1 Alterations in ER expression or function

Since all endocrine therapies target ER, the expression of the latter is the main predictor of the outcome of such therapies. The *de novo* resistance is clearly caused by the lack of ER expression which can be due to histone deacetylation (Parl, 2003) or associated with aberrant methylation of ER CpG islands that deactivates chromatin (Ottaviano et al., 1994; Weigel & deConinck, 1993). Interestingly, ER expression can be restored in ER-ve breast cancer following co-treatment with DNMT1 and HDAC inhibitors (Robertson et al., 2000; Rountree et al., 2000; Yang et al., 2001).

It was initially thought that acquired resistance might be due to missing or non-functional variants of ER. However, only 17–28% of patients with acquired endocrine resistance lack ER expression (Gutierrez et al., 2005; Johnston et al., 1995). Furthermore, approximately 20% of tamoxifen-resistant patients will eventually respond to second-line treatment with aromatase inhibitors or fulvestrant (Howell et al., 2005; Osborne et al., 2002). Although a number of exon-deleted receptor isoforms have been described, their frequency is insufficient to account for resistance.

Furthermore, these mutations have been detected in ER-ve tumours (Herynk & Fuqua 2004). A mutation that results in a hypersensitive receptor that shows enhanced binding of co-activators in the presence of low estrogen levels (a single amino acid substitution changing lysine 303 to arginine) was found in 20 of 59 hyperplastic breast lesions (Fuqua et al., 2000). However, the role and frequency of such mutations in primary breast carcinomas and their relation to endocrine resistance needs to be explored in a larger number of patients.

Patients carrying inactive alleles of cytochrome P450 2D6 (CYP2D6) (approximately 8% of Caucasian women) fail to convert tamoxifen to its active metabolite, endoxifen (4-hydroxy-N-desmethyl-tamoxifen), and are consequently less responsive to tamoxifen, which is considered to be a significant factor in resistance to therapy (Hoskins et al, 2009). The baseline levels of endoxifen are elevated in patients carrying the wild-type CYP2D6 and who

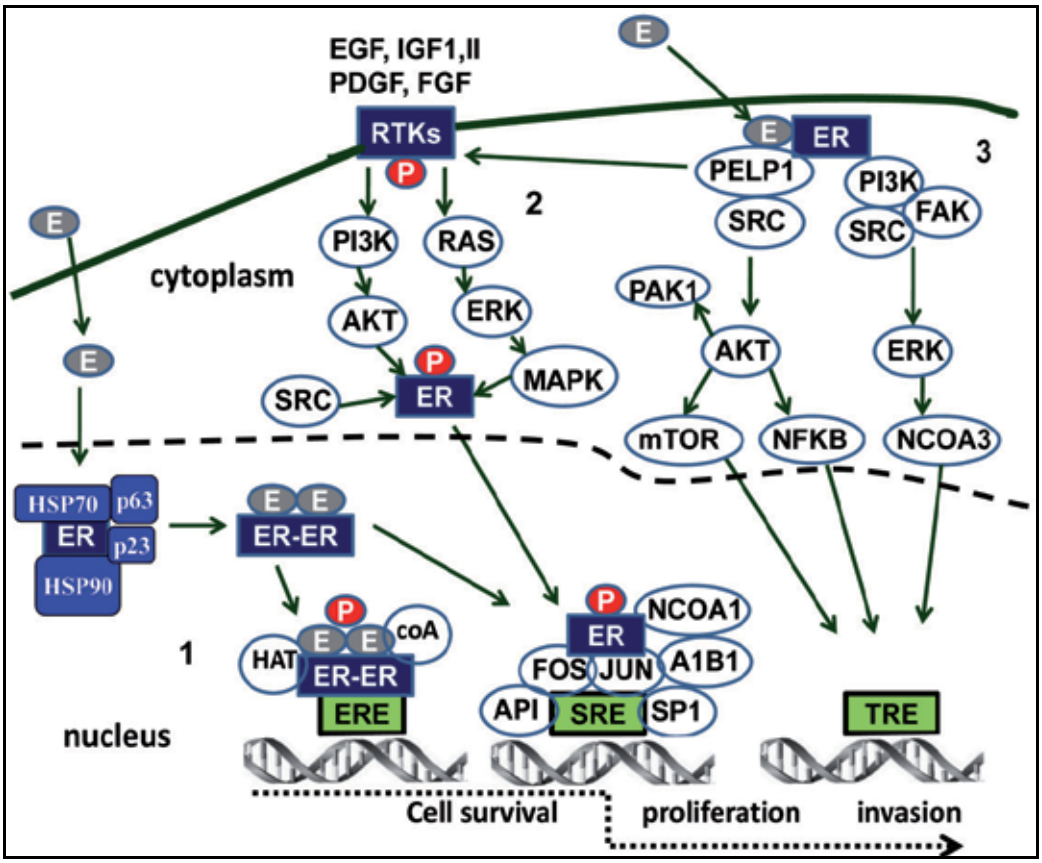

Fig. 2. Proposed cellular mechanisms mediated through the estrogen receptor. 1. Classical genomic mode of action, in which estrogen binds to an inactive ER complex, causing dissociation from heat shock and other cognate proteins, receptor dimerisation and phosphorylation (P). This can then interact directly with estrogen response elements (ERE) on target genes in concert with histone acetyl transferase (HAT) and several other co-activators (coA) or by association with the ubiquitous transcriptional factors FOS/JUN and with NCOA1 and AIB1 co-activators at API/SPI sites termed serum response element (SRE). 2. Cytoplasmically located ER can be phosphorylated by the action of AKT, SRC and ERK/MAPK serine/threonine kinases, downstream of signalling events initiated by various growth factors' interaction with their respective tyrosine kinase containing receptors and mediated through RAS or PI3K. This ligand- independent activated receptor can initiate transcription through the SRE. 3. Binding of estrogen to membrane–associated ER may induce assembly of complexes with either PI3K/FAK/SRC leading to activation through ERK of the transcriptional activator NCOA3 or with PELP1/SRC resulting in up-regulation of mTOR and NFKB through AKT. These mediate an action through other transcriptional response elements (TRE) on a variety of target genes without involving direct interaction of ER with chromatin. The latter mechanisms are referred to as the non-genomic pathways, that are postulated to explain those observed effects of estrogen which are too rapid to be accounted for by mechanism 1. Further 'crosstalk' between ER and RTKs may involve participation of PELP1.

had low levels of the metabolite when co-treated with paroxetine, a selective serotonin re-uptake inhibitor (prescribed to alleviate tamoxifen-associated hot flashes) that can inhibit CYP enzymes. Heterozygous patients showed a better outcome when treated with tamoxifen, as compared with untreated patients suggesting a role for cytochrome P450 enzyme variants in regulating the response to tamoxifen (Wegman et al., 2005).
The presence of ER variants was also hypothesized to have a role in endocrine resistance. A reduced response to endocrine therapy has been associated with the presence of a new truncated variant of ER, ER36, in addition to the full-length receptor (Shi et al., 2009).

3.2 Estrogen receptor β

It has been reported that ERß transcript levels were about 2-fold higher than those of ERα in tamoxifen-resistant as compared with tamoxifen-sensitive patients (Speirs et al., 1999) and that ERß bound to tamoxifen, raloxifen or the anti-estrogen ICI 164 384, increased transcription of AP-1-dependent genes (Paech et al., 1997). Other studies show that ERβ has a negative effect on ERα-promoted transcription (Hall & McDonnell 1999; Pettersson et al., 2000) or no correlation with response or resistance to endocrine treatment (Cappelletti et al., 2004). Development of antibodies distinguishing between the ER types and their variants has led to identification of responses in ERβ+ve but ERα-ve cancers and a potential role for the carboxy-terminally truncated variants of ERβ (ERβ2 and ERβ5) in tamoxifen responsiveness (Honma et al., 2008; Murphy and Watson, 2006). In addition to ERβ, the oestrogen-related receptor ERRγ was found to be over-expressed and mediated tamoxifen resistance in lobular invasive breast cancer models (Riggins et al., 2008).

3.3 Adaptation to estrogen withdrawal

Breast cancer cells can acquire a state of hypersensitivity to estrogen that renders them resistant to endocrine therapy. MCF7 cells cultured in estrogen-free medium to produce long-term estrogen deprived cells (LTED) mimics the effects of ablative endocrine therapy (Santen et al., 2003) and produces cells that are highly sensitised to substantially lower concentrations of estrogen as compared with wild-type MCF-7 cells (Masamura et al., 1995). Growth factor signalling and ER expression was significantly higher in these cells. Treatment with estrogen resulted in rapid association of ER and phosphorylation of SHC, an adaptor protein involved in tyrosine kinase receptor signalling, and increased activation of both SRC and the RAS/RAF/MEK/MAPK signalling pathways (Song et al., 2002a,b; Song et al., 2004). Exposure of these cells to fulvestrant blocked MAPK activation indicating that this pathway may be a downstream effector of the ER non-genomic pathway (Santen et al., 2003; Song et al., 2002a,). However, a high AKT and MAPK level in LTED cells was associated with increased resistance to endocrine therapy and a worse outcome.
In another version of MCF7 LTED cells, enhanced transcriptional activity of ER was associated with increased activation of growth factor pathways that in turn trans-activate ER (Johnston & Dowsett, 2003). After prolonged culture in the absence of estradiol, the ER in these cells functions independently from exogenous estradiol, which was suggested to be due to a super-sensitivity of LTED to residual estrogen present in the medium (Chan et al., 2002; Martin et al., 2003). These cells also showed increased levels of phosphorylation of ER at serine 118, a known target for several intracellular kinases. Furthermore, IGF-1R and ERBB2 signalling was significantly increased in these cells concurrently with increased MAPK activation. Interestingly, the phosphorylation of ER at serine 118 was blocked by

MAPK or EGFR/ERBB2 blockade but not by blocking MEK/MAPK or PI3K/AKT signalling, indicating that additional kinases might be involved in this hypersensitive state. Nicholson et al., (2004) also developed an MCF7 cell line (MCF-7X cells) that is resistant to estrogen withdrawal but not hypersensitive to it. These cells could be growth inhibited by fulvestrant, implying that the ER pathway is still involved in their proliferation. However, the PI3K/AKT pathway was demonstrated to be the main factor promoting their growth without the involvement of EGFR/ERBB2 or IGF-1R signalling, suggesting that the adaptation to estrogen withdrawal can occur in the absence of increased sensitivity to estrogen and does not require activation of classical growth factor receptors.

3.4 Estrogen receptor and co-regulators

Since ER action is mainly controlled through transcriptional factors and co-regulator molecules, it seems likely some of these may be implicated in endocrine resistance. For example, increased AP1 and NFKB transcriptional activity has been associated with endocrine resistance (Johnston et al., 1999; Zhou et al., 2007). And similarly when ER co-activators are over-expressed or phosphorylated. For example, NCOA3 (A1B1 or SRC3) over-expression leads to constitutive ER-mediated transcription, which confers resistance both *in vitro* and in xenograft models and is associated with reduced responsiveness to tamoxifen in patients (Ali & Coombes, 2002; Osborne et al., 2003; Ring & Dowsett, 2004). Another ER co-activator associated with resistance is PELP1 (Fig 2) which is a cytoplasmic scaffold protein that modulates ER interaction with SRC, leading to activation of SRC and the ERK family kinases and also promotes oestrogen activation of PI3K (Gururaj et al., 2006). Interestingly, ER cytoplasmic complex composed of ERα, PI3K, SRC and focal adhesion kinase (FAK; also known as pTK2) is formed as a result of the transient methylation of ER at R260 by protein arginine N-methyltransferase 1 (pRMT1). This complex activates AKT and could confer resistance to endocrine therapy but this methylation event which is frequent in breast cancer has yet to be linked to resistance (Le Romancer et al., 2008).

3.5 Growth factor receptor pathways

Perhaps the most important factors that affect the response to endocrine therapy are those that can modulate alternative proliferation and survival in the tumours in which the ER signalling pathway is effectively inhibited. These alternative growth pathways can do so by the establishment of a bidirectional cross talk with ER signalling. These pathways will act as ER-independent drivers of cancer proliferation and survival and are involved in both *de novo* and *acquired* resistance (Normanno et al., 2005). Increased expression of EGFR, ERBB2 and IGF1R along with their downstream components such as ERK and PI3K can modulate tamoxifen resistance (Faridi et al., 2003; Hutcheson et al., 2003; McClelland et al., 2001). ERBB2 has been reported to be over-expressed in association with down regulation of the X-linked tumour suppressor forkhead box p3 (FOXP3) and the zinc finger transcription factor GATA4 (Hua et al., 2009; Zuo et al., 2007). Other factors that might affect ERBB2 expression are the presence of the paired-domain transcription factor PAX2 and the ER co-activator NCOA3 which compete for binding and regulating ERBB2 transcription and, in turn, responsiveness to endocrine therapy. However, like GATA4 and FOXP3, PAX2 was also shown to be down-regulated in tamoxifen resistant breast cancers in the presence of NCOA3 and an over-expressed ERBB2 (Hurtado et al., 2008). The SRC substrates BCAR1 and BCAR3 have both been reported to elicit endocrine resistance *in vitro* (Dorssers et al., 1993). BCAR1

binds and activates SRC leading to phosphorylation of EGFR and the signal transducer and activator of transcription 5B (STAT5B) (Riggins et al., 2007). On the other hand, BCAR3 is believed to activate RAC and p21-activated kinase 1 (pAK1), which is a mediator of endocrine resistance itself through ER phosphorylation, and through the activation of SRC in association with BCAR1 (Cai et al., 2003; Rayala et al., 2006; Riggins et al., 2003; van Agthoven et al., 1998).

The de-regulation of several growth pathways including EGFR, ERBB2 and IGF1R are implicated in endocrine resistance (Faridi. et al., 2003; Miller et al., 2009). Many events might trigger this de-regulation such as activating mutations in PIK3CA and loss of heterozygosity or methylation of PTEN, activation of AKT, over-expression of ERBB2 and activation of IGF1R and ERBB3 following the loss of PTEN (Arpino et al., 2008; Miller et al., 2009; Riggins et al., 2007). However, following de-regulation of these pathways acquisition of endocrine resistance might be effected by a number of possible activities as summarised by Musgrove & Sutherland, (2009): "decreased ER expression mediated by ERK activation; loss of ER-mediated repression of EGFR and ERBB2 and consequent activation of mitogenic signalling cascades; ligand-independent activation of ER or its co-activators through phosphorylation; up-regulation of key cell cycle regulators, for example MYC and the D and E-type cyclins, through constitutive activation of mitogenic signalling pathways; and the inhibition of apoptosis through constitutive activation of survival signalling".

3.6 Cell cycle signalling molecules

In order for cancer cells to bypass the inhibition of cell proliferation elicited by endocrine agents, one would expect down-regulation of effector molecules involved in the induction of apoptosis while those involved in proliferation, especially during G1 phase, are up regulated. Over-expressed cell cycle regulators include MYC, cyclin E1, cyclin D1, cyclin D1b, as well as p21 and p27, and a de-activated RB gene (Prall et al., 1998; Wang et al., 2008). Over-expression of MYC and cyclin D1 leads to an abundance of CDK complexes that are directly associated with increased cellular proliferation and/or relief of the inhibitory effects of the negative cell cycle regulators p21 and p27, a phenomenon that is also achieved through activation of ERBB2, AKT and SRC (Caldon et al., 2009; Chu et al., 2008; Hui et al., 2002; Perez-Tenorio et al., 2006). Cyclin D1 can also interact with several transcription factors including ER and STAT3 (Coqueret et al., 2002). Tamoxifen actually enhances the binding of cyclin D1 to ER at the expense of STAT3, hence activating both transcription factors and consequently establishing endocrine resistance (Ishii et al., 2008). Other important molecules are those involved in apoptosis. In particular, the pro-apoptotic molecules such as BIK (BCL2-interacting killer) and caspase 9 are down regulated in endocrine resistant cancers while those which are considered as anti-apoptotic molecules such as BCL-XL and its second messenger ceramide, are up regulated (Mandlekar et al., 2001; Riggins et al., 2005) . The expression of these molecules is also affected by signalling through PI3K/AKT, TNF, IFN and NFKB.

4. Epithelial mesenchymal transition

The phenomenon of epithelial cells undergoing a transition towards a mesenchymal phenotype was first identified as programmed events occurring during embryonic developmental processes (Greenberg & Hay, 1982). Since then EMT has since been described in various pathological conditions. During the process of cancer metastasis, a minority of

epithelial cells lose their apico-basal polarity, detach from adjacent cells, scatter and acquire increased motility and are able to invade into the extracellular matrix with subsequent penetration into the vasculature. This process is facilitated by a morphological transformation into a fibroblastoid structure that has all the hallmark features of EMT, Both processes share remarkable similarities, with characteristic phenotypic changes. These include the loss of cell-cell adhesion as a result of reduced E-cadherin in adherens junctions, occludins (OCLN) and claudins (CLDN) in tight junctions and desmoplakin (DSP) in desmosomes and down regulation of epithelial cytokeratins (KRT8, KRT18, and KRT19) and up-regulation of mesenchymal proteins most notably vimentin (VIM) and fibronectin and sometimes alpha smooth muscle actin (ACTA2) along with many other changes.
Fig 3 depicts the changes occurring during EMT. Multiple molecular mechanisms underlie EMT initiation and its reversal process, MET, which cancer cells are thought to undergo at sites where they form metastases, in order to re-establish cohesive colonies and initiate neo-vascularisation.

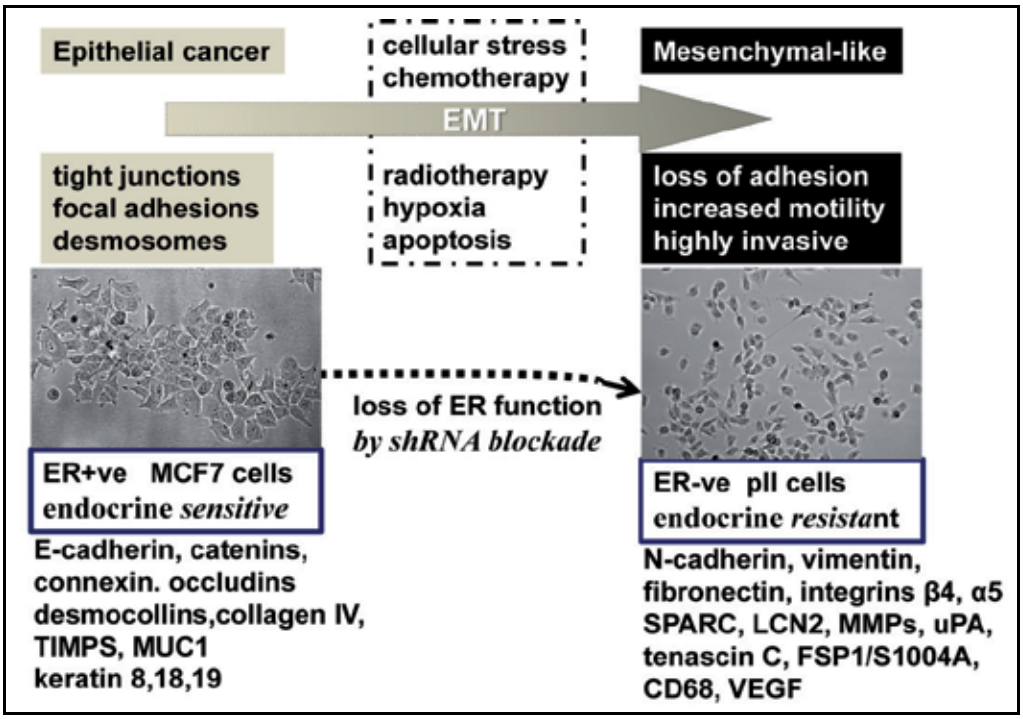

Fig. 3. Epithelial to mesenchymal transition. Loss of epithelial characteristics and breakdown of tissue architecture through dissolution of cell-cell junctions and loss of apico-basal polarity by detachment from the basement membrane can be initiated through a variety of diverse cellular insults which lead to transformation into a cell type that displays mesenchymal–like features. At a molecular level there is a certain uniformity of changes. Cells that have lost ER function and consequently acquired endocrine independence, in this case by shRNA- induced down-regulation (Al Saleh, 2010), show both the morphological appearance as well as the phenotypic changes that are characteristic of cells undergoing EMT. Several differences are indicated between MCF7 and pII cells that parallel those seen during EMT.

The transformation of epithelial cells into a mesenchymal-like form requires the participation of a complex network of both extra- and intra-cellular signals., Amongst the many identified are TGFβ, HGF, FGF, EGFR family members, IGF1 and 2, and PDGF (Thiery et al., 2002). An array of embryonic transcription factors such as the homeobox protein GOOSECOID (GSC), TCF3 (E47), the zinc-finger proteins SNAIL1 and SNAIL2 (previously SLUG), the basic helix-loop-helix protein TWIST1, the forkhead box proteins FOXC1 and FOXC2 , and the zinc-finger E-box-binding proteins ZEB1 and ZEB2 (SIP1) , are generated by the activity of these growth factor pathways, each of which is capable, on its own, of inducing an EMT.

There is increasing evidence of extensive crosstalk between these molecules, permitting the formation of an extensive signalling network responsible for establishing and maintaining a mesenchymal phenotype. (Moreno-Bueno et al., 2008; Peinado et al., 2007). In addition, some of these transcriptional activators such as TWIST are pivotal factors in overcoming cellular senescence (Ansieau et al., 2008) and in generating tumourigenic cancer stem cells (Mani et al., 2008). Interestingly, EMT-inducing transcription factors also confer stem cell characteristics on epithelial cells. For example, the receptor KIT which is an important factor for maintaining the stem cell state in the haematopoietic system has been shown to induce SNAIL2 expression in both mice (Perez-Losada et al., 2002) and humans (Sanchez-Martin et al., 2002). Many of these transcription factors exert repressive functions by binding to conserved E-box sequences in the promoter regions of such critical genes as CDHI (Gilles et al., 2003; Pieper et al., 1992).

4.1 Transforming Growth Factor β

TGFβ can independently promote an EMT phenotype in mouse mammary epithelial cells (Thuault et al., 2006; Waerner et al., 2006). This cytokine induces EMT by both SMAD-dependent and independent signalling events (Berx et al., 2007; Das et al, 2009; Santisteban et al., 2009). In advanced disease, TGF-β can stimulate invasion and metastasis of tumours that have become TGF-β insensitive which can be inhibited by ectopic expression of dominant negative TGF-β receptors (Ozdamar et al., 2005). TGF-β1 ligand activates a heteromeric receptor of two transmembrane serine/threonine kinases, type I and II receptors (TβRI and TβRII). TβRII transphosphorylates TβRI, activating its kinase function to exert its signalling effects. Activated TβRI phosphorylates the intracellular proteins SMAD 2 and 3 which then associate with SMAD 4, translocating to the nucleus where the complex interacts with other transcriptional co-activators and co-repressors to regulate expression of several genes (Onder et al., 2008). This type of signalling that depends on SMAD, up-regulates the expression of many transcription factors such as SNAIL1, SNAIL2, TWIST, and members of the ZFH family, ZEB1 and ZEB2 (Sarrio et al., 2008; Vandewalle et al., 2005; Yang et al., 2004) that are considered to be primary transcriptional inducers of EMT. TGFβ can also phosphorylate certain cytoplasmic proteins regulating cell polarity and tight junction formation. These include RAS/MAPK (Xue et al., 2003), integrin β-1 (Blanco et al., 2002), integrin-linked kinase (Hartwell et al., 2006), p38 MAPK (Mani et al., 2007), RHOA kinase (ROCK) (Moody et al., 2005), PI3K (Martin et al., 2003), JAGGED1/NOTCH (Come et al., 2006), SARA (Laffin et al., 2008), NFKB (Lester et al., 2007), PAR6 (Berx et al., 2001; Storci et al., 2008), pAR66A and ERK (Wu et al., 2009). Furthermore, EMT induced by the oncogenic stimulation by RAS and/or RAF activation in mammary, kidney and skin epithelial tissue was found to depend almost completely on TGF-β signaling (Moustakas and Heldin, 2009). TGFβ can also induce the activation of other signalling pathways that

might participate in initiation of EMT such as the WNT and NOTCH pathways (Polyak and Weinberg, 2009). Figure 4 illustrates the major events that are thought to be critical in the trans-differentiation of epithelial cells.

4.2 AXL

As mentioned earlier, receptor tyrosine kinase activity is altered in breast cancer and is considered to be an important factor in endocrine resistance. These molecules are also implicated in EMT since they already play a pivotal role in embryogenesis. One interesting member of the TAM (Tyro-AXL-MER) receptor tyrosine kinases is AXL which exerts diverse effects in regulating cellular responses that include cell proliferation, cell survival, migration, autophagy, angiogenesis, natural killer cell differentiation and platelet aggregation (Linger et al., 2008). AXL was reported to be associated with EMT since it is activated in many signal transduction pathways including AKT, MAPK, NFKB, and STAT. (Hafizi et al., 2006). Furthermore, AXL expression alone is considered as a predictive marker for poor overall patient survival. It has also been reported that elevated AXL levels are needed for maintaining breast cancer invasiveness, growth in foreign microenvironments and metastatic potential. Endocrine-resistant breast cancer cells show highly elevated expression of AXL (Al Saleh et al., 2010).

4.3 E-cadherin and its transcriptional repressors

E-cadherin is a critical switch in EMT during early embryonic development. Its down-regulation in epithelial cells triggers acquisition of a fibroblastic phenotype, dissociation from the epithelium sheets and migration, vital steps in gastrulation, neural crest formation and organ development (Thiery, 2003). E-cadherin expression is often lost in aggressive breast cancers acquiring EMT which would result in the disassembly of inter-cellular adhesion complexes, loosening contacts between neighbouring epithelial cells and thus disrupting the overall tissue architecture. E-cadherin loss also causes the liberation of β-catenin to the nucleus and its subsequent activation of WNT signalling of other EMT inducers as described above. Furthermore, E-cadherin loss mediates EMT through the induction of its own transcriptional repressors, SNAIL, TWIST and ZEB1 (EF1), in a feed-forward loop that sustains E-cadherin repression and potentiates EMT (Onder et al., 2008).

An interesting connection between endocrine resistance and EMT is established through the connection between SNAIL, E-cadherin and metastasis-associated protein 3 (MTA3). MTA3, which is directly activated by ER, is a repressor of SNAIL, thereby also repressing EMT (Al Saleh et al., 2011). We have recently shown that down-regulation of ER in MCF7 cells leads to a reduction in both MTA1 and MTA3 and a concurrent rise in SNAIL2 (Al Saleh et al., 2011a).

Reduction of E-cadherin expression correlates with poor differentiation, invasiveness, aggressive metastatic behaviour, and an unfavourable prognosis (Berx et al., 2001; Wheelock et al., 2003); experimental knockdown of E-cadherin is sufficient to establish metastasis but not fully reverse EMT by itself. Interestingly, the down regulated expression of E-cadherin during EMT is a reversible process that arises through hypermethylation of the E-cadherin promoter or transcriptional repression although many lobular breast cancers appear to have lost the expression of E-cadherin through inactivating mutations and loss of heterozygosity (Berx et al, 2001).

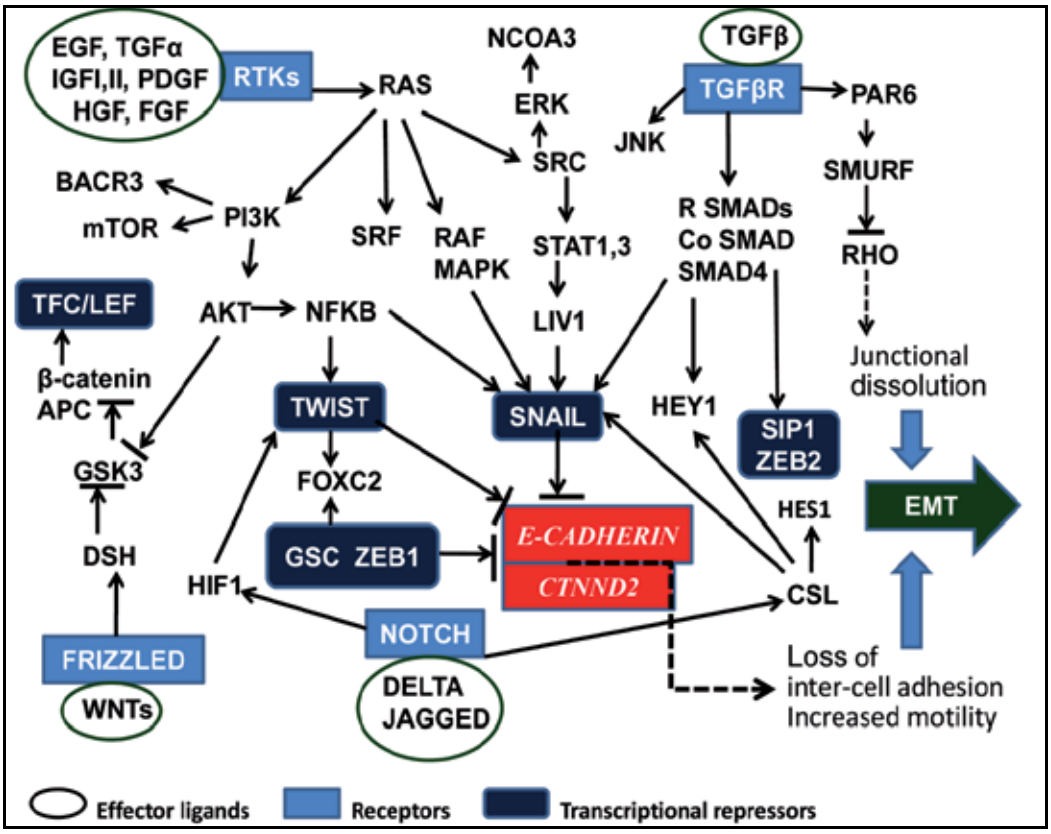

Fig. 4. Transduction pathways and effectors contributing to processes leading to EMT. A variety of growth factors (EGF, TGFα, IGFI, II, PDGF, HGF, FGF) binding to receptor tyrosine kinases (RTK) activate the central RAS pathway to promote transcription of SNAIL through the RAF/MAPK, the PI3K/AKT/NFKB or the SRC/LIV pathways. AKT, as well as WNTs acting through the FRIZZLED receptor, promote inhibition of GSK3 through DSH to promote re-localisation of β-catenin and generate TCF/LEF that also increases SNAIL. DELTA/JAGGED signalling through NOTCH also increases SNAIL via CSL as well as TWIST through HIF1. TGFβ signals through its receptor to increase SMAD family members that co-operatively promote both SNAIL as well as SIP1/ZEB2. It also acts through PAR6 to up-regulate the ubiquitin ligase SMURF that degrades RHO which is a key promoter of tight junctions, The transcriptional repressors SNAIL, TWIST, GSC, ZEB1,2 and TFC/LEF effectively down-regulate E-cadherin and associated molecules, which leads to loss of cell adhesion, permitting cell scattering, cellular motility and invasion through the action of up-regulated proteases. Not shown here, for clarity, is HEDGEHOG signalling which through GLI integrates with the RTK and WNT pathways to up-regulate SNAIL family members Evidence for the interactions illustrated is summarised in excellent reviews by Huber et al., 2005; Moustakes & Heldin, 2007 and Sabbah et al., 2008 and references therein.

The appearance of another mesenchymal marker, N-cadherin (CDH12) and/or cadherin-11 (CDH11), in a process termed 'cadherin switching', is also a well documented event in EMT (Gjerdrum et al., 2010; Sarrio et al., 2008; Sphyris and Mani, 2009; Wheelock et al., 2008). The expression of these mesenchymal markers during EMT is induced by SNAIL, ZEB2/SIP1 and SNAIL2 (Cano et al., 2000; Sarrio et al., 2008; Vandewalle et al., 2005). N-cadherin is reported to be highly expressed in invasive and metastatic human breast cancer cell lines and tumours and to correlate with aggressive clinical behaviour. Nevertheless, N-cadherin expression can be triggered in E-cadherin expressing cells and it could in fact cause EMT, impacting on their epithelial phenotype, suggesting a dominating role for this cadherin over the other, possibly in synergy with FGF2 (Hazan et al., 2000, 2004). MCF7 cells that have acquired endocrine independence through induced loss of ER expression also display cadherin switching which is accompanied by increased motility, F-actin cytoskeletal rearrangement and the loss of cellular adhesion molecules. It is suggested that endocrine resistance is a major event influencing the cells to move and invade into the surrounding tissues (Al Saleh, 2010; Al Saleh et al., 2011a).

4.4 Vimentin

A marker that is commonly used to characterise EMT is vimentin, a component of type III intermediate filaments and the archetypal mesenchymal marker (Trimboli et al., 2008). Elevated vimentin expression correlates well with increased cell migration, invasion and EMT induction in several breast cancer cell lines (Al Saleh, 2010; Al Saleh et al., 2011a; Gilles et al., 2003) in co-ordination with other mesenchymal markers such as tenascin C (Dandachi et al., 2001; Polette et al., 2007), which has been associated with over-expressed ERBB2 and down-regulated ER. The molecular events triggering vimentin expression during EMT are less well delineated in comparision to the mechanisms inducing E-cadherin down-regulation. The expression of vimentin is considered to be a late occurrence in EMT in a temporal sequence of genetic events starting from loss of epithelial markers followed by appearance of mesenchymal markers (Polette et al., 2007). Direct activation of vimentin expression in human breast tumour cells (Gilles et al., 2003) by β-catenin/T-cell factor/lymphocyte enhancer factor-1 is consistent with the activation of β-catenin as a downstream event from consequential loss of E-cadherin. The indirect promotion of vimentin expression by ZEB2/SIP1 during EMT in a β-catenin-independent manner (Bindels et al., 2006) suggests the existence of some trans-activators driving EMT which are associated with vimentin expression.

4.5 Matrix metalloproteinases and lipocalin

In order for cancer cells to metastasise, they need to penetrate into and through the extracellular matrix (ECM). This process is facilitated by the activity of matrix metalloproteinases (MMPs). A family of more than 28 MMPs have been reported to be up-regulated in nearly every tumour type and are closely involved in cancer progression through cleavage and release of bioactive molecules that inhibit apoptosis and stimulate cancer invasion and metastasis. For example, treatment of cells with MMP-3 results in an increased expression of the activated splice variant RAC1b, elevating the levels of cellular reactive oxygen species which, in turn, lead to increased expression of SNAIL and EMT initiation (Orlichenko et al., 2008). An MMP-9 associated protein, Lipocalin2 (LCN2), was

also found to play a major role in cell regulation, proliferation, differentiation and regulation of EMT. It's over-expression in human breast cancer cells can cause up-regulation of vimentin and fibronectin while E-cadherin is down regulated (Yang et al., 2009). Furthermore, LCN2 over-expression significantly increases cell motility and invasiveness in previously non-invasive MCF-7 cells. Interestingly, siRNA-mediated LCN2 silencing inhibited cell migration and development of the mesenchymal phenotype in aggressive breast cancer cells. It was also reported that reduced expression of ER and increased expression of SNAIL2 was correlated with LCN2 expression while over-expression of ER in LCN-2 expressing cells was able to reverse EMT and reduce SNAIL2 expression, suggesting that ER negatively regulates LCN2-induced EMT (Yang et al, 2009).

4.6 Hypoxia

An interesting physiological mechanism that can cause EMT is hypoxia. It has been reported that tumour progression and metastasis is promoted by the stabilisation of the hypoxia-inducible factor-1α (HIF-1α). This transcription factor was shown to be associated with TWIST in inducing both EMT and tumour metastasis by hypoxia or over-expression of the former. Furthermore, the expression of TWIST was found to be regulated by HIF-1 binding to the hypoxia-response element (HRE) in the TWIST proximal promoter and is associated with it in inducing EMT or metastasis (Yang et al, 2008). Interestingly, the HIF-1α null mice phenotype resembles TWIST deficient mice. In addition, patients with head and neck cancer whose tumours co-express TWIST and HIF-1 had very poor prognosis suggesting a major role for these two genes in regulating EMT.

4.7 HOX genes

Another important set of genes in regulating EMT is the homeobox (HOX) gene family, master players in regulating embryonic development and maintaining homeostasis through strictly regulated expression in various tissues and organs during adult life. Several studies have demonstrated the association of HOX genes in the pathogenesis of multiple cancers. For example, HOXA7 and HOXD13 have been associated with lung cancer (Lechner et al., 2001), HOXC4 and HOXC8 in prostate cancer (Miller et al., 2003), HOXB7 in ovarian cancer (Naora et al, 2001) and HOXA10 in endometrial cancer (Yoshida et al., 2006). In one study 60% of their breast cancers had no HOXA5 expression (Raman et al., 2000) which causes p53-dependent apoptosis. HOXA5 was reported to cause cell death through the activation of the caspase pathways in HS578T cells expressing mutant p53 (Chen et al., 2004). HOXD10 was extensively reduced as malignancy increased in epithelial cells, and restoring its expression in MDA-MB-231 could significantly reduce the migration capacity of these highly aggressive cells (Carrio et al., 2005). HOXB13 over-expression was associated with increased MCF10A cell motility and invasion *in vitro*, while its ratio to interleukin-17β receptor was predictive of tumour recurrence during adjuvant tamoxifen monotherapy. HOXB7 is involved in tissue remodeling of the normal mammary gland (Ma et al., 2004) and is expressed at higher levels in metastatic breast tumours (Care et al., 1998, 2001). Furthermore, regulation of the expression of several growth and angiogenic factors, including basic FGF, VEGF, IL8, ANG1, ANG2, and MMP9 in SKBR3 breast cancer cells, depends on the over-expressed levels of HOXB7 which can result in the formation of vascularised tumours when grown as xenografts in nude mice. HOXB9 like HOXB7 can lead to increased cell motility and EMT (Hayashida et al., 2010).

4.8 NOTCH

DELTA/JAGGED acting through the NOTCH pathway are implicated in both cell fate in the normal human mammary gland (Raouf et al., 2008) and regulation of cancer stem cells (CSCs) in both ductal carcinoma *in situ* and in invasive carcinoma of the breast (Dontu et al., 2004; Stylianou et al., 2006). This pathway is known to be transcriptionally induced by TGFβ/SMAD signalling and contributes to EMT (Zavadil et al., 2004). This pathway is cell type specific and can be either oncogenic through activation of the NKFB pathway or it can be tumour suppressive. Wang et al., (2006) provided evidence demonstrating that NOTCH receptor signalling regulates SNAIL 1 and 2, ZEB1 and vimentin.

4.9 WNT

The WNT signalling pathway mediates several vital processes such as cell proliferation, migration, differentiation, adhesion and death (Vincan et al., 2008). In addition, this pathway can promote migration and EMT in breast cancer cells through the stabilisation or increased expression of SNAIL1 and 2 and TWIST (Onder et al., 2008; Vogelstein et al., 2004). SNAIL has been implicated in regulating WNT-1-induced EMT in MCF-7 cells. Furthermore, WNT signalling can also lead to the translocation of β-catenin to the nucleus where it can drive the expression of several EMT inducing transcription factors through the WNT induced inhibition of glycogen synthase kinase-3β (GSK3β)-mediated phosphorylation. However, β-catenin alone usually is not enough to induce EMT although in colorectal cancer WNT is indeed a silencer of its negative regulators SOX17 (Zhang et al., 2008), SFRPS18, 19 and DKK1 (Aguilera et al., 2006). Interestingly, both SFRP1 and DKK1 are frequently silenced by methylation in breast cancer.

4.10 miRNA

It is well established that non-protein coding micro (mi) RNAs play a significant role in regulation of gene expression and cellular protein levels. They are now also being increasingly recognised as major regulators of EMT and metastasis, specifically the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429 and miR-205 (Gregory et al, 2008; Park et al., 2008). Members from the miR-200 family and miR-205 are associated with increased expression of E-cadherin and decreased vimentin. In addition, these miRNAs also target the expression of ZEB1 and ZEB2, the E-cadherin transcriptional repressors. Expression levels of miR-205 and of some members of the miR-200 family were also found to vary inversely with vimentin expression in primary serous papillary carcinomas of the ovary (Park et al., 2008). In another study, EMT was induced through either TGFβ or the tyrosine phosphatase pEZ in Madin–Darby canine kidney (MDCK) cells. The levels of both miR-205 and miR-200 family members was down-regulated after EMT induction while their ectopic expression induced MET (Gregory et al., 2008).

One way that natural antisense transcripts can play a major role in EMT is by targeting the regulation of ZEB2 expression. This was documented when EMT was induced in a human colorectal cancer cell line by SNAIL. ZEB2 levels were found to be directly increased after EMT initiation which was explained as the result of the action of a natural antisense transcript that prevented the splicing of a large intron in the 5′ untranslated region (UTR) that contains an internal ribosomal entry site which lowers ZEB2 levels in epithelial cells through the inhibition of ribosome scanning. During EMT activation, the antisense transcript levels are increased. They bind to the 5′UTR and inhibit splicing, preserving the

internal ribosomal entry site sequence and thereby increasing the translational efficiency of ZEB2 which then directly inhibits E-cadherin expression, maintaining an EMT state (Beltran et al., 2008).

Although these RNA molecules are associated with the regulation of EMT and MET, other miRNAs such as miR-10b are reportedly associated with metastasis and invasion. It inhibits HOX10 translation while increasing RHOC when induced by TWIST (Ma et al., 2007). Another miRNA that seems to increase the metastatic potential of cancer cells is miR-29a; up-regulated in a mesenchymal metastatic RASXT mammary cell line compared to epithelial EpRas cells. In addition, over-expression of miR-29a suppresses expression of tristetraprolin, a regulator of epithelial polarity and metastasis, and leads to EMT and metastasis through RAS signalling. This correlates with data from breast cancer patients showing enhanced miR-29a and reduced tristetraprolin levels (Gebeshuber et al., 2009). In contrast to miR-10b, miR-335 was found to be a suppressor of invasion and metastasis through modulation of the expression of the 'six gene signature' set: COL1A1, MERTK, PLCB1, PTPRN2, TNC and SOX4 which are considered predictive markers of metastasis and invasion. miR-335 was also reported to suppress invasion and metastasis in MDAMB231, a highly metastatic and invasive ER-ve breast cancer cell line (Tavazoie et al., 2008).

4.11 Epithelial to mesenchymal transition and breast cancer stem cells

An interesting idea that has emerged recently suggests the possibility that cancer cells undergoing EMT acquire stem cell-like characteristics. The breast cancer stem cell (BCSCs) hypothesis contends that breast cancer is derived from a single tumour initiating cell with stem cell-like properties.

BCSCs are characterized as $CD24^{-/low}$ and $CD44^{+}$ cells which are associated with basal subtype breast cancer. It was first reported by Al-Hajj et al., (2003) when they showed that a $CD44^{+}/CD24^{-/low}$ sub-population of breast cancer cells could produce tumours in a xenograft model more effectively. These cells are regarded as the 'metastatic component' of the cancer, particularly in breast neoplasms as they are the only subset of cells with potential to initiate new tumour growth. This was further supported by analysis of genetic profiles of $CD44^{+}$ breast cancer cells which showed enrichment with stem-cell markers and displayed activated TGFβ signalling with lung metastasis and poor clinical outcomes (Sheridan et al., 2006; Shipitsin et al., 2007). Furthermore, it has been reported that metaplastic and claudin-low breast cancers are enriched with markers of EMT and display stem cell characteristics suggesting that cancer cells undergoing EMT exhibit stem cell-like characteristics (Prat et al., 2010). In addition to that, inducing EMT in immortalized human mammary epithelial cells with either TGFβ, SNAIL1 and TWIST confers stem cell characteristics with increased formation of mammospheres in three dimensional culture and ductal outgrowths in xenotransplants (Mani et al., 2008; Morel et al, 2008). Interestingly, BCSCs isolated from primary tumors and normal breast tissue showed an increased expression of the mesenchymal markers TWIST1 and 2, FOXC2, SNAIL1, ZEB2, vimentin and fibronectin while epithelial cells (which are $CD44^{-}/CD24^{+}$) isolated from differentiated carcinoma do not (Mani et al., 2008). Furthermore, hypoxia-induced SNAIL2 expression has also been associated with acquisition of a basal-like breast cancer phenotype with high levels of the stem cell regulatory genes CD133 and BMI1 (Storci et al., 2008). Inhibition of WNT signalling through LRP6 was found to reduce stem cell-like properties and cause EMT reversal, restoration of the epithelial phenotype, and suppression of SNAIL2 and TWIST expression (DiMeo et al., 2009) in a mouse model of breast cancer metastasis to the lung.

It has also been reported that a $CD24^{-/low}/CD44^{+}$ *in vivo* tumour out- growth which is enriched with EMT markers results from CD8 T-cell-mediated immune response to epithelial breast cancer which would develop characteristics of aggressive carcinomas including potent tumourigenicity, ability to re-establish an epithelial tumour, and enhanced resistance to drugs and radiation (Sheridan et al., 2006; Santisteban et al., 2009). Moreover, breast cancer cells disseminated into the circulation and bone marrow are enriched with $CD44^{+}CD24^{-}$ antigen phenotype (Balic et al., 2006)

EMT induction may be a contributory factor to the decreased efficacy of chemotherapy in breast (Cheng et al., 2007), colorectal (Yang et al., 2006) and ovarian cancer (Kajiyama et al., 2006) while introduction of TWIST into breast cancer cells has been shown to induce paclitaxel resistance. In addition, AKT2 expression, which was amplified in breast cancer has also been correlated with acquired paclitaxel resistance (Cheng et al., 2007). Interestingly, acquisition of enhanced EGFR/ERBB2 signalling in ER+ breast cancer with tamoxifen resistance has been suggested to result from the selection of a more stem cell-like phenotype. EGFR expression is seen in stem cells of the normal mammary gland in both mice and humans (Asselin-Labat et al., 2006; Hebbard et al., 2000) whilst ER is predominantly expressed in the more differentiated luminal cells (Hebbard et al., 2000; Shipitsin et al., 2007). Furthermore, the EGFR pathway is also activated in CSCs of DCIS of the breast and there is emerging evidence for a role of the ERBB2 pathway in the function of CSCs. Expression of ERBB2 and presence of ALDH1+ CSCs was positively correlated in one series of 491 breast cancer patients (Ginestier et al., 2007). The CSC populations of four ERBB2+ breast cancer cell lines have been shown to express more ERBB2 mRNA and protein in comparison to the non-CSC population. Furthermore, trastuzumab was also shown to reduce mammosphere-forming ability and tumourigenicity on serial xenotransplantation (Magnifico et al., 2009). Interestingly, ERBB2+ tumours that received treatment with lapatinib showed decreased EMT related genes in comparison to $CD24^{low/-}/CD44^{+}$ post treatment tissues from patients that received standard anthracycline-taxane chemotherapy. In addition, the γ secretase inhibitor DAPT or a NOTCH 4 neutralizing antibody significantly reduced mammosphere formation in DCIS. NOTCH pathway antagonism has been reported to enhance the reduction of mammosphere formation in ERBB2 over-expressing cell lines induced by trastuzumab (Magnifico et al, 2009).

Colorectal and lung tumours undergoing EMT display decreased sensitivity to EGFR kinase inhibitors, possibly by the activation of downstream targets PI3K and AKT (Barr et al., 2008). In breast cancer, $CD44^{+}/CD24^{-/low}$ CSCs acquire resistance against the chemotherapeutic agents docetaxel, doxorubicin and cyclophosphamide (Li et al., 2008). Furthermore, a proportion of $CD44^{+}/CD24^{-/low}$ cells increase in breast cancer patients following treatment with these anti-cancer drugs suggesting that breast cancer cells may acquire resistance to both conventional and targeted therapies upon conversion to a mesenchymal-like phenotype. This in turn would suggest that any EMT inducing factors such as TWIST and ERBB2 are crucial players in inducing cancer stem cells.

An analysis of a panel of breast cancer cell lines of luminal, intermediate and basal phenotypes showed a significant increase in the fraction of CSCs ($CD44^{+}/CD24^{low/-}/ESA^{+}$) in basal type breast cancers compared to hormone-sensitive luminal cancers (Fillmore & Kuperwasser, 2008). In addition, the number of CSCs and cell line tumourigenicity in *in vivo* models was correlated positively (Fillmore et al., 2008).

A functionally redundant ER in endocrine-resistant breast cancer might promote a more mesenchymal stem-cell-like phenotype based on the observation that ER negatively regulates the expression of the key EMT transcription factors including SNAIL1 and SNAIL2 (Dhasarathy et al., 2007; Ye et al., 2008). Furthermore, tamoxifen resistant MCF7 cells have been reported to show an enhanced mammosphere formation capacity in comparison to the tamoxifen sensitive cells which suggests an increased CSC fraction (Storci et al., 2008). EMT may facilitate the generation of CSCs with mesenchymal and self-renewal properties necessary for dissemination and initiation of metastasis. (Hollier et al., 2009; Mani et al, 2008). An immunohistochemical analysis of 479 invasive breast carcinomas showed a high expression of the EMT-induced markers vimentin, α-smooth muscle actin, N-cadherin, CDH1, SPARC, laminin and fascin, in comparison to the low expression of E-cadherin in these $CD44^+/CD24^-$ basal-like breast tumours. These tumours have the ability to form distant metastases hence exhibiting a worse prognosis (Perou et al., 2000; Sorlie et al., 2001). In a study on 117 samples of primary invasive breast carcinomas, nuclear staining of the EMT inducing transcription factor FOXC2 showed a significant correlation with $CD44^+CD24^-$ basal-like subtypes (Mani et al., 2007). Another study on 226 blood samples from 39 patients with metastatic breast cancer showed that the majority of the circulating tumour cells (CTCs) exhibited EMT and CSC characteristics (Aktas et al., 2009). CTCs were present in 69 of 226 (31%) blood samples taken from patients with metastatic breast cancer to investigate the expression of TWIST, AKT2, and PI3Kα and ALDH1 which is considered to be a stem cell marker. In the CTC-positive group, 62% were positive for the EMT markers and 69% for ALDH1, while in the CTC-negative group the proportions were 7 and 14%, respectively (Aktas et al., 2009). The CTCs have also been shown to have a reduced expression of epithelial-specific cytokeratins (Pantel et al., 2008). Interestingly, disseminated tumour cells (DTCs) over-expressed TWIST. Assessment of occurrence of bone marrow metastases indicated that TWIST+ cells were present prior to chemotherapy and this was significantly associated with relapse (Watson et al., 2007).

EMT undergoing CTCs have also been shown to resist apoptosis. One study reported that following the induction of EMT by TGFβ in the EpH-4 and nMuMG murine mammary epithelial cell lines, they tended to acquire resistance to ultraviolet light induced apoptosis (Robson et al., 2006). Likewise, down regulation of the expression of LET-7 miRNA in breast cancer cell lines increased their metastatic potential and the resistance to therapy, in association with the acquisition of stem cell characteristics and EMT-associated gene expression profiles (Yu et al., 2007). Furthermore, the factors that can induce a full EMT; TGFβ, WNT, HEDGEHOG, NOTCH, and RAS signaling pathways, are all considered to be involved in the induction and maintenance of stem cell niches (Fuxe et al., 2010). There is however some data showing that TGFβ stimulation of transformed human breast epithelial cells can result in the loss of stem cell-like properties including the ability to form mammospheres (Tang et al., 2007).

5. Endocrine resistance and EMT

It is becoming increasingly apparent that acquired endocrine resistance is a multi-factorial stepwise progression that can be triggered through a number of distinct pathways, that *in vitro*, can be manipulated. Whether it is the actual loss of ER due to transcriptional or translational down-regulation, or functional redundancy of ER (which seems to be the more frequent occurrence *in vivo*), either scenario would have the same end result in terms of

independence from estrogen. It is therefore pertinent to ask what happens to a cell that experiences loss of ER. As described in preceding sections this issue has been addressed by various cell models that have been made endocrine resistant by exposure to antiestrogens or by deprivation of estradiol, but rarely by the direct prevention of ER synthesis.

We have explored this avenue by modifying MCF7 cells by transfection with shRNA generating plasmids targeting the ER mRNA (Al Azmi, 2006; Luqmani et al., 2009; Al Saleh et al., 2011a). As expected, stably transfected cell lines with constitutive reduction of ER (termed pII) exhibit a loss of response to either estradiol or tamoxifen/fulvestrant and hypersensitivity to EGF and IGF1 (Salloum, 2010). There is reduction in the classical ER-regulated markers such as pS2, cathepsin D, PR and PRLR. Like the tumour-derived naturally ER-ve MDAMB231 cell line, these (acquired) endocrine resistant cells show increased motility and ability to invade simulated components of the ECM mimicking the behaviour of aggressive ER-ve/EGFR+ve tumours. Both of these activities as well as cellular proliferation are reduced by various tyrosine kinase inhibitors that are known to block, in particular, EGFR and VEGFR phosphorylation (Al Saleh, 2010) supporting the data mentioned in preceding sections. However, the most striking features of pII cells was initially noted in their morphological appearance (see Fig 3), assuming a more elongated spindly shape and failure to form the compact colonies characteristic of MCF7 cells, with re-arrangement of the actin cytoskeleton giving rise to increased incidence of lamellipodia and microspikes, features closely associated with cellular motility (Parker et al., 2002).

Microarray analysis confirmed that pII cells had assumed a phenotype that is generally seen for mesenchymal cells, with transcriptional loss of genes normally associated with epithelial cells. Lack of colony formation can be explained by loss of E-cadherin and many other factors responsible for normal cell-cell adhesion including catenins, laminin, type IV collagen, desmogleins, desmocollins, occludins, connexion 2b claudins and MUC1. Likewise, archetypical epithelial components such as keratins 8, 18 and 19 and tissue inhibitors of metallo-proteinases are all reduced. On the other hand, we observed an increased expression of mesenchymal markers such as N cadherin, vimentin, fibronectin, integrins β4 and α5, tenascin, SPARC, PLAU, VEGF, CD68, FSP1/S100A4, LCN2 and various metalloproteinases In short, we are seeing all the hallmarks of cells undergoing EMT with acquisition of the phenotype characterising the group of basal-like 'claudin low' tumours such as the triple negative (ER-ve, PR-ve, ERBB2-ve) metaplastic tumours described by Hennessy et al., (2009). A similar conclusion was reached by Gadalla et al., (2005) who observed an EMT-like transition with loss of E-cadherin and reduction in CD24 induced by ER silencing. However, they did not observe the increase in CD44 that we and others have widely reported.

An interesting molecule whose expression was found to be substantially repressed in our pII cells (Al Saleh et al., 2011a) is GATA3, a zinc finger transcription factor that plays an important role as a regulator of mammary gland formation and development (Kouros-Mehr et al., 2008) and has been implicated in both EMT and breast cancer metastasis. GATA3 is a positive transcriptional regulator of ER expression whilst simultaneously itself being a target gene for the ER complex. Its expression has been linked to favourable outcome of endocrine therapy (Parikh et al., 2005). Several studies have shown association of GATA3 with ER+ tumours (eg, Mehra et al., 2005). Yan et al., (2010) recently demonstrated that not only was GATA3 expression abolished in ER-ve cell lines but also correlated with E-cadherin. siRNA-induced silencing of GATA3 resulted in fibroblastic-like transformation of MCF7 cells. On the other hand, restoration of GATA3 expression in ER-ve cells led to

renewal of epithelial characteristics as typified by increased levels of E-cadherin and decrease of N-cadherin, vimentin and MMP9 with parallel reduction of tumour forming capacity of MDAMB231 cells injected into xenografted mice. These studies elegantly support the notion that ER regulated events is intimately involved in the same processes that lead to EMT and very crucially, that these events are reversible.

Another significant group of genes variously implicated in EMT that is elevated in pII cells is included in the '24 gene signature' of genes proposed as predictive of invasiveness (Zajchowski et al., 2001): integrin, TIMP-2 and TIMP-3, MT1-MMP, PAI-1, Osteonectin/SPARC, thrombospondin-1, collagen (VI) α1 and collagen (I) α2. pII also display the '9 gene signature' of down-regulated or low expressing genes (E-cadherin, CLDN7, CRB3, KRT8, TACSTD1, IRF6, SPINT2, MAL2 and MARVELD3) that was found by Katz et al., (2011) to be common between their C35 transfected cells and claudin-low tumours. Evidence that the latter represent EMT is now substantial and supported by *in vitro* observations (Prat et al., 2010; Taube et al., 2010).

Substantial reduction in ER expression has been observed in modified MCF7 sub-lines resistant to the mitotic inhibitors paclitaxel and docetaxel and the anthracycline doxorubicin (Iseri et al., 2011). Microarray analysis showed up-regulation of SNAIL2, CDH2, VIM, CLDN1, CLDN11, EGFR, FGFR1, SMAD3 and TGFBR2 and down-regulation of E-cadherin, OCLN, CLDN3, CLDN4, and CLDN7. This data bears remarkable resemblance to the profile for pII cells with the common denominator being loss of ER.

This brings us finally to the group of transcriptional repressors that have been coined as the 'mediators of EMT' and discussed above, so far a relatively smaller group that unify a much larger and diverse array of signalling molecules involved in their regulation. Of the key factors identified in cadherin switching, ZEB1, ZEB2/SIP1 and SNAIL2 (Onder et al, 2008) are all significantly elevated in our endocrine resistant pII cells. These observations lead us to conclude that there is a high degree of synonimity between endocrine resistance and EMT, both effected by functional loss of ER and both resulting in increased propensity for tumour dissemination through the actions of a common set of mediators. The repression of SNAIL by the ER dependent MTA3 (Fujita et al., 2003), a subunit of the Mi-2/NuRD histone deacetylase complex, which could well be regarded, among others, as a guardian of the epithelial phenotype (?) may be worthy of further attention. Interestingly, another family member, MTA1, is described as a potent inhibitor of nuclear ER function through cytoplasmic sequestration of the receptor and this may provide an explanation for resistance in ER+ cells as MTA1 would indirectly reduce the levels of MTA3 thereby relieving SNAIL repression.

There have also been intriguing suggestions regarding the origin of the mesenchymal-like cells, with the attractive view of these as a possibly slow growing pre-existing CSC sub-population within the tumour (Lim et al., 2010; May et al., 2011). In such a scenario there is no induced EMT as such, but a gradual emergence of a group of cells already bearing these properties, to become the dominant group. Similar ideas have often been suggested to explain the re-emergence of 'drug–regressed' tumours as an expansion of a pre-existing intrinsically resistant cell population once the sensitive cells have been eliminated. However, attractive as this may be, in the alternative scheme elaborated by May et al., (2011) there would be a reversion of such 'MaSCs' back to an epithelial phenotype at the site of metastatic growth in a reverse MET transition, which raises the question that If cells can undergo MET then why not EMT, and there is no necessity to postulate the existence of *a priori* mesenchymal cells. Moreover, the *in vitro* data demonstrates quite clearly that an

actual EMT transition does take place as the initial population of cells is relatively homogeneous with respect to being epithelial in nature. Most if not all of the cells in culture can simultaneously undergo EMT whereas it is very likely that only a very small fraction of cells in a tumour acquire all of the characteristics enabling them to undergo a full transition, which may be why such mesenchymal-like cells have not been routinely reported by pathologists (Thompson et al., 2008).

6. Summary

The persistent problem of drug resistance and in particular the therapeutic failure of endocrine agents presents serious therapeutic issues especially in view of the success of this type of intervention in a significantly large proportion of women with breast cancer. Many studies have focused on elucidating the mechanisms responsible for *de novo* and *acquired* independence from estrogen. Consensus of opinion favours the view that signaling pathways mediated through a variety of peptide growth factors is largely responsible for the aggressive proliferation of tumours that have ceased to depend upon the ER, although no single unifying or even major factor has been identified. Somewhat in parallel, the last few years have witnessed an increasing number of reports describing the relatively recently recognized phenomenon of EMT, highlighting its similarity to the events leading to tumour invasion and vascular dissemination. Many of the key mediators of EMT particularly the transcriptional repression of E-cadherin by SNAIL appear to be critical steps in tumour progression. The association of mesenchymal-like features such as cadherin switching, loss of adhesion proteins and CD24, increased vimentin and fibronectin, with ER-ve tumours, have been sporadically, almost anecdotally reported in the literature over the last decade or more. We have now found evidence to show that the acquisition of endocrine independence, due to induced ER loss, by previously ER+ breast cancer cells, is accompanied by all the hallmark features of EMT. Although it is still far from clear whether the two processes are occurring side by side or whether either is causal of the other, it seems reasonable to conclude that loss of ER can directly trigger EMT. It remains to be seen whether restoration of ER in the trans-differentiated cells can reverse EMT and allow the cells to regain estrogen dependence.

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23

Junctional Adhesion Molecules (JAMs) - New Players in Breast Cancer?

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1. Introduction

1.1 Global incidence of breast cancer

Worldwide, breast cancer remains a leading cause of death amongst women. Annually, it is estimated that breast cancer is diagnosed in over a million women (Kasler *et al*., 2009) with over 450,000 deaths worldwide (Tirona *et al*., 2010). The incidence of the disease is highest in economically-developed countries, with lower rates in developing countries. Despite continual advances in breast cancer care which have led to reduced mortality, however, the incidence of the disease is still rising. The decrease in breast cancer-specific mortality has been attributed to improvements in screening techniques which permit earlier detection, surgical and radiotherapy interventions, better understanding of disease pathogenesis and utilization of traditional chemotherapies in a more efficacious manner. Consequently, early stage breast cancer is now a curable disease while advanced breast cancer remains a significant clinical problem.

Breast cancer is a heterogeneous disease encompassing many subtypes, which differ both in terms of their molecular backgrounds and clinical prognosis. These breast cancer subtypes range from pre-invasive early stage disease to advanced invasive disease. The simplest classifications of disease subdivide breast cancer into pre-invasive and invasive forms; with the pre-invasive forms being ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). Carcinoma *in situ* is proliferation of cancer cells within the epithelial tissue without invasion of the surrounding stromal tissue (Bland & Copeland, 1998). DCIS arises in the terminal ductal lobular units (TDLU) and in extra-lobular ducts while LCIS occurs in the breast lobules, and is recognisable histopathologically by the presence of populations of aberrant cells with small nuclei (Hanby & Hughes, 2008). Invasive breast cancers are sub-classified into invasive ductal breast cancer, invasive lobular breast cancer, inflammatory breast cancer and Paget's disease. Invasive ductal carcinoma (IDC) is the most common form of invasive breast cancer, accounting for around 85% of all cases.

DCIS is frequently considered as an obligate precursor to IDC, progressing from lower to higher grades and then onto invasive cancer with progressive accumulation of genomic changes (Farabegoli *et al*., 2002). However it has alternately been suggested that there exist genetically-distinct subgroups of DCIS, only some of which have the potential to progress to invasion (Shackney & Silverman, 2003). Long-term natural history studies of DCIS have provided supportive evidence for both possibilities (Page *et al*., 1995; Collins *et al*., 2005; Sanders *et al*., 2005). Despite such controversies, the large extent to which the genome is

altered in DCIS strongly suggests that genomic instability precedes phenotypic evidence of invasion (Hwang *et al*., 2004). This serves to underline the fact that malignant transformation in a heterogeneous disease like breast cancer is a dynamic process evolving through multiple multi-step pathway models.

Many factors are thought to be responsible for the development of breast cancer. Genetic factors play a vital role in the predisposition to breast cancer, with mutations of *BRCA1* and *BRCA2* genes accounting for 5–10% of breast cancer cases and being responsible for 80% of inherited breast cancers (Nathanson *et al*., 2001). On a more complex level, much insight has been gained from the genetic profiling of thousands of tumours to generate gene signatures of prognostic value (Sorlie *et al*., 2001; van 't Veer *et al*., 2002; van de Vijver *et al*., 2002), which have spurred the development of commercially-available diagnostic tests. The importance of reproductive factors in the aetiology of breast cancer is also well recognised with early onset of menarche, nulliparity, late menopause, endogenous and exogenous hormones representing the main risk factors (Reeves *et al*., 2000; Key *et al*., 2001; Howell & Evans, 2011). Several other studies have reported an increased risk of breast cancer with lack of physical activity (especially in pre menopausal women), as well as increasing age and obesity (Clarke *et al*., 2006; Walker & Martin, 2007; Harrison *et al*., 2009; Rod *et al*., 2009; Awatef *et al*., 2011). These risk factors accentuate the abnormal growth control of cells by increasing the circulating levels of oestrogen thereby promoting tumourigenesis within the breast microenvironment. A proper understanding of the breast cancer microenvironment is essential for understanding breast cancer, and will be explored in detail in the next sections.

1.2 Breast structure and breast cancer microenvironment

The breasts are modified sweat glands with a specialized function to produce milk. In the adult, the mature breast extends from the second ribs to the seventh rib and from the lateral border of the sternum to the midaxillary line and projects into the axilla at the axillary tail of Spence (Monkhouse, 2007). The breast is located within the superficial fascia of the anterior thoracic wall and is made up of 15-20 lobes of glandular tissue (Bland & Copeland, 1998). Fibrous connective tissue forms the framework that supports the lobes and adipose tissue which fills the space between the lobes. Each lobe of the mammary gland terminates in a lactiferous duct which opens onto the nipple and is lined with breast epithelial tissue. These ducts have a sinus at the base beneath the areola called the lactiferous sinus (Figure 1).

Breast cancers are characterised by abnormal proliferation of breast epithelial cells and mostly originate in milk ducts (Sainsbury *et al*., 2000). Normal milk ducts consist of an outer myoepithelial cell layer and an inner luminal epithelial layer. Myoepithelial cells, which are of ectodermal origin, lie between the surface epithelial cells and the basal lamina. Both the epithelial and myoepithelial cells of the breast duct lie on a basement membrane composed of extracellular matrix factors secreted by those cells (Figure 2). The basement membrane is important for defining the barriers of the normal duct, and thus alterations in the basement membrane have been implicated in abnormal cell differentiation and the formation of metastases (Kleinman *et al*., 2001).

Proliferation of cells within the breast ducts is controlled by growth-promoting proto-oncogenes and growth-inhibiting tumour suppressor genes. In most cases, normal cells divide as many times as needed and then stop. Carcinogenic mutations in either (or both)

oncogenes and tumour suppressor genes (along with subsequent interactions between defective genes and the breast microenvironment) alter not just cell proliferation, but also differentiation, survival and genome stability (Hahn & Weinberg, 2002) of breast cells, leading to abnormal cell growth and potentially cancer.

Much evidence supports the contention that the pathogenesis of breast cancer is influenced by complex interactions between ductal epithelial cells and the cells that compose the tumour microenvironment (Weaver *et al.*, 1996; Polyak & Hu, 2005; Hu *et al.*, 2008). The next section will focus on the cells of the microenvironment with respect to normal breast tissue structure and also their possible involvement in breast tumourigenesis.

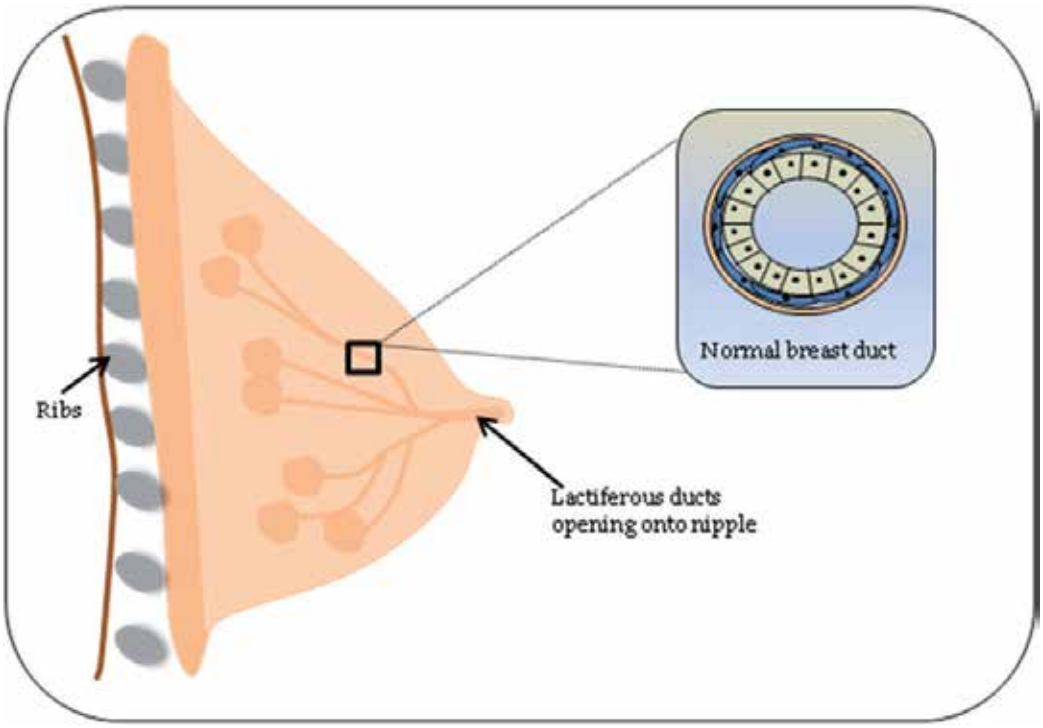

Fig. 1. Structure of the breast showing lobules and lactiferous ducts terminating at the nipple

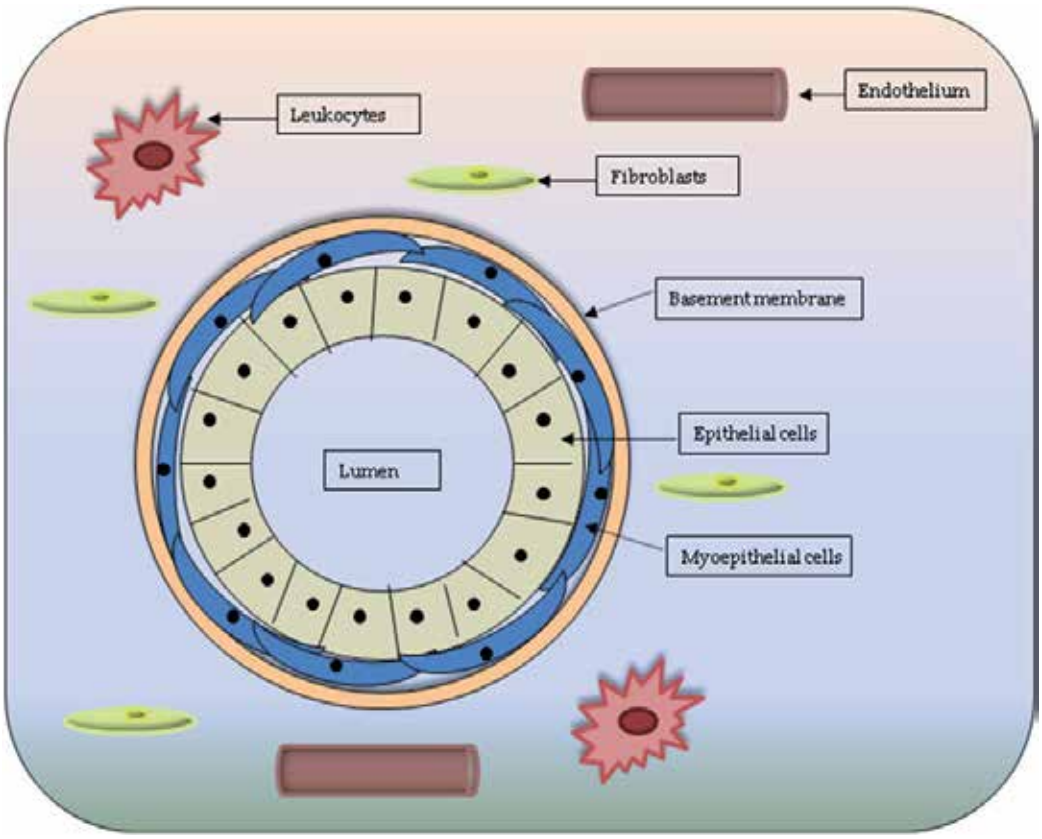

Fig. 2. Diagram of a normal breast duct depicting cells of the microenvironment.

1.2.1 Cells of the breast microenvironment

The abnormal epithelial cells composing a breast carcinoma form only one component of a complex microenvironment which influences the success or failure of a developing tumour. In fact the breast tumour microenvironment consists also of multiple cell types; including myoepithelial cells, fibroblasts, endothelial cells and immune cells such as macrophages (Figure 2). In terms of their likely contributions to breast tumourigenesis, fibroblasts and macrophages are often considered as tumour promoters through downstream signalling from various secreted factors, while the endothelial cells which develop in tumour-associated blood vessels also support cancer development. In contrast, myoepithelial cells exert functions broadly considered as tumour-suppressive.

Fibroblasts are an important structural component of the extracellular environment in the normal breast, where they help control the development of the breast epithelium (McCave *et al*. 2010). Their secretion of extracellular matrix components and cytokines has also implicated them in tumorigenic growth associated with invasive breast cancer (Orimo *et al*., 2005), and differences in cellular responsiveness to normal versus tumour-derived fibroblasts have been noted (Sadlonova *et al*., 2005). Many studies have highlighted the

potential involvement of fibroblasts in promoting tumour progression both at genomic and transcriptomic levels, with reports of altered genetic signatures between normal and tumour-associated fibroblasts supporting a complex role for fibroblasts in influencing tumour progression (Hu *et al.,* 2005; Hu *et al.,* 2008; Ma *et al*., 2009).

Macrophages within the breast cancer microenvironment have been shown to enhance tumour growth through the secretion of pro-angiogenic factors like vascular endothelial growth factor (VEGF); (Murdoch *et al*., 2004; Lamagna *et al*., 2005 ; Lewis & Hughes, 2007). They have also been implicated in promoting a metastatic phenotype, via the secretion of pro-migratory factors such as EGF (Wyckoff *et al*., 2004) which enhance cellular dissemination from a primary tumour. Accordingly, the enhanced physical juxtaposition of macrophages, tumour cells and endothelial cells has been proposed as a new prognostic histopathological marker associated with increased risk of metastases in human breast cancer (Robinson *et al*., 2009).

Endothelial cells which line the blood vessels are derived from angioblasts forming the vascular network. Enhanced vessel density occurring as a result of tumour-associated angiogenesis is a major contributor to both the survival of primary breast tumours (via the delivery of systemic growth factors) and the risk of metastasis (via increased access of disseminated tumour cells to a circulatory source). Expression of pro-angiogenic factors such as VEGF has been shown to increase in haematological malignancies (Fiedler *et al*., 1997; Molica *et al*., 1999) in addition to solid tumours including breast, renal, ovarian, gastric and lung cancer (Patel *et al*., 2009; Burger, 2011; Gou *et al*., 2011; Sharma *et al*., 2011). VEGF promotes neovascularisation via mitogenic and pro-migratory effects on endothelial cells (Asahara *et al*., 1999).

Finally, myoepithelial cells are known to play a role in the formation of the basement membrane and thereby assist in maintaining polarity of the breast ductal epithelium. They also interact with epithelial cells to regulate the cell cycle and suppress breast cancer cell growth, invasion and angiogenesis (Weaver *et al*., 1996; Alpaugh *et al*., 2000; Barsky, 2003). Tumour and non-tumour primary myoepithelial cells have been described to differ in functional properties relating to the secretion of extracellular matrix components such as laminin-1 (Gudjonsson *et al*., 2002), and accordingly myoepithelial cells reportedly lose their established tumour-suppressive properties during tumour progression (Polyak & Hu, 2005).

Taken together, the many cell types within the breast tumour microenvironment can both individually and coordinately regulate several functions relevant to tumour progression. In order to better understand their relative contributions to breast cancer, it is necessary to dissect the signals that regulate their own functions. Since adhesive functions are central to the behaviour of all of these cell types, the remainder of this chapter will focus on their potential regulation by a family of adhesion proteins termed the Junction Adhesion Molecules (JAMs), whose role in breast cancer initiation and progression is just emerging.

2. Cell-cell adhesion and the functional roles of JAMs in epithelial/endothelial cells

2.1 Introduction to cell-cell adhesion complexes and JAMs

Cells within the breast tumour microenvironment physically interact with each other and with the extracellular matrix through a range of cell adhesion proteins. Cell adhesion proteins play fundamental roles in normal physiology (such as the control of cell polarity and epithelial barrier function), but their dysregulation has been shown to participate in

tumour cell migration, invasion and adhesion (for review, see Brennan *et al.*,2010). Adhesion proteins rarely exist in isolation from each other on the cell membrane, rather they form components of multi-cellular adhesion complexes containing a network of adhesion, scaffolding and signalling proteins. Breast epithelial cells express various types of adhesion complexes, namely hemidesmosomes and focal adhesions at the cell-matrix interface, with tight junctions, adherens junctions, desmosomes and gap junctions at the cell-cell interface. Collectively, adhesion complexes are composed of integral membrane proteins and cytoplasmic scaffolding proteins that organise signalling complexes and anchor cell-cell contacts to intermediate filaments (at desmosomes and hemidesmosomes) or to actin filaments (at adherens junctions, tight junctions and focal adhesions).

Tight junctions (TJs) play a vital role in regulating the paracellular flux of ions, small molecules and inflammatory cells as well as defining distinctly-polarized membrane domains and facilitating bi-directional signalling between the intracellular and extracellular compartments. These functions of the TJ are regulated by the balance of three different types of integral membrane proteins; (1) Occludins and Tricellulin, (2) Claudins and (3) Immunoglobulin Superfamily (IgSF) members. Of most interest in this chapter is the Junctional Adhesion Molecule (JAM) subfamily of the IgSF, and its potential contribution to cancer initiation and progression.

The JAM family consists of 5 proteins (JAM-A, -B, -C, -4, -L) which are major components of TJs in endothelial and epithelial cells in a variety of vertebrate and invertebrate tissues (Martin-Padura *et al.*, 1998; Liang *et al.*, 2000; Liu *et al.*, 2000; Arrate *et al.*, 2001; Aurrand-Lions *et al.*, 2001; Itoh *et al.*, 2001; Hirabayashi *et al.*, 2003; Tajima *et al.*, 2003). JAM proteins are also expressed on the surface of haematopoetic cells such as platelets, neutrophils, monocytes, lymphocytes, leukocytes and erythrocytes; in addition to connective tissue cells such as fibroblasts and smooth muscle cells (Azari *et al.*, 2010; Kornecki *et al.*, 1990; Naik *et al.*, 1995; Malergue *et al.*, 1998; Williams *et al.*, 1999; Cunningham *et al.*, 2000; Palmeri *et al.*, 2000; Arrate *et al.*, 2001; Aurrand-Lions *et al.*, 2001; Moog-Lutz *et al.*, 2003; Morris *et al.*, 2006). JAMs are type I transmembrane proteins consisting of an N-terminal signal peptide, an extracellular domain (consisting of two immunoglobulin-like domains), a single membrane-spanning domain and a short cytoplasmic tail (Martin-Padura *et al.*, 1998; Liu *et al.*, 2000; Sobocka *et al.*, 2000; Aurrand-Lions *et al.*, 2001; Naik *et al.*, 2001; Santoso *et al.*, 2002). The cytoplasmic tail is thought to play a major role in the assembly of adhesion signalling complexes, since it has been reported to bind to PDZ domain-containing scaffold proteins such as ZO-1 (Bazzoni *et al.*, 2000; Ebnet *et al.*, 2000), AF-6 (Ebnet *et al.*, 2000) and MUPP1 (Hamazaki *et al.*, 2002).

JAMs -A, -B and -C exhibit a short cytoplasmic tail of 45–50 residues that ends with a type II PDZ binding motif, while JAM-4 and JAM-L have longer cytoplasmic tails (of 105 and 98 residues respectively). JAM-4 and JAM-L differ in that the cytoplasmic tail of the former ends in a canonical type I PDZ binding motif, while that of the latter lacks a PDZ-binding motif (Mandell & Parkos, 2005). The cytoplasmic tails of JAM proteins also contain consensus phosphorylation sites that may serve as substrates for protein kinase C, protein kinase A and Casein Kinase II (Naik *et al.*, 1995; Cunningham *et al.*, 2000; Ozaki *et al.*, 2000; Sobocka *et al.*, 2000; Arrate *et al.*, 2001; Naik *et al.*, 2001). Indeed, evidence suggests that specific phosphorylation sites may be critical for targeting of JAMs to intercellular junctions (Ozaki *et al.*, 2000; Ebnet *et al.*, 2003).

JAM proteins have been implicated in a diverse array of physiological functions involving cell–cell adhesion/barrier function (Liang *et al.*, 2000; Liu *et al.*, 2000; Mandell *et al.*, 2004), leukocyte migration (Martin-Padura *et al.*, 1998; Palmeri *et al.*, 2000; Johnson-Leger *et al.*, 2002; Ostermann *et al.*, 2002), platelet activation (Kornecki *et al.*, 1990; Naik *et al.*, 1995; Gupta *et al.*, 2000; Ozaki *et al.*, 2000; Sobocka *et al.*, 2000; Naik *et al.*, 2001; Babinska *et al.*, 2002; Babinska *et al.*, 2002) and angiogenesis (Naik *et al.*, 2003; Naik *et al.*, 2003). These functions will be further discussed in the next sections.

2.2 JAM proteins regulate epithelial/endothelial cell–cell adhesion and barrier function

JAM proteins are well-known to be important for cell-cell adhesion in both epithelial and endothelial cells (for review see Mandell & Parkos, 2005), but emerging evidence supports the possibility that they also regulate cell-matrix adhesion complexes. Interestingly, JAM-A knockdown in endothelial cells and MCF7 breast cancer cells has been shown to reduce adhesion to fibronectin and vitronectin (McSherry *et al.*, 2011; Naik & Naik, 2006), while JAM-C overexpression in endothelial cells reportedly decreases attachment to fibronectin, vitronectin, and laminin (Li *et al.*, 2009). This apparent incongruity may relate to the fact that JAM-A may activate β1 integrins (McSherry *et al.*, 2011), while JAM-C has conversely been described to inactivate β1 integrins (Li *et al.*, 2009). An inverse relationship between JAMs –A and –C has also been observed in terms of tight junction function, with JAM-A promoting tight junction sealing while phosphorylated JAM-C increases paracellular leakiness due to its redistribution away from TJs (Li *et al.*, 2009). Furthermore, adhesion of the lung carcinoma cell line NCI-H522 to endothelial cells was significantly blocked by soluble JAM-C (Santoso *et al.*, 2005).

The contribution of JAM proteins to cell-cell adhesion and the assembly of epithelial/endothelial TJs relates to their ability to promote the localization of ZO-1, AF-6, CASK and occludin at points of cell-cell contact. Evidence suggests that both homophilic and heterophilic interactions, as well as an intact PDZ binding motif, are important for such protein functions of JAMs. Accordingly, JAMs have been shown to physically interact with the PDZ proteins, ZO-1 (Bazzoni *et al.*, 2000; Ebnet *et al.*, 2000), AF-6 (Ebnet *et al.*, 2000), CASK (Martinez-Estrada *et al.*, 2001), PAR-3 (Ebnet *et al.*, 2001; Itoh *et al.*, 2001) and MUPP-1 (Hamazaki *et al.*, 2002); which are involved in actin cytoskeletal rearrangement (Fanning *et al.*, 2002), cell signalling (McSherry *et al.*, 2011; Boettner *et al.*, 2000) and the control of cell polarity. However JAMs can also bind to non-PDZ proteins such as cingulin (Bazzoni *et al.*, 2000), and indirectly bind occludin (Bazzoni *et al.*, 2000) and claudin 1 via their interactions with ZO-1 (Hamazaki *et al.*, 2002). Although the manner in which JAMs interact with some of these proteins is incompletely understood, it appears that homo-dimerisation of JAM proteins is important for regulating some key downstream functions. This has been illustrated by the fact that dimerisation-blocking anti-JAM-A antibodies (Liu *et al.*, 2000) and soluble Fc–JAM-A (Liang *et al.*, 2000) delay the recovery of electrical resistance (a marker of TJ function) in epithelial cells following transient depletion of extracellular calcium.

2.3 JAM proteins regulate epithelial/endothelial migration

In general cell adhesion and cell migration are inversely related, and serve to control important physiological functions and pathophysiological events. However, in the case of JAM family members, close functional associations with cell polarity proteins may act as a switch between increased adhesion (predisposing to slow, directional migration) and decreased

adhesion (predisposing to faster, more random motility). For example, JAM-A re-expression in JAM-A-/- mouse endothelial cells has been shown to reduce the occurrence of spontaneous and random motility. This ability of JAM-A to influence the polarised movement of cells was reliant on its interaction with polarity proteins through its PDZ binding motif (Bazzoni & Dejana, 2004). JAM-A deletion mutants lacking their PDZ-binding residues have been shown to have increased availability of Par3 (Ebnet *et al*., 2001), resulting in PKCζ inactivation and the loss of contact-dependent inhibition of cell motility (Mishima *et al*., 2002; Bazzoni & Dejana, 2004). These data show that loss of functional JAM-A results in faster random motility with reduced cell-cell contact inhibition of migration. Interestingly, JAM-C redistribution away from TJs stimulates β1 and β3 integrin activation, resulting in increased cell migration and adhesion (Aurrand-Lions *et al*., 2001). Furthermore, JAM-A and JAM-4 have been found to induce the formation of actin-based membrane protrusions, an essential part of cell migration, in endothelial and COS-7 cells (Mori *et al*., 2004). Together these data suggest loss of JAM-A promotes random motility, while JAM-A, JAM-C and JAM-4 promote directional cell migration through their effects on integrin function and cytoskeletal reorganization.

In the context of cancer, knockdown of JAM-A has been shown to enhance invasiveness of the breast cancer cell lines MDA-MB-231 and T47D, and the renal cancer cell line RCC4 (Naik *et al*., 2008; Gutwein *et al*., 2009). Conversely, the overexpression of JAM-A in MDA-MB-231 cells reportedly inhibits both migration and invasion through collagen gels (Naik *et al*., 2008), suggesting that loss of JAM-A expression increases cancer cell dissemination and invasion. However, the specific contribution of JAM-A to breast cancer progression remains controversial. McSherry *et al* showed a significant association between *high* JAM-A gene or protein expression and poor survival in 2 large cohorts of patients with invasive breast cancer, and concurrently a decrease in the migratory abilities of high JAM-A-expressing MCF-7 cells upon knockdown or functional inhibition of JAM-A (McSherry *et al*., 2009). Reduced motility after JAM-A loss was subsequently linked to reduced interactions between JAM-A, AF-6 and the Rap1 activator PDZ-GEF2, resulting in reduced activity of Rap1 GTPase (McSherry *et al*., 2011), a known activator of β1-integrins (Sebzda *et al*., 2002) and a regulator of breast tumourigenesis (Itoh *et al*., 2007). Complementary evidence in a recent publication by Gotte *et al.* has also supported the theory that JAM-A overexpression is of more functional relevance in breast cancer than JAM-A loss, since over-expression of micro RNA (miR)-145 in breast cancer cells led to a decrease in cellular migration and invasion via downregulation of JAM-A expression (Gotte *et al*., 2010). Still more recently (during the proofing stage of this chapter), additional histopathological evidence has been provided for a link between JAM-A over-expression and poor prognosis in breast cancer patients (Murakami *et al.,* 2011). This, along with the finding that JAM-A promotes the survival of mammary cancer cells (Murakami *et al.,* 2011), strongly suggests that JAM-A depletion or antagonism could offer promise in reducing breast tumour progression. Furthermore, depletion of JAM-A has been found to inhibit bFGF-induced migration of human umbilical vein endothelial cells (HUVEC) on vitronectin, through effects on integrin function (Naik & Naik, 2006). In other cell systems, silencing of the JAM-A gene has been shown to block the migration of inflamed smooth muscle cells (Azari *et al*., 2010) and to increase the random motility of dendritic cells (Cera *et al*., 2004). JAM-A has also been shown to be required for neutrophil directional motility (Corada *et al*., 2005), and to promote neutrophil chemotaxis by controlling integrin internalization and recycling (Cera *et al*., 2009). Thus while the area remains controversial, increasing evidence is suggesting that JAMs promote migration and

invasion through the regulation of integrin expression and activation (McSherry *et al.*, 2011; Naik & Naik, 2006; Li *et al.*, 2009; McSherry *et al.*, 2009).

In breast cancer, the formation of metastases at distant sites is the leading cause of cancer-related death. In order for breast cancer cells to metastasize, they must first migrate out of the primary tumour before ever reaching a distant organ and potentially proliferating into a secondary tumour. While JAMs are already known to regulate migration, the possibility that they are also involved in the regulation of proliferation will be referred to in section 3.3 of this chapter.

All together these data highlight the role of JAM family members in controlling the balance between cell adhesion and migration. Although much remains to be understood about the exact role of JAMs in breast cancer cell migration, the classic description of tumours as "wounds which do not heal" (Riss *et al.*, 2006) suggests that the migratory mechanisms employed by JAMs in physiological responses (such as wound healing) may also be utilised by cancer cells to promote tumour progression or survival.

2.4 Potential role of JAM proteins in epithelial/endothelial differentiation

In previous sections we discussed the biphasic role of JAM family members in regulating cell adhesion and migration. In this section we will outline the emerging contribution of the JAM family to cellular differentiation. Cell differentiation in the context of normal tissue usually involves the transition from an undifferentiated stem/progenitor cell to a terminally-differentiated cell such as an epithelial, muscle or nerve cell.

JAM-A, JAM-B, JAM-C and JAM-4 have been found to be highly expressed on hematopoietic stem cells (HSCs) in the bone marrow, with their expression decreasing during the acquisition of a more differentiated state (Nagamatsu *et al.*, 2006; Sakaguchi *et al.*, 2006; Sugano *et al.*, 2008; Praetor *et al.*, 2009). Furthermore JAM-A expression has been reported to be high on undifferentiated HC11 mammary epithelial cells relative to differentiated cells (Perotti *et al.*, 2009). In support of a potential association between high JAM-A and poor differentiation status, high JAM-A gene or protein expression has been associated with a poorer grade of differentiation in tissues from patients with invasive breast cancer (McSherry *et al.*, 2009). Conversely, JAM-A has been found to mediate the differentiation of CD34+ progenitor cells to endothelial progenitor cells and to facilitate CD34+ cell-induced re-endothelialization *in vitro* (Stellos *et al.*, 2010). This suggests that JAM-A is required for circulating CD34+ progenitor cells to recognise a site of injury, differentiate into endothelial cells and proliferate to repair the injured endothelium. In addition, JAM-A is reportedly upregulated during the differentiation of pancreatic AR42J cells (Yoshikumi *et al.*, 2008), while JAM-A mRNA and protein levels have been shown to be increased during differentiation of human monocytic cell THP-1 into mature dendritic cells (Ogasawara *et al.*, 2009). JAM-L is also induced during differentiation of myeloid leukaemia cells, with expression of JAM-L in myeloid leukaemia cells resulting in enhanced cell adhesion to endothelial cells (Moog-Lutz *et al.*, 2003). This upregulation of JAM-A during differentiation is reportedly followed by increased expression of the polarity proteins par3 and PKCλ (Yoshikumi *et al.*, 2008), which have been previously shown to affect cell polarity and migration. While these data suggest conflicting roles for JAMs in stem cell populations versus their role in differentiation, at this early stage the exact role(s) of JAMs in stem cell renewal or differentiation can only be speculated upon. Fundamentally, it is also unknown whether the expression of JAMs is actively required or

passively upregulated in stem cell populations. However, based on the increased expression of JAM-A in poorly-differentiated breast cancers (McSherry *et al*., 2009) and the emerging role of JAM-A in regulating proliferation and apoptosis (Azari *et al*., 2010; Nava *et al*., 2011; Naik *et al*., 2003; Murakami *et al*., 2011), it will be interesting to determine if JAM-A is upregulated on cancer stem cell populations and whether its expression promotes self-renewal.

3. Functional regulation of cells in the breast cancer microenvironment by JAMs

3.1 JAM proteins regulate endothelial angiogenesis

As already alluded to, JAM proteins are highly expressed on endothelial cells and have been crucially implicated in the control of barrier function and cell motility. In the context of cancer, however, endothelial cells assume a new importance via the development of neovascularisation sites to support growing tumours (Hanahan & Folkman, 1996). This section will review the evidence currently linking JAM proteins to angiogenesis as a contributory mechanism to cancer progression.

Angiogenesis in response to enhanced growth factor signalling is of particular relevance in tumour microenvironments. A body of work from Naik *et al* has convincingly shown an important role for JAM-A in angiogenesis induced by basic fibroblast growth factor (bFGF). Specifically, bFGF signalling facilitates the disassembly of an inhibitory complex between JAM-A and αvβ3 integrin, permitting JAM-A-dependent activation of MAP kinase which leads to endothelial tube formation, a surrogate for angiogenesis (Naik *et al*., 2003). JAM-A has also been shown to activate extracellular signal-related kinase (ERK) signalling in response to bFGF, facilitating endothelial migration (Naik *et al*., 2003) in a matrix-specific context (Naik & Naik, 2006). *In vivo*, JAM-A expression has been linked with the very early stages of murine embryonic vasculature development (Parris *et al*., 2005), and although deletion of JAM-A appears to be dispensable for vascular tree development, homozygous JAM-null mice were found to be incapable of supporting FGF-2-induced angiogenesis in isolated aortic ring assays (Cooke *et al*., 2006). In the context of tumour neovascularisation, others have reported reduced angiogenesis in a model of pancreatic carcinoma in JAM-A-null mice (Murakami *et al*., 2010).

Other JAM family members appear to contribute similarly to angiogenesis; with functional blockade of JAM-C being shown to decrease aortic ring angiogenesis and block angiogenesis in hypoxic vessels of the murine retina (Lamagna *et al*., 2005; Orlova *et al*., 2006). Furthermore, soluble JAM-C shed into the serum of patients with inflammatory conditions (presumably following cleavage by ADAM enzymes) was noted to induce endothelial tube formation in a Matrigel model (Rabquer *et al*., 2010). An interesting dichotomy, however, is that amplification of JAM-B in a trisomy-21 mouse model of Down's syndrome has been linked with reductions in VEGF-induced angiogenesis and thus anti-tumour effects in a lung carcinoma model in these mice (Reynolds *et al*., 2010).

Taken together, these studies illustrate that by influencing angiogenic functions in endothelial cells, JAMs may indirectly influence the ability of tumours to survive and progress. While there appears to be a consensus that JAMs –A and –C activate signalling cascades that promote angiogenesis, it is possible that clear roles for the other family members in the regulation of angiogenesis will also emerge in time. It is tempting to speculate that pharmacological antagonism of JAMs will show promise as an option for

blocking tumour progression, similar to the VEGF-A-neutralizing antibody bevacizumab (avastin) (Van Meter & Kim, 2010).

3.2 JAM proteins regulate trafficking of leukocytes

In addition to the potential regulatory roles of JAM proteins on the vascular endothelium, effects exerted on JAM-expressing leukocytes within the breast tumour microenvironment may also have relevance to cancer progression. For instance, JAMs are known to play important roles in the transendothelial migration of monocytes, which differentiate into macrophages once in the breast tissue. Accordingly, a function-blocking monoclonal antibody directed against JAM-A (BV11) has been described to inhibit spontaneous and chemokine-induced monocyte transmigration both *in vitro* and *in vivo* (Martin-Padura *et al.*, 1998). Furthermore, treatment of mice with a monoclonal antibody directed against JAM-C has been shown to reduce macrophage infiltration into a murine lung tumour model (Lamagna *et al.*, 2005), and to promote reverse transmigration of monocytes back into the bloodstream from inflamed tissue sites (Bradfield *et al.*, 2007). Given the existence of a breast tumour-promoting paracrine loop between epidermal growth factor secreted by macrophages and colony-stimulating factor-1 secreted by tumour cells (Goswami *et al.*, 2005), this implies that JAM-based regulation of monocyte transmigration could have a profound and self-amplifying influence on macrophage trafficking and tumour proliferation.

In the context of leukocytes other than monocytes/macrophages, many studies have implicated JAMs in the functional control of neutrophil transmigration across both epithelial (Zen *et al.*, 2004; Zen *et al.*, 2005) and endothelial (Sircar *et al.*, 2007; Woodfin *et al.*, 2007) barriers. As yet nothing is known about JAM-dependent events that might control neutrophil trafficking or activation within the breast tissue, despite the fact that neutrophils accumulate in highly aggressive inflammatory breast cancers. In other tissues, JAM-A has been shown to be required for efficient infiltration of neutrophils into the inflamed peritoneum or into the heart upon ischemia–reperfusion injury; as evidenced by increased adhesion and impaired transmigration in JAM-A-deficient mice (Corada *et al.*, 2005). Interestingly, in this model JAM-A expression on the neutrophil appears to be more important than that on the endothelium; since selective loss of endothelial JAM-A did not phenocopy the transmigration deficits (Corada *et al.*, 2005). In addition, soluble JAM-A shed from cultured endothelial cells has been shown to reduce *in vitro* transendothelial migration of neutrophils and to decrease neutrophil infiltration *in vivo* (Koenen *et al.*, 2009).

Recent evidence also proves that family members other than JAM-A can participate in leukocyte trafficking, with JAM-C over-expressing mice exhibiting an increased accumulation of leukocytes into inflammatory sites or during ischaemia/reperfusion injury, while JAM-C neutralization or loss reduces leukocyte recruitment in models of lung, kidney or muscular inflammation (Aurrand-Lions *et al.*, 2005; Scheiermann *et al.*, 2009). Finally leukocytic expression of JAM-L has been shown to promote attachment to endothelium (Luissint *et al.*, 2008), and functional inhibition of JAM-B is reported to decrease migration of peripheral blood lymphocytes across cultured human umbilical vein endothelial cells (HUVECs) (Johnson-Leger *et al.*, 2002).

Collectively these data highlight an important role for JAMs in the migration of immune cells across endothelia, a mechanism that could be hijacked by JAM-overexpressing cancer cells as they leave the breast and invade into blood vessels.

3.3 JAM proteins and the regulation of stromal cells

The final grouping of breast cancer microenvironmental cells which will be discussed are stromal cells, broadly including fibroblasts and myoepithelial cells. Although little is known about JAM-mediated control of breast stromal cells specifically, insights from other cellular systems may suggest that this multifunctional family of proteins could have a hand in influencing the mesenchymal element of tumourigenic processes.

JAM-C expression has been noted on the surface of primary fibroblasts derived from human lung, skin and cornea (Morris *et al*., 2006). The same authors observed JAM-A and JAM-C expression on the widely-studied NIH-3T3 fibroblast cell line. Interestingly, high JAM-C expression on synovial fibroblasts has been associated with the pathology of murine experimental arthritis, and JAM-C antagonism shown to have functional benefits in reducing the severity of inflammation (Palmer *et al*., 2007). An immunohistochemical study in human arthritis has also demonstrated JAM-C expression on the synovial fibroblasts of both osteoarthritis and rheumatoid arthritis patients, in conjunction with JAM-C-dependent adhesion of myeloid cells to these fibroblasts (Rabquer *et al*., 2008). Enhanced expression of JAM-A has also been described on the skin of patients with the inflammatory disorder systemic sclerosis, in comparison to that on normal dermal fibroblasts (Hou *et al*., 2009).

Aside from facilitating adhesion of leukocytic cells to stromal elements such as fibroblasts, another way in which JAM family members could influence the breast cancer microenvironment is by altering proliferation of fibroblasts or other accessory cells. JAM-A has been reported to be required for proliferation of vascular smooth muscle cells, since JAM-A gene silencing exerted anti-proliferative effects in this system (Azari *et al*., 2010). Whether this is through direct or indirect mechanisms remains uncertain, particularly in light of conflicting evidence in intestinal epithelial cells suggesting that JAM-A expression restricts proliferation by inhibiting Akt-dependent Wnt signalling (Nava *et al*., 2011). However functional inhibition of the extracellular domain of JAM-A has been shown to inhibit bFGF-induced endothelial cell proliferation, and overexpression of JAM-A was also found to increase endothelial cell proliferation (Naik *et al*., 2003). Accordingly, very recent evidence has suggested that JAM-A expression exerts a negative tone on apoptosis in the mammary epithelium (Murakami *et al*., 2011). It is likely that processes as crucial as proliferation are strictly regulated in a spatial manner, which could account for tissue-specific differences as observed from the little available evidence to date. Whether or not JAM family members may influence proliferation of breast stromal cells like fibroblasts and the myoepithelium remains to be investigated. However, it is tempting to speculate that the acquisition of a proliferative phenotype in tumours may be co-ordinately linked to the pro-migratory "mesenchymal" phenotypes observed in many aggressive, poorly-differentiated breast cancers, to which evidence has already linked members of the JAM family. Co-culture models which better recapitulate the complexity of the breast cancer microenvironment than mono-cultures (Holliday *et al*., 2009) may offer promise in dissecting the relative cellular contributions of JAMs to tumour progression at a reductionist level.

4. JAMs as novel potential drug targets in breast cancer

The pleiotrophic roles of JAM family members in regulating both the breast epithelium and cells of the microenvironment may suggest JAMs as novel therapeutic targets for the future management of breast cancer. Whether by aiming to block migratory behaviour, angiogenesis, proliferation or to promote polarisation and differentiation, selective

pharmacological targeting of JAM molecules could prove particularly useful in cancers that overexpress one or more JAMs. This naturally pre-supposes that JAMs are causally involved in the disease process rather than simply acting as passive biomarkers, a fact that remains to be solidified. However, irrespective of the last caveat, another facet worth exploring is the potential of targeting JAMs to promote drug delivery. Since tight junctions (TJs) as a whole are primary regulators of paracellular transport across epithelial cells (Gonzalez-Mariscal *et al.*, 2005), successful drug delivery may require modulation of TJ proteins to allow drug molecules to pass (Matsuhisa *et al.*, 2009). However disruption of TJ proteins for drug delivery purposes is a double-edged sword, given the risk of disrupting homeostatic mechanisms of polarity, differentiation and migration which are tightly regulated by TJs in normal tissues and whose dysregulation may themselves promote tumourigenesis.

As yet, there are no cancer therapies on the market which specifically target tight junctions. However several tight junction proteins have been described as receptors for specific molecules or organisms, and as such, these might provide valid and novel targets for drug delivery. A particular precedent exists with the claudin family of TJ proteins; Claudins-3 and -4 having been suggested as drug delivery targets since they act as the receptor for *Clostridium perfringens* enterotoxin (CPE). The ability of CPE to rapidly and specifically lyse cells expressing claudin-3 or -4 could potentially be exploited in the treatment of breast cancers over-expressing these proteins (Katahira *et al.*, 1997; Morin, 2005; Santin *et al.*, 2007; Santin *et al.*, 2007). Sub-lytic doses of CPE could alternatively be used to compromise TJs thus enhancing the influx of drug molecules across the epithelium. This could be of particular benefit in accessing hypoxic tumour cores, around which the tumour cells may be very tightly packed and thus relatively inaccessible to chemotherapeutic drugs. To date CPE administration has been shown to reduce growth of claudin-4 overexpressing pancreatic tumour cells (Michl *et al.*, 2001; Michl *et al.*, 2003), but their potential use in other cancer settings remains an open question.

How JAM molecules might be therapeutically targeted also remains an unanswered question, but one could predict value in using monoclonal antibodies or small molecule inhibitors to block the signalling functions which contribute to processes such as migration and angiogenesis. However, to date, the role of JAMs as chemotherapeutic targets (or even prognostic/predictive biomarkers) in the clinical setting of breast cancer has yet to be elucidated and validated. Following the lead of JAM-A as a potential biomarker and therapeutic target for breast cancer (McSherry *et al.*, 2009; Gotte *et al.*, 2010; McSherry *et al.*, 2011; Murakami *et al.*, 2011), we speculate that this will be a lucrative area of research in the future.

5. Conclusion

To conclude, breast cancer remains a leading cause of cancer worldwide (Jemal *et al.*, 2008), and the search for new targets of prognostic and therapeutic relevance will continue particularly in this era where semi-personalised medicine is becoming more of a likelihood than an aspiration.

This chapter has attempted to summarize the known roles of the JAM family in controlling cell adhesion, polarity and barrier function, and their emerging roles in controlling functional behaviours within cells of the breast tumour microenvironment which promote cancer progression. Finally, it introduced the topic of JAM as a potential drug target in breast cancer; whether to directly influence JAM-dependent oncogenic signalling or indeed to interfere with cell-cell adhesion for the purposes of enhancing drug delivery. Continued

expansion in our understanding of the cell and molecular biology of JAMs and their roles in tumour progression may open up new horizons supporting their evaluation as breast cancer biomarkers and drug targets of the future.

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Breast Cancer Metastasis: Advances Through the Use of In Vitro Co-Culture Model Systems

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1. Introduction

Worldwide, breast cancer is the most frequent cancer diagnosed in women and is the second-most leading cause of cancer related deaths in women (Jemal, Bray et al. 2011). Death from breast cancer is most often the result of the spread of the primary tumour to distant sites, where the cancer cells lodge and develop into metastases. Depending on the site of the metastasis, the patient may live for years with reduced quality of life and needing increased health care resources. There is clearly a need for a greater understanding of the molecular events involved in breast cancer metastasis in order to improve treatment options for breast cancer patients and develop therapies aimed at preventing breast cancer metastasis.

Here we will summarize what is known about the molecular basis of breast cancer metastasis and discuss the use of *in vivo* and primarily *in vitro* model systems to study it.

2. Current knowledge

2.1 Metastasis

As early as 1889, Stephen Paget observed that some cancers metastasized preferentially to specific organs, and developed his theory of "seed and soil"(Paget 1889). The essential tenet of this theory was that cancer cells (seeds) disseminate throughout the body from their point of origin but can only develop metastatic satellites in appropriate stromal environments (soils). The many advances in our understanding of the molecular and cellular bases of breast cancer metastasis has led to a somewhat more complex picture, and the processes involved are still not completely understood. Breast cancer can spread to any secondary site in the body but metastases appear preferentially in bone, lung and liver (Rabbani and Mazar 2007). Presumably these sites provide a microenvironment favourable for the growth and development of breast cancer cells (Nguyen, Bos et al. 2009).

There are two prevailing models of breast cancer metastasis; one suggesting a linear progression and the other a parallel progression. The linear progression model advances the idea that cells in the primary tumour accumulate progressive mutations in a stepwise manner in genes regulating some aspect of cell growth and division such as oncogenes and tumour suppressor genes. Some cells eventually become able to proliferate autonomously; they expand clonally and leave the primary site to travel through lymphatic or vascular systems to a distant organ where they develop into a secondary metastatic growth. This

model implies that cells at the primary site must undergo a number of rounds of division before they become autonomous and so development of metastasis is linked to primary tumour size with metastases more likely to develop from larger primary tumours. In support of this model it has long been known that there is a close association between tumour size and the possibility of development of metastasis, and tumour size is used as part of histological classification (1983; Rakha, Reis-Filho et al. 2010). The model also suggests that cells being shed by the primary tumour are fully metastatic and that cells that have metastasized to a secondary site should also be able to leave that site to set up at a tertiary site (Klein 1998; Klein 2009). Mutations in genes such as BRCA1, BRCA2, p53 and RB and amplification of the HER-2 receptor at the site of the primary tumour have been identified as being predictive of poorer outcome for breast cancer patients, consistent with this model (Slamon, Clark et al. 1987; Ross and Fletcher 1999; Bordeleau, Lipa et al. 2007; Bosco and Knudsen 2007; Kumar, Walia et al. 2007; Baker, Quinlan et al. 2010).

The parallel progression model suggests that tumour cells may disseminate from the site of the primary tumour very early in its development and may be subsequently genetically modified in the metastatic niche where they later settle (Klein 2009). This model predicts that disseminated tumour cells in the blood or lymph should be detectable very early in development of the primary tumour and that cells at the site of metastasis could be genetically divergent from those at the site of the primary tumour. In support of this model it has been shown in a HER-2 mouse model and in women with ductal carcinoma *in situ*, that disseminated tumour cells in bone and micro metastases could be detected from the time of earliest epithelial alterations at the site of the primary tumour. The numbers of disseminated tumour cells in this study were found to be the same for small and large tumours (Husemann, Geigl et al. 2008), suggesting that shedding of cells from the tumour mass was independent of primary tumour size. In a qualitative and quantitative study of 12,423 women with breast cancer, J. Engel *et. al.* (Engel, Eckel et al. 2003) determined that systemic disease was already present at the time of diagnosis in women who went on to develop metastases, again suggesting cells left the primary tumour early during its development.

The advent of single-cell genomics has allowed comparison of the characteristics of disseminated tumour cells in the blood and lymph and cells at the site of the primary tumour and these have been found to be genetically divergent in some cases (Klein, Seidl et al. 2002; Klein 2003; Fuhrmann, Schmidt-Kittler et al. 2008; Klein 2009; Klein and Stoecklein 2009), indicating that early clonal divergence and parallel progression may occur in some breast cancers. Disseminated tumour cells may also differ genetically from cells that eventually develop into a metastasis in the same patient (Stoecklein and Klein 2010). This could reflect the requirement for the disseminated tumour cells to undergo whatever genetic changes are necessary for them to adapt and be able to successfully grow in the new microenvironment. If that is the case it follows that the genetic aberrations found in the primary tumour may not reflect those seen in the metastasis and this has been found to occur (Tortola, Steinert et al. 2001; Albanese, Scibetta et al. 2004; Gow, Chang et al. 2009; Stoecklein and Klein 2010). In colorectal cancer, mutations in B-raf, K-ras and p53 seen in the primary tumour may be absent or altered in the metastasis. In some cases mutations in the metastasis may be absent in the primary tumour (Tortola, Steinert et al. 2001; Albanese, Scibetta et al. 2004; Stoecklein and Klein 2010). In a study of non-small-cell lung cancer where EGFR mutation status is used as a determinant for treatment with tyrosine kinase inhibitors, 27% of paired primary/metastasis samples (n=67 patients) were found to be

discordant with respect to EGFR mutation status (Gow, Chang et al. 2009). This is of concern in a time of more personalized treatment, where often it is the genetic signature of the primary tumour alone on which outcome predictions or treatment options are based.

2.2 Metastasis suppressor genes

Evidence suggests that less than 1% of breast cancer cells that enter the circulatory system are capable of generating metastatic foci (Fidler 1970; Fidler and Nicolson 1977). Often disseminated breast tumour cells that have settled in the microenvironment at the site of metastasis will lie dormant for years in patients with no evidence of disease before developing into a clinically significant metastatic focus, indicating they are capable of escaping early systemic therapies that target rapidly proliferating cells at the site of the primary tumour (Pantel, Schlimok et al. 1993; Klein, Seidl et al. 2002; Riethdorf, Wikman et al. 2008; Morgan, Lange et al. 2009). As they remain quiescent for some period of time this also suggests that they, or the cells in their microenvironment, or both undergo genetic changes which allow them to progress to a metastatic phenotype (Riethdorf, Wikman et al. 2008) (Riethdorf, Wikman et al. 2008; Klein 2009; Nguyen, Bos et al. 2009; Smith and Theodorescu 2009; Rose and Siegel 2010; Stoecklein and Klein 2010). A class of genes that has been implicated in the regulation of this process is metastasis suppressor genes (Smith and Theodorescu 2009). These are genes that inhibit metastasis but do not affect the ability of cells to produce a primary tumour, and they play key roles in invasion, dissemination, arrest, survival and colony formation. Their function must be lost or inhibited for a metastasis to develop and they represent fertile new ground for the development of anti-metastatic therapeutics.

A number of metastasis suppressor proteins have been reported to inhibit breast cancer metastasis. Reduced levels of nm23 family proteins in the primary tumour have been reported to correlate with more aggressive phenotype in breast cancer patients (Galani, Sgouros et al. 2002; Terasaki-Fukuzawa, Kijima et al. 2002; Steeg, Ouatas et al. 2003; Peihong and Perry 2007), although conflicting results have also been presented (Charpin, Garcia et al. 1998; Belev, Aleric et al. 2002; Sgouros, Galani et al. 2007). The results seen in mouse models are more straightforward, where breast cancer cells with low expression of nm23 are more metastatic than those with high levels (Leone, Flatow et al. 1993; Bhujwalla, Aboagye et al. 1999; Tseng, Vicent et al. 2001). *In vitro* models have revealed that nm23 acts by reducing breast cancer cell motility and invasiveness (MacDonald, Freije et al. 1996; Russell, Pedersen et al. 1998; Steeg, Ouatas et al. 2003; Horak, Lee et al. 2007).

For Breast Cancer Metastasis Suppressor-1 (BRMS1), the clinical data reporting it to be a metastasis suppressor protein in breast cancer tumour samples is also conflicting (Kelly, Buggy et al. 2005; Hicks, Yoder et al. 2006; Lombardi, Di Cristofano et al. 2007). Again, its role in mouse models is clearer, where higher expression in breast cancer xenografts clearly resulted in reduced metastasis (Hedley, Vaidya et al. 2008; Hurst, Xie et al. 2008; Phadke, Vaidya et al. 2008). The stage at which BRMS1 suppresses metastasis is less clear, as it appears to affect a number of steps in the process of metastasis (Stafford, Vaidya et al. 2008). At least two of its functions appear to be increasing anoikis of cells free in the vascular system and inhibition of colonization of disseminated cells (Phadke, Vaidya et al. 2008).

KAI1 (CD82, Tetraspannin), has also been clearly verified as a breast cancer metastasis suppressor in clinical samples, where decreased expression correlates with poor outcome (Yang, Welch et al. 1997; Christgen, Christgen et al. 2009; Malik, Sanders et al. 2009). Similar to BRMS1, KAI1 appears to act in multiple ways to inhibit metastasis and reduce breast

cancer cell adhesion, migration and invasion *in vitro* (Malik, Sanders et al. 2009) and metastasis in mouse models *in vivo* (Yang, Wei et al. 2001). Other metastasis suppressor genes implicated in inhibiting breast cancer metastasis include KISS1 (Harms, Welch et al. 2003), MTSS1 (Parr and Jiang 2009) and alpha2beta1 integrin (Ramirez, Zhang et al. 2011), although their roles, at least in breast cancer have been less well studied.

As can be seen, the determination of the role of metastasis suppressor genes in metastasis using clinical samples is often confusing. This seemingly conflicting data may be a result of the many different experimental approaches to examining clinical samples; whether the samples are frozen or paraffin embedded and formalin fixed, whether mRNA or protein levels are the final determinant of expression (and these do not always correlate well), the type of extraction procedures used, and the source of the antibodies and staining methods for immunohistochemistry. The other difficulty with clinical samples is that they are almost exclusively derived from the primary tumour site, as biopsies of metastases are rarely carried out. Metastasis suppressor genes by definition do not inhibit events at the site of the primary tumour but must be inhibited for metastasis to take place. This inhibition may allow invasion of the circulatory system from the site of the primary tumour, survival through the process of transportation to the site of metastasis and evasion of the immune system, arrest within the metastatic niche, extravasion from the circulatory system or growth in the new environment (Kaplan, Psaila et al. 2006; Rabbani and Mazar 2007). Inhibition of expression at any step following detachment from the primary tumour would not likely be detected in the primary tumour.

3. Model systems of metastasis

It is evident that disseminated tumour cells in the blood and lymph and cells at the site of metastasis may diverge in phenotype from cells at the site of the primary tumour and from each other. Metastasis suppressor genes represent some of the genes with altered expression and one possibility of targeting metastasis therapeutically is to induce their re-expression or reiterate their function at the site of metastasis (Steeg, Ouatas et al. 2003; Stafford, Vaidya et al. 2008; Smith and Theodorescu 2009). To effectively select therapeutic targets it will be important to better understand their functions in the microenvironment of the site of metastasis. It is also known that the stromal environment surrounding the tumour cell is an active collaborator in the development of the metastasis. It is imperative to examine interactions between tumour cells and the stromal cells in the metastatic niche to understand what changes the stromal cells induce in the tumour cells and what changes the tumour cells induce in the stromal cells. *In vivo* and *in vitro* model systems have long been used as pre-clinical models to study breast cancer metastasis and ways of treating or preventing it.

3.1 In vivo mouse models

The use of mouse models in studying human breast cancer metastasis has the very great advantage of being able to study the entire process of metastasis from development of the primary tumour to the final development of the metastasis. It is possible to label the tumour cells with a variety of probes including green fluorescent protein (GFP) or luciferase and there are many excellent imaging techniques such as magnetic resonance, computed tomography and ultrasound available for live animal imaging to follow the progress of the tumour cells in the mouse. Live imaging is an advantage as progress can be monitored in one mouse over a period of time rather than sacrificing a number of mice at different time

points. One of the major disadvantages in using mouse models to study human cancer metastasis is that mice are not human, and there is no guarantee that the metastasis will develop in them in a way that recapitulates what happens in a human body. Mice do develop breast cancer as a heterogeneous disease, similar to humans (Andrechek and Nevins 2010), but there are significant differences between mice and humans in the capacity of the primary cells for transformation, the size of tumours, expression of hormone receptors and preferential sites of homing for breast cancer. For researching metastasis of human cells, immunodeficient mouse strains need to be used, taking the model a further step away from what happens in a human host. In addition human breast cancer cell lines may not accurately reflect the biological characteristics of in-vivo breast cancer such as natural evolution and tumor diversity. Given that caveat, mouse models are very important for testing pre-clinical data before moving on to clinical trials or human tumour tissue samples.

There are many technical issues to take into account when considering the use of a mouse model to study breast cancer metastasis. Those are beyond the scope of this chapter but are very fully reviewed by Danny Welch (Welch 1997). Mouse models have been particularly useful in identifying molecules important for a number of steps in metastasis, such as epithelial to mesenchymal transition (EMT), invasion, extravasation and intravasation (Vernon, Bakewell et al. 2007). One approach to modeling metastasis is to use xenografts, where human tumour cells are injected subcutaneously or into the mammary fat pad of a mouse and allowed to develop a primary tumour that spontaneously metastasizes. A second approach is to inject tumor cells directly into the venous system, using tail vein injection or cardiac puncture. Tail vein injection results primarily in metastasis to the lung, but cardiac puncture results preferentially in bone metastasis. This approach obviates the need for development of the primary tumour but is not useful for studying some of the early steps of metastasis. The artificial injection of tumor cells directly into the venous system may produce pseudometastasis through a process of embolization rather than true physiological metastasis. A third approach is to utilize genetically engineered mice that have had a tumour suppressor gene deleted or an oncogene activated in an organ specific manner.

Xenograft models and venous injections most generally use breast cancer cell lines, many of which are maintained and sold by the American Type Culture Collection (ATCC). These cell lines have a variety of gene expression profiles that identify them as similar to luminal, basal A or basal B [subtypes initially defined in tumour samples in 2006 (Fridlyand, Snijders et al. 2006)] and they show a variety of receptor and p53 profiles (Neve, Chin et al. 2006). Gene expression in these cell lines can be modified by over expression or deletion and the effect of the altered gene on metastasis can be monitored following injection. Although the resulting tumours are considered to metastasize "spontaneously", the injected cell lines are an artificial starting material as they have been cultured for long periods of time *in vitro* and do not resemble a spontaneously arising tumour. One of the advantages of xenograft models is that the cells of the primary tumour must interact with the stromal cells surrounding the tumour and must also interact with the stromal cells at the site of the metastasis for a productive metastasis to develop. A great deal of information about the interactions of human tumour cells and stromal cells has been accumulated by injecting human tumour cells and human mesenchymal stem stromal cells together in xenograft models [reviewed in (El-Haibi and Karnoub 2010)]. By using the same cell lines and different routes of injection it is possible to determine whether a gene is necessary for early steps of metastasis or whether it is involved in later steps (Chabottaux, Ricaud et al. 2009).

Genetically engineered mice, whether transgenic or gene knockout animals, have an advantage over xenograft models in that they illustrate the metastasis of tumours that arise in the mouse mammary gland as a result of internal genetic changes and not exogenously injected cells. This is more representative of how tumours develop from the very beginning of the metastatic process. To limit expressed genes to the breast of the transgenic mouse a breast-specific promoter such as the Mouse Mammary Tumour Virus (MMTV) promoter or the Whey Acidic Protein promoter (WAP) is generally utilized (Kim and Baek 2010). As the tumour arises from mouse cells, immunocompetent mice can be used and the effects of an intact immune system on the process of metastasis can be determined. There are many strains of mice available with well defined genetic backgrounds, enabling researchers to study the effects of differing genetic backgrounds on the development of metastasis arising from gain or loss of the gene of interest (Husemann and Klein 2009). Mice carrying different transgenes or knockouts can be crossed with each other to determine if there is an additive or synergistic effect of the different genes on development of metastasis (Vernon, Bakewell et al. 2007). Gene expression or deletion can be temporally regulated, using an MMTV promoter either alone or directing expression of the Cre/loxP system for somatic deletion. The MMTV promoter becomes active only after puberty, preventing the oncogene or tumour suppressor gene of interest from causing embryonic lethality. One of the drawbacks to using a genetically engineered mouse model is in testing therapeutic compounds. These mice develop subtypes of breast cancer similar to, but not identical with the subtypes seen in human breast tumours. Also, most tumours arising in genetically engineered mice lack expression of the estrogen receptor and thus fail to recapitulate human tumours that are estrogen receptor positive. Cytogenetic and genetic backgrounds are different between mice and humans as well and this could lead to misinterpretation of the usefulness and safety of a therapeutic compound (Kim and Baek 2010).

3.2 In vitro co-culture systems

3.2.1 Three-dimensional co-culture

Normal breast epithelial cells grown in three dimensional cultures will spontaneously aggregate to form hollow, cyst-like acini. The cells develop apicobasal polarization and are tightly regulated with respect to growth and proliferation thus reiterating several important features of glandular epithelium *in vivo*. For this reason these models represent a physiologically relevant system that is a reasonable alternative to expensive *in vivo* experimental systems. Some breast cancer cell lines such as MDA-MB-435 also form acinar structures in three dimensions (Glinsky, Huflejt et al. 2000) but others (DU4475) form clusters and cords (Langlois, Holder et al. 1979).

For cells to grow as aggregates in three dimensions they need to be in an environment where the adhesive forces between the cells are greater than their affinity for the substrate they are plated on. Some of the commonly used techniques include embedding the cells completely in a reconstituted basement membrane substrate such as Matrigel or collagen I, or growing them on a thin layer of solidified reconstituted basement membrane in a dilute solution of basement membrane in medium (liquid overlay) (Hebner, Weaver et al. 2008). Three dimensional aggregates can also be obtained using spinner culture flasks, where they are maintained in suspension by constant rotation. Some of the recently developed methods include growing the cells on pre-fabricated scaffolds of extracellular matrix that recreate the natural structure of a living tissue, and a NASA developed Rotary Cell Culture System

where the cells are grown in simulated microgravity in liquid medium (Kim, Stein et al. 2004).

Monotypic three-dimensional cell cultures have been the primary model used in the study of human breast cancer. These studies have revealed a great deal about the functions of oncogenes, tumour suppressor genes, reversion of tumour phenotypes, how cells escape from proliferative arrest, invasive and migratory behaviour and epithelial to mesenchymal transition [reviewed in (Weaver, Fischer et al. 1996; Debnath and Brugge 2005)]. Fewer researchers have used heterotypic co-culture models in three dimensions. Some of the approaches are summarized here.

Some studies have concentrated on the relationship between breast tumour cells and stromal fibroblasts as it has long been known that alterations in the stroma can alter tumour cell behaviour and disease progression. A research group at the Lawrence Berkeley Laboratory in Berkeley, California used three dimensional co-cultures to determine the origin of myofibroblasts in breast cancer. These are interstitial cells frequently found in the stroma of breast neoplasias that were, at that time, of uncertain origin. They isolated fibroblasts, vascular smooth muscle cells and pericytes from normal stroma and grew them in collagen gels with MCF-7 or HMT-3909 S13 breast cancer cell lines in co-culture for fourteen days. They found that it was primarily the fibroblasts that were converted to myofibroblasts and that only five percent of the fibroblasts closest to the spherical colonies made by the tumour cells were converted, suggesting a concentration gradient of factors released by the tumour cells was responsible for the conversion (Ronnov-Jessen, Petersen et al. 1995). A second group in Regensburg, Germany grew tumour cell lines and normal, breast tumour derived or skin fibroblasts as separate spherical colonies in three dimensional liquid overlay co-cultures. Interestingly, only two of the breast cancer cell lines tested, MCF-7 and SK-BR-3 cells, could infiltrate either the breast or the skin fibroblast spheroids under these experimental conditions. MCF-7 cells are normally considered to have low metastatic potential and only occasionally invaded the fibroblast spheroids whereas SK-BR-3 cells are highly metastatic and extensively infiltrated the fibroblast spheroids. Induction of the myofibroblastic phenotype by the tumour cells was only induced in the normal or tumour-derived fibroblasts, and not the skin fibroblasts (Kunz-Schughart, Heyder et al. 2001).

Another research group at Universitat Halle in Halle, Germany investigated the properties of mesenchymal stem cells in three dimensional co-cultures with MCF-7 or MDA-MB-231 breast cancer cell lines. In their experiments they found that within two hours of plating mesenchymal stem cells with MCF-7 spheroids or MDA-MB-231 aggregates the mesenchymal stem cells could invade the cancer cell masses. Using a Transwell assay, with breast cancer cell lines grown in the bottom well, they were able to show that the breast cancer lines attracted the mesenchymal stem cells indicating they were secreting a chemoattractant (Dittmer, Hohlfeld et al. 2009).

Researchers at The Pennsylvania State University have developed a specialized bioreactor for long term (up to ten months) co-culture of MDA-MB-231 breast tumour cells with murine osteoblasts. They have determined that the osteoblast cultures develop over time in the same way as in natural bone including development of ossification and phenotypic transformation into osteocytes. They differentially labelled the bone cells and the breast cells with green fluorescent protein and Alexa Fluor 568 respectively and were able to follow the real-time cancer cell invasion and colonization of the osteoblast tissue. They observed that important pathologic events such as cancer cells infiltrating the bone cells in single file and microtumour formation that are seen clinically were reproduced their *in vitro* system. They

also observed that breast cancer cell colonization of the bone cells depended strongly on the maturity of the osteoblastic culture (Dhurjati, Krishnan et al. 2008; Mastro and Vogler 2009; Krishnan, Shuman et al. 2010).

A novel approach to three dimensional co-culturing of cells was developed by researchers at the University of Wisconsin-Madison in Madison, Wisconsin. They used a ninety-six arrayed single channel microchannel plate for co-culturing cells in 2ul collagen matrices and compared their results to conventional co-culturing of cells in collagen in six-well tissue culture plates. T47D breast cancer cells were co-cultured with human mammary fibroblasts and growth properties and inhibition of growth by small molecule inhibitors were compared between the two systems and found to be the same. The microchannel model has a number of advantages over conventional three dimensional co-culture systems in that it requires fewer resources, uses fewer cells, creating the possibility of using patient samples, and it is amenable for using high throughput screening of potential therapeutics (Bauer, Su et al. 2010). It will be interesting to follow future developments in the use of three dimensional heterotypic co-cultures in breast cancer research as this model system appears to have great potential.

3.2.2 Two-dimensional co-culture

By far the most commonly used *in vitro* co-culture model in the study of breast cancer metastasis is two-dimensional. Cells of various origins are cultured directly with breast cancer cells or in separate layers, as in Transwell plates. The measured outcomes in two dimensional co-cultures relate to breast cell growth, proliferation, adhesion, colony formation, migration and invasion. Signalling between cell types can be modified using gene overexpression or knock-down assays, or by adding inhibitory or stimulatory antibodies or other soluble compounds or drugs to the assay system. Some of the many and varied approaches are outlined below.

Researchers in Munster and Witten, Germany were interested in the role played by the HER-2 receptor in extravasation from the primary tumour through the venular wall. They modeled the venular wall using human umbilical vein endothelial cells grown on porous membranes coated with basement membrane extracellular matrix. They co-cultured these calls with breast cancer cell lines and with disaggregated tumour cells from twenty-three patients. They found that cell lines or patient samples with higher levels of HER-2 expression were significantly more invasive than cells with lower HER-2 expression. Interestingly, they also noted that there were subpopulations within individual breast cancers that had high HER-2 expression, and presumably high metastatic potential (Roetger, Merschjann et al. 1998).

A study was carried out in Milan, Italy, to investigate the interactions between hormone-dependent MCF-7 and ZR75.1 cells and hormone-independent MDA-MB-231 or BT20 breast cancer cells. Using a modified Transwell plate and measuring cell growth in the bottom well under serum-free conditions, they determined that the hormone-independent cell lines were capable of inducing cell growth in the hormone-dependent cells, in the absence of estrogen. Growth of the hormone-dependent cell lines could be further stimulated by the addition of transforming growth factor alpha to the medium. Their results confirmed the importance of paracrine interactions between cells in heterogeneous tumours and suggested an important role for transforming growth factor alpha in these interactions (Cappelletti, Ruedl et al. 1993).

Two dimensional co-culture systems are amenable to the use of primary tumour cells. A research group in Manchester, UK used primary epithelial cells from tumorous, benign or normal breast tissue in co-culture with human bone marrow or mammary fibroblasts from normal or malignant breast tissue. They found that breast epithelial cells from tumour tissue adhered preferentially to bone stroma over breast fibroblasts. The epithelial cells from normal or benign breast showed no preference for any of the stromal substrates. Interestingly, although breast tumour epithelial cells adhered preferentially to bone cells, this stromal environment did not provide a preferential growth platform (Brooks, Bundred et al. 1997). A similar study was carried out in Marseilles, France to determine the effect of stromal and epithelial cells from normal and tumorous breast tissue on growth of breast cancer cell lines. Fibroblasts from normal breast tissue but not conditioned medium from normal breast tissue were able to inhibit the growth of MCF-7 cells suggesting complex paracrine interactions between the two cell types. Normal fibroblasts did not inhibit the growth of immortalized S2T2 cells. Normal breast epithelial cells or the conditioned medium from them could inhibit a number of breast cancer cell lines suggesting that both fibroblasts and epithelial cells could have growth regulatory roles in the breast.

Many researchers co-culture breast cancer cell lines with bone-derived mesenchymal stem cells (MSCs) as these have been shown to have a profound effect on breast cancer metastasis. These cells were first observed by Friedenstein in 1976 (Friedenstein, Gorskaja et al. 1976) and have come to be defined as non-hematopoietic cells derived from bone stroma that are spindle-shaped and can be separated from other bone stromal cells by their tendency to adhere to plastic tissue culture plates. They have the stem cell characteristics of being able to differentiate into multiple cell lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts and they express a consistent set of marker proteins on their surface (Brooks, Bundred et al. 1997; Pittenger, Mackay et al. 1999). Mesenchymal stem cells have been used in a number of laboratories in co-culture experiments with breast cancer cell lines or primary tumour cells and have been found to influence breast cancer cell adhesion, morphology, gene expression, proliferative capacity and growth characteristics (Brooks, Bundred et al. 1997; Hombauer and Minguell 2000; Fierro, Sierralta et al. 2004; Oh, Moharita et al. 2004). They have been shown *in vivo* to be able to migrate to sites of tissue damage and to primary tumour sites, and to modify the ability of breast cancer tumours to metastasize to other organs, making them potentially interesting vehicles for cell-based anti-tumour agents (Ferrari, Cusella-De Angelis et al. 1998; Hall, Dembinski et al. 2007; Rhodes and Burow 2010). They have also been shown in one research study to stimulate epithelial to mesenchymal transition in breast cells which may make them less suitable for use in drug delivery (Martin, Dwyer et al. 2010).

We use MSCs in a two dimensional co-culture model designed to determine factors that affect breast cancer cell behaviour in a microenvironment resembling breast cancer metastasis to bone; one of the most common sites of breast cancer metastasis. Our source of bone cells is from reamings from hip and knee replacement surgeries that are carried out on a regular basis in local hospitals rather than the more commonly used bone marrow aspirates that are more difficult to obtain. We wanted to determine that bone marrow cells that we derived from bone reamings resembled bone cells that were normally biologically involved in breast cancer metastases to bone. Breast cancer bone metastases are frequently characterized by the presence of a desmoplastic response, where normal haematopoietic tissue is replaced by activated fibroblastic cells. Adherent fibroblastic cells were isolated from both hip and knee bone reaming samples with a

successful recovery rate of approximately 62% (8/13 patients). Microscopically, recovered cells that grew as a monolayer were observed to be morphologically heterogeneous, spindle shaped and fibroblast-like in appearance (Figure 1A) similar in appearance to mesenchymal stem cells previously reported in the literature (Wagner and Ho 2007; Wagner, Roderburg et al. 2007).

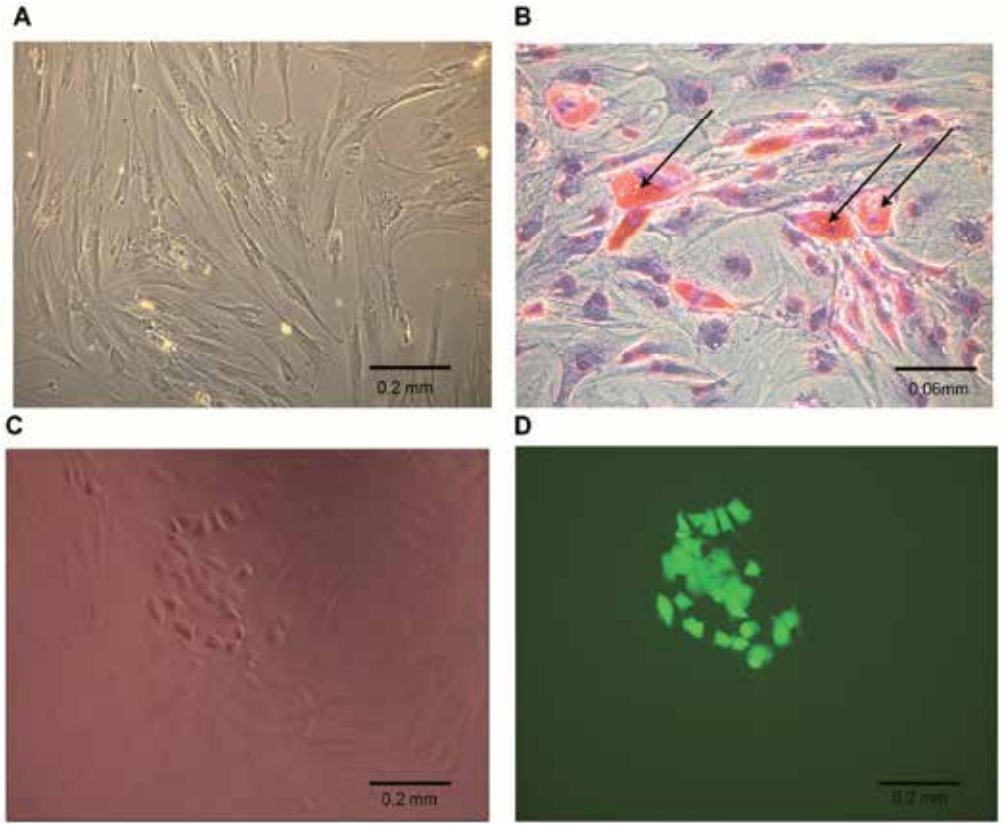

Fig. 1. Characterization of mesenchymal stem cells (MSCs) and breast cancer cell colonies plated on MSCs. MSCs growing as a monolayer are morphologically heterogeneous, spindle-shaped and fibroblast-like (A). MSCs can be induced to differentiate into adipocytes (B). Arrows indicate accumulations of lipid-rich vacuoles. Colonies of breast cancer cells growing on a lawn of MSCs can be visualized by light microscopy by their different refractive index (C) or, if they are stably transfected with GFP can be visualized using fluorescent microscopy (D).

To determine the multipotent potential of the bone cells adipogenic differentiation was induced in the isolated bone marrow derived cell cultures by treatment with Adipogenic Differentiation Medium (Fisher Scientific, SH3088602) according to the manufacturer's instructions. Induction was apparent by the accumulation of lipid-rich vacuoles within cells (Figure 1B). The content of the observed vacuoles was stained with Oil Red O dye and was localized to inside the cells where cell nucleus and membrane were counterstained with haematoxylin. This is consistent with our bone cell cultures having some of the multipotent characteristics of mesenchymal stem cells and we will refer to them as MSCs.

One of the usual characteristics to measure when breast cancer cells are grown on any stroma includes the ability of the breast cancer cells to form colonies on that stroma cell type. This can be determined using limiting dilution analysis. We used statistical analysis for limiting dilution assays adapted from the method described by Lefkovits and Waldmann (Lefkovits and Waldmann 1999). Limiting dilution analysis software developed by P. Rovenksy, J. Rubes, and T. Beran and included with the Lefkovits and Waldmann textbook was used for chi square and frequency calculations.

A modified limiting dilution analysis (LDA) method was used to evaluate the frequency of a given event in a population. We evaluated proliferation/survival of individual cancer cells, where binomial colony formation events were defined as 1) a *positive event* being the presence of colony ≥ 8 cells in size after the indicated time (days) period, and a 2) a *negative event* being the absence of any colonies or a colony <8 cells after the indicated time (days) period.

For co-cultures, 1000 cells/well of substratum cells were seeded in 96-well plates and allowed to attach and grow over 2 day period. After 2 days, the wells were washed twice using PBS and various dilutions of the breast cancer cell lines (1, 2, 4, 5, 7, 10 cells/well) were added in 100uL volume of serum-free Opti-MemI media per well (Gibco cat. #31985). The plates were incubated for an indicated time period at 37°C and 5% CO_2. Each well was analysed for the presence of colonies using an inverted microscope (100X magnification). Breast cancer cells were identified by morphology and a different refractive index when compared to the large flattened MSCs. An example is presented in Figure 1C and D.

Each well was scored as positive or negative based on the above established criteria. The data was tabulated and frequencies were determined using a Poisson distribution:

$$F_r = \frac{\mu^r}{r!} e^{-\mu}$$

Frequencies were calculated using the aforementioned LDA software package with a linear regression through the origin. Graphical representations of the distributions were also plotted on the μ vs. -lnF_0 graphs. The accuracy of the fitted line was evaluated using a chi-squared test for goodness of fit

$$(X^2 = \frac{df \times V}{\sigma^2})$$

based on a 95% confidence interval of accepting the null hypothesis that the best line of fit accurately represents the observed data. The null hypothesis was accepted (line of best fit accurately represents the data that follows single-hit kinetics) when the p-value was greater than 0.05.

Inter-trial frequencies were compared based on the overlap of the 95% confidence limits of the slopes based upon evaluation of the reliability of the regression line estimates. Confidence limits of the slope (*a*) were calculated using the following equations,

$$a_{upper} = a + t_{\frac{\alpha}{2},n-1} \sqrt{\frac{1}{(n-1)(\frac{\sum y_i^2}{\sum x_i^2} - a^2)}} \ ,$$

$$a_{lower} = a - t_{\frac{\alpha}{2},n-1}\sqrt{\frac{1}{(n-1)(\frac{\sum y_i^2}{\sum x_i^2} - a^2)}}$$

so that new slopes (a_{lower}, a_{upper}) define the boundaries of the fan. The values for the area α or α/2 were obtained from the Student's *t*-test table. The overall frequency for each cell line was calculated using pooled data from experiments using the above described analysis.
Using limiting dilution analysis we determined, for example, that one MCF-7 cell in every fourteen could develop a productive colony on MSCs but only one MCF-7 cell in every thirty could develop a colony on HS68 fibroblasts, indicating that bone stroma was the preferential stroma to colonize (Figure 2).
Another characteristic very often measured in two dimensional co-cultures involves the ability of one cell type or conditioned medium from a cell type to influence the migratory capacities of another cell type. This is sometimes done with a wound healing assay where a confluent culture of one type of cells is disrupted by scratching cells off the tissue culture plate surface in a straight line and then measuring how long it takes the "wound" to fill in with new cells under conditions of differing types of conditioned media.

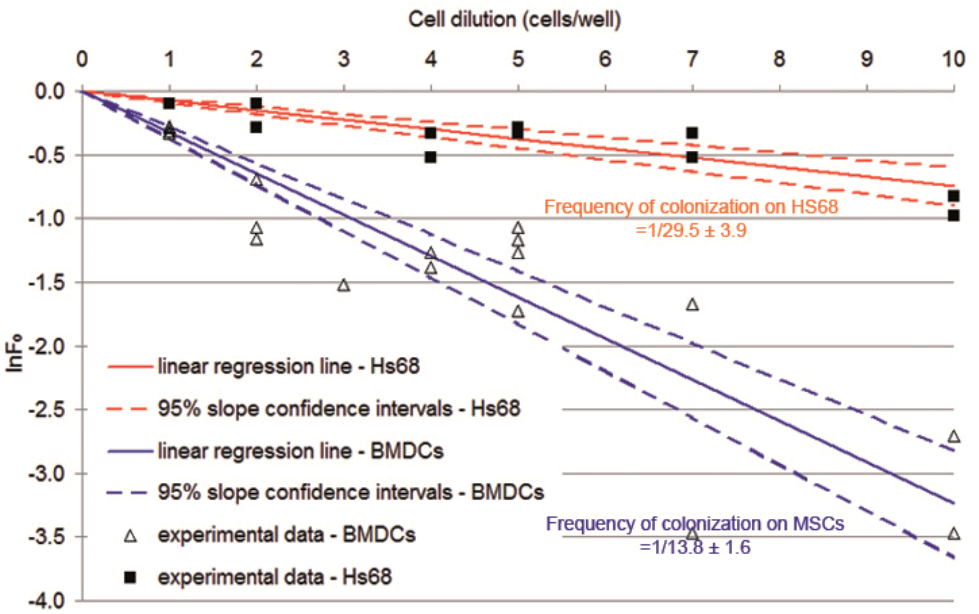

Fig. 2. Limiting Dilution Analysis (LDA) of MCF-7 Breast cancer cells grown on MSCs (blue) or HS68 fibroblast cells (red) indicates that the breast cancer cells can colonize wells having MSCs as a substrate at a significantly higher frequency than they can colonize wells in which HS68 cells have been plated as a substrate.

An alternative and more quantitative way to measure cell migration is in a Transwell or Boyden Chamber assay. Here, cells of interest are placed in the lower well of a Transwell plate and allowed to grow for some time to provide conditioned medium (Figure 3A). Cells to be tested for migratory capacity are placed on a porous membrane in the upper chamber

and are allowed to migrate through the membrane for a given period of time. An example of MCF-7 breast cancer cells migrating through pores in response to MSCs or HS68 fibroblasts is given in Figure 3, where it can be seen that the breast cancer cells migrate preferentially in response to bone stromal cells.

A variation on the Transwell migration assay is an invasion assay where the cells must invade through a Matrigel layer before migrating through the pores. Usually an invasion assay and a migration assay are carried out at the same time under the same conditions and invasion is measured as a percentage of number of cells invading/migrating.

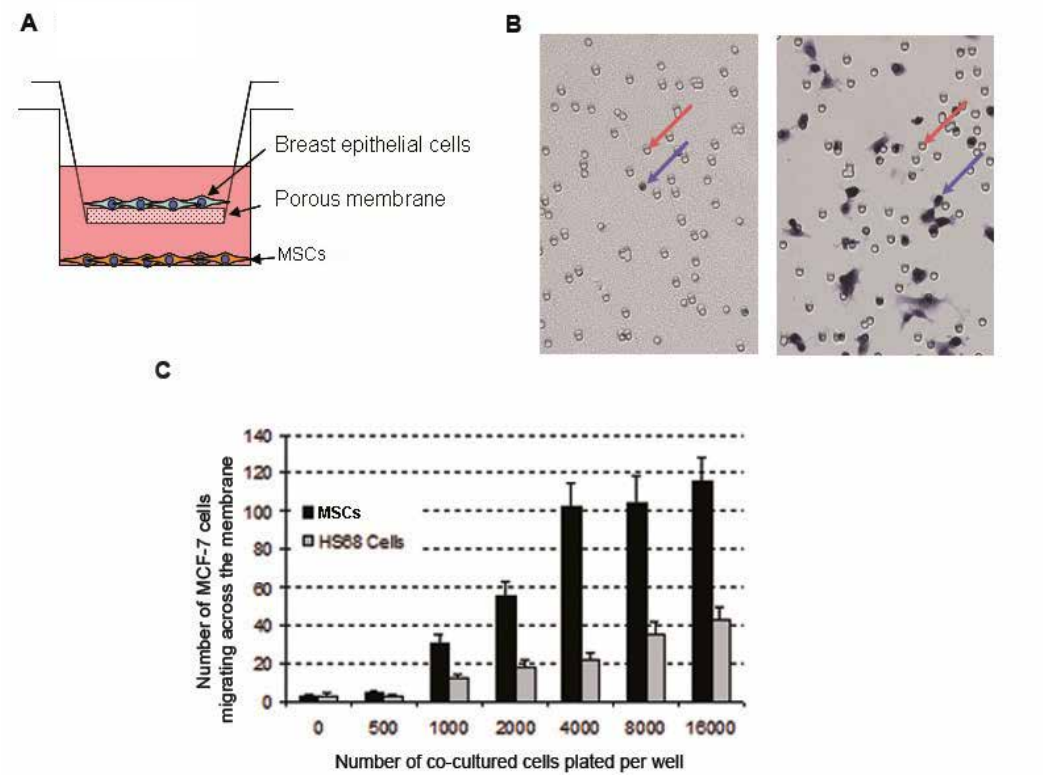

Fig. 3. Use of a Transwell Assay to determine the migration of MCF-7 breast cancer cells in response to MSCs or HS68 cells. A schematic diagram to illustrate the assay design (A). The Transwell membrane when stained and photographed from an inferior aspect has 8um pores (red arrows) and cells that have migrated through the pores can be visualized and counted (blue arrows). More cells migrate through the Transwell membrane in response to MSCs than in response to HS68 cells (C). Error bars indicate standard error of the mean.

One of the advantages of using a two dimensional co-culture system using Transwell plates is the ability to separate the cells after exposure to each other for analysis by western blot, PCR analysis or microarray analysis of differentially expressed genes. Another advantage is the ability to separate cells to determine which cell type is expressing a factor that regulates invasion or migration. For example, the bone remodeling protein Osteopontin is produced by bone cells and breast cancer cells. There are a number of reports in the literature suggesting Osteopontin produced by breast cancer cells regulates their migratory properties

and contributes to the aggressiveness of the disease (Sharp, Sung et al. 1999; Chakraborty, Jain et al. 2008; Hedley, Welch et al. 2008; Patani, Jouhra et al. 2008; Ribeiro-Silva and Oliveira da Costa 2008). In our co-culture model we found, at least in the breast/bone microenvironment, that it was Osteopontin produced by the bone cells, not the breast cells that increased breast cancer cell migration (Koro, Parkin et al. 2010).

4. Future directions

It is becoming evident that gene expression at the site of breast cancer metastasis may not be the same as at the site of the primary tumour and we need better ways to treat metastases. It will likely be important to biopsy more metastatic tissue to provide the type of designer therapeutics aimed at pathways known to be targetable at the site of the metastasis as we currently do with the primary tumour. Currently, biopsies of metastases are rare. As stroma is known to be an active contributor to the metastasis we also need to develop therapeutic approaches aimed at targeting the stroma. The recent development of new technologies for capture and analysis of circulating tumour cells may help to improve our understanding.

5. Conclusions

Breast cancer is a complicated disease and progression to metastasis may occur by clonal expansion or parallel progression. Changes in gene expression may occur between the primary tumour and the site of metastasis and development of therapeutics aimed at either the breast or stromal cells at the site of the metastasis will likely be needed to develop better therapeutics against breast cancer metastasis. Some of these new therapeutics may be aimed at reconstituting the expression of breast cancer metastasis inhibitor genes and much research is being done in this field. *In vivo* and *in vitro* model systems have contributed in many ways to our understanding of breast cancer metastasis and will surely continue to do so.

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Breast Cancer Metastases to Bone: Role of the Microenvironment

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1. Introduction

Bone is the preferred site for breast cancer metastasis, which leads to altered mineral metabolism, disruption of bone architecture, and considerable pain burden. Prior to homing to the bone, the primary breast tumour releases soluble factors that lead to the creation of a pre-metastatic niche in the bone, which then serves to attract and maintain invading breast cancer cells. Breast cancer cells actively influence resident bone cells, altering both the action of and cross-talk between bone forming osteoblasts and bone-destroying osteoclasts. Breast cancer cells inhibit osteoblast differentiation and prevent them from creating and mineralizing new bone. Immature osteoblasts act as part of a hematopoietic stem cell niche and provide an attachment site for breast cancer cells. Breast cancer cells also produce factors, such as parathyroid hormone-related protein (PTHrP), which induce osteoblasts to stimulate the production of the pro-resorptive cytokine RANKL and to inhibit the production of RANKL inhibitor, OPG. RANKL, together with other osteoclastogenic factors released from breast cancer cells, promotes the fusion and differentiation of osteoclasts, resulting in bone destruction. As a result of bone resorption, growth factors stored in the bone matrix, such as TGFβ, are released and can further stimulate the proliferation and survival of tumour cells. Thus, the complex interactions between breast cancer cells and the bone microenvironment underlie the homing of the breast cancer to bone and the subsequent progression of osteolytic lesions. Current therapeutics against bone metastases aim to prevent osteoclastic bone resorption by blocking osteoclast differentiation or stimulating their apoptosis. The osteoblast provides a valuable potential target, as a source of osteoclastic differentiation factors, and a platform for cancer cell attachment. Recent results from basic and clinical research provide new targets to prevent the interactions between breast cancer cells and the bone microenvironment at different stages of the metastatic cascade.

2. Chapter outline

- Physiological regulation of breast and bone
  - Breast Growth and Development
    - Interactions of normal breast tissue with bone
    - Breast carcinoma
  - Bone Microenvironment

    - Bone structure and composition
    - Bone functions
    - Osteoclasts, osteoblasts and osteocytes: origins, differentiation, function, physiology, pathology
    - Bone marrow and hematopoietic stem cell niche
    - Bone cell communications during normal bone remodelling
- Homing of breast cancer cells to bone
  - Creation of pre-metastatic niche
  - Migration of breast cancer cells to bone
  - Attachment proteins between breast cancer cells and the bone
  - Osteomimicry
- Establishing of a metastatic tumor in the bone microenvironment
  - Interactions with osteoblasts
    - Inhibition of osteoblasts by breast cancer cells
    - Contribution of osteoblasts to the creation of an osteolytic environment
    - Role of osteoblasts in supporting breast cancer cells
  - Interactions with osteoclasts
    - Stimulation of osteoclasts by breast cancer cells
    - Role of osteoclasts in supporting breast cancer cells
- Therapeutic targets in the bone microenvironment
- Conclusions
- References

3. Physiological regulation of breast and bone

3.1 Breast growth and development

Interactions of normal breast tissue with bone

The interactions of normal breast tissue with bone arise during childbearing and breastfeeding. A normal human fetus needs approximately 30 g of calcium to mineralize its skeleton during gestation (1), that leads to significant changes in calcium homeostasis during pregnancy, including adjustments in levels of parathyroid hormone (PTH), calcitonin and 1,25 dihydroxy-vitamin D [1,25[OH]D] (2). These hormones exhibit their effects through three main target tissues – the intestines, kidneys and bone (3). Parathyroid hormone related peptide (PTHrP) is a hormone closely related to PTH, but which is produced by local tissues, such as breast, and is important for its differentiation (4). In addition to its role in local tissue development, PTHrP can substitute for PTH in the tissues expressing their common receptor, and thus participate in calcium homeostasis by elevating 1,25(OH)D and suppressing PTH, regulating placental calcium transport, and affecting bone resorption in the maternal skeleton (3). The regulation of calcium homeostasis by the lactating mammary gland may be of critical importance, since nursing humans secrete 300-400 mg of calcium into milk each day (5). The hormonal balance changes again during lactation, with still-reduced PTH levels, but normalized calcitonin and 1,25(OH)D, and increased PTHrP (2). During this time, increased prolactin concentrations allow for the release of breast milk, and also act to enhance bone turnover (6,7). Suckling stimulates prolactin secretion and inhibits GnRH production, both of which reduce estradiol levels, leading to bone resorption (8). Bone resorption has been shown to increase during lactation, and bone formation to decrease, resulting in a loss of 5-10% of trabecular mineral

content per month (9). Lactation-induced fragility fractures have been reported as a result, but are not common (10). Of interest, other important molecular mediators for the developing of lactating mammary gland are receptor activator of nuclear factor κB (RANK) and its ligand RANKL, which are better known for their key role in regulating the formation of osteoclasts. Expression of RANKL in the mammary epithelium is induced by hormones increased during pregnancy, such as prolactin, progesterone, and PTHrP, and mice lacking RANKL or RANK cannot form lobuloalveolar mammary gland structures, resulting in complete inability to develop a lactating mammary gland (11). Thus, normal breast tissue can interact with bone through a system of hormonal regulators that are important during lactation, and it expresses molecular machinery that employs the same mediators to perform locally distinct functions (Figure 1).

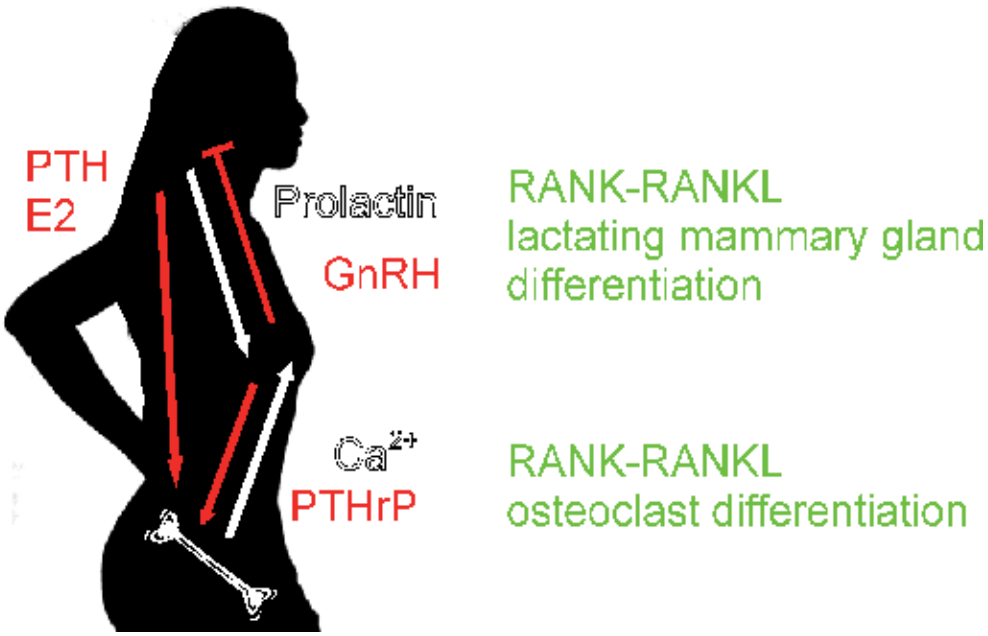

Fig. 1. Physiological interactions between the functions of breast and bone. Lactation involves secretion of large amounts of calcium. Bone is a key participant in calcium homeostasis. PTH is reduced during lactation while PTHrP production by the breast tissue is increased. Suckling stimulates prolactin secretion and inhibits GnRH production, both of which reduce estradiol levels, leading to bone resorption. Prolactin and PTHrP induce breast expression of RANKL, necessary for normal lactating mammary gland function. In the bone tissue, osteoblast-produced RANKL is key regulator of osteoclastogenesis.

Breast carcinoma

Breast carcinomas may arise from the inner lining of the milk ducts or from the lobules, known, respectively, as ductal carcinomas or lobular carcinomas (12). Once a tumour exceeds 1-2 mm in diameter, it requires extensive vascularization in order to survive (13), but the speed of cancer growth often exceeds its capability to form normal vascular organization. Poor angiogenesis results in an under-vascularized microenvironment, which leads to hypoxia, acidic pH and nutrient depletion in the tumour (14). Some cancer cells may

develop the ability to detach from the primary tumour and invade other areas to form secondary tumours, in a process called metastasis. Breast cancer cells favour regional lymph nodes as well as the liver, lungs, brain and bone as sites of metastasis (15). The metastatic process occurs in a complex series of interrelated steps. An epithelial-to-mesenchymal-transition (EMT) may occur whereby epithelial breast cancer cells take on a mesenchymal phenotype of reduced attachment to neighbouring cells and increased migratory capabilities (16). This may assist in their intravasation process, where the cell breaks through the epithelium into a blood vessel (17). From here, the cell migrates to a distant site, which is driven by chemotaxis and the communication between the cancer cell and a secondary site where it aims to establish (18-20). Instead of combating cancer cells, tumor-associated macrophages and T-cells may assist in the survival and dissemination of cancer cells by mitigating the immune response and promoting cancer progression (21,22). When the cell has reached its destination, it will then undergo extravasation to exit the blood vessel and establish in a new tissue (23). Bone is a preferred site for breast cancer metastases, therefore specific interactions are likely to establish between breast cancer cells and bone cells.

3.2 Bone microenvironment

Bone is a dynamic tissue that provides support and protection for organs and maintains body mineral homeostasis. All 213 bones are constantly remodelled by the coordinated action of specialized bone cells—osteoclasts that destroy bone and osteoblasts that build bone. Bone remodelling contributes to the many functions that bones provide and occurs at different rates in different areas. Higher rates of bone turnover are observed in trabecular bone compared to cortical bone (24), and at bone sites adjacent to actively hematopoietic bone marrow in the axial skeleton, where bone metastases also commonly occur (25). High bone turnover has been found to correlate to poor prognosis in patients with bone metastases (26), and prostate cancer cells have been shown to preferentially metastasize to sites of active bone turnover (27), making bone homeostasis an essential part of understanding cancer progression.

Structure

The adult skeleton is composed of 80% solid and dense cortical bone, surrounding the remaining 20% trabecular bone, a network of plates and rods through the bone marrow (28). Bone is composed of an organic phase of extracellular matrix containing collagen-1 triple-helical chains and non-collagenous proteins, and mineral phase of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$]. Osteogenesis occurs by two distinct mechanisms – endochondral ossification, and intramembranous bone formation. Endochondral ossification occurs in most bones of mesodermal origin that form the axial skeleton, including long bones, skull, ribs and vertebrae, and involves the formation of initial mineralized cartilage template, which is first degraded by osteoclasts and then replaced with bone matrix by osteoblasts (29,30). Intramembranous ossification occurs in the flat bones and the mandible, maxilla and clavicle, where an ossification centre is created when mesenchymal stem cells condense, and directly differentiate into bone-forming osteoblasts (31).

Functions

The mechanical functions of bone are probably their best recognized. Bones protect internal organs from damage and support the structure of the body. Bones provide anchorage for

muscles, ligaments and tendons to allow movement in three-dimensional space. Hearing is also attributed to the mechanics of bones, with several of the body's smallest bones involved in the transmission of sound in the ear. Bone is the body's major reservoir of calcium, storing approximately 99% of it in the bone's mineral phase. Plasma calcium levels are strictly regulated in the range of 2.2-2.6 mmol/L total calcium. Such regulation is achieved by regulating calcium exchange with the environment through the kidney and intestine, and, in the absence or insufficiency of environmental sources, by regulating calcium exchange between plasma and bone through osteoblastic bone formation and osteoclastic bone destruction (32). The coordination of calcium fluxes is achived through complex hormonal regulation. Parathyroid hormone and 1,25 dihydroxy-vitamin D act to increase calcium by increasing calcium reabsorption from the kidneys and small intestine, respectively, and both act by enhancing the mobilization of calcium from bone through resorption (33). Calcitonin acts to reduce blood calcium by suppressing renal calcium reabsorption and inhibiting the mobilization from bone by preventing bone resorption (34). The combined work of these systems ensures that hypo- or hyper-calcemia is corrected, and ingested calcium is stored or eliminated as waste.

Bone tissue also interacts with other functionally diverse systems in the body. The endosteal surface of the medullary cavity of bones houses the haematopoietic stem cell niche, the specific location where blood stem cells best differentiate. Osteoblasts are well known to support the haematopoietic stem cell niche directly (35), and haematopoietic cells in turn regulate osteogenesis (36). Adipocyte-derived leptin regulates both appetite and bone mass accrual (37), and osteoblast-derived osteocalcin affects insulin secretion and sensitivity, as well as energy expenditure (38,39). It has most recently been shown that the skeleton regulates male fertility through osteocalcin (40), extending the breadth of bone's influence into reproduction as well.

Bone cells

The three cell types critical to bone's structure and function are the bone-resorbing osteoclast, the bone forming osteoblast, and the mechanosensory osteocyte. These cells work in concert to build bones, maintain mechanically sound bone tissue by replacing it on average every 10 years, and repair bones in the incidence of trauma.

Osteoclasts: The destruction of bone, both physiological in the case of morphogenesis and replacing old or damaged bone, and pathological in the case of osteolytic diseases such as osteoporosis, breast cancer metastasis to bone and rheumatoid arthritis, occurs through the activity of the osteoclast. Osteoclasts are cells of hematopoietic origin. The key molecular mediators of osteoclast formation from monocytic precursors are macrophage colony-stimulating factor (M-SCF) acting through its receptor c-fms, and RANKL which binds to its receptor RANK (41-43). Osteoprotegerin (OPG) is the high affinity decoy receptor for RANKL and is able to prevent osteoclast differentiation by inhibiting RANK-RANKL interactions (44). RANKL binding to RANK in the presence of M-CSF induces the recruitment of adaptor molecules including TRAF6 by RANK (45), resulting in the activation of transcription factor NFκB. One of the early targets of NFκB is another transcription factor essential for osteoclastogenesis, nuclear factor of activated T-cells c1 (NFATc1), which later undergoes auto-amplification with the assistance of an activator protein-1 complex containing c-Fos (46-48). NFATc1 nuclear localization is regulated by

calcium signalling, which also activates calmodulin-dependent kinase, critical for further osteoclast differentiation (49). These events lead to the expression of osteoclast-specific genes including tartrate-resistant acid phosphatase (TRAP), cathepsin K, and b3 integrin (50), which are important for the degradation of bone tissue. Osteoclasts resorb bone by creating a unique microenvironment localized between this cell and bone tissue. Osteoclasts first recognize and bind to the bone matrix with integrin receptors β1 that bind collagen, fibronectin and laminin, and αvβ3 that binds osteopontin and bone sialoprotein (51). This border forms a sealing zone over the area of bone to be resorbed, and the polarization of osteoclasts results in the formation of a ruffled border between the osteoclast and matrix (52). Targeted secretion of H^+ ions through the ruffled border H^+ ATPase, accompanied by movement of Cl^- through chloride channels, acidifies the sealed space to a pH of approximately 4.5 (53,54), resulting in dissolution of the mineral phase of bone, and proteolytic enzymes cathepsin K and matrix metalloproteinase-9 (MMP-9) are released and activated to digest the organic matrix (55).

Osteoblasts: Osteoblasts are differentiated from the mesenchymal stem cells (MSC) that can also give rise to progenitors of myoblasts, adipocytes and chondrocytes (56). Commitment of MSC to become osteoprogenitors results in the upregulation of receptors for hormones, cytokines and growth factors, including PTH, prostaglandin, interleukin-11, insulin-like growth factor-1 and transforming growth factor-β (57). Next, osteoprogenitor cells differentiate into preosteoblasts, cells that exhibit limited proliferation and start to express extracellular matrix proteins, such as collagen type I, bone sialoprotein and osteopontin. Preosteoblasts are also active in the production of pro-resorptive cytokine RANKL (58). Finally, mature osteoblasts do not proliferative, but actively produce and secrete collagen type I, bone sialoprotein and osteopontin as well as osteocalcin. In addition, mature osteoblasts switch to produce the RANKL inhibitor, OPG (58). Osteoblastogenesis commitment is driven by the downstream activities of Wingless-ints (Wnt) singling, the closely associated Hedgehog signalling pathway (Sonic Hedgehog, Indian Hedgehog) and bone morphogenetic proteins (BMPs), which determine where mesenchymal stem cells condense during embryonic patterning and cross-talk to induce osteoblast differentiation (59,60). Another developmentally important pathway, Notch signalling, has been shown to negatively regulate osteoblast differentiation (61-63). Important signalling events during osteoblast differentiation include the activation of the runt-related transcription factor 2 (Runx2) transcription factor, which regulates the expression of the zinc finger-containing transcription factor Osterix (64). Osterix interacts with nuclear factor for activated T cells 2 (NFATc2), and in collaboration, controls the transcription of osteoblastic target genes osteocalcin, osteopontin, osteonectin and collagen-1 (65,66). Osteoblasts anchor to newly formed bone matrix by cadherin-11 and N-cadherin, and secrete type 1 collagen and non-collagenous matrix proteins (57). The osteoblasts then regulate the subsequent mineralization of extracellular matrix (67-69).

Osteocytes: While each cell type is essential for the maintenance of bone homeostasis, osteocytes are the most populous and account for over 95% of all cells in the skeleton, covering 94% of all bone surface (70). Osteocytes are differentiated from osteoblasts embedded in the bone matrix. During differentiation, the osteocyte cell body size decreases, and the number of long dendrite-like cell processes increases and they extend, connecting the cell with other osteocytes (70,71). Osteocyte-specific genes are activated, including phosphate-regulating gene with homologies to endopeptidases on the X chromosome

(PHEX), matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein 1 (DMP1), and fibroblast growth factor-23 (FGF23) (72,73). Osteocyte networks in the bone tissue are implicated in regulating the maintenance and mineralization of bone tissue (70,74), through expression of sclerostin, a negative regulator of bone formation (75), as well as in sensing mechanical load in part through sheer stress generated by interstitial fluid moving through the lacuno-canalicular network (76). It has also been suggested that osteocytes participate in mineral homeostasis by resorbing the lacunar walls in which they are embedded (77-79).

Communication between bone cells during normal bone remodelling

Osteoblasts, osteoclasts and osteocytes must work in concert to maintain bone homeostasis (Figure 2). In normal bone physiology, the osteoclast will resorb worn or damaged bone, and then the osteoblast will form new bone in its place. The best studied example of the crosstalk between bone cells involves the RANK-RANKL-OPG triangle, where osteoblasts and osteocytes produce RANKL to promote osteoclast differentiation and survival, and OPG to prevent it, while osteoclasts express RANK, allowing them to respond to these regulatory cues. Many hormonal regulators of bone remodelling, such as PTH and estrogen, were demonstrated to act through changing the ratio of RANKL and OPG expression by osteoblasts (80). Interestingly, production of RANKL and OPG by osteoblasts is also regulated by their developmental stage, with immature osteoblasts producing more RANKL and mature osteoblasts produce more OPG, (58). Osteocytes also, at least in part, affect osteoclastogenesis through production of RANKL, which is induced in mechanically-stimulated osteocytes (81). Osteoclasts are in turn able to influence osteoblast activity. The concept of osteoclast-mediated osteoblastogenesis arose from the finding that 97% of new bone formation occurs in resorption pits (82). Several studies where osteoclasts have been genetically altered to have impaired function demonstrated diminished bone formation (83), and studies have begun to find mediators of this reversal coupling. Cardiotrophin-1 is among the first identified, and is expressed by osteoclasts and increases osteoblast activity (84). Sphingosine-1-phosphate has been shown to act earlier and induce osteoblast precursor recruitment and subsequent mature cell survival (85). Ephrin-B2/EphB4 bidirectional signaling between osteoclasts and osteoblasts, has also been identified as a key mediator of contact-dependent communication. Forward signalling by ephrin-B2 on osteoclasts to EphB4 on osteoblasts activates bone formation, whereas reverse signalling from EphB4 on osteoblasts binding to ephrin-B2 on osteoclasts inhibits osteoclastogenesis (86). Since the ability for bone cells to communicate is essential for the maintenance of bone homeostasis, it can be anticipated that disruptions in these the complex networks would lead to profound consequences. Indeed, the RANKL/OPG ratio represents one of the key mediators of pathological bone destruction (87).

4. Homing of breast cancer cells to bone

4.1 Creation of the pre-metastatic niche

Recent evidence has led to the idea that the bone marrow supports a pre-metastatic niche - a site that receives signals from the primary tumour mass before dissemination, and changes the landscape of the target tissue to be conducive to tumour growth. It has been shown in mice treated with medium conditioned by tumour cells of different origin, the potential to home to different organs of subsequently injected cancer cells can be altered (88). In

particular, in bone, bone marrow derived hematopoietic stem cells have been implicated in mediating the establishment of pre-metastatic niche (19,88). Molecular mediators such as vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1) and integrin α4β1 have been implicated in this process. VEGFR1 positive haematopoietic progenitor cells are recruited to sites of future metastasis (88). VEGF receptors are expressed by breast cancer cells as well as osteoclasts and osteoclast precursors, and VEGF expression correlates to increased tumour size and grade in humans (89). In addition, we have shown that breast cancer cells secrete factors that support the subsequent attachment of breast cancer cells acting at least in part through γ-secretase-mediated Notch signalling (20).

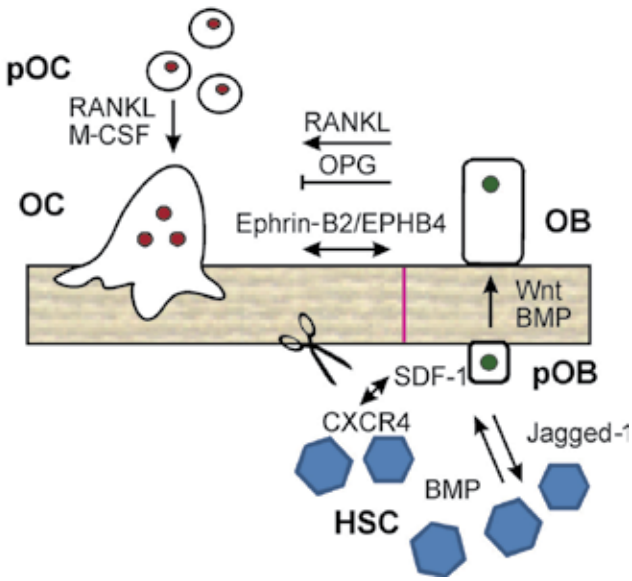

Fig. 2. Cell-cell interactions in the bone microenvironment. Osteoclast differentiation from monocytic precursors is induced by M-CSF, RANKL produced by osteoblastic cells. Osteoblasts are derived from mesenchymal stem cells through Wnt and BMP signalling pathways. Osteoblasts and osteoclasts communicate through osteoblast-derived RANKL/OPG and bidirectional Ephrin-B2/EphB4 signalling. Haematopoietic stem cells (HSC) support osteoblasts in the HSC niche through BMPs, while osteoblasts support HSCs through upregulated Notch signalling through Jagged-1. Osteoclasts cleave SDF-1 to mobilize HSCs from the endosteal niche.

4.2 Migration of breast cancer cells to bone

Breast cancer cells express receptors that direct their movement towards fertile sites where they may establish into secondary tumors. These proteins are generally expressed in normal cells, and are often involved in developmental pathways. Several chemokines have been suggested to be released from the bone microenvironment, implicating chemoattraction through G-protein-coupled chemokine receptors in driving the movement of tumour cells towards bone (90). Interactions between stromal-derived factor-1 (SDF-1) and CXCR4 are essential for the correct localization of lymphocytes and haematopoietic cells in physiological states. Breast cancer cells express higher levels of CXCR4 compared to normal

breast tissue (15), and SDF-1 is strongly expressed in lung, liver, bone marrow and lymph nodes, the primary sites of secondary breast tumours, leading to the identification of the role of the SDF-1/CXCR4 in promoting breast cancer metastasis to bone (91). In addition to directional migration, chemokines have been shown to promote cancer cell survival, proliferation, and adhesion (92). In keeping, the inhibition of CXCR4 limited breast cancer metastases in mice (93), and the overexpression of CXCR4 indicates poor prognosis in both human and murine breast cancer (92,94). Another chemokine implicated in metastases of breast cancer cells expressing high levels of CCL21, is CCR7 that is expressed highly in metastatic sites, such as lymph nodes (15). Since haematopoietic stem cells (HSCs) use these chemokine and receptor interactions to home to the HSC niche in the bone marrow, it has been suggested that cancer cells use this same mechanism to parasitize these microenvironments and harvest the resources of HSCs (95). Another pertinent means of cancer cell migration towards bone relies on the cancer cell expression of RANK (96), which mediates directional migration of breast, melanoma and prostate cancer cells towards RANKL, produced in bone by osteoblasts (97,98).

Breast cancer cells may also stimulate the action of matrix metalloproteinases that support cancer cell migration and invasion. The murine orthologue of Glycogen Nonmetastatic Melanoma Protein B (GPNMB) is called osteoactivin and has been identified as a key modulator of osteolysis. Its forced expression leads to increased tumour grade and enhanced bone metastasis by upregulated MMP3 through ERK signaling (99,100). Furthermore, GPNMB was identified as a poor prognostic marker in patients with breast cancer (101). Most recently, this group has identified ADAM10 as a sheddase that releases osteoactivin from the cell, which induces endothelial cell migration and subsequent angiogenesis (102). ADAMTS1 and MMP1 are also tumour-derived metalloproteinases able to degrade the matrix. The stimulated action of these enzymes by breast cancer cells enhances osteoclast differentiation by suppressing OPG expression, and their expression in human samples correlates to a greater incidence of bone metastases (103).

4.3 Attachment proteins between breast cancer cells and the bone

Cancer cells express or induce the expression of adhesion molecules that may facilitate their interactions with the bone microenvironment. The best studied family of proteins that bind cancer cells to bone cells are integrins, heterodimeric transmembrane glycoproteins whose α and β subunits combine to form 24 known combinations with unique specificity for binding, signaling and regulatory mechanisms (104). Integrins have been demonstrated to be involved in several stages of cancer dissemination, with highly metastatic cancer cells displaying a different integrin profile than cells from the primary tumour (105). Several integrins have been shown to interact with extracellular matrix proteins during bone metastasis, with the most important being αvβ3, a receptor for osteopontin, fibronectin and vitronectin (106). Adhesion molecules engaged between breast cancer cells and bone cells may overlap with those that bind haematopoietic stem cells (HSC) to osteoblasts. HSC preferentially home to areas with more fibronectin (88). Breast cancer cells can attach to fibronectin, in an integrin-dependent manner (107). The interaction of cancer cells with fibronectin increases the production of matrix metalloproteinase-2 from fibroblasts to facilitate invasion (108). Another molecule involved the adhesion of HSC to the endosteal niche is annexin II (95). By serving as an anchor for SDF-1/CXCL12, it has been shown to regulate the homing of HSC as well as prostate cancer cells to the HSC niche (109,110).

Blocking annexin II or its receptor limited the localization of prostate cancer cells to osteoblasts and endothelial cells (111). In keeping, the inhibition of the SDF-1/CXCL12 and annexin II signaling was shown to inhibit breast cancer progression (112,113). Bone matrix proteins, such as bone sialoprotein (BSP) or osteopontin (OPN) have been shown to exhibit a potential to regulate the attachment of breast cancer cells to bone (114). Early reports have argued that BSP inhibits breast cancer cell binding to bone cells (115). However, breast cancer cells have been shown to express both BSP and OPN, and to upregulate BSP expression in pre-osteoblasts through BMP signalling; and OPN was found localized between cancer cells and bone cells at sites of metastasis (116,117). Moreover, the expression of BSP has been found to correlate with bone metastasis development (118), and OPN expression and serum concentrations have been shown to be poor prognosis markers in breast cancer patients (119,120). As osteopontin is also a mediator of the hematopoietic stem cell niche, directing migration and acting as an adhesion molecule to HSC via β1 integrin (121), it represents a potentially valuable therapeutic target against bone metastases.

4.4 Osteomimicry

Osteomimicry describes the phenomenon where osteotropic cancer cells express proteins and receptors found on bone cells and the bone matrix. It was speculated that such measures allow cancer cells to evade the immune system and/or establish in the bone microenvironment (122,123). These proteins include but are not limited to osteocalcin, osteopontin, alkaline phopsphatase and Runx2 (124). Osteoblast transcription factor Runx2 is ectopically expressed by breast cancer cells and stimulates their proliferation, motility, and invasion through increased MMP9 expression from both cancer cells and osteoblasts (125,126). Runx2 has also been shown to regulate TGFβ-influenced PTHrP levels, as well as upregulate Indian hedgehog (127). Breast cancer cells express Hedgehog ligands that activate osteopontin expression in osteoclasts, promoting osteoclast maturation and resorptive activity through upregulated Cathepsin K and MMP9 (128,129). Of interest, expression of anti-resorptive OPG has been demonstrated to correlate with increased bone-specific homing and colonization potential in breast cancer cells (122), and to promote cancer cell survival (130,131). Osteoclastic intergrin αvβ3 (54), has been shown to be upregulated in metastatic versus primary tumour cells, and has been identified as a critical mediator of breast cancer metastasis to bone (107,132). It is unclear whether cells from the primary tumour display osteomimetic features that allow their metastasis to bone, or whether secondary tumour cells established in the bone marrow and matrix receive environmental factors that give them their osteomimetic features. Regardless, the ability of cancer cells to produce many of these factors has been beneficial to thrive in the bone microenvironment.

5. Establishing of a metastatic tumour in the bone microenvironment

5.1 Interactions of breast cancer cells with osteoblasts

Inhibition of osteoblasts by breast cancer cells

Breast cancer metastasis to bone is associated with a reduction in bone formation markers in patients with bone metastases (133). In vitro, breast cancer cells have been shown to produce soluble factors able to inhibit osteoblast differentiation (20,134), the effect that may be mediated at least in part by the dysregulation of Notch and Wnt developmental signalling

pathways. Notch signalling is essential in embryogenesis but has distinct roles in bone homeostasis, regulating the proliferation of immature osteoblasts (135) and suppressing osteoblast differentiation (62,63). Upregulated Notch signalling in breast cancer, through ligand Jagged-1, has been shown to correlate with increased bone metastases (136), and breast cancer cells have been shown to induce Jagged-1 expression and upregulate Notch signalling by osteoblasts (20). Wnt signaling is also a highly conserved developmental pathway, well studied in bone and essential for osteoblast and osteoclast differentiation, as well as for the production of pro-resorptive cytokine RANKL and anti-resorptive OPG (137). Wnt inhibitor DKK-1 has been shown to be upregulated in diseases associated with bone destruction, such as osteoarthritis (138), myeloma (139), and potentially in Paget's disease (140). Blocking DKK-1 in a breast cancer metastasis model has also been shown to reverse breast cancer-mediated suppression of osteoblast differentiation and reinstate OPG expression (141). Breast cancer cells have also been shown to induce osteoblast apoptosis, through increased Bax/Bcl-2 ratio and caspase expression in osteoblasts (142,143). In addition to preventing the formation of new bone, breast cancer-induced inhibition of osteoblast differentiation likely indirectly contributes to the change in production of cytokines regulating osteoclast formation and function.

Contribution of osteoblasts to the creation of an osteolytic environment

The formation of an osteoclast-supportive microenvironment is critical for the successful establishment of an osteolytic lesion during breast cancer metastasis to bone. It has been previously shown that an increase in the ratio between a pro-resorptive RANKL and anti-resorptive OPG is a key change induced by breast cancer cells (reviewed in (144,145)). Since osteoblasts are the primary source of both pro-resorptive and anti-resorptive cytokines, they represent a critical target for cancer-derived factors. Osteoblast production of RANKL is stimulated by tumour-derived PTHrP, Il-8 , Il-6 and Monocyte Chemoattractant Protein (MCP-1) (reviewed in (146)). Moreover, under the influence of breast cancer cells, undifferentiated osteoblasts express higher levels of RANKL and lower OPG, resulting in an increase in osteoblast-mediated osteoclastogenesis (20), an effect that was reversed when osteoblastic cultures were treated with the inhibitors of γ-secretase – an enzyme implicated in Notch signalling (20,136). One of the mediators of these changes was shown to be the tumour-overexpressed CCN3, that can inhibit osteoblast differentiation and shift the RANKL/OPG ratio to favour osteolysis (147). Another osteoblast-produced osteoclastogenic factor, MCSF, has also been implicated in breast cancer metastases to bone (148).

Role of osteoblasts in supporting breast cancer cells

An emerging area of interest is the role of osteoblasts in supporting the haematopoietic stem cell niche and how cancer cells parasitize this relationship. Haematopoiesis occurs on the endosteal surface of the bone marrow, where haematopoietic stem cells (HSCs) are maintained by the supporting cells, including osteoblasts. The main functions of the interaction between these cell types are *i*) the maintenance of HSC quiescence through osteoblast-derived osteopontin, and *ii*) modification to expand the progenitor population through Notch signaling (35,121). Several osteoblast-expressed receptors, cytokines and growth factors have been found to regulate an haematopoietic stem cell niche (149,150), including PTH/PTHrP receptors and BMPs acting to expand the osteoblast population, and Notch ligand Jagged-1 to expand the population of HSCs (35,151). Cancer cells disseminated from the primary tumour may also lay dormant for long periods of time before being

activated to form metastases (152), so it is plausible that cancer cells harvest resources from the HSCs niche to maintain their survival and to induce expansion at the right environmental cues.

5.2 Interactions of breast cancer cells with osteoclasts

Stimulation of osteoclasts by breast cancer cells

Breast cancer cells have been found to produce many factors capable of simulating osteoclastogenesis, both by inducing RANKL expression by osteoblasts and stromal cells, and by producing osteoclastogenic factors themselves. PTHrP was one of the first factors identified to be secreted by breast cancer cells and to promote osteolysis through the stimulation of RANKL by stromal cells (153). Although the expression of PTHrP in primary tumours has been associated with a lower incidence of bone metastasis (154,155), it was shown that increased PTHrP expression by cancer cells present in the bone metastatic lesion positively correlates with increasing osteoclast activity and subsequent osteolysis (155), suggesting that the expression pattern of the cancer cells can change during metastasis, and implicating local factors, such as TGFβ derived from osteoclastic bone resorption in affecting metastasizing breast cancer cells. Osteoclastogenesis may also be stimulated by IL-8 secreted from breast cancer cells and acting both directly on osteoclasts and through osteoblastic RANKL signalling (156,157). Although the mechanisms of IL-8 action are not fully understood, the expression of IL-8 correlated with a higher incidence of bone metastasis in mice *in vivo* (158).

It has also been shown that during differentiation osteoclast precursors may acquire sensitivity to cancer-derived factors that are ineffective in inducing osteoclast formation from naive monocytes (159). Several signalling pathways in osteoclast precursors have been implicated in these effects, including calcium signalling, NFATc1 activation and MAPKs ERK1/2 and p38 (159,160). Tumour-produced CCN3 was demonstrated to stimulate osteoclast formation from RANKL-primed osteoclast precursors (147). These effects can be relevant to the propensity of cancer cells to metastasize to bone sites undergoing active bone remodelling, and thus containing increased numbers of RANKL-primed osteoclast precursors. At such sites, breast cancer cells can promote further osteoclast formation, and can affect the survival of mature osteoclasts, increasing their resorptive capacity. In this regard, M-CSF secreted from breast cancer cells was shown to be responsible for the delayed apoptosis in osteoclasts (146,161). Anti-apoptotic effects of breast cancer-derived factors included PLCγ-mediated suppression of pro-apoptotic protein BIM, and M-CSF-mediated inhibition of caspase cleavage (146).

Role of osteoclasts in supporting breast cancer cells

During osteoclastic resorption, the bone matrix components, including many growth factors stored in the bone, such as TGFβ, BMPs, IGFs, fibroblast growth factors (FGFs), and platelet-derived growth factors (PDGF) are released into extracellular space, where they are free to act on surrounding cells, including metastasizing cancer cells (162). Matrix released- TGFβ activated by osteoclastic resorption (163), is one of the most commonly studied matrix-derived growth factors, which was shown to stimulate cancer cell growth, modify cell invasion, and affect immune regulation (164,165). Considerable research has linked increased TGF-β in the microenvironment to the progression of metastasis, with TGFβ

altering both the growth and phenotype of breast cancer cells (166), and increasing their expression of CTGF, CXCL11 and PTHrP (167) via Smad and MAPK signalling in breast cancer cells (153,168,169). PTHrP increases VEGF production, leading to stimulated osteoclastogenesis through the ERK1/2 and p38 signalling pathways (170). TGFβ also acts on other cells present in the bone microenvironment, such as osteoclasts themselves by sensitizing them to other breast cancer derived factors (159), through the ERK1/2, p38 and c-Jun-NH_2 kinase signalling pathways (160,171). In keeping with a key role of TGFβ in bone metastases, pharmacological inhibition of TGFβ signalling through the TβRI kinase inhibitor SD-208 resulted in decreased bone metastasis and tumour burden, and improved bone quality (172). The self-accelerating cycle of osteoclast stimulation by breast cancer cells, resulting in release of matrix growth factors due to osteoclastic resorption, leading to further stimulation of breast cancer cells and further increase in osteoclastic resorption was coined the name of "vicious cycle" (173), underlying the strong rationale for the use of anti-resorptive drugs for the treatment of cancer metastases to bone.

6. Therapeutic targets in the bone microenvironment

The bone microenvironment presents multiple targets for developing therapeutic treatments targeting the homing of breast cancer cells to bone, as well as progression of bone metastatic lesions (Figure 3). Molecular mediators of critical events underlying the stimulation of bone resorption and inhibition of bone formation, as well as tumour supportive environmental changes and cellular targets have been explored for their benefits in treatment of osteolytic bone metastases.

Since its discovery, the RANKL pathway has been considered to be of important therapeutic value given its role in osteoclastogenesis mediating osteolysis and subsequently discovered breast cancer cell migration, underlying pre-metastatic homing. Fully human monoclonal antibody against RANKL, Denosumab, was approved for major North American and European markets in 2010 for the prevention of osteoporosis and skeletal related events in patients with bone metastases from solid tumours. Compared to the most potent osteoclast-targeting drug in the market, bisphosphonate zoledronic acid, Denosumab treatment further delayed the occurrence of the first skeletal related event (SRE), and provided a further reduction in bone turnover markers in breast cancer patients (174). In non-metastatic breast cancer patients additionally receiving adjuvant aromatase inhibitors, bone mineral density gains were greater with Denosumab treatment (175). Bisphosphonate-resistant patients with bone metastases from breast or prostate cancer also benefitted from Denosumab treatment, with most having normalized serum markers of bone resorption after 13 weeks of treatment (176). Although Denosumab proves an effective treatment option, long-term use and toxicity data remains unknown.

DKK-1 was identified as a key mediator of myeloma-induced inhibition of bone formation, and was demonstrated to play important role in breast cancer induced inhibition of osteoblastogenesis. Neutralizing anti-DKK-1 antibodies have demonstrated significant benefits in preclinical studies in mouse models of myeloma-induced bone disease, resulting in increased osteoblast numbers, reduced osteoclast numbers and increased bone volume, and stimulating interest in further development of this approach (177). Bortezomib, a proteasome inhibitor that among other proteins affects DKK-1 and BIM (a pro-apoptotic protein that mediates osteoclast apoptosis) (178,179), was shown to inhibit

osteoclastogenesis (180) and has been successful in combating the osteolytic effects of multiple myeloma (181), making it an attractive candidate for the prevention and treatment of breast cancer-induced osteolysis.

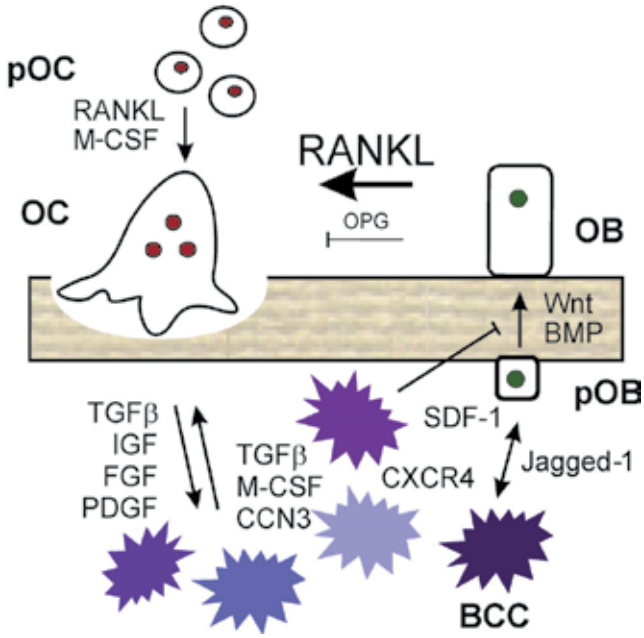

Fig. 3. Breast cancer cells alter normal bone homeostasis. Breast cancer cells maintain osteoblasts in an immature state and stimulate RANKL production by osteoblasts, while inhibiting OPG. Breast cancer cells stimulate osteoclastogenesis directly through TGFβ, M-CSF and CCN3 production. Increased bone resorption by activated osteoclasts releases matrix-derived growth factors TGFβ, IGF, FGF, PDGF, which act back on breast cancer cells to stimulate their growth and survival.

VEGF represents an interesting target potentially affecting breast cancer cell homing, development of pre-metastatic niche and new vasculature formation. Many anti-VEGF therapies exist to prevent vascularization of tumours and inhibit their growth (182). There have been several hindrances in the progress of this therapy due to drug resistance and toxicity (183), and the increased incidence of osteonecrosis of the jaw in combined bisphosphonate-antiangiogenic agent therapy (184). Notwithstanding, the use of VEGF-A monoclonal antibody Bevacizumab in combination with chemotherapy has proven beneficial in reducing breast cancer growth (185) and osteolysis (186). Other targets based on the in vitro and in vivo studies, such as TGFβ, GPNMB, and CXCR4 are being explored in preclinical and clinical studies, providing the basis for the next generation of treatments.

Osteoclasts are commonly targeted therapeutically for osteolytic disease, with one of the most widely used drugs being bisphosphonates. Analogs of mineralization-inhibiting pyrophosphate (187), bisphosphonates are a class of synthetic compounds composed of two phosphate groups covalently linked to carbon with a P-C-P backbone and side groups that vary their properties and pharmacokinetics. Bisphosphonates attach selectively to bone and induce osteoclast apoptosis when they are ingested during resorption. In osteoporosis studies, all bisphosphonates given daily have been shown to reduce osteoporotic vertebral

fracture rates by 40-50% (188), and zoledronic acid and risedronate have been shown to significantly reduce non-vertebral fracture risk in pivotal trials (189). Bisphosphonates are widely used in prevention and treatment of breast cancer metastases to bone, resulting in delay and reduction in skeletal related events (190). In addition to their effects on osteoclasts, bisphosphonates have been shown to inhibit tumour growth, induce tumour cell apoptosis, and stimulate the immune response against tumour cells (191). However, some patients do not tolerate bisphosphonates well, and low but significant incidences of osteonecrosis of the jaw have been observed in patients that have undergone dental extraction procedures while treated with bisphosohonates (192). In addition, significant proportion of patients failed to normalize bone resorptive indices in response to bisphosphonate treatment (176), demonstrating the need for new therapeutic approaches.

7. Conclusion

Breast cancer is the most commonly diagnosed cancer in women, which may lead to bone metastasis resulting in altered mineral homeostasis, the disruption of bone microarchitecture, pain and pathological fractures. Recent studies have demonstrated that breast cancer cells start affecting the bone microenvironment prior to their dissemination from the primary tumour by secreting circulating soluble factors that prepare bone for the future arrival of metastasizing cancer cells, a process that likely involves mediators of the hematopoietic stem cell niche. Multiple mediators of directional migration of breast cancer cells have been identified, as well as mediators of breast cancer cells anti-osteoblastic and pro-osteoclastic actions. Breast cancer-stimulated RANKL, M-CSF, PTHrP, TGFβ, GPNMB, Runx2 and CXCR4 remain among the most critical mediators of cancer-induced osteoclastic bone resorption. Yet, they are not the whole picture, and new players are being identified, providing more complex and comprehensive description of the events leading from the formation of primary tumour to the establishment of progressive osteolytic bone lesions. However, while considering the multitude of molecular mediators, it is important to remember the heterogeneity of breast cancer disease in patients, suggesting that treatments targeting different molecular mediators should develop in parallel with the testing capabilities able to implicate a particular mediator in disease progression in a specific patient. An alternative approach is to target the processes and cellular targets similarly altered through different molecular mediators. An example of such approach is the clinical success of bisphosphonates, which broadly target osteoclast formation and activity. Nevertheless, both approaches need to be developed to provide clinicians with the set of tools for broad preventive measures, as well as for targeted personalized medicine for non-responsive or atypical cases.

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Rho GTPases and Breast Cancer

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1. Introduction

The Rho GTPases is a subfamily of molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state within the Ras superfamily. In the past, members of the Rho subfamily were mainly thought to be involved in the regulation of cytoskeletal organization in response to extracellular growth factors. However, a number of studies over the past few years have revealed that the Rho GTPases play crucial roles in a wide spectrum of cellular functions related to cell adhesion to the extracellular matrix, cell morphology, cell cycle progression, malignant transformation, invasion and metastasis. Alterations of the expression levels to Rho GTPases have been detected in many types of human tumors and, in some cases, up-regulation and/or overexpression of Rho protein correlates with poor prognosis. This article reviews the evidence of aberrant Rho signaling and the cellular effects elicited by Rho GTPases signaling in human breast tumors.

2. Categorization

Rho GTPases belong to the Ras superfamily of low molecular mass (~21 kDa) proteins that are widely expressed in mammalian cells (DerMardirossian and Bokoch 2001). In mammals, the Rho family of GTPases contains 22 members which can be classified into six groups: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG), Cdc42 (Cdc42, TC10, TCL, Chp, Wrch-1), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoBTB (RhoBTB1, RhoBTB2) and Miro (Miro-1, Miro-2) (Wennerberg and Der 2004). RhoD, Rif and RhoH/TTF have not been grouped yet. RhoA, Rac1 and Cdc42 are the best-characterized family members of Rho family GTPases. Each controls the formation of a distinct cytoskeletal element in mammalian cells. Activation of Rac induces Actin polymerization to form lamellipodia (Ridley, Paterson et al. 1992), whereas activation of CDC42 stimulates the polymerization of actin to filopodia or microspikes (Nobes and Hall 1995). In contrast, Rho regulates bundling of actin filaments into stress fibers and the formation of focal adhesion complexes (Keely, Westwick et al. 1997).

3. Regulators and effectors in Rho GTPases signaling

3.1 Regulators of the Rho GTPases

Like all members of the Ras superfamily, the activity of the Rho GTPases is tightly controlled by the ratio of their GTP/GDP-bound forms in the cell (Fig. 1)(Scheffzek and Ahmadian 2005).

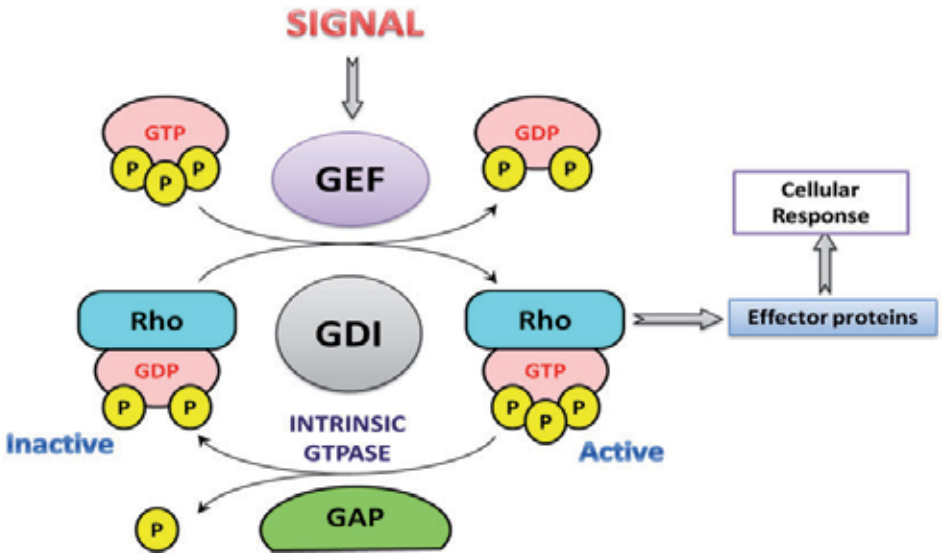

Fig. 1. Regulation of Rho family proteins.

The cycle of activation/inactivation of Rho family GTPases is under the regulation of three distinct families of proteins: GEFs, guanine nucleotide exchange factors catalyze nucleotide exchange when activated by upstream signals; GAPs, GTPase-activating proteins promote the GTP hydrolisis; GDIs, guanine nucleotide dissociation inhibitors block both nucleotide hydrolisis and exchange and participate in Rho GTPase movement between cytosol and membranes.

Rho-specific guanine nucleotide exchange factors (RhoGEFs) activate Rho proteins by facilitating the exchange of GDP for GTP. Rho GTPase activating proteins (RhoGAPs) stimulate the intrinsic rate of hydrolysis of Rho proteins, thus converting them into their inactive state. While Rho-specific guanine nucleotide dissociation inhibitors (RhoGDIs) compete with RhoGEFs for binding to GDP-bound Rho proteins, and sequester Rho in the inactive state (Olofsson 1999).

3.1.1 GEFs

GEFs for Rho GTPases belong to a rapidly growing family of proteins that share common minimal functional units, including a Db1-homolog (DH) domain followed by a pleckstrin homology (PH) domain (Cerione and Zheng 1996). The DH domain is the catalytic site required for GDP-GTP exchange, whereas the PH domain contributes to protein-protein, protein-cytoskeleton, and protein-lipid interactions that help regulate the intracellular localization of GEFs as well as their catalytic activity. Db1 oncogene product is the prototype for the DH domain, and was originally discovered because of its ability to induce focus

formation and tumorigesis when expressed in NIH-3T3 cells (Eva and Aaronson 1985). It has 29% sequence identity with the *Saccharomyces cerevisiae* cell division protein Cdc24, which is found upstream of the yeast small GTP-binding protein Cdc42 in the bud assembly pathway (Ron, Zannini et al. 1991). This was the first clue that DB1 functions as a GEF. Biochemical study has confirmed that Db1 is able to release GDP from the human homolog of Cdc42 *in vitro*. Further study suggested that the DH domain is essential and sufficient for the catalytic activity and that this domain was also necessary to induce oncogenicity (Zheng, Zangrilli et al. 1996).

After the discovery of Dbl, a number of mammalian proteins containing DH and PH domain have been studied (Cerione and Zheng 1996). Many of these have been identified as oncogenes in transfection assays. Tiam, however, was first identified as an invasion-inducing gene using proviral tagging and *in vitro* selection for invasiveness (Habets, Scholtes et al. 1994). Two other members of the DH/PH-containing protein family, Fgd1 and Vav, have been shown to be essential for normal embryonic development (Pasteris, Cadle et al. 1994; Tarakhovsky, Turner et al. 1995). Moreover, some members of the DH protein family (such as Dbl) have been shown to exhibit exchange activity *in vitro* for a broad range of Rho-like GTPases, whereas others appear to be more specific. For example, Lbc and oncoproteins Lfc and Lsc, are specific for Rho, whereas Fgd1 is specific for Cdc42 (Glaven, Whitehead et al. 1996). Although Vav was originally identified as an activator of Ras (Gulbins, Coggeshall et al. 1993), it has been demonstrated more recently to function as a GEF for members of the Rho family (Crespo, Schuebel et al. 1997; Han, Das et al. 1997).

3.1.2 GAPs

The first GAP protein specific for the Rho family GTPases was purified from cell extracts using recombinant Rho. This protein, designated p50Rho-GAP, was shown to have GAP activity toward Rho, Cdc42 and Rac *in vitro* (Hall 1990; Lancaster, Taylor-Harris et al. 1994). Since then, a growing number of proteins that present GAP activity for Rho GTPases have been identified in mammalian cells, all of which share a related GAP domain that spans 140 amino acids without significant resemblance to Ras GAP. In addition to accelerating the hydrolysis of GTP, Rho GAPs also mediate other downstream functions of Rho proteins in mammalian systems. For example, it has been reported that the p190GAP plays a role in cytoskeletal rearrangement (Chang, Gill et al. 1995).

3.1.3 GDIs

The ubiquitously expressed protein Rho GDI was the first GDI identified for the members of the Rho family. It was isolated as a cytosolic protein that preferentially associated with the GDP-bound form of RhoA and RhoB and thereby inhibited the dissociation of GDP (Fukumoto, Kaibuchi et al. 1990; Ueda, Kikuchi et al. 1990). Rho GDI was found to be active on Cdc42 and Rac as well (Abo, Pick et al. 1991; Leonard, Hart et al. 1992). Further studies demonstrated that Rho GDI also associated weakly with the GTP-bound form of Rho, Rac, and Cdc42 (Hart, Maru et al. 1992; Chuang, Xu et al. 1993), leading to an inhibition of the intrinsic and GAP-stimulated GTPase activity of the Rho GTPases. Therefore, Rho GDI appears to be a molecule capable of blocking both the GDP/GTP exchange step and the GTP hydrolytic step. It was also reported that the Rho GDIs play a crucial role in the translocation of the Rho GTPases between membranes and the cytoplasm. In resting cells, the Rho proteins are found in the cytosol as a complex with Rho GDIs, which inhibit their

GTP/GDP exchange ratio, but are released from the GDI and translocated to the membranes during the course of cell activation (Takai, Sasaki et al. 1995).

3.2 Effectors of the Rho GTPases

The Rho GTPases have been implicated in a wide varity of cellular processes, including cytoskeletal organization, cell adhesion to the substratum, cell polarity, and transcriptional activation. Several lines of evidence indicate that Rho GTPases link plasma membrane receptors to the assembly and organization of the actin cytoskeleton. Rho GTPases control individual aspects of the actin cytoskeleton through distinct effector proteins. In fact, over 60 targets of the three common Rho GTPases (Rho, Rac, Cdc42) have been found (Fig. 2).

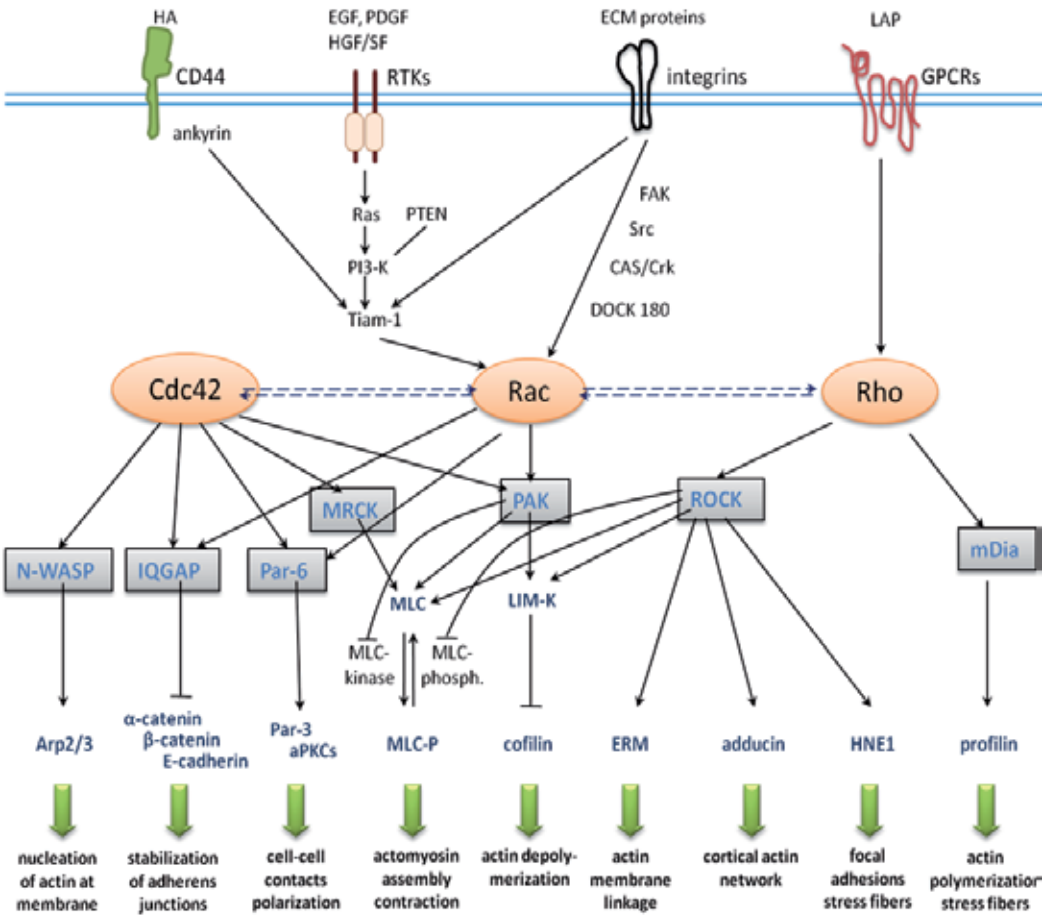

Fig. 2. Regulators and mammalian targets of the Rho family GTPases.

Transmembrane receptors activate Rho GTPases through GEFs such as Tiam-1 or adaptor proteins. Activated Rho GTPases bind to and activate protein kinases, including these of the MRCK, PAK and ROCK families. The effector proteins then interact with several proteins with distinct effects on the actin cytoskeleton and cellular morphology. See text for details.

3.2.1 Rho signaling

Rho was originally studied for its role in regulate the formation of stress fibers and focal adhesion (FA) complexes (Nobes and Hall 1995) which precursors actomyosin assembly and contractile potential, both of which are required for the cellular movement. Rho is also involved in cell-cell adhesion. In particular, inactivation of RhoA by C3 transferase disrupts the organization of actin filaments at cell-cell contact, leading to the inhibition of the proper formation of both adherens junctions (AJs) and tight junctions (TJs) (Braga, Machesky et al. 1997; Takaishi, Sasaki et al. 1997). For example, in normal mammary epithelial cells, MCF10 cells, E-cadherin cytoskeletal links in AJs was disrupted by C3 transferase. In addition, inhibition of Rho blocks the formation of new AJs in MCF10 cells (Zhong, Kinch et al. 1997). It has been suggested that the function of Rho can be either promoted or antagonized by Rac and Cdc42, depending on different variables, such as cellular context, stimulus, and extracellular matrix (ECM) (Zhang, Nie et al. ; Narumiya and Morii 1993; Nobes and Hall 1995). In Swiss 3T3 fibroblasts, the Rho GTPases have been placed in a hierarchical order where Cdc42 activates Rac, and Rac activates Rho (Nobes and Hall 1995); however, in N1E-115 neuroblastoma and Madine-Darby canine kidney (MDCK) cells, constitutively activated Rac down-regulates Rho (Leeuwen, Kain et al. 1997; Michiels and Collard 1999).

Rho is widely studied for its involvement in the acquisition of migratory, invasive, and metastatic phenotypes. Expression of a dominant negative form of RhoA led to the attenuation of membrane ruffling, lamellipodia formation and migration (O'Connor, Nguyen et al. 2000). In addition, RhoA localization to lamellipodia was blocked by inhibiting phosphodiesterase activity while enhanced by inhibiting cAMP-dependent protein kinase activity (O'Connor, Nguyen et al. 2000). Furthermore, activation of Rho either by LPA treatment or by stimulating the actomyosin system has been associated with the migratory ability of tumor cells. For example, in an experimental metastasis model, NIH3T3 fibroblasts expressing a constitutively active form of RhoA were injected into the tail vein of nude mice and formed increased number metastasis nodules in the lung (del Peso, Hernandez-Alcoceba et al. 1997). Moreover, in the absence of serum, activated RhoA is capable of promoting invasion of cultured rat MM1 hepatoma cells through a mesothelial cell monolayer (Yoshioka, Matsumura et al. 1998). Although these are not oncogenes by themselves, RhoA and RhoC are frequently found to be overexpressed in clinical cancers (Sahai and Marshall 2002), and RhoC has been repeatedly associated with metastasis. For example, the expression of RhoA, RhoB and RhoC in 33 pancreatic ductal adnocarcinoma cases were examined in a study (Suwa, Ohshio et al. 1998), it was found that the expression level of RhoC was higher in tumors than in non-malignant tissues, higher in metastatic lesions than in primary tumors, and correlated with perineural invasion and lymph node metastasis as well as poorer prognosis. Although early studies showed that RhoB has a positive role in cell growth, more recent studies suggested that RhoB is down-regulated in human tumors, and its expression inversely correlates with tumor aggressiveness. For example, RhoB protein is found expressed in normal lung tissue and is lost progressively throughout lung cancer progression (Mazieres, Antonia et al. 2004). In line with this, higher expression of RhoB is associated with favorable prognosis in bladder cancer (Kamai, Tsujii et al. 2003). It has been suggested that RhoB can act as a tumor suppressor, since it is activated in response to several stress stimuli, such as DNA damage and hypoxia, inhibits tumor growth, cell migration, and invasion, and has proapoptotic functions in cells (Huang and Prendergast 2006).

3.2.2 Effectors of Rho

There are two major effectors that are downstream of Rho: Rho associated coiled-coil forming kinase (ROCK/Rho kinase/ROK) (Leung, Manser et al. 1995; Ishizaki, Maekawa et al. 1996) and mammalian homolog of Drosophila diaphanous (mDia) (Watanabe, Madaule et al. 1997; Wasserman 1998). While mDia is a formin molecule that can catalyze actin nucleation, polymerization,and produce long, straight actin filaments (Goode and Eck 2007), ROCK is a serine/threonine kinase that phosphorylates a number of substrates (Riento and Ridley 2003). The actions of ROCK and mDia on actin and myosin are believed to work together to induce actomyosin bundles in cells. Expression of an active form of mDia induces stress fibers in cultured cells, and treatment of these cells with a specific ROCK inhibitor, Y-27632 (Narumiya, Ishizaki et al. 2000), causes dissolution of the bundles, leaving the cells with diffusely distributed actin filaments (Watanabe, Kato et al. 1999). It has also been reported that ROCK and mDia are required in contractile ring formations (Kosako, Yoshida et al. 2000; Watanabe, Ando et al. 2008).

At least six substrates of ROCK are known to play roles in actin cytoskeletal reorganization, including myosin light chain (MLC), myosin-binding subunit of MLC phosphatase, LIM-kinase, adducin, ezrin/radixin/moesin (ERM) family of proteins, and Na^+/H^+ exchange protein (NHE1). Among the six substrates, MLC-phosphatase, MLC, and LIM-kinase, are the three best studied ROCK effectors and have been found to play important roles in driving ROCK's physiological function on the actin cytoskeleton. ROCK inactivates myosin-binding subunit of MLC-phosphatase by phosphorylation (Kimura, Ito et al. 1996; Uehata, Ishizaki et al. 1997). ROCK is also able to phosphorylate myosin light chain directly (Maekawa, Ishizaki et al. 1999). These two actions of ROCK increase the myosin light chain phosphorylation, stimulate cross-linking of actin by myosin and enhance actomyosin contractility. ROCK also phosphorylates and activates LIM-kinase, which in turn phosphorylates and inactivates actin-depolymerizing and severing factor, cofilin (Amano, Ito et al. 1996). The later action of ROCK results in stabilization of existing actin filaments and increase in their content.

The ROCK effectors adducin and the ERM family of proteins regulate actin cytoskeleton in a more direct way. ROCK has been shown to phosphorylate adducin (Kimura, Fukata et al. 1998; Fukata, Oshiro et al. 1999), which, together with spectrin, is an important component of the cortical actin network underlying the plasma membrane (Gardner and Bennett 1987). ROCK-phosphorylated adducin interacts with filamentous-actin (F-actin), and its localization suggests a role in regulating cellular migration. In HGF/SF-stimulated MDCK cells, phosphoadducin localizes to membrane ruffles, and ROCK-phosphorylated adducin localizes to the leading edge of migrating NRK49F fibroblasts in wound healing assays (Fukata, Oshiro et al. 1999); while the introduction of nonphosphorylatable adducin into MDCK and NRK49F cells inhibited membrane ruffling and migration, as did a dominant negative ROCK mutant (Fukata, Oshiro et al. 1999). ROCK can also phosphorylate the ERM proteins that are important for linking actin filaments to the plasma membrane (Matsui, Maeda et al. 1998). Interestingly, it has been demonstrated that the TSC1 tumor suppressor hamartin regulates cell adhesion to cell substrates through the ERM family of actin-binding proteins and RhoA (Lamb, Roy et al. 2000). Finally, NHE1 is well known as a ubiquitous Na^+/H^+ exchange protein that enables stress fiber formation (Tominaga, Ishizaki et al. 1998).

3.2.3 Rac and Cdc42 signaling

In classical Swiss 3T3 fibroblast model, activation of Cdc42 leads to filopodia formation, Rac results in lamellipodia formation and membrane ruffling, and Rho results in stress fibers formation (Nobes and Hall 1995). The cytoskeletal rearrangements caused by Rho GTPases activation play a key role in cell motility. In addition to their effects on the actin cytoskeleton and motility, Rac and Cdc42 also play a role in cell-cell adhesion in epithelial cells. Expression of a constitutively active form of Rac in MDCK cells or keratinocytes leads to an increase in E-cadherin complex members and F-actin at cell-cell contacts, while a dominant negative mutant was found to disrupt cell-cell adhesions (Braga, Machesky et al. 1997; Takaishi, Sasaki et al. 1997; Jou and Nelson 1998). A number of studies have suggested that Cdc42 plays an important role in establishing the initial polarization of epithelial cells, which is required for the proper formation of cell-cell adhesions. For example, transfection of a dominant negative form of Cdc42 in MDCK cells results in the selective depolarization of basolateral membrane proteins due to inhibition of membrane transport (Kroschewski, Hall et al. 1999). Expression of a constitutively active form of Cdc42 in MDCK cells increased AJs and blocked cellular migration induced by HGF/SF (Kodama, Takaishi et al. 1999).

Given the importance of Rac and Cdc42 in the regulation of cell cytoskeletal, adhesion and motility, it has been widely considered that they play important roles in cellular processes related to invasion and metastasis. The first evidence of Rac's role in invasion was obtained when Rac-specific GEF T-lymphoma invasion and metastasis (Tiam-1) was identified in a retroviral insertional mutagenesis screen. Virus-infected T-lymphoma cells were repeatedly selected for *in vitro* invasion through a layer of fibroblasts and the proviral insertions in invasive clones were used to identify the Tiam-1 gene (Habets, Scholtes et al. 1994). Subsequently, Rac, and later Cdc42, were shown to also confer an invasive potential to these T-lymphoma cells (Michiels, Habets et al. 1995; Stam, Michiels et al. 1998). More evidence for Rac and Cdc42's involvement in invasion and metastasis has been provided since then. Expression of the laminin-receptor α6β4 integrin in the melanoma cell line MDA-MB-435 promotes invasiveness in a Rac and PI3-kinase-dependent manner (Shaw, Rabinovitz et al. 1997). In addition, constitutively active forms of Rac and Cdc42 in breast carcinoma cell line T47D promote invasion through a collagen matrix. However, this invasion can be blocked by PI3-Kinase inhibitors, indicating that PI3-kinase acts downstream of Rac and Cdc42 (Keely, Westwick et al. 1997).

3.2.4 Effectors of Rac and Cdc42

A number of Rac and Cdc42 effectors have been identified. Some of these have been found to specifically mediate cell motility, whereas others play a more prominent role in mediating cell adhesion. It is well established that WASP and MRCKs are Cdc42 specific effectors that regulate actin organization and filopodia formation which promotes a more motile phenotype (Aspenstrom, Lindberg et al. 1996; Miki, Miura et al. 1996). In addition, members of the p21-activated kinase family (PAK), downstream of Rac and Cdc42, play important roles in cytoskeletal-mediated changes that affect motility (Manser, Leung et al. 1994). The scalffold proteins IQGAP and Par-6, both of which can be activated by Cdc42 and rac, promote cell polarization and contribute to cell-cell adhesion.

The scaffold protein N-WASP binds to Arp2/3 complexes that are crucial for the assembly of within filopodia (Kolluri, Tolias et al. 1996). It has been shown that both N-WASP and Arp2/3 complexes are required for Cdc42 to trigger actin filament assembly (Welch, DePace

et al. 1997; Miki, Sasaki et al. 1998). Therefore, N-WASP may promote cellular motility through proper filopodia formation. MRCKs α and β are Cdc42 specific effectors that can phosphorylate MLC via a ROCK-like kinase domain (Leung, Chen et al. 1998). It is well accepted that phosphorylation of MLC is required for actomyosin complex assembly and contraction. Overexpression of MRCKα and Cdc42 synergizes to promote filopodia formation, while a MRCKα kinase-deficient mutant inhibits the formation of Cdc42-induced filopodia (Leung, Chen et al. 1998). Therefore, MRCDs are believed to play important roles in cytoskeletal organization and contraction, and contribute to migration. PAK, a protein kinase downstream of Rac and Cdc42, plays a crucial role in actin dynamics and adhesion (Manser, Leung et al. 1994). PAK has been demonstrated to phosphorylate and inactivate MLCK, subsequently causing a decrease in MLC phosphorylation (Sanders, Matsumura et al. 1999). Thus, inactivation of MLCK leads to stress fiber and focal adhesion disassembly. Moreover, PAK controls the actin cytoskeletion through the phosphorylation and subsequent activation of LIM-kinase. Phosphor-LIM-kinsae can further phosphorylate and inactivate the actin-depolymerizing protein cofilin, thus inhibiting actin depolymerization when Rac is activated and causing extreme membrane ruffling (Arber, Barbayannis et al. 1998; Yang, Higuchi et al. 1998). The IQGAP1 and IQGAP2 scaffolding effectors of Cdc42 and Rac regulate cell-cell adhesion through actin polymerization and sequestration of β-catenin (Kuroda, Fukata et al. 1996; Erickson, Cerione et al. 1997). *In vitro*, IQGAP oligomerizes and cross-links F-catin it has also been found to complex with Cdc42 and F-actin *in vivo* (Fukata, Kuroda et al. 1997). In addition, one study has shown that the IQGAP protein also competes with α-catenin for binding to β-catenin, thus preventing E-cadherin/α-catenin/β-catenin complex from attaching to the actin cytoskeleton, and thereby disrupting cell-cell contacts (Erickson, Cerione et al. 1997). Another scaffolding protein, Par-6, was identified using activated Cdc42 and TC10 mutants as baits in yeast two-hybrid screens (Joberty, Petersen et al. 2000; Qiu, Abo et al. 2000). It is known that Par-6 binds to a second scaffolding protein, Par-3, and both Par-6 and Par-3 bind independently to atypical protein kinase C (aPKC) isioforms (Lin, Edwards et al. 2000). In addition, endogenous Par-3 localizes to TJs in MDCK cells, overexpression of Par-6 or the N-terminal portion of Par-3 (the Par-6-interaction responsible region) disrupts TJ formation (Joberty, Petersen et al. 2000).

4. Expression of Rho GTPases in breast tumors

Aberrant Rho signalling resulting from alterations in Rho GTPase protein level, changes in activation status, and abnormal quantity of effector proteins are found in a large variety of human tumors. of GTPases: the Rho family (RhoA, RhoB and RhoC), the Rac family (Rac1, Rac2 and Rac3) and the Cdc42 family, in order to avoid repetitions.

4.1 Rho GTPases in breast tumors

Overexpression of RhoC has been found in inflammatory breast cancer (IBC), an aggressive form of breast cancer that is highly infiltrative and metastatic with poor prognosis for the patients, using in situ hybridization (van Golen, Davies et al. 1999). Compared to normal untransformed parental cells, RhoC-transformed cells produce and secrete high levels of proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and interleukin-8 (IL-8). when compared to normal untransformed parental cells (van Golen, Wu et al. 2000). In addition, microarray

analysis has shown that MCF10A breast cells stably transfected with wild type RhoC or a constitutively active mutant of RhoC overexpress genes associated with invasion and metastasis (Wu, Wu et al. 2004). Other RhoGTPases are also involved in breast tumors. RhoA is found overexpressed in breast tumor tissues but not in the normal tissue (Fritz, Brachetti et al. 2002). The expression of dominant negative RhoA in rat mammary adenocarcinoma cells affects tumor cell growth *in vivo* and reduces intravasation into the peripheral blood, resulting in decrease in lung colonization ability (Fritz, Just et al. 1999). Other studies have indirectly shown an important role of RhoA in breast carcinogenesis. For example, highly metastatic MDA-MB-231 cells that were treated with HMG-CoA reductase inhibitor, namely cerivastain, showed reduced proliferation and invasion through Matrigel, in a RhoA- but not Ras-dependent manner (Denoyelle, Vasse et al. 2001). However, poorly metastatic breast cancer cells such as MCF-7 are less sensitive to cerivastain treatment, indicating that RhoA might be more significantly overexpressed in late stages of breast cancer as with other tumors.

Rho proteins are also important players in breast tumor progression and metastasis exerted by the CD44 hyaluronan receptor (Bourguignon 2001). CD44 is expressed in human breast tumors and promotes cell growth and metastasis in tumor cells. Studies have found that RhoA and CD44 directly interact with each other *in vivo* in highly metastatic human breast cancer cell lines. Accordingly, inhibition of Rho signaling results in the abrogation of the metastatic phenotype elicited by CD44 (Bourguignon, Zhu et al. 1999). RhoA has also been found to be involved in insulin signaling via Shc in human breast cancer (Finlayson, Chappell et al. 2003). Overexpression of insulin receptors correlates with development, progression and outcome of breast cancer, and insulin signaling involves hyperphosphorylation of Shc. Hence, Shc leads to the activation of geranyl transferases, which results in an increased amount of prenylated RhoA in the tumor tissue compared with normal mammary tissue (Finlayson, Chappell et al. 2003). Furthermore, RhoA has been reported to increase the metastatic potential of tumor cells via its ability to promote tumor angiogenesis through the downregulation of thrombospodin-1 (Tsp-1) (Watnick, Cheng et al. 2003). Rho pathway is part of the downstream signaling cascade that is activated by PI3K and leads to ROCK stimulation, Myc phosphorylation and Tsp-1 repression.

4.2 Rac GTPases in breast tumors

The involvement of Rac GTPases in breast cancer was first reported in rodents (Bouzahzah, Albanese et al. 2001). Expression of a dominant negative Rac1 mutant indicated that Rac1 affects tumor cell growth and metastasis *in vivo*. Deregulation of Rac3, closely related to Rac1, has also been detected in breast cancer (Mira, Benard et al. 2000). Rac3 maps to chromosome band 17q25.3, a region known to contain candidate tumor suppressor genes both in breast and ovarian cancers (Morris, Haataja et al. 2000). Highly proliferative breast cancer cells, T47D and MCF-7, but not normal breast cell lines, contain constitutively active Rac3 in a Ras-independent manner (Morris, Haataja et al. 2000). It has also been shown that expression of a dominant negative mutant Rac3 (N17) leads to inhibition of S-phase entry and cellular proliferation in breast tumor cells, which indicate that Rac3 may promote cell growth (Leung, Nagy et al. 2003). Further, the Rac-PAK signaling pathway is essential for receptor tyrosine kinase ErbB2-mediated transformation of human breast epithelial cancer cells (Mazieres, Antonia et al. 2004). Activation of Rac-PAK1 pathway by ErbB2 homodimers can induce growth factor-independent proliferation and promote disruptions to the three-dimensional (3D) mammary acinar-like structures, via activation of the Erk and

Akt pathways (Mazieres, Antonia et al. 2004). Moreover, Rac1 enhances estrogen receptor α (ERα) transcriptional activity, resulting in increased proliferation in breast cancer cells (Rosenblatt, Garcia et al. ; Folkman 1972).

4.3 Cdc42 family in breast tumors

Cdc42 is overexpressed in some breast cancers and there is accumulating evidence that activated Cdc42 contributes to the accumulation of ErbB1 in cells through the regulation of c-Cbl function (Abraham, Kuriakose et al. 2001; Marionnet, Lalou et al. 2003). The view that Cdc42 is involved in human breast carcinogenesis is supported by a rodents model of breast carcinoma where the expression of a dominant negative mutant of Cdc42 reduced the number of focal contacts, inhibited colony formation in soft agar and affected cell growth *in vivo* (Fritz, Just et al. 1999). The dominant negative Cdc42 also reduced intravasation of tumor cells into peripheral blood and ability to form lung metastasis. In addition, through the activation of Cdc42, transforming growth factor α (TGF-α) mediates the invasion of MDA-MB-231 cells into 3-D collagen matrices by initiating the formation of protrusions into collagen. (Kamai, Tsujii et al. 2003; Fisher, Sacharidou et al. 2009). Further, another study has shown that membrane-type-1 matrix metalloproteinase (MT1-MMP) and Cdc42 are fundamental components of a co-associated invasion-signaling complex that controls directed single-cell invasion of 3D collagen matrices (Fisher, Sacharidou et al. 2009).

5. Multiple functions mediated by Rho GTPases in breast cancer

Rho GTPases mediate housekeeping aspects of cell biology including cell growth, cell polarity, cell adhesion, membrane trafficking and motility. They function as signaling switches that regulate lipid metabolism, microtubules- and actin-based structures, epithelial cell-junctions, cell cycle and apoptosis regulatory proteins, and transcription factors.

5.1 Rho GTPases and cytoskeleton organization

Eukaryotic cellular morphology and attachment to the substratum in response to extracellular signals are largely dependent on rearrangements of the actin cytoskeleton. Cell motility, cytokinesis and phagocytosis all rely on coordinated regulation of the actin cytoskeleton (Small 1994; Zigmond 1996). Filamentous actin can be organized into several discrete structures: (a) filopodia, finger-like protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. These are found primarily in motile cells and neuronal growth cones. (b) lamellipodia, thin protrusive actin sheets that dominate the edges of cultured fibroblasts and many other motile cells. Membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substrate and fold backward. (c) actin stress fibers, bundles of actin filaments that traverse the cell and are linked to the ECM through focal adhesion (Van Aelst and D'Souza-Schorey 1997). The actin polymerization is tightly regulated by Rho GTPases.

Rho activation in fibroblasts is known to stimulate the assembly of contractile actin/myosin filaments, the formation of stress fibers, and the clustering of integrins involved in the formation of focal adhesion complexes. Activation of Rac facilitates actin polymerization at the cell periphery to generate protrusive actin-rich lamellipodia and membrane ruffling. And activation of Cdc42 results in actin polymerization to form peripheral actin microspikes and filopidia. As described previously, a number of proteins have been identified as targets

of Rho, Rac and Cdc42 (Fig. 3). Most of them are involved in Rho GTPases mediated cytoskeletal rearrangements (Tang, Olufemi et al. 2008).

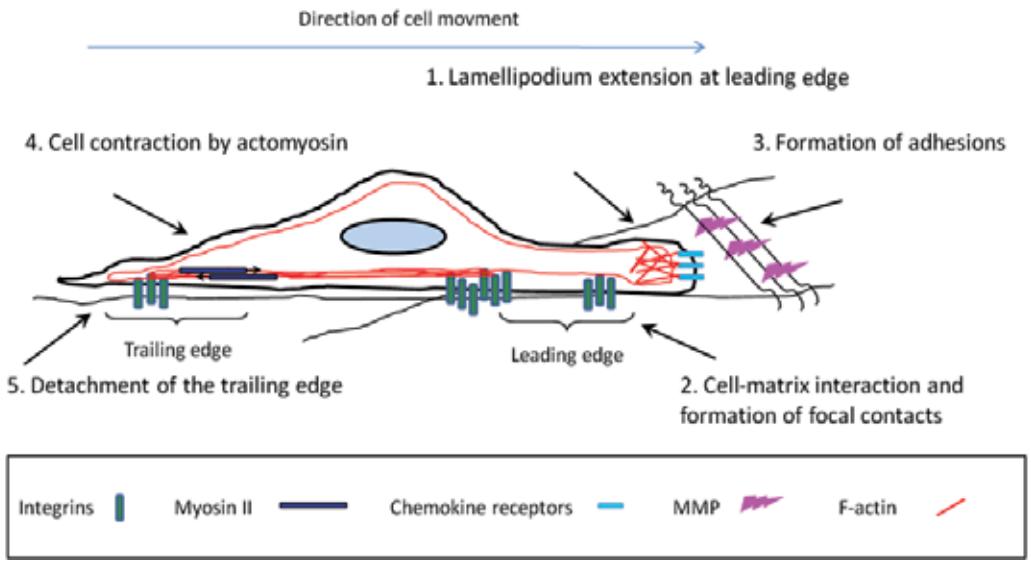

Fig. 3. A model of the cellular migratory process. See text for detailed explanation of motility phases.

5.2 Rho GTPases in cell migration

Cell migration is a multistep process involving polarization, sequential cell protrusion and adhesion formation in the direction of migration, cells body contraction, and tail detachment (Pinner and Sahai 2008). During the migration process, cells move with extending protrusions at the front and a retracting tail at the rear, both regulated by members of the Rho GTPases family (Ridley, Schwartz et al. 2003). The idea that Rho family GTPases could regulate cell migration derives from observations that they mediate the formation of specific actin containing structures. In addition, Rho proteins regulate several other processes that are relevant to cell migration, including cell-substrate adhesion, cell-cell adhesion, protein secretion, vesicle trafficking, and transcription.

5.2.1 Cell polarization and lamellipodium extension at the leading edge

An asymmetrical organization of intracellular activities is required for a cell to move, that means the molecular processes at the leading and trailing edges of a moving cell must be different. Establishing and maintaining cell polarity in response to extracellular stimuli appear to be mediated by Rho family GTPases.

Cdc42 is well accepted as a master regulator of cell polarity in eukaryotic organisms ranging from yeast to human.Cdc42 was first studied in a budding yeast model for its involvement in cell polarity. During the cell cycle, yeast cells adopt alternative states of growth to non-focused isotropic growth. In the absence of Cdc42, *Saccharomyces cerevisiae* fail to establish focused apical growth and, cells expand isotropically (Pruyne and Bretscher 2000). Cdc42 regulates cell polarity by deciding the location of lamellipodia formation (Srinivasan, Wang et al. 2003). In addition, Cdc42 directs the localization of the microtubule-organizing center

(MTOC) and Golgi apparatus to the front of the nucleus, oriented toward the direction of movement. MTOC orientation at the leading edge then facilitates the delivery of Golgi derived vesicles to the leading edge and microtubule growth into the lamellipodium (Rodriguez, Schaefer et al. 2003). It has been further studied that Cdc42 exerts its effect on MTOC through its downstream effector, PAK1 (Li, Hannigan et al. 2003).

5.2.2 Protrusion formation

Inherent polarity drives the formation of membrane protrusions, and the organization of filaments depends on the type of protrusion. Actin filaments form a branching dendritic network in lamellipodia, but form long parallel bundles in filopodia (Pollard, Blanchoin et al. 2000). The dendritic organization of lamelipodia that provides a tight brush-like structure, formed via the actin-nucleating activity of the actin-related proteins 2/3 (Arp2/3) protein complex (Urban, Jacob et al.). Rac stimulates new actin polymerization by acting on Arp2/3 complexes, which binds to pre-existing filaments (Campellone and Welch). Activation of Arp2/3 complexes by Rac is carried out through its target IRSp53. Upon activation, IRSp53 interacts with WAVE through its SH3 domain, it then binds to and activates Arp2/3 complexes (Chesarone and Goode 2009). It has also been reported that IRSp53 binds to Cdc42 through a separate domain (Miki, Yamaguchi et al. 2000). So, IRSp53 can serve as a direct link between Cdc42 and Rac, which explains how Cdc42 induces Rac involvement in lamellipodium formation. Furthermore, IRSp53 can bind to a Rho target, Dial, which might underlie the capability of Rho to facilitate lamellipodium extension (Cox and Huttenlocher 1998; Fujiwara, Mammoto et al. 2000).

5.2.3 Cell-substrate adhesions

Newly formed focal adhesion complexes are localized in the lamellipodia of most migrating cells. Once the lamellipodium attach to the ECM, integrins come into contact with ECM ligands and cluster in the cell membrane where they interact with FAK, α-actin, and talin (Cox and Huttenlocher 1998). All these proteins can bind to adaptor proteins through Src-homologous domain 2 and 3 (SH2, SH3) as well as proline rich domains to more actin binding proteins (vinculin, paxillin and α-actin) and regulatory molecules PI3K to focal complexes (Zamir and Geiger 2001). Rac is required for focal complex assembly, and Rac itself can be activated by cell-substrate ECM adhesion (Rottner, Hall et al. 1999). It is suggested that the adhesion assemblies in migrating cells begin with small-scale clustering and the speed of the cell migration is dependent on ECM composition, which determines the relative activated levels of Rho, Rac and Cdc42 (Price, Leng et al. 1998). Therefore, interactions between ECM and integrins at the leading edge of cells play an important role in maintaining the level of active Rac. This indicates the existence of a positive feedback loop that allows continuous crosstalk between integrins and Rac, and allows cells to respond to changing ECM composition.

5.2.4 Cell body contraction by actomyosin complexes

Cell body contraction is driven by actomyosin contractility and the force transmitted to sites of adhesion derives from myosin II. Myosin II, which is predominantly induced by Rho and its downstream effector ROCK, controls stress fiber assembly and contraction. Rho acts via ROCKs to affect MLC phosphorylation by inhibiting MLC phosphatase or the MLC phosphorylation. MLC phosphorylation is also regulated by MLCK, which is controlled by both intracellular calcium concentration and ERK MAPKs (Fukata, Amano et al. 2001).

ROCKs and MLCK have been suggested to act in concert to regulate different aspects of cell contractility, since ROCK appears to be required for MLC phosphorylation which are associated with actin filaments in the cell body, and MLCK is required at the cell periphery (Totsukawa, Yamakita et al. 2000).

5.2.5 Adhesion disassembly and tail detachment

Tail detachment occurs when cell-substrate linkages are preferentially disrupted at the rear of a migrating cell, while the leading edge remains attached to the ECM and continues to elongate (Palecek, Huttenlocher et al. 1998). Mechanisms underlying the focal complex disassembly and tail detachment depend on the type of cell and strength of adhesion to the extracellular matrix at the trailing edge (Wear, Schafer et al. 2000). In slow moving cells, tail detachment depend on the action of a calcium-dependent, non-lysosomal cysteine protease calpain that cleaves focal complex components like talin and cytoplasmic tail of β1 and β3 integrins along the trailing edge (Potter, Tirnauer et al. 1998). Strong tension forces exerted across the cells at the rear adhesions is required to break the physical link between integrin and the actin cytoskeleton. Rho and myosin II are involved in this event. Furthermore, Rho plays important roles in reducing adhesion and promoting tail detachment in fibroblasts, which have relatively large focal adhesion complexes (Cox and Huttenlocher 1998).

5.3 Rho GTPases and transcriptional activation

A number of studies have suggested that Rho family GTPases are involved in the regulation of nuclear signaling. Rac and Cdc42, but not Rho, have been demonstrated to regulate the activation of JNK and reactivate kinase p38RK in certain cell types (Seger and Krebs 1995). Expression of constitutively active forms of Rac and Cdc42 in HeLa, NIH-3T3, and Cos cells stimulates JNK and p38 activity (Coso, Chiariello et al. 1995). Furthermore, these same effects were observed with oncogenic GEFs for these Rho proteins (Minden, Lin et al. 1995). However in human kidney 293 T cells, Cdc42 and the Rho protein, but not Rac, induces the activation of JNK (Teramoto, Crespo et al. 1996). Upon activation, JNKs and p38 translocate to the nucleus where they phosphorylate transcription factors, including c-Jun, ATF2, and Elk (Derijard, Hibi et al. 1994; Gille, Strahl et al. 1995). Further, Rac has been shown to activate PEA3, a member of the Ets family, in a JNK-dependent manner (O'Hagan, Tozer et al. 1996). Activated p38 phosphorylates ATF2, Elk, Max, and the cAMP response element binding protein.

PAKs are the farthest known upstream kinases that connect Rho GTPases to JNK and p38 through GTP-dependent bindings to Rac and Cdc42 *in vitro* and are activated after binding to activated Rac and Cdc42. (Manser, Chong et al. 1995). In addition, certain constitutively active forms of PAK can activate JNK and p38 (Zhang, Han et al. 1995). Further, a mutant effector of Rac that cannot bind to PAK remains a potent JNK activator (Westwick, Lambert et al. 1997). These observations suggest that other kinases, in addition to PAK, participate in the signalling from Rho GTPases to JNK. Supporting this, MLK3 and MEKK4 are found to be regulated by Cdc42 and Rac, and selectively activate the JNK pathway (Gerwins, Blank et al. 1997). It has also been reported that Cdc42/Rac can bind to MLK3 both *in vitro* and *in vivo* and that the coexpression of activated Cdc42/Rac mutants elevates the enzymatic activity of MLK3 in Cos-7 cells (Teramoto, Coso et al. 1996; Gerwins, Blank et al. 1997). In addition, Rho, Rac and Cdc42 stimulate the activation of the serum responsive factor (SRF) (Hill, Wynne et al. 1995). SRF forms a complex with TCF/Elk proteins to stimulate transcription

with serum response elements (SREs) at their promoter enhancer regions, for example the Fos promoter (Treisman 1990).

5.4 Rho GTPases and cell growth control

Several lines of evidence have suggested that Rho family members play important roles in several aspects of cell growth. The Rho proteins have been shown to increase expression of cyclin D1, a cell cycle regulator that controls the transition from G_1 phase to S phase, in Swiss 3T3 fibroblasts (Yamamoto, Marui et al. 1993; Olson, Ashworth et al. 1995) and in mammary epithelial cells (Liberto, Cobrinik et al. 2002). Overexpression of RhoE inhibits cell cycle progression by inhibiting translation of cyclin D1 mRNA (Villalonga, Guasch et al. 2004). In fibroblasts, RhoA is involved in ERK activation and subsequent cyclin D1 expression (Roovers and Assoian 2003). RhoA also downregulates cdk inhibitors p21 and p27 during the G1 phase of the cell cycle (Weber, Hu et al. 1997). Rac 1 is capable of regulating the cell cyle through the activation of a number of distinct intra-cellular pathways, including the NFκB pathway. In contrast to other Rho proteins, Rac1 can directly activate cyclin D1 expression (Page, Li et al. 1999).

Furthermore, Rho, Rac, and Cdc42 have been demonstrated to possess transforming and oncogenic potential in some cell lines. For example, cells with constitutively active forms of Rac and Rho display enhanced anchorage independent growth ability, and initiate tumor formation when inoculated into nude mice (Khosravi-Far, Solski et al. 1995). The observation that Tiam, a Rac GEF, can transform NIH-3T3 cells suggests a role for Rac in transformation (van Leeuwen, van der Kammen et al. 1995). While expression of constitutively activated Rac is sufficient to cause malignant transformation of rodent fibroblasts (Qiu, Chen et al. 1995), this is not the case with Rho (Qiu, Chen et al. 1995), suggesting that the growth-promoting effects of the Rho GTPases are specific to cell type. Evidence of Cdc42's role in cell growth has been provided in fibroblasts. The constitutively active mutant of Cdc42 stimulates anchorage independent growth and proliferation in nude mice (Qiu, Abo et al. 1997). Using a Cdc42 mutant, Cdc42(F28L), that can undergo GTP-GDP exchange in the absence of GEF, one study demonstrated that cells stably tranfected with Cdc42(F28L) exhibited not only anchorage-independent growth but also lower dependence on serum for growth (Lin, Bagrodia et al. 1997). A role for Cdc42 in Ras transformation has also been established in Rat 1 fibroblasts. Coexpression of a dominant negative form of Cdc42, Cdc42N17, with oncogenic Ras results in inhibition of RasV12-induced focus formation and anchorage-independent growth, and reversed the change in morphology in RasV12-transformed cells (Qiu, Abo et al. 1997).

5.5 Rho GTPases and angiogenesis

Beside their roles in multiple processes of cellular control, tumor growth, progression and metastasis, the Rho proteins have also been shown to be involved in angiogenesis, a process Where new blood vessels arise from existing mature vessels. This process is controlled by a number of pro-angiogenic and anti-angiogenic factors at different stages (Folkman 1972). The major pro-angiogenic factors are comprised of vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), platelet derived growth factor-β (PDGFβ), angiopoietins 1 and 2 (Ang-1 and 2), tumor necrosis factor (TNF), interleukin 6 and 8 (IL-6 and 8), and epidermal growth factor (EGF). The main anti-angiogenic foctors include the thrombospondins (TSPs), angiostatin, and endostain (Merajver and Usmani 2005). The Rho

proteins are believed to be capable of altering the expression and activity of pro-angiogenic and anti-angiogenic factors during angiogenesis.

5.5.1 Regulation of VEGF and hypoxia inducible factor-1 (HIF1)

It has been reported that hypoxia increases the expression and activity of Cdc42, Rac1 and RhoA in renal cell carcinoma cell lines and a human microvascular endothelial cell line (Turcotte, Desrosiers et al. 2003). This study demonstrated that reactive oxygen species (ROS) are responsible for the upregulation of Rho proteins and that RhoA is required for the accumulation of HIF-1α (Turcotte, Desrosiers et al. 2003), a transcription factor induced by hypoxia that plays important roles in the process of angiogenesis by inducing the transcription of crucial mediators, including VEGF, PDGFβ and Ang-2 (Gleadle and Ratcliffe 1998). In contrast, Rac1 is shown to be involved in hypoxia-induced PI3K activation of HIF-1α through a different mechanism (Hirota and Semenza 2001). Hypoxia-induced expression of Rac1 also contributes to the upregulation of HIF-1α and, subsequently, VEGF in gastric and hepatocellular cancer cells (Xue, Bi et al. 2004). VEGF has been reported to increase RhoA activity and localization to the cell membrane, and the RhoA /ROCK pathway has been implicated in the VEGF-mediated angiogenesis (van Nieuw Amerongen, Koolwijk et al. 2003). In addition, RhoA activation also increases tyrosine phosphorylation of the primary VEGF receptor, VEGFR-2 (Gingras, Lamy et al. 2000).

Overexpression of RhoC in human mammary epithelial cells (HME) and a highly aggressive breast cancer cell line, SUM-149, increases VEGF expression (van Golen, Wu et al. 2000). Similar finding were found in the MCF10A cells (Wu, Wu et al. 2004), further suggesting that RhoC plays a role in, further suggesting that RhoC plays a role in increasing VEGF in mammary neoplasis.

5.5.2 IL-6 and IL-8 expression

IL-6 is a multifunctional cytokine that is involved in many different biological process, including immunological and inflammatory processes, tumor growth and angiogenesis (Hirano, Akira et al. 1990; Mateo, Reichner et al. 1994). IL-8 is another important cytokine that acts as a pro-angiogenic factor. Both of these cytokines can be induced by hypoxia (Yan, Tritto et al. 1995; Mizukami, Jo et al. 2005) and have been shown to upregulate VEGF mRNA expression (Cohen, Nahari et al. 1996). Studies indicate that active Rho proteins upregulate the expression of NFκB components in NIH-3T3 cells (Perona, Montaner et al. 1997; Montaner, Perona et al. 1998). Consistent with Rho-mediated activation of NFκB, HKG-CoA reductase inhibitors had been reported to reduce IL-6 expression by inhibiting Rho proteins (Ito, Ikeda et al. 2002). Rac1 has been shown to mediate the activation of a potential oncogen, STAT3, through NFκB regulated IL-6 signaling (Faruqi, Gomez et al. 2001).

IL-8 expression has also been found to be regulated by Rho proteins. In human endothelial cells, it has been shown that inhibition of RhoA, Rac1 and Cdc42 decreases NFκB activation and, therefore, decreases IL-8 mRNA and IL-8 protein expression (Hippenstiel, Soeth et al. 2000; Warny, Keates et al. 2000). In addition, RhoC has been shown to increase IL-6 and IL-8 expression in aggressive breast cancer cell lines (Xue, Bi et al. 2004). These evidences suggest that different Rho proteins modulate IL-6 and IL-8 through distinct signaling pathways.

5.5.3 FGF activation

FGF1 and FGF2 are the two earliest characterized members of the FGF family of growth factors. FGF is an angiogenic factor that is frequently overexpressed in breast and prostate cancers. Rac1 and Cdc42 have been reported to increase FGF1 expression by stimulating the FGF1 gene promoter region (Chotani, Touhalisky et al. 2000). One study demonstrated that Rac1 activity is required for FGF2-induced activation of Ras/MAPK signaling in human breast cell line MCF7 (Liu, Chevet et al. 1999). In addition, medium collected from RhoC stably transfected HME and SUM149 cells present higher level of FGF2, in comparison to those collected from control transfected HME cells (van Golen, Wu et al. 2000). However, how Rho proteins regulate FGF expression remains unclear.

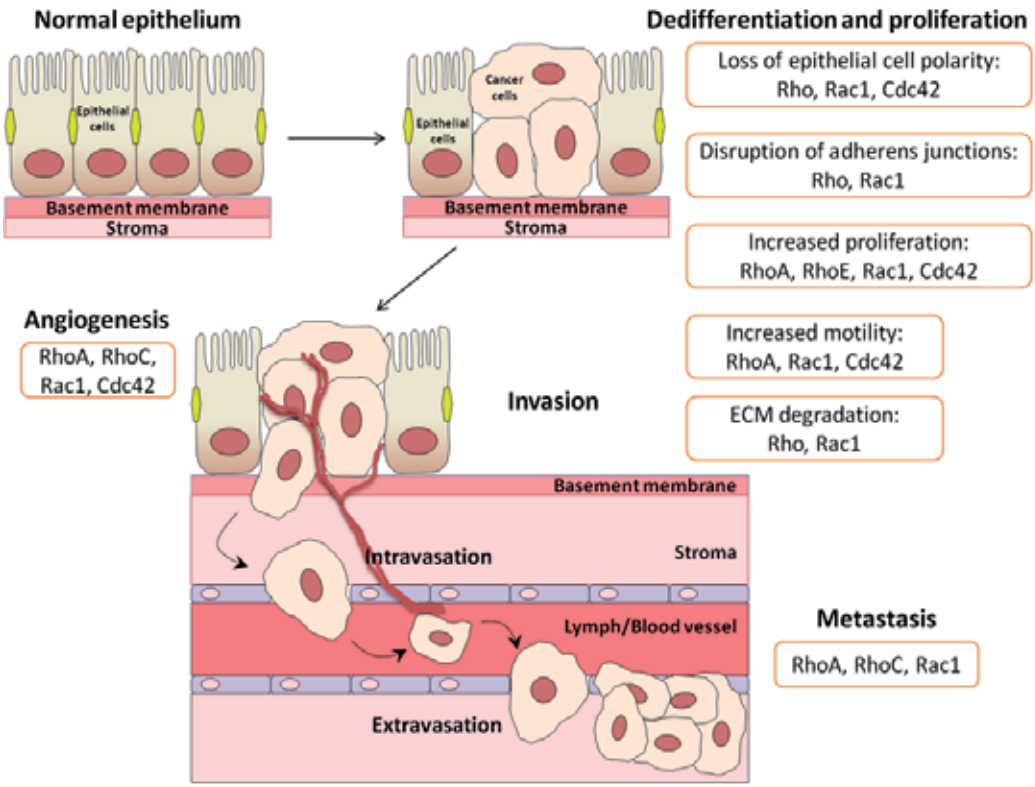

Fig. 4. Rho family GTPases are involved in different stages of breast cancer progression: dedifferentiation and upregulation of uncontrolled proliferation, angiogenesis, invasion and metastasis.

5.5.4 Repression of Tsp-1

The anti-angiogenic molecule Tsp-1 is capable of inhibiting metalloproteinase-9 (MMP9) from releasing the VEGF sequestered in ECM. The oncoprotein Ras has been reported to increase VEGF expression and inhibit Tsp-1 expression. One study showed that the inhibitory function of Ras on Tsp-1 via PI3K pathway also involve RhoA and RhoC in human embryonic kidney cell lines, human mammary cell lines, and breast cancer cell lines

(Watnick, Cheng et al. 2003). And the suppression of Tsp-1 always correlates with promotion of angiogenesis.

6. Conclusion

It is apparent that individual members of Rho GTPases play specific roles in different aspects in breast cancer development (Fig. 4). Aberrant expression and activity of Rho proteins contribute to the transformation from normal epithelial phenotype, increases in proliferation, the promotion of angiogenesis, elevated motility, and metastasis to distant organs. RhoA, RhoC and Rac1 are frequently overexpressed in metastatic breast cancers. Manipulating the Rho GTPases' regulatory proteins and their effectors can induce activation of Rho proteins, , leading to aberrant transcription factor activation, including that of NFκB, that contribute to invasive phenotypes. All this evidence suggests that Rho GTPases could be targets in cancer therapy. Therefore, better knowledge of the the regulation mechanisms of Rho GTPases in breast cancer may be critical for a more in-depth understanding of tumor biology, facilitating development of novel approaches for cancer treatment.

7. References

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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