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Embryonic Stem Cells

Recent Advances in Pluripotent Stem Cell-Based Regenerative Medicine

Edited by Craig Atwood

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Notice

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Meet the editor

Craig S. Atwood, Ph.D. is an Associate Professor of Medicine at the University of Wisconsin and a Health Science Specialist with the Geriatric Research, Education and Clinical Center at the William S. Middleton Memorial Veterans Administration Hospital in Madison. Dr. Atwood completed his Ph.D. in Biochemistry from the University of Western Australia in Perth, Australia prior to post-doctoral fellowships at the National Cancer Institute, NIH, Bethesda, and Massachusetts General Hospital, Charlestown. He held faculty positions at Harvard Medical School and Case Western Reserve University prior to his current appointment where he directs the research program of the Laboratory for Endocrinology, Aging and Disease. Dr. Atwood has broad research interests related to the endocrinology of embryogenesis, adulthood and senescence as elaborated upon in 'The Reproductive-Cell Cycle Theory of Aging'. He has published over 200 scientific articles, has served on numerous review boards and is an Editor of 20 scientific journals including the Journal of Biological Chemistry. In 2006 he received the Zenith Fellows Award from the Alzheimer's Association in recognition of his research.

Contents

Preface

The hope that one day human pluripotent stem cells (hPSCs) can be utilized for the treatment of various diseases has swept throughout the world since the isolation and in vitro maintenance of human embryonic stem cells (hESCs) just before the end of the last millennium (Thomson et al. 1998). Contrasting with this tremendous hope of hESCs for regenerative medicine and continued mortality lies the moral issues related to the use of pluripotent ESCs obtained from the discarded embryos of in vitro fertilization (the termination of life). In the first section of this book, 'Clinical Applications and Ethical Considerations' the first chapter by Malgieri et al. highlights the potential for stem cell technologies, including hESC technologies, in regenerative medicine. The second chapter by Birnbacher delves into the complex bioethical and biopolitical issues of using hESCs for basic and applied applications. Birnbacher provides a thoughtful and in depth assessment of complicity- the role of scientists as accomplices in the propagation of a "wrong doing". As Birnbacher points out, the wrong doing is not so much the use of ESCs, themselves not human embryos, "but moral concerns as to the source from which the stem cells are derived and the methods by which they are retrieved (or the life terminated)." As the authors continue, "Complicity is the core of the criticism levelled against research on pluripotent hESC in many quarters and has even, in some countries, become the basis of legal prohibitions. Complicity is a problem only for those who are torn between the conviction that embryo research is (for intrinsic or extrinsic reasons) a moral evil and the conviction that hESC research is worth pursuing either for its medical or for its scientific prospects or both. The class most likely to face this uncomfortable dilemma is the class of conservative politicians in countries such as Germany and Italy in which embryo research is strictly prohibited by law, but in which hESC research is nevertheless permitted or even encouraged, although only with raw material imported from countries with more permissive laws. For these politicians, complicity is, and should be, the stumbling block lying in the way of pragmatic compromise".

The potential for using PSCs (e.g. hESCs and induced pluripotent stem cells (iPSCs) for specific diseases and conditions is reviewed in the next 16 chapters. Heart disease is the biggest killer in the Western world (USA - 26% of all deaths), and in the next section, 'Tissue-specific Regeneration of the Heart', Hao et al. examine the possibility of one day engineering autologous replacement cardiomyocytes via iPSCs, and review the small molecules that to date direct differentiation of stem cells down the cardiomyogenic pathway. Sasaki and Okano next discuss the advantages and disadvantages of different methods for the generation of cardiomyocytes from ESCs – namely the hanging drop method, suspension culture method and the cell-patterning method.

They end their chapter with a discussion of the cell sheet method for transplantation of cardiomyocytes into the patient.

Age-related neurodegeneration leading to stroke, dementia and Parkinson's disease accounts for around 10% of deaths in the United States. In the Section on 'Tissue-specific Regeneration of the Brain and Sensory Organs', Kujoth and Başkaya report on the use of ESC-derived multipotent mesenchymal stromal cells (MSC; also known as mesenchymal stem cells) for the treatment of stroke from studies of focal ischemia in the rat. These authors discuss the mechanisms by which MSC may be trophic, as well as the potential pitfalls - allograft rejection and limited long-term cell survival/replacement – in the hostile post-ischemic environment, issues that likely pertain to all replacement therapies in all tissues of the aging individual. In the next chapters, Fan et al. discuss the use of ESC transplantation for a range of neurological diseases, while Daadi focuses on the cellular and molecular control of neural stem cell derivation from adult and pluripotent stem cells and their differentiation into dopaminergic lineage for the treatment of Parkinson's disease. Hargus and Bernreuther next weigh in on the practical issues that need to be overcome in order to successfully transplant neurons into the brain in order to restore function. As the authors elaborate, these factors include adequate differentiation, survival, migration, and integration of transplanted cells, as well as the prevention of teratomas. The authors next provide examples, including from their own research, of how cell adhesion molecules (L1) and extracellular matrix molecules (tenascin-R) can be applied (transfected) to successfully modify ESCs for cell therapy approaches in animal models of neurological diseases. These molecules provide important support to cells, participate in the control of cell development (neurite outgrowth, synapse formation, and cell migration), and mediate cell survival both *in vitro* and *in vivo*. Neuron support and surveillance also is provided by microglia, and in the next chapter, Roy et al. describe protocols for the differentiation of human and mouse PSCs into microglia, and how such cells can be expanded in number for drug screening and cell therapies (combating cancer, neurodegeneration and repair of brain lesions).

Familial and age-related sensory loss (that affect nearly all with age) is the focus of the next 3 chapters. Sensorineural hearing loss is a major public health problem caused by the loss or damage of sensory hair cells in the organ of Corti and degeneration of spiral ganglion neurons (SGNs) or neurons in the auditory brainstem. Hashino and Fritsch and Nishimura report on the present status of development of stem cell-based therapies aimed at inner ear regeneration. Importantly, Hashino and Fritsch describe the how T cell leukemia 3 (Tlx3) confers ESCs differentiation into neurons with a glutamatergic neurotransmitter phenotype, which is accompanied by establishment of proper synaptic assembly and axon outgrowth. The authors also highlight the fact that Tlx3-expressing ESCs can migrate towards degenerating SGNs in the inner ear of host animals and how such cells may be used to replace damaged SGNs, which cause irreversible hearing loss in humans. Both authors discuss technical issues related to surgical approaches for safe and efficient transplantation of stem cells in the human cochlea, as well as, non-invasive monitoring of stem cell engraftment in the cochlea. The potential use of PSCs for the treatment of retinal diseases such as diabetic retinopathy and age-related macular degeneration are examined in the next chapter by Messina, who examines current protocols (and hurdles) for the differentiation of mouse iPSCs into photoreceptors and the functional integration of the transplanted cells. As suggested

by the author, taking cues from the hormonal signals regulating retinal development and differentiation will help improve *in vitro* protocols for photoreceptor production.

The tissues with the most immediate potential for replacement are various hematopoietic cell types. In the next 3 chapters of the section 'Tissue-specific Regeneration of Hematopoietic Systems', Feng et al., Yokoyama et al. and Nakamura report on the various methods that have been used to generate almost all types of blood cells from hESCs (and mESCs), including functionally mature erythrocytes and neutrophils, platelets, megakaryocytes, eosinophils, monocytes, dendritic cells (DCs), nature killer (NK) cells, mast cells, and B-, T-lineage lymphoid cells. As the authors note, these advances will surely translate into clinical applications in the short-term in the field of transfusion therapies (erythrocytes and platelets) and immune therapies (NK cells and DCs).

Regenerative medicine approaches are described for the liver, thymus and gonads in the next three chapters of the section 'Tissue-specific Regeneration of Other Tissues'. Imamura describes research on the generation of hepatocytes for liver disease; the culturing of embryoid bodies in collagen scaffolds for 24 days with exogenous growth factors and hormones associated with liver development results in cord-like structures containing immature hepatocytes. They demonstrate that these cells within collagen scaffolds form hepatic lobule-like aggregates in the livers of partially hepatectomized mice. Similarly, Lai demonstrate that murine ESCs can be selectively induced to differentiate into thymic epithelial progenitors with specific growth factors. Age-dependent thymic involution, various genetic and infectious diseases, and protracted T cell deficiencies following chemotherapy or radiotherapy and preparative regimens for foreign tissue or organ transplants, are some of the conditions that would benefit from this research. Kerkis et al. next describe the remarkable progress that has been made in the differentiation of mouse ESCs in the derivation of germ cells and male and female gametes, although further research is required to generate functional gametes that could be used for reproductive applications.

In the final section of this book, 'Side Effects of Pluripotent Stem Cell Therapies', 2 groups review the potential of pluripotency for normal tissue differentiation, but the consequences of the loss of ESCs genome stability. Li and Tanaka review how intrinsic and extrinsic factors can promote the uncontrolled differentiation of PSCs leading to tumorigenesis, while Moriguchi et al. find that induction of p21 is necessary to avoid malignant transformations of human iPSCs. Silván et al. examine the similarities between cancer stem cells and ESCs and the potential for the Yamanaka genes to induce cancer stem cells. Finally, Rebuzzini et al. point out that propagation of ESCs for extended culture periods leads to recurrent abnormalities in hESCs and random karyotypic changes in all chromosomes, changes that must be continually monitored. These authors describe chromosome abnormalities in ESC lines of human, primates and rodents and the possible causes of karyotype variation during culture.

It is hoped that the research and reviews described here will help to update the ESC research community on recent advances in the generation of tissue specific cell types for regenerative applications. The next few decades will see the realization of the potential for hESCs to treat certain diseases and conditions. However, it is clear from these reviews that much progress is required in the areas of PSC differentiation into tissue-specific cells, transplantation and immune rejection, and genomic stability before PSCs

can fulfill their promise. While certain small FDA clinical trials are currently underway, if the history of another 'Cinderella' technology, gene therapy, is any marker of the progression of PSC research for clinical treatment, it will likely be decades before PSCs are routinely used to treat age-related diseases, with perhaps the exception of hematopoietic applications.

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Part 1

Clinical Applications and Ethical Considerations

1

The Role of Complicity in the Ethics of Embryonic Stem Cell Research

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1. Introduction

In everyday language, complicity means taking part, in one way or the other, in a crime or wrongdoing done by another person. In general, the role of the accomplice is not strictly of the same quality as that of the main actor or, in legal terminology, of the principal. As a rule it is judged to of minor gravity. Nevertheless, if the offense is grave enough, the accomplice may be found guilty, in law, as an accessory to the crime, or, in general morality, become the object of moral reproach as standing in a relation to the wrongdoer analogous to that of accessory and principal.

During the last decades, bioethical discussion has considerably extended the scope of the concept of complicity. Quite a number of further semantic facets were added to its standard meaning, making the concept extremely fruitful for a fine-grained ethical analysis of certain moral constellations involving a plurality of agents (cf. Singer 1993, 165 f., Luna, 1997, Friele 2000). These extensions concern weaker kinds of collaboration or collusion with a morally wrong act than the legal concept, in particular practices of making use of the results or products of a morally wrong or morally problematic activity. This kind of "eating fruit from a forbidden tree" is not usually subjected to the same moral criticism as the wrong act itself (henceforth "W"). But "complicity" continues to imply some kind of moral offensiveness. The concept keeps its negative connotation, though the degree to which the wrong of the "accomplice" (henceforth "C") is held be condemnable is usually significantly lower than that of the act to which it is accessory. Though the act of the "accomplice" is much less grave than the original act, it nevertheless leaves a "moral stain", however weak, on the accomplice.

2. Five types of complicity

Before discussing how complicity is exemplified, under certain premises, by embryonic stem cell research, let us first pass in review the most important of the possible relations that can obtain between W, the wrongdoer, and the "accomplice" C, in the standard and in the extended sense. As far as I see, five types of complicity can be distinguished (Table 1).

Whereas the first variant constitutes the most clear-cut and indeed "classical" case of complicity, complicity in the other variants is more indirect. In variant 2, complicity consists in achieving the goals of an act W that is itself blameworthy but that would not achieve its end without the collaboration of some other agent. C as it were brings the process to the end it was intended for from the start. Whoever knowingly sells stolen goods is not a thief.

However, he completes, as it were, the act of stealing and realizes the end for which, as a rule, the original act was undertaken. The same holds for the seller and the buyer of goods produced under morally problematic conditions, such as children's labour, or, from the perspective of the ethical vegetarian, for sellers and buyers of meat from animals. In these cases, although the fact that there are "accomplices" who realize the purposes for which W is undertaken is not necessary for W, which would constitute an offense in its own right, C is necessary for W to have a point. Furthermore, the "accomplice" is necessary if W is to become a practice and to establish an expectation on the side of the doer of W that he will be able to profit from what he does. In many cases, a general abandonment of C would stop the practice of W. Without the seller of stolen goods, stealing would lose much of its attraction, as would slaughtering animals without meat-eating customers.

	Act constituting complicity	*Complicity relation*
1	C actively participates in carrying out W	Direct active participation
2	C achieves the end for which W was carried out	Completion of W
3	C contributes to W becoming a practice by encouraging others, including the original agent, to carry out W	Imitation effect
4	C encourages others to become less critical of W (and, possibly, to co-operate with W-doers)	Re-evaluation effect
5	C is, or is seen as, incongruent with the moral rejection of W	Expressive dissonance

Table 1. Types of complicity

In variant 3, complicity lies in the effect C has on the tendency of others to imitate W, either in its own right or because of what can be gained from its direct or indirect effects. By making use of W for positively valued aims, C may encourage others to risk W despite its moral offensiveness, especially if W is the only or the only available means to achieve these aims. Even if W lies in the past, making use of its results has, as a rule, a certain probability to encourage similar acts in the future, where the probability of this happening depends on several factors, among them the publicity of C and the reputation of the agent. A convinced vegetarian with a certain influence on his social environment thereby has an additional reason to abstain from buying meat at the supermarket. Indeed, professed vegetarians like Albert Schweitzer have been rightly criticized by ideologists of vegetarianism for not being consistent in their behaviour given their role as moral models. By breaking the rules they are perceived to stand for they invite imitation and tend to weaken the moral judgements underlying the rejection of W.

In variant 4, this weakening takes a different turn. The role of complicity consists in inviting not an imitation but a re-evaluation of W given its favourable effects. This type of complicity is exemplified by cases in which C is judged to have an influence on the moral evaluation of W irrespective of whether C is thought to have a direct or indirect influence on the practice of W itself. A relevant example is the use of cells and tissues from aborted embryos. It is unlikely that using tissues from aborted embryos has a significant effect on the frequency with which abortions are carried out, and most of the commentators on the practice know that perfectly well. After all, the international guidelines for the transplantation of embryonic neural tissue explicitly demand that the woman's decision to have an abortion be independent from her decision to allow using the aborted embryo for research or therapeutic purposes. Nor is it reasonable to expect that the part played by gynaecologists in abortion is significantly changed by the perspective of using the results of the procedure for

purposes such as the experimental treatment of patients in late stages of Parkinson's disease. In these cases, then, there is again no direct causal relation between C and W. However, putting the results of W to some further use might nevertheless be seen as constituting an act of complicity to the extent that the morally laudable aims of using the results of W might tend to weaken the moral condemnation of W, and thus lower the psychological threshold that now exists for W and similar acts. The reasoning behind this argument is obvious. If W is morally wrong, it must be good that it meets with widespread moral disapproval. It must be bad if the practice of using its results for morally laudable purposes makes it seem more acceptable than it is.

This kind of reasoning seems to have been the basis of the recommendation of the Central Ethics Committee of the Bundesärztekammer, the German Medical Association, in July 1998, in which the Committee declared its opposition to the retrieval of fetal brain tissue for purposes of neurotransplantation. One of its arguments was that this practice might weaken the moral rejection of abortion for non-medical reasons in society at large (Zentrale Ethikkommission, 1998). Partly as a consequence of this, neurotransplantation of embryonic tissue, which started in the early 1990s in Hanover, came to a halt in Germany (later, its problematic success was a further reason not to pursue this line further). However, the reasonableness of this step was doubtful from the start, since the sociological hypothesis underlying the Committee's statement does not seem plausible. Attitudes to abortion are a matter of fundamental moral outlook. It is unlikely that these attitudes will be significantly changed by the recognition that some good may come from evil.

In many ways, variant 5 is the most interesting one. In this case, complicity involves an incongruence, as one might call it, between rejecting W and making use of results which were made possible by W. In this case, even an indirect causal relation between C and W is absent. It need not be assumed, that is, that acts similar to W are encouraged by C or that attitudes will be changed in a way that raises the probability of W being carried out. The complicity relation in this case consists in a purely internal relation between C and the qualification of W as morally objectionable. The conflict is not one between C and its consequences but between C and the beliefs held by the person carrying out C. Ronald Green has adequately described this case as one of "appearance of endorsing, conferring legitimacy on or diluting the condemnation of a wrongful deed" (Green, 2001, 146). There is an obvious incongruence, as one might call it, between rejecting W and using results which were made possible by W. One important additional variable on which the tendency to attribute this last type of complicity depends seems to be the extent to which the person carrying out C *co-operates* with the person carrying out W in using the results or products of the latter's wrongdoing. Using the organs of a murder victim for purposes of transplantation is not seen as an act of complicity precisely because there is normally no co-operation between murderer and transplanting physician. The physician is in no way involved in the act that lead to the transplant, nor is there any kind of contact between the physician and the murderer. The situation is different if the transplants come from prisoners executed in China for political reasons. It is hard to imagine circumstances under which these organs could be made available without at least an indirect co-operation between those interested in making use of these organs and the authorities responsible for the executions. For the overall moral evaluation of complicity, co-operation is at least one crucial factor. The point is that transplanting organs from Chinese political prisoners would seem to be incompatible with an honest moral disapproval of the death penalty for political prisoners, whereas transplanting organs from murder victims seems to be fully compatible with a honest moral

disapproval of murder. Although disapproval of W and disapproval of making use of the results of W are separate items, they cannot be "isolated" against each other. There remains what might be called an *expressive dissonance*.
Expressive dissonance is largely a symbolic relation. Complicity in this case is primarily something "in the head" and nothing in the "outer world". This is immediately clear when one looks at the controversy, in American bioethics of the 1990ies, about whether results of the cruel experiments carried out in German and Japanese concentration camps during the Second World War should be used in medical research (cf. the contributions of Freedman, Greene, Kor and Segal in Caplan, 1992). The focus of this debate was not the fear that making use of these results might function as an incentive to carry out similar experiments in the future (in the sense of variant 3) or that psychic thresholds against this kind of research might be lowered (in the sense of variant 4), but a purely symbolic relation of incongruence between thought and action, valuation and motivation.

3. Types of complicity involved in ESC research

Complicity in one or more of the variants distinguished above is the core of the criticism levelled against research on pluripotent human embryonic stem cells (hESC) in many quarters and has even, in some countries, become the basis of legal prohibitions. It is clear that hESC research is not *inherently* morally problematic, at least not in ways that are peculiar to this kind of research. Though there is some amount of misunderstanding in the general public on the point, embryonic stem cells are not themselves human embryos and therefore should not be subsumed under the umbrella term "embryo research". Whatever is morally problematic about this research concerns the sources from which the stem cells are derived and the methods by which they are retrieved. Three of these sources and four procedures are held to raise moral problems:

	Type of research	*Source of stem cells*	*Morally problematic procedure*
1	Research on human embryonic germ cells	Aborted embryos	Abortion for non-medical reasons
2	Research on hESC	Supernumerary embryos produced in the course of IVF	Destruction of human embryos due to extraction of stem cells
3			Making the human embryo a "mere" means to extraneous ends
4			Generating supernumerary human embryos with the the conditional intention of using them as sources of stem cells
5		Supernumerary embryos resulting from PGD	Selection of living human embryos
6	Research on hESC	Embryos produced specifically for research by IVF or stem cell nuclear transfer	Production of human embryos by nuclear transfer; destruction of human embryos due to extraction of stem cells

Table 2. Sources and methods of the retrieval of hES cells

1. Aborted embryos: In the retrieval of embryonic germ cells, the morally problematic point of the procedure is the abortion for non-medical reasons that precedes the derivation of germ cells. At least a substantial proportion of the population rejects abortion for non-medical reasons. Furthermore, abortion is unlawful in some countries. In German law, for example, abortion in the first trimester is not punishable, but nevertheless unlawful. On the other hand, no further moral problem seems to arise provided that the couple validly consents to the use of the aborted material for research or therapeutic purposes. Although using an aborted embryo or parts of it for research or therapeutic purposes amounts to using it as a means to an end, this is not generally thought to be morally objectionable. The crucial consideration is that the embryo is dead and incapable of further development. The therapeutic use to which the embryonic tissue is put is commonly held to carry enough moral weight to compensate for the lack of piety involved in using the dead embryo as a means to an end.

2/3. Supernumerary embryos from IVF: The extraction of hESC from supernumerary embryos exhibits two morally controversial features: the killing of a living embryo by the extraction of the inner cell tissue, and the fact that the embryo is instrumentalized. Since the embryo is utilized for purposes neither related to the preservation of its own life or health nor to its own development, it is used as a "mere" means to an end in the Kantian sense. For this reason, using IVF embryos for the derivation of cells (or, more generally, to do research with them) is legally forbidden especially in countries where either the principles of the protection of human life is understood to cover the complete prenatal period (as Roman Catholicism does) or where the principle of human dignity is interpreted as ruling out "instrumentalizing" the human embryo even in its first stages of development. In addition, the German Embryo Protection Act enjoins that all embryos generated in the course of IVF be transferred to the uterus, thereby precluding that supernumerary embryos are generated with the conditional intention of using them as sources of stem cells.

4. Given the availability of stem cell extraction techniques, a further problem might be seen to arise from the conditional intention to make use of this technique in the course of IVF. This is relevant not only to cases in which supernumerary embryos are generated with the explicit purpose of later using them as raw material for stem cell research, but also when this possibility is accepted as a *potential* consequence of generating more embryos than are transferred to the woman.

5. Pre-implantation diagnosis with selective transfer of IVF embryos is often seen as morally problematic because it involves the conditional intention to let some living embryos perish, i. e. those that prove to be unsuitable for implantation in the course of genetic diagnosis. Selection of offspring is, in bioethics as in the general population, often rejected out-of-hand, irrespective of context and purpose. There is a widespread tendency to associate selection of offspring with "eugenics", which by itself is predominantly used with a negative connotation. It is interesting, in this context, to contrast selective procedures such as pre-implantation diagnosis (PGD) with germ-line intervention. Even if practised for the same purposes, both procedures have a completely different *gestalt*. The *gestalt* of germ-line intervention is that of doing something good to something living, whereas the *gestalt* of selection is that of a test by which the right to life is reserved to whatever meets certain criteria. A similar contrast can be drawn with pre-natal diagnosis (PND). Though it might be said that the physician's role as a "gatekeeper" to existence is common to PGD and PND, whoever

holds a strong principle of human dignity will see an important difference between the two methods: the fact that PGD involves a higher degree of selectivity than PGD. In both cases a choice is made, but the choices are of different sorts. In PND, there is a choice between yes and no, acceptance or non-acceptance, whereas the choice involved in PGD is of a "pick-and-mix"-kind, a selection of the best candidates out of a larger collective. Therefore, PGD might be seen as "instrumentalizing" embryos to a higher degree than PND. It is, therefore, no accident that according to the German Stem Cell Law, the permission to import hES cells from countries in which the extraction of hES cells from embryos is legal is restricted to embryos produced from IVF without the additional employment of selective techniques such as PGD.

6. Specific-for-research embryos: This procedure is often seen as posing moral problems of an even higher gravity than the above two procedures. In this case human embryos are not only killed and instrumentalized, but are explicitly generated in order to be instrumentalized for research or therapeutic purposes. This further step is widely held to constitute an additional and independent moral problem. The intention of generating human life solely for purposes other than for development to maturity is, in this case, unconditional and unavoidable. Although it cannot be excluded that the same unconditional intention may be present in some cases in which supernumerary embryos are generated in IVF, there is an objective difference between the procedures. While in IVF reproductive purposes are dominant (no matter what further intentions come in), reproductive purposes are explicitly ruled out in research cloning. In consequence, the production of embryos by nuclear transfer is illegal even in many countries in which hES cell derivation is legal.

It should by now be obvious that hESC research involves the researcher in quite a number of complicity relations with the procedures seen as morally problematic by many. The first variant of complicity is involved whenever a researcher directly participates in the extraction of stem cells from embryos (resulting in their death) or participates in generating embryos by nuclear transfer. The first variant of complicity is also exhibited by instigation. Instigation is present whenever the researcher makes others carry out these procedures with the (unconditional or conditional) intention to use the products or the results of these procedures for research, his own or other's. In this case, he is not himself directly causally involved in carrying out the procedures, but nonetheless acts as one of the causally necessary conditions of their being carried out by others. Instigation implies that – at least in the normal course of events – the procedures would not have been undertaken but for the intervention of the researcher. A paradigm case is the "ordering" of hES cells for research from countries in which retrieval of these cells is legal.

In contrast to this "strong" variant of complicity, the other, weaker variants of complicity are ubiquitous and are characteristic of the relevant research activity even in countries in which the extraction of hES cells is illegal. All kinds of hESC research depend on the availability of material derived from embryos, and some kind of co-operation, possibly mediated by third parties, seems inevitable, with the physicians carrying out the abortion and with other physicians or biologists extracting stem cells from blastocysts. Complicity of some kind seems unavoidable. However, that the one variant of complicity is illegal in these countries whereas the others are not, is in itself an interesting fact and throws light on the compromise character of the permission to do this kind of research. On the one hand, one does not want to stay behind other countries in this line of research. On the other hand, one wants to pay tribute to the moral conviction of those sectors of the population who are strictly against the

use of human embryos, however, indirect, in research. There are many forms these compromises may take. Mostly they exclude certain kinds of complicity (for example the use of hES cells generated by procedures 5 and 6 in the above list) but allow others that are seen to be more acceptable (such as sources 1 and 2 in the above list). It is clear, however, that all procedures involve one or more variants of (weak) complicity.

Research on embryonic germ cells (1) presupposes that an abortion has been carried out for non-medical reasons. Even if the mother decides on the abortion independently of the decision to make the aborted embryo available for research such an abortion is unlawful in may legislations (even if not punishable) and is regarded by many people as morally problematic if not worse. Though the researcher who makes use of the cells derived from the embryo does not actively participate in the abortion he inevitably stands to it in a complicity relation that, from the perspective of those with strong moral reservations against abortions, confers part of the blame to him as the one who profits from it. Though his purposes are quite different from the purposes of the original wrong (and possibly morally neutral or even meritorious) he participates in an overall constellation that is predominantly morally wrong (cf. Vawter 1991). Furthermore, the more his purposes are morally laudable, the more he risks imitation and re-evaluation effects.

Research on hESC retrieved from supernumerary embryos (2-4) involves complicity at least in sense 2 in all cases in which the research is carried out in a country (like, at present, Germany or Italy) in which derivation of hES cells is illegal but in which importing hES cells is legal under certain conditions. As a rule, further variants of complicity are involved at the same time. Thus, though the German Stem Cell Law rules out that research activities in Germany are directly causal for the derivation of hES cells in countries with a more liberal legal situation by instituting a "Stichtagsregelung" similar to that established by President Bush in the US which provides that imported hES cells must be already available at the point of time at which they are "ordered", it cannot prevent that these cells are extracted by those who provide them with, among others, an intention to sell them in the future to researchers in countries where derivation is illegal. Furthermore, the expectation that the "Stichtagsregelung" will be handled in a sufficiently flexible way to allow future imports has considerably grown since the German Bundestag decided on extending the "Stichtagsregelung" in a way that allows researchers to buy updated cell lines in order to keep abreast of new international developments. Thus, though the Law effectively rules out complicity of type 1, the active participation of German researchers in the killing of human embryos by the extraction of hES cells in the form of instigation, it is unable to rule out complicity of type 2. Even in the absence of a direct causal relation between W and C, carrying out the research means to achieve the aims for which W was done in the first place.

Whether complicity of type 3 and 4 are also present in this case, depends on the success, or rather the expected success of the experimental use of hES cells. Apart from the growing scientific interest of hESC research, especially as a model serving as a measuring rod for the potentialities of induced pluripotent human stem cells, therapeutic uses in humans have not yet become visible and are increasingly seen with scepticism, not least because of the considerable risks of cancerogenity and the risks that transplanted hES cells will be rejected by the host organism.

Analogous considerations apply to the two remaining forms of hESC research, research on hESC retrieved from supernumerary embryos by others in the context of PGD (5) or produced by nuclear transfer (6). In both cases, complicity comprises additional factors whenever not only the extraction of hES cells from embryos is seen as morally wrong but

also the methods by which the embryos are produced. The fact that (5) and (6) are explicitly ruled out, e. g. by the German Stem Cell Law shows that not only considerations of direct or indirect causal role enter into the legal response to these procedures, but also considerations of complicity. The legal situation is only adequately understood if, besides the strong form of complicity (variant 1), the weaker forms of complicity are taken into account.

4. When does complicity amount to a "moral stain"?

Complicity can amount to a "moral stain", but this is not necessarily so. In general, certain further conditions must be fulfilled in order to make complicity with a morally problematic act itself a problematic act. Since whether an act is morally problematic is not a yes-or-no-affair but allows of degrees, the discussion of these factors gives us an opportunity to see on what the extent to which an act of complicity seems condemnable depends.
In the following I will, for reasons of simplification, make two presuppositions:

1. I will only refer to acts that constitute weak complicity (variants 2-5). Whereas complicity in its strong sense (1) is an established topic in action theory and in the philosophy of law, it is exactly these weaker meanings of complicity that stand in need of philosophical elucidation.
2. I will take it as understood that the purposes to which the act constituting complicity is carried out are not only morally neutral but morally good purposes and that the act in question has a reasonable chance to achieve these purposes. Unless one of these conditions is fulfilled there is no real moral conflict. (There might, however be a legal or even constitutional conflict, e. g. with the fundamental right of researchers to free inquiry.) If, for example, hESC research were carried out with highly problematic intentions (e. g. for reasons of biological warfare) or had absolute no chance to achieve any of its scientific or therapeutic aims, the (potential) complicity of this research would not constitute the problem it in fact is. The research would have to be viewed with scepticism even in the absence of complicity.

On which factors does the intuition of a "moral stain" from complicity depend? Which variables are crucial for the perception that making use of bad practices of others for good ends is in some way morally tainted? I have already referred to the fact that it seems to be an inherent feature of the concept of complicity that complicity can only be attributed to an act C if the agent stands in some kind of co-operative relation to the original wrong W. It seems impossible, for example, to ascribe complicity to the various uses of pharmaceuticals that were developed in the past under circumstances that by modern standards would be morally unacceptable. Many standard medications were originally tested under conditions in which, for example, the requirement of informed consent in human subjects research was more or less unknown. It might be asked, however, whether this condition is also fulfilled in cases exemplifying the "weakest" variant of complicity in which the connection between W and C is mainly of a symbolic nature. I think, however, that even in these cases, some kind of co-operation between the agents involved is necessary for complicity even though this relation may be thin and indirect. In cases in which the relation is too thin to constitute even an indirect form of co-operation the concept of complicity seems to become inapplicable, for example, if a researcher makes use of the results of morally indefensible experiments published fifty years ago. The situation is different if he makes use of unpublished results of morally indefensible experiments as an employee of the same company that carried out the experiments. In this case there is a tighter relation of co-operation, mediated by the identity

of the company and the exclusive nature of the relevant information. This gives us one of the criteria on which the perceived moral significance of acts of complicity seems to depend, the perceived extent of co-operation involved in using the "fruits of a bad tree". The perception of complicity is considerably weakened if no co-operation at all is necessary to get the desired information or products, as in the case of the published scientific results of morally indefensible experiment, or if W lies in a psychically remote past so that the process of tradition is no more regarded as co-operative even in a thin way.

Another factor, of at least the same importance, seems to be the relation between the extent to which W is morally unacceptable and the extent to which C is morally desirable. The intuition of complicity seems adequate only if there is an obvious disproportion between these factors. Complicity can be present only if there is a clear imbalance between the moral badness of W and the moral goodness of C, however good and bad are weighted in this kind of balancing. (Some ethicists, for example so-called prioritarian utilitarians think that the bad should systematically carry more weight than the good, in accordance with widespread popular moral perception.) Apart from this systematic point of dissent, even more dissent is to be expected about where to strike the balance between good and bad and whether, in individual cases, the good to be expected from C is at all able to compensate for the evil of W. A striking example of such dissent is presented in the contributions to Arthur Caplans book on the question whether the results of concentration camp experiments should be used in medical research. In this case, opinions were sharply divided between former victims (and their descendants) on the one hand, and researchers and doctors interested in having unrestricted access to these results (see, e. g. Kor 1992, 7 versus Freedman 1992, 147ff.). This question was already inconclusively discussed during the Nuremberg trials in 1946 (cf. Friele 2000, 127).

If the bad done by W is balanced by the good done by C, or if the good done by C clearly outbalances the bad done by W, attributions of complicity are, as a rule, absent. For example, complicity is no issue in the context of importing organs explanted in countries with an opting-out regulation into countries in which this regulation is rejected. The reasoning is clear. Even those who seriously, and for moral reasons, object to the opting-out system do not feel that the moral blemish of this system is weighty enough to restrict the exchange of transplants, say in the Eurotransplant network in which two countries with an opting-out regulation are co-operating partners. The moral stakes of transplantation are simply too weighty to fuss about the "deviant" system of organ procurement in neighbouring countries. An imbalance is, however seen to exist in the case of countries in which organs are commercialized or in which regulation and control of organ procurement are held to be inadequate. Even in these cases, however, the willingness to fight commercial organ procurement is limited in view of the situation of the organ buyer. The German Transplant Law, for example, contains an explicit mitigating clause that considerably lowers the probability that the buyer will be held to be punishable if he is identical with the patient needing the organ.

Concerning this criterion, the situation in hESC research is controversial and fundamentally unclear. There is controversy both about the extent to which the practice of making use of early human embryos as providers of stem cells is morally problematic and about the extent to which the prospects of hESC are thought to be sufficiently favourable to justify a positive overall judgement on this line of research. At the extremes, opinions are diametrically opposed. Scientists and physicians, even if not directly or indirectly involved in hESC research, typically judge the moral opprobrium of embryo research to carry, if at all, less

weight than the moral prospects of ESC research. Representatives of the Christian churches, especially Roman Catholics, give more weight to the morally objectionable features of embryo research and are more sceptical of the prospects of hESC research. A further factor is that scientists tend to give significantly more moral weight to basic research than the Churches and their followers. This is an important factor since the justification of hESC research on the part of research organizations has recently considerably shifted to the scientific side. The more the potential therapeutic applications of hESC research recede into a far and uncertain future, the more this research is defended by its scientific rather than by its therapeutic merits.

5. The status problem – once again

The crucial factor in the differing assessments of hESC research continues to be the so-called status problem, the problem of the measure of protection owed to the human embryo. On the view that the embryo is due the same protection that is owed to newborns, say, extraction of stem cells from blastocysts is a serious crime, indeed murder. On the view that the embryo is due no or only minimal protection, the balance will, as a rule, go down on the side of research and no problem of complicity arises.

There are two respects in which the status problem is involved in judgements of complicity in hESC research that should be clearly distinguished: protection of life and dignity. The derivation of hES cells from human embryos constitutes a violation of the principle of sanctity of life, understood in a sense that comprises all phases of human existence, in each of the specific forms it may assume, and exactly to the same degree. With dignity, understood as a comprehensive principle of non-instrumentalization, this is different. It is relevant to all methods mentioned above, but in different degrees. Extracting stem cells from PGD embryos is commonly seen as a more objectionable violation of the principle of human dignity than extraction of stem cells from supernumerary embryos from IVF because it involves selection, and the same holds for the retrieval of stem cells by research cloning because it constitutes the production of a human being with the only purpose of destroying it and using its parts as a means to an end. Protection of life and dignity differ in other respects as well. Though both are components of the "status" of the embryo, taken together in the expression "status problem", they exhibit a very different logic and, to the extent that they are applied to prenatal human existence, are far from being correlatives. A "right to life" may be thought to admit of grades, whereas this seems impossible with dignity. An embryo either possesses dignity or not, whereas its right to life may be thought to be negotiable against other kinds of goods and other rights. And not all violations of a potential prenatal right to life are necessarily violations of dignity. Abortion is clearly a violation of a potential embryonic right to life, but it is a violation of dignity only if dignity is interpreted as implying a right to life, an interpretation that is by no means the only one possible. On the other hand, manipulations of the embryo for research purposes might be seen as violations of its dignity even if the embryo is not thereby destroyed or damaged. There are, then, good reasons to keep the issues of protection of life and the protection of dignity separate and discuss both issues each by each.

Are there reasons for an embryonic *right to life* sufficiently strong to dominate the good that comes from hESC research? I take it that the most plausible conception of prenatal protection of life is a gradualist conception according to which the "right to life" of the embryo/foetus is a matter of degrees, starting at a very low value and then gradually rising

until it reaches its peak at the time of birth. As this rising curve may be drawn in very different ways, this conception is really a bundle of conceptions and not strictly one. But this bundle is held together by the fundamental idea that the duties owed to the human embryo are not a constant but correlated with the developmental stages of the embryo, with the later stages requiring more protection than the earlier ones. It is true, this general conception has not so far been theoretically explicated in any systematic form. It is hard to deny, however, that it is implicit in most of the normative views about the status of prenatal human existence both in bioethics and in general morality. For example, the embryo is nearly always held to be less worthy of protection in the first few days of its existence than in later stages (see, e. g., Veerger et al. 1998, 11), a fact that is of some relevance in the discussion of the disparity in the legal handling of prenatal diagnosis with subsequent selective abortion on the one hand and of pre-implantation diagnosis with subsequent selective implantation on the other (cf. Birnbacher 2007). Furthermore, gradualist thinking is clearly mirrored in most criminal codes by the apportionment of punishments for taking the life of embryos and foetuses. In many legislations, termination of pregnancy within the first 14 days of gestation is not punishable, as well as the destruction or non-implantation of IVF embryos before the conjunction of sperm and egg cell though they have the same potential to become full-fledged human beings under suitable conditions as embryos in the full sense. Finally, abortion is nearly everywhere punished to a significantly lesser degree than infanticide or manslaughter/murder.

All this is plainly incompatible with a constant-protection view like that of the Roman Catholic Church that ascribes the same right to life to the embryo/foetus in all stages of development, either in the form of a right not be destroyed by interventions from outside or even in the sense of a right to be saved from death by natural causes. A constant-protection view is usually based on a potentiality principle in conjunction with an identity principle according to which any human embryo has a right to life that has the potentiality to develop into a fully developed human being and (in order to exclude pre-conjunction embryos) is numerically identical with this being. Such a strong principle does not seem at all plausible. The reason for this is that a potentiality argument in the case of humans would only be plausible if it were plausible in the general case, i. e. that it would be true that if x has the right to life, a potential x has a right to life not only for humans but for any organism whatsoever. It does not seem plausible, however, that such a general principle can be accepted. A bird's egg or the shoot of a tree are both potential birds or trees, but I do not think it acceptable that they have the same moral status as fully developed birds and trees. Normative properties of organisms like the possession of rights are supervenient properties. They supervene on certain descriptive properties of these organisms. As the human embryo shares only some of the properties of a born human being, it shares only some of its normative properties. In the same gradual way the embryo/foetus acquires the properties of the born child, so it gradually acquires its normative properties, among them a right to life that is only fully developed at a rather late stage of gestation, with viability or birth.

Apart from that, the combination of a strong potentiality principle with the identity principle according to which a potential x shares the normative properties of the actual x seems to imply that even the pre-conjunction embryo should be ascribed a right to life simply because potentiality implies identity, with the consequence that the principle of identity cannot serve as a limiting principle, restricting the right to life to the fully developed embryo. If the embryo that will become x is identical with x, then the pre-conjunction embryo that is to become this embryo is also identical with x. Even it is not

identical with the future child as a human being, it is identical with the future child under some broader concept, for example as an assemblage of organic matter. The consequence is that even the pre-conjunction embryo should have a right to life. This is not only highly implausible, it is also plainly incompatible with the legal practice in countries that prohibit the destruction or non-implantation of IVF embryos but permit the discarding of cryoconserved pre-conjunction embryos that have been fertilized but are not used for implantation.

According to a gradualist view (or rather: the variant of gradualism I would like to suggest, there may be others), there is, then, no real problem of complicity in hESC research as far as a purported right to life of the embryo is concerned, provided that the conditions stated above are met: that some good is to be expected from this research and that the hopes accompanying this research cannot be discarded as illusory. The reason is that the extraction of stem cells which is often taken as the crucial ethical stumbling block of this research takes place in a very early stage of embryonic development, within a few days after fertilisation. According to a gradualist conception of embryo protection the protection of life due to the embryo at this stage is minimal. This is in agreement with the legal practice of most societies which do not sanction abortions (e. g. by abortive pills) in the first two weeks of gestation or, as in the German criminal law, posit a legal definition of abortion that excludes abortion within the first two weeks of development to count legally as abortion.

This is not the end of the matter. Even if the complicity attributed to hESC research cannot plausibly be based on arguments pertaining to the life of the embryo from which the stem cells are taken, there is another dimension of the protection of the embryo to be considered, the dimension of *dignity*. It is an essential and uncontested component of the concept of dignity that dignity excludes what may be called total instrumentalization, i. e. dealing with a human being like a thing or a commercial good, like in slavery or in forced labour. Though dignity is a normative dimension clearly distinguishable from the protection of life, dignity is compromised most blatantly whenever instrumentalization (making someone a "mere" means to extraneous ends) takes the form of the deliberate taking the life of someone in order to achieve ends that have nothing to do with the ends of the person sacrificed. Sacrificing the life of a person for the ends of others seems a particularly clear case of a violation of the dignity of that person, and this is reflected in many military laws that prohibit sending soldiers on missions on which they are certain to lose their lives however clearly these missions would serve important strategic ends. On the same line, the German constitution which makes human dignity the highest and even non-negotiable constitutional value and imposes on the state a similarly non-negotiable obligation not only not to violate but also to actively protect the dignity of all human individuals is commonly interpreted as excluding any "sacrifice" of the life of one person for the life (or other fundamental goods) of others, with the exception of cases in which the life of an innocent victim can be saved only at the expense of the life of the perpetrator of the crime by which he has been made a victim. The question arises whether the destruction of human embryos for the sake of the retrieval of stem cells is not exactly an exemplification of this kind of "sacrifice" of one life for the lives of others, or, more realistically, for the scientific and remotely therapeutic purposes by which hESC is currently justified. Since the principle of human dignity is rapidly gaining ground at present and is increasingly introduced into constitutions and into international treaties and conventions the resistance to hESC is much more to be expected from this quarter than from that of the protection of embryonic life. An additional reason why resistance is to be expected on grounds of human dignity rather than on grounds of the

sanctity of life is the pragmatic one that restrictions on account of instrumentalization are feasible whereas restrictions on account of sanctity of life are not. It does not seem possible to abolish abortion whereas it is perfectly possible to close the door to new developments in biomedicine such as hESC research, PGD, and human cloning.

6. Do human embryos possess dignity? The German debate on hESC research

A characteristic feature of German bioethics and especially of German biopolitics is the outstanding role assigned to the principle of human dignity in biopolitical debate. One of the reasons for this is the distinctly Kantian character of the German constitution. The reference to Kantian principles was seen as a common denominator on which the politically strongly heterogeneous parties to the founding assembly of the *Bundesrepublik* in 1949 could find a consensus. Moreover, the principle of dignity which had just been introduced as a leading principle into the Universal Declaration of the Rights of Man by the United Nations in 1948, was seen as a a safeguard against tyranny and particularly against the atrocities of the Nazi regime from which most members of the assembly had suffered in one way or other. Since then, the principle of respecting human dignity laid down in article 1 of the German constitution became a kind of creed that had an important supporting function for the cohesion and identity of German society. However, while the parties deciding on the wording of the constitution were strongly divided over the question whether human dignity (and the fundamental right to life) is to be understood in an inclusive sense, comprising prenatal human forms of existence alongside with the existence of born human individuals, the Constitutional Court and, subsequently, constitutional law increasingly made the inclusive interpretation the standard interpretation and held the principles of dignity and of protection of life to be applicable to prenatal forms of existence in roughly the same way as to born individuals. Though there is, at the moment, an unmistakable tendency in constitutional law to revise this interpretation and especially to re-interpret the principle of human dignity in such a way that it cannot further function (because of its non-negotiability) as an effective check on embryo research and the introduction of reproductive technologies such as single-embryo transfer and PGD, a majority of politicians continue to think that the principle is incompatible with the use of human life as a means to an end in all possible forms, irrespective of the stage to which human life has developed.

According to this view, the moral necessity to preserve and to protect human life starts with the conjunction of egg and sperm. However, all controversial practices in reproductive medicine at present discussed in law and politics involve, in one way or another, "instrumentalizing" early human life. Pre-implantation diagnosis and pre-implantation sex selection involve the selective discarding of unwanted blastocysts, retrieval of stem cells involves the destruction of blastocysts, research cloning even the production of human embryos with the explicit intention to destroy them at a later stage. These practices, therefore, cannot be justified as forms of well-intentioned paternalism. In all cases, human life in its early forms is made a means to ends other than the life or well-being of the embryo concerned, no matter how important and respectable these other ends may be. Furthermore, the introduction of any one of these new methods is seen as a potential door-opener to embryo research, which in itself is a realistic perspective, given that the physicians practising pre-implantation diagnosis in the context of a University clinic will hardly be

satisfied with practising it without making it at same time the object of research, not least to improve its success rate.

The ethical premises underlying this stance are essentially two: 1. The concept of human dignity applies to prenatal human life in the same way as it applies to postnatal human life. 2. The principle of the protection of human dignity applies to prenatal human life with the same force as it applies to postnatal human life, i. e. it is as little negotiable against other rights and obligations as it is in its application to human beings at a postnatal stage.

Both premises have a relatively firm backing in law. The first premises was confirmed in the second judgement of the Constitutional Court on abortion in 1975. One of the famous quotes from this judgement is that "human dignity is a property of human life wherever it exists". Not human *persons* (in whatever sense of "person"), but human *life* is the proper object of protection in the name of human dignity. The second premise is a more or less undisputed principle of constitutional law. Differently from the other basic rights formulated in the Constitution, including the right to life, the right to protection of human dignity is absolute and non-negotiable. It has to be given a *minimalistic* interpretation in order to prevent that conflicts of basic rights become ubiquitous. These two premises taken together do not seem to leave much room for alternatives. The Embryo Protection Act with its strict verdict on "instrumentalizing" human embryos, for research or other non-reproductive purposes, seems a more or less logical conclusion from the constitutional situation.

It is not surprising that the legal situation has been a major factor in the estrangement between biopolitics and bioethics in the field of beginning-of-life issues. Bioethical discussion has throughout been much more open than biopolitics and biolaw to arguments in favour of liberalisation. In bioethics, and especially in secular, non-theological bioethics, the nearly absolute ban on embryo research and on the consumption of embryos for non-reproductive purposes has rarely been upheld with the same strictness as it has been upheld in politics and law. When, in 1990, the Embryo Protection Law was passed by the Bundestag, it came as a surprise to most bioethicists, since bioethical discussion had already moved quite a long way from the moral extremism of the Roman Catholic Church. Even the so-called Benda commission that had been investigating the ethical and legal issues of reproductive medicine prior to the Embryo Protection Act (and which did not consist of particularly "progressive" experts) had held embryo research to be permissible within limits.

There are basically two groups of bioethicists in Germany who are not prepared to follow the Constitutional Court in its application of a strong principle of human dignity to human embryos regardless of their stage of development. A minority of bioethicists has raised doubts about the very possibility of applying the concept of human dignity to prenatal human life. According to this opinion, human dignity is primarily a political and social concept, a "Kampfbegriff", guiding the struggle against such practices as torture, slavery, capital punishment, and the persecution of racial, ethnic or religious minorities. Its historical roots are located essentially in the emancipation movements of the Enlightenment and in the workers' movement of the 19th century, with the double focus on personal autonomy and social security. Its main content is identified as liberty, non-discrimination, social rights, and such elements of self-respect as freedom from humiliation and persecution. This concept, in consequence, is simply not held to be relevant to practices such as embryo research and embryo selection which affect human life at a stage at which it is neither sentient nor capable of aims which might be frustrated by political or social pressure.

Another group of bioethicists thinks that the principle of human dignity *is* applicable to prenatal human life, and even to the early human embryo, but that in this context it has neither the same *sense* nor the same *force* as the concept that is applied to born human beings. According to this opinion, the Constitutional Court's mistake is to treat the concept of human dignity as univocal. There is not one, but a family of interrelated concepts, each one having its own domain and its own peculiar force. There is, first, the *strong* concept applying to born human beings, which indeed may be treated as non-negotiable for practical purposes. (Theoretically, it remains possible that one is forced to choose between two atrocities each of which violates human dignity.) It comprises a number of basic rights such as minimal liberty, minimal self-respect, and basic social services. This concept is central to modern democracies and an undisputed achievement of the secular emancipation process started by the Enlightenment. This strong concept, however, is not relevant to human embryos, at least not to the temporal stages which are affected by the practices under discussion.

Apart from this strong concept of human dignity this group of bioethicists recognize two other, derivative concepts of human dignity: a *secondary* concept which applies to everything human in the biological sense, irrespective of its stage of development or decay, and a *generic* concept which applies to the human species as such and which is often invoked in non-consequentialist arguments against such practices as the production of man-animal-hybrids and reproductive cloning. Both concepts are considerably weaker in normative force and differ from the strong concept semantically and syntactically. While human dignity in its primary meaning needs an individual subject as bearer, this is not necessary with the two derivative concepts. With them, there need not be a real subject to correspond to the grammatical subject. This is evident where human dignity is applied to the species as such, but it is also the case in its application to human zygotes and early embryos, entities that cannot reasonably be assumed to be "real subjects". With human dignity in its primary sense the object of respect and protection is the concrete human being. With human dignity in its derivative senses it is something more abstract: humanity, human life, or the identity and dignity of the human species defined by its specific potentialities.

According to this second position, which is taken by the present author, human dignity is in fact applicable to the early embryo but in a different and specific sense in which it is does not carry the quasi-absolute moral force of the primary concept. Prenatal human life deserves respect, but not the absolute respect an adult person deserves. It deserves respect because it is a form of specifically *human* life, irrespective of whether it is viable or not, whether it is destined to be discarded anyway (as most "spare" embryos from in-vitro-fertilisations are) or whether there is some chance that it will develop into a full-blown human being. What is important, however, is that this respect is a *weak* form of respect which is not incompatible, as the respect owed to born human beings is, with treating embryos as a means to an end, provided these ends are themselves sufficiently respectable. The basis of this respect is straightforward speciesism, or, to use a more sympathetic term, "generic solidarity". It is one of the forms by which a feeling of unity with everything human is expressed, no matter how this feeling of unity is philosophically construed, in a deep, metaphysical way, or in an everyday, naturalistic way. One of the implications of this concept is that it applies independently of whether the human being in question is among the living or the dead. Human corpses qualify as objects of this kind of weak respect no less than human embryos. In this way, the close link that has been established between the principle of human dignity and the principle of "sanctity of life" is weakened. Respect for

human dignity, in its secondary sense, is a principle different from that of respect for life, even if respect for life is one of the forms by which respect for human dignity can be expressed.

7. Conclusion

Does this mean that complicity is, after all, not really a problem in the controversy about hESC research? In fact, for those on the pro-side, complicity does not seem to be a real issue because they generally do not think that embryo research is sufficiently problematic to conflict with what they see as morally desirable in the development of new therapies and in basic research. For those on the anti-side, on the other hand, complicity does not seem to be an issue either, because they condemn the embryo manipulation preceding hESC research and are less optimistic on the side of therapeutic prospects and less enthusiastic on the side of the intrinsic moral value of scientific research. They do not need to confront the complicity problem because they are up against hESC research anyway.

Complicity is a problem only for those who are torn between the conviction that embryo research is (for intrinsic or extrinsic reasons) a moral evil and the conviction that hESC research is worth pursuing either for its medical or for its scientific prospects or both. The class most likely to face this uncomfortable dilemma is the class of conservative politicians in countries such as Germany and Italy in which embryo research is strictly prohibited by law, but in which hESC research is nevertheless permitted or even encouraged, although only with raw material imported from countries with more permissive laws. For these politicians, complicity is, and should be, the stumbling block lying in the way of pragmatic compromise.

This result is only one of the facets of the deep gulf that separates bioethics from biopolitics. This gulf depends on the fact that biopolitics follows, and has to follow, criteria which go far beyond the criteria of bioethical judgement. A solution to a bioethical problem may be ethically and rationally acceptable without being politically acceptable. There are at least two other criteria that have to be satisfied. First, biopolitical solutions must conform to certain procedural norms. They must have been arrived at on the basis of accepted democratic procedures. There is no guarantee that solutions arrived at in this way are in full conformity with the norms of intellectual rigour, coherence and adequacy to which bioethicists are professionally committed. Second, the solutions found by politics have to take into account pragmatic considerations such as conserving social harmony, which again may conflict with considerations of ethical adequacy. Biopolitics cannot contradict public opinion to the same extent that bioethics can. If it does, it risks losing the acceptance of substantial sections of the population. Since biopolitical problems often touch quite fundamental ethical and religious beliefs, these risks are substantial. As a rule, political decisions will have to be taken in a way that allows even those whose attitudes and interests have been deeply frustrated, to accept the decision, at least in principle. In a pluralistic society, this means that political decisions touching deep convictions will often assume the form of compromise solutions by which none of the parties concerned is fully satisfied but which nevertheless minimise the net sum of frustrations on all sides.

As far as Germany is concerned, legislation with regard to beginning-of-life issues has throughout followed the policy of committing itself to rather strong principles in order to satisfy the adherents of "pro-life" positions, and to make room, at the same time, for a wide range of exceptions in order to satisfy the adherents of "pro-choice" positions. More

concretely, the strategy of satisfying both sides at the same time and by the very same law has consisted mainly in officially condemning a practice more strongly than it has been condemned before, and at the same time extending the availability of the practice so that all those who might benefit from it have free access to it. This is illustrated not only by the German abortion law but also by the Stem Cell Law. The abortion law declares abortion during the first trimester to be illegal, thus satisfying the adherents of the "pro-life" position on abortion. At the same time it exempts it from penal sanctions, thus enabling pregnant women to have an abortion in the first trimester even for trivial reasons. A similar strategy is followed by the Stem Cell Law. On the one hand, it is stricter even than the Embryo Protection Act in regulating not only the retrieval of stem cells in Germany (which continues to be illegal) but also the use of imported stem cells legally retrieved in other countries. At the same time it does not only make room for research with embryonic stem cells in Germany, it also reduces the possibility that this kind of research might be restricted by ethics committees and internal review boards. The law's demand that a special ethics commission be installed to control stem cell research with imported stem cells partakes of the same dialectic characteristic of the law as such: On the one hand, it expresses the political will to have a particularly keen eye on whether stem cell research is conducted in an ethically defensible way. On the other hand, the functions of the commission are reduced to a purely symbolic one. The commission is given no discretion to reject a submitted research protocol that is scientifically sound. Its function is merely to examine whether the protocol is scientifically plausible and whether the stem cells have been imported by the ways specified by the law. There is no room for restricting research with stem cells for genuinely ethical reasons. Thus, the law is a good example of what has been called "symbolic politics". It shows the political will to promote stem cell research in Germany. At the same time, it expresses this will in a way that misleads pro-lifers into thinking that legal control in a sensitive and controversial field of research is tightened rather than loosened.

The price to pay for this kind of political compromise is a considerable lack of transparency and consistency. This is a high price because transparency is a central democratic virtue. It prevents that a policy is understood and found to be intelligible by the general population. In biopolitics, compromises seem inevitable. At they same time, they inevitably seem to involve some form of moral opportunism.

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2

Potential Clinical Applications of Embryonic Stem Cells

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1. Introduction

Embryonic stem cells (ESC) have been reported for different mammalian species (i.e. hamster, rat, mink, pig, and cow), but only murine ES cells have successfully transmitted their cell genome through the germline. Recently, interest in stem cell technology has intensified with the reporting of the isolation of primate and human ES cells.
In developing this chapter, some conventions have to be established to describe consistently what stem cells are, what characteristics they have, and how they are used in biomedical research. Also, we intend to describe and distinguish the details of foetal and adult stem cells. In between lie important information describing what researchers have discovered about stem cells and a newly developed autologous ES cell–like stem cells, called induced pluripotent stem (iPS) cells. These reprogrammed stem cells (iPS) could be generated from any patient, thus removing both ethical and immunological issues at one time.
A stem cell is a special kind of cell that has a unique capacity to renew itself and to give rise to specialized cell types. Although most cells of the body, such as heart or skin cells, are committed to conduct a specific function, a stem cell is uncommitted and remains uncommitted, until it receives a signal to develop into a specialized cell. Their proliferative capacity combined with the ability to become specialized makes stem cells unique.
Stem cells can originate from embryonic, foetal, or adult tissue and are broadly categorized accordingly.
Embryonic Stem Cells (ESCs) are commonly derived from the inner cell mass (ICM) of a blastocyst, an early (4–5 days) stage of the embryo. Embryonic germ cells (EGCs) are isolated from the gonadal ridge of a 5–10 week foetus.
Adult stem cells differ from ESCs and EGCs in that they are found in tissues after birth, and to date, have been found to differentiate into a narrower range of cell types, primarily those phenotypes found in the originating tissue. An adult stem cell is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ that can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found, because they are able to self-renew and yield differentiated cell types.
They are thought to reside in a specific area of each tissue (called a "stem cell niche"). Stem cells may remain quiescent (non-dividing) for long periods of time until they are activated

by a normal need for more cells to maintain tissues, or by disease or tissue injury. Typically, there is a very small number of stem cells in each tissue, and once removed from the body, their capacity to divide is limited, making generation of large quantities of stem cells difficult. Today, donated organs and tissues are often used to replace those that are diseased or destroyed. Unfortunately, the number of people needing a transplant far exceeds the number of organs available for transplantation. Adult stem cells, such as blood-forming stem cells in bone marrow (called hematopoietic stem cells, or HSCs), are currently the only type of stem cell commonly used to treat human diseases.

Scientists in many laboratories are trying to find better ways to manipulate them to generate specific cell types so they can be used to treat injury or disease. Pluripotent stem cells offer the possibility of a renewable source of replacement cells and tissues to treat a myriad of diseases, conditions, and disabilities including Parkinson's disease, Amyotrophic Lateral Sclerosis, spinal cord injury, burns, heart disease, diabetes, and arthritis. This pluripotency represents both advantages and disadvantages in cell-based therapies. In fact for culture *in vitro*, their ability to generate the large number of cells often required for therapies, as well as their potential to yield whichever phenotype may be of interest, is considered beneficial. For implantation *in vivo*, however, the concern arises that these same attributes will either allow ESCs to proliferate limitlessly and form teratomas or differentiate uncontrollably into undesirable cell phenotypes.

Several are the applications of ESCs in human medicine: tissue repair, gene therapy, drug discovery and toxicological testing.

Stem cells are promising tools for studying the mechanisms of development and regeneration and for use in cell therapy of various disorders as cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, stroke, diabetes and cartilage.

Although hESC are thought to offer potential cures and therapies for many devastating diseases, research using them is still in its early stages.

In late January 2009, the California-based company Geron received FDA clearance to begin the first human clinical trial of cells derived from human embryonic stem cells.

But some scientific hurdles to hESCs application have to be deeply considered:

- the rejection of transplanted tissues (originating from donor embryos);
- the risk of teratoma formation due to any residual rogue undifferentiated pluripotent hESCs in the hESC-derived tissue (after the differentiation process);
- the inadequate number of cells available for treatment (for obtaining a large numbers of cells, large-scale cell production strategies are needed utilizing bioreactors and perfusing systems);
- the safety measures to be taken when a whole cell is administered because a variety of impurities may be administered with it (cells cells must be generated under cGMP current good tissue culture practice conditions using xenofree protocols to prevent the risk of transmission of adventitious agents and rogue undifferentiated hESCs that may induce teratomas);
- the best route and the frequency of administration (direct cell injections into the malfunctioned organ would be preferred to peripheral or portal vein administration to prevent the cells homing in unwanted sites, thus inducing cancers).

For the above reasons a long-term *in vivo* functional outcome after hESC-derived tissue transplantation also needs to be properly worked out.

2. Origin and classification of stem cells

Human stem cells can be classified into many types based on their source of origin. More recently, they have been classified based on the presence or absence of a battery of CD and embryonic stem cell (ESC) markers.

The male and female gonads contain stem cells referred to as spermatogonia and oogonia, respectively. Through their self-renewal and subsequent meiosis they are responsible in producing the cells of the germ line and eventually spermatozoa and oocytes. These two haploid gametes eventually fertilize to establish diploidy and produce the zygote. The zygote remains at the top of the hierarchical stem cell tree, being the most primitive cell, and the germ cells therefore possess the unique feature of developmental totipotency (Yoshimizu T et al. 1999; Pesce et al 1998). The zygote undergoes cleavage in the human through a period of 5–6 days, producing two to four blastomeres on day 2, eight on day 3, fusing or completely fused blastomeres (compacting or compacted stage) on day 4, and blastocyst stages on days 5 and 6 (Bongso et al 2005; Fong et al 2004). Each of the blastomeres is considered totipotent because it has the potential to produce a complete organism, as demonstrated when blastomeres are placed into the uterus of rabbits or mice. The first stem cell to be produced in the mammal is in the inner cell mass (ICM) of the 5-day-old blastocyst. These cells self-renew and eventually produce two cell layers: the hypoblast and epiblast. The hypoblast generates the yolk sac, which degenerates in the human, and the epiblast produces the three primordial germ layers (ectoderm, mesoderm, and endoderm). These germ layers produce all the various tissues of the organism. For this reason hESCs are considered pluripotent and not totipotent because they cannot produce complete human beings but have the potential to produce all the 210 tissues of the human body.

During embryogenesis and fetal growth such embryonic stem cells that have not participated in organogenesis remain as adult stem cells in organs during adulthood. It can thus be hypothesized that the function of adult stem cells residing in specific organs is to be dedifferentiated and be recruited for repair of injury incurred by the specific organ. Unfortunately, such adult stem cells in the organs are few in number.

It has been shown that fetal and adult stem cells, referred to as somatic stem cells or non-embryonic stem cells, are able to self-renew during the lifetime of the organism and to generate differentiated daughter cells. Moreover they could cross boundaries by trans-differentiating into other tissue types and are thus referred to as multipotent [Solter et al., 2006, Bjornson CR, et al 1999; Jackson KA, et al 1999;Clarke DL et al 2000 ; Krause DS et al 2001].

Adult tissues, even in the absence of injury, continuously produce new cells to replace those that have worn out. For this reason, adult stem cells can be found in a metabolically quiescent state in most specialized tissues of the body, including brain, bone marrow, liver, skin, and the gastrointestinal tract. Therefore, multipotency is restricted to those mesenchymal stem cell types that can differentiate into a small variety of tissues.

Those stem cells that are unable to trans-differentiate but differentiate into one specific lineage are referred to as unipotent. An example of such unipotency is the differentiation of bone marrow hematopoietic stem cells to blood. Thus as embryogenesis shifts to organogenesis, infancy, and then adulthood, stem cell plasticity shifts from pluripotency to multipotency.

Recently there has been tremendous interest in the derivation from embryonic, fetal and adult tissues and, more recently, also from extra-embryonic adnexa such as umbilical cord,

placenta, fetal membranes and amniotic fluid.[Zhao et al 2006; McGuckin CP et al. 2005 ; Fong CY ,et al. 2007] . These tissues possess both CD and some ESC markers, and thanks to their "intermediate" properties, are considered useful for transplantation therapy [Fong et al 2007] . The umbilical cord, for example, has three types of stem cells localised in cord blood, in the Wharton's jelly, and in the perivascular matrix around the umbilical blood vessels within the cord itself [Sarugaser et al. 2005] .

3. Stem cells characteristics

The term "stem cell" originated from botanical monographs where the word "stem" was used for cells localised in the apical meristem, and responsible for the continued growth of plants [Kaufman et al 2002]. In mammals, given the vast variety of stem cells isolated from pre-implantation embryos, fetus, amniotic liquid, umbilical cord, and adult organs, it becomes necessary to provide a more general definition for the term "stem cell" and a more specific definition based on the type of stem cell.

In general, stem cells differ from other kinds of cells in the body, and have dual ability to proliferate indefinitely (i.e. self renewal) and to differentiate into one or more types of specialized cells (i.e. potency) [Mimeault and Batra 2006].

Stem cells are capable of dividing and renewing themselves for long periods. Unlike muscle, blood, or nerve cells—which do not normally replicate themselves—stem cells may replicate many times, or proliferate. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

Stem cells are unspecialized. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. However, unspecialized stem cells can give rise to specialized cells, including heart, muscle, blood or nerve cells.

Stem cells can give rise to specialized cells. When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are just beginning to understand the signals inside and outside cells that trigger each step of the differentiation process. The internal signals are controlled by cell's genes carrying coded instructions for all cellular structures and functions. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules within the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that restrict DNA expression in the cell and can be passed on through cell division.

The degree of differentiation of stem cells to various other tissue types varies with the different types of stem cells, and this phenomenon is referred to as plasticity.

The plasticity of stem cells and differentiated cells in the postnatal organism poses important questions concerning the role of environmental cues. What mechanisms allow a stem cell to escape developmental pressures and maintain its "stemness"? What macro- or micro-environmental cues maintain a cell in its differentiated state? Other important questions to solve are related to the developmental origin of postnatal stem cells, to their possible relationships, as well as the role of symmetrical and asymmetrical cell divisions that maintain stem cell compartments but allow for differentiation in the same time [Booth, and Potten 2000; Morris, R. 2000]

4. Embryonic stem cell: hESCs and mESCs

Embryonic stem (ES) cells were first isolated in the 1980s by several independent groups [Cole et al., 1965, 1966; Evans and Kaufman, 1981; Martin, 1981; Bongso et al., 1994; Thomson et al., 1995, 1998; Axelrod,1984; Wobus, et al.1984; . Doetschman et al. 1985]. These investigators recognized the pluripotential nature of ES cells to differentiate into cell types of all three primary germ lineages. Gossler et al. described the ability and advantages of using ES cells to produce transgenic animals [Gossler et al 1986]. Thomas and Capecchi reported the ability to alter the genome of the ES cells by homologous recombination (Thomas et al 1987). Smithies and colleagues later demonstrated that ES cells, modified by gene targeting when reintroduced into blastocysts, could transmit the genetic modifications through the germline [Koller at al 1989]. Today, genetic modification of the murine genome by ES cell technology is a seminal approach to understanding the function of mammalian genes *in vivo*. Successively, interest in stem cell technology has intensified with the reporting of the isolation of primate and human ES cells [Thomson et al., 1995, 1998; Shamblott et al 1998; Reubinoff et al. 2000].

Embryonic Stem Cells (ESCs) continue to grow indefinitely in an undifferentiated diploid state, when maintained in optimal conditions. ES cells are sensitive to pH changes, overcrowding, oxygen and temperature changes, making it imperative to care for these cells daily. ES cells that are not cared for properly will spontaneously differentiate, even in the presence of feeder layers and leukemia inhibitory factor (LIF).

Embryonic stem cells have the advantages of possessing pluripotent markers, producing increased levels of telomerase, and being coaxed into a whole battery of tissue types. On the other side they have the disadvantages of potential teratoma production, their derived tissues have to be customized to patients to prevent immunorejection, and their numbers have to be scaled up *in vitro* for clinical application.

Since the first report of ESC derivation in mice was published in 1981, [Evans and Kaufman 1981] various findings have emerged to explain the basic properties of ESCs. Recent advances in our understanding of ESC biology have included the identification of several master regulators of ESC pluripotency and differentiation. However, intensive study of ESC growth conditions has yet to produce a complete picture of the unique transcriptional and epigenetic state that is responsible for pluripotency and self-renewal in ESCs.

In summary, genuine hESC have the following characteristics: (1) self-renewal in an undifferentiated state for very long periods of time with continued release of large amounts of telomerase, (2) maintenance of "stemness" or pluripotent markers, (3) formation of teratoma containing tissues from all three primordial germ layers when inoculated in SCID mice, (4) maintenance of a normal stable karyotype, (5) clonality, (6) stem cells marker expression (e.g., NANOG), and (7) ability to produce chimeras when injected into blastocysts in the mouse model.

hESCs have many applications in human medicine. First of all the production of hESC-derived tissues in regenerative therapy.

5. Using pluripotent stem cells in clinic issues

A number of scientific and medical issues need to be addressed before stem cells can be considered safe for clinical applications. The first difficulty is the tumorigenic potential of pluripotent cells (hESCs and iPSCs). Because pluripotency is evidenced by the ability to

form teratomas when transplanted in immunodeficient mice, the concern exists that these cells could form malignant tumors in the host. One strategy for dealing with this problem is to select pure populations of more committed cells for transfer. Therefore it is important demonstrating the genetic and epigenetic stability before these cells are used clinically. In fact is imperative that controlled, standardized practices and procedures be followed to maintain the integrity, uniformity, and reliability of the human stem cell preparations. Because in many studies stem cells are both maintained and expanded *in vitro* before transplantation, culture conditions compatible with human administration must be used. Feeder cells and sera of animal origin have to be avoided to reduce the potential risk of contamination by xenogeneic protein and pathogens. Also karyotypic abnormalities, might be at least partially dependent on culture techniques [Mitalipova et al. 2005]. Accordingly hESCs must be produced under current Good Manufacturing Practices (cGMP) quality. That is defined by both the European Medicines Agency and the Food and Drug Administration, as a requirement for clinical-grade cells, offering optimal defined quality and safety in cell transplantation. In Europe, the requirement for cell therapy products is outlined in several directives and guidelines that are pertinent as regards hESCs (Directive 2004/23/EC, Commission Directives 2006/17/EC and 2006/86/EC).

Finally, transplantation of hESCs into patients is also limited by potential HLA incompatibility. Consequently, life-long immunosuppressive therapy, which can lead to infections and organ-based toxic side effects, such as nephropathy, might be required to prevent graft rejection.

In this regard induced Pluripotent Stem Cells (iPSCs) hold great promise because they are histocompatible with the patient from which they are derived and their use avoids one of the major ethical concerns associated with hESCs.

6. ESCs cell therapy *in vivo* and *in vitro*

The NIH funded its first basic research study on hESCs in 2002. Since that time, biotechnology companies have built upon those basic foundations to begin developing stem cell-based human therapies.

Cell therapy, including the disciplines of regenerative medicine, tissue-, and bio-engineering, is dependent on cell and tissue culture methodologies to generate and expand specific cells in order to replace important differentiated functions lost or altered in various disease states (i.e. no insulin production in diabetes). Central to the successful development of cell based therapies is the question of cell sourcing. Thus, advances in stem cell research have a vital impact on this problem.

The use of human ESCs as resource for cell therapeutic approaches is currently performed for several diseases. Among these we are going to describe myocardium diseases and lung disease.

The Landmark's study is the first to document the potential clinical utility of regenerating damaged heart muscle by injecting hESC–derived cardiomyocytes directly into the site of the infarct [Laflamme MA et al., 2007]. Researchers have demonstrated the proof-of-concept of this approach in mice. Mouse embryonic stem cells have been used to derive mouse cardiomyocytes. When injected into the hearts of recipient adult mice, the cardiomyocytes repopulated the heart tissue and stably integrated into the muscle tissue of the adult mouse heart. After that, they have derived human cardiomyocytes from hESCs (GRNCM1) using a process that can be scaled for clinical production. GRNCM1 cells shown normal contractile

function and responded appropriately to cardiac drugs. These cells have been transplanted into animal models of myocardial infarction in which the cells engraft and improve the left ventricular function compared to those animals receiving no cells. The ability of hES cell–derived cardiomyocytes to partially regenerate myocardial infarcts and attenuate heart failure encouraged their study under conditions that closely match human disease.
In 2007 another study showed that intramyocardial injection of hESC-CMs performed few days after infarction in immunodeficient rodents seemed to enhance left ventricular ejection fraction (LVEF) compared to a control group [van Laake LW et al 2007].
Unfortunately, this enhancement was not sustained after 12 weeks of follow-up. Another study suggested that a coinfusion of hESC-CMs and MSCs in mice was of benefit because a ''synergistic trophic effect that enhanced repair of injured host tissue'' was brought about. Importantly, no teratoma was found in animals receiving hESC-CMs [van Laake LW et al 2007 ; Puymirat et al 2009].
Respiratory diseases are a major cause of mortality and morbidity worldwide. Current treatments offer no prospect of cure or disease reversal. Transplantation of pulmonary progenitor cells derived from human embryonic stem cells (hESCs) may provide a novel approach to regenerate endogenous lung cells destroyed by injury and disease. In a study researcher examine the therapeutic potential of alveolar type II epithelial cells derived from hESCs (ATIICs) in a nude mouse model of acute lung injury (Spitalieri P. et al. submitted). The capacity of hES to differentiate *in vitro* into ATIICs was demonstrated together with the ability of the above committed cells to repair *in vivo* lung damage in a pulmonary fibrosis disease models, obtained by Silica inhalation in mice. After injection of committed cells into damaged mice, a significant recovery of inflammation process and fibrotic damage, was obtained and demonstrated by the restoration of lung functionality (measurement of blood oxygen saturation levels).
Up to date in human only one trial based on hESCs has been initiated. During July 2010, the FDA notified the biotechnology company Geron that they could begin enrolling patients in the first clinical trial of a hESC-derived therapy. The phase I of this multi-center trial is designed to establish the safety of using hESCs to achieve restoration of spinal cord function. To do this, they have derived oligodendrocyte progenitor cells (GRNOPC1) from hESCs. GRNOPC1 is a population of living cells containing precursors to oligodendrocytes, otherwise known as oligodendrocyte progenitor cells (OPC). Oligodendrocytes are naturally occurring cells in the nervous system that have several functions, they produce myelin (insulating layers of cell membrane) that wraps around the axons of neurons to enable them to conduct electrical impulses.
In collaboration with researchers at the University of California, Geron have shown in animal models that GRNOPC1 can improve functional locomotor behaviour after cell implantation in the damaged site, seven days after injury. Histological analysis also provided evidence for the engraftment and function of these cells [Keirstead HS et al 2005].
In additional studies, GRNOPC1, when injected into the injury site of spinal cord, migrated throughout the lesion site matured into functional oligodendrocytes that remyenilated axons and produced neurotrophic factors [Zhang YW et al. 2006], resulting in improved locomotion of the treated animals. These above observations served as the rationale for the use of GRNOPC1 in treating spinal cord injuries in humans.
The clinical hold was placed following results from a single preclinical animal study in which Geron observed a higher frequency of small cysts within the injury site in the spinal cord of animals injected with GRNOPC1, respect to previous studies. In response to those

results, Geron developed new markers and assays, completed an additional confirmatory preclinical animal study to test the new markers and assays, and subsequently submitted a request to the FDA for the clinical hold to be lifted.
Another biotech company, ACT, has recently filed the paperwork with FDA to request permission to begin another hESC-derived stem cell safety test. The trial regard the treatment of patients with an eye disease called Stargardt's Macular Dystrophy (SMD), using hES-derived retinal cells.

7. Adult stem cell

For many years, researchers have been seeking to understand the body's ability to repair and replace the cells and tissues of some organs. Scientists have now focused their attention on adult stem cells. It has long been known that stem cells are capable of renewing themselves and that they can generate multiple cell types. Today, there is new evidence that stem cells are present in far more tissues and organs than once thought and are capable of developing into more kinds of cells than previously imagined. Efforts are now underway to harness stem cells and to take advantage of this capability, with the goal of devising new and more effective treatments. What lies ahead for the use of adult stem cells is unknown, but it is certain that there are many research questions to be answered and that these answers hold great promise for the future.
Adult stem cells share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies and specialized functions.
Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Such cells are usually regarded as "committed" to differentiate along a particular cellular development pathway, although this characteristic may not be as definitive as once thought [Marcus A. et al. 2008].
Unlike embryonic stem cells, which are defined by their origin, adult stem cells share no such definitive means of characterization. In fact, no one knows the origin of adult stem cells in any mature tissue. Some have proposed that stem cells are somehow set aside during fetal development and restrained from differentiating. The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.
In the next part of the chapter we will refer only to fetal and adult stem cells.

8. Fetal Stem Cells

In recent years, foetal stem cells (FSCs) and stem cells isolated from cord blood or extraembryonic tissues have emerged as a potential 'half way house' between ES cells and adult stem cells. FSCs can be found in foetal tissues such as chorionic villus sampling (CVS) blood, liver, bone marrow, pancreas, spleen and kidney. They are also found in cord blood and extraembryonic tissues such as amniotic fluid, placenta and amnion [Marcus A et al 2008]. Their primitive properties, expansion potential and lack of tumorigenicity make them

an attractive option for regenerative medicine in cell therapy and tissue engineering settings. While extraembryonic tissues could be used with few ethical reservations, the isolation of FSCs from abortuses is subject to significant public unease. We review here the characteristics of stem cells from foetal, cord blood and extra embryonic tissues, their application in cell therapy and their potential for reprogramming towards pluripotency.

Fetal stem cells are advantageous for research for some relevant reasons.

First, they could be obtained from minimally invasive techniques during the gestation, for prenatal diagnosis. A number of studies followed, reporting that preparations of amniotic epithelial cells (AECs), amniotic mesenchymal cells (AMCs), and cells collected from amniotic fluid (AFCs), seem to contain cells with certain stem cell properties. These cells possess a high proliferation potential, express markers (such as OCT4) specific to pluripotent stem cells, and display the potential to differentiate *in vitro* into cells of all three germ layers [Alviano F. et al. 2007;De Coppi P. et al. 2007; Ilancheran S., et al 2007 Kim, J. et al 2007; Miki, T et al 2005;Tamagawa T. et al 2007; Zheng Y.B et al. 2008].

Second, fetal stem cells have a higher potential for expansion than cells taken from adults. Mesenchymal cells from umbilical cord blood can be induced to form a variety of tissues when cultured *in vitro*, including bone, cartilage, myocardial muscle, and neural tissue [Bieback et al 2004]. Third, the ability to isolate pluripotent autogenic progenitor cells during gestation may be advantageous for the timely treatment of congenital malformations or genetic diseases in newborns (in utero therapy). Fourth, their use is devoid of the ethical issues associated with embryonic stem cells [Weiss, M.L., and Troyer, D.L.2006]. Recently, a new source of human amniotic fluid stem cells (hAFSC) has been isolated [De Coppi et al 2007]. These cells represent 1% of the population of cells obtained from amniocentesis and are characterized by the expression of the receptor for stem cell factor c-Kit (CD117). hAFSC are multipotent, showing the ability to differentiate into lineages belonging to all three germ layers, and can be propagated easily *in vitro* without the need of a feeder layer. hAFSC express the markers OCT4 and SSEA-4, both of which are typical of the undifferentiated state of embryonic stem cells (ESC). However, hAFSC do not express some of the other typical markers of ESC, such as SSEA-3, and instead express mesenchymal and neuronal stem cell markers (CD29, CD44, CD73, CD90, and CD105) that are normally not expressed in ESC. Therefore, hAFSC can be considered as an intermediate type of stem or progenitor cell between ESC and adult stem cells resident in differentiated organs.

Although AFS cells have been recently discovered and many questions concerning their potential are still open, they appear to harbour specific advantages in comparison to other stem cell populations: (1) they can be easily harvested through amniocentesis, which is a safe procedure routinely performed for the antenatal diagnosis of genetic diseases [Caughey AB et al 2006]; (2) they do not form tumours after implantation *in vivo* [De Coppi et al 2007]; (3) obtaining them during pregnancy is harmful neither to the mother nor to the foetus [Caughey AB, et al 2006;Eddleman KA,et al., 2006; Cananzi M, et al 2009]. Moreover, recent papers have demonstrated that, when injected in models of organ damage and development, AFS cells are able to: integrate into the developing kidney and express early markers of renal differentiation [Perin L et al 2007]; repopulate the bone marrow of immunocompromised mice after primary and secondary transplantation [Ditadi A et al. 2009], and engraft into the lung, differentiating into pulmonary lineages [Carraro G et al 2008] respectively.

A recent study reported for the first time a detailed characterization of the differentiation capability of fetal cells obtained from chorionic villus sampling (CVS) [Spitalieri P et al 2009]. CVSs can be routinely obtained during early pregnancy for prenatal diagnosis

purposes, can be easily cultured *in vitro* and modified by gene targeting protocols for cell therapy applications [D'Alton, M.E. 1994.; d'Ercole, C.,et al. 2003;Sangiuolo, F.et al 2005]. The study investigated whether cells with phenotypic and functional characteristics of stem cells are present within human CVSs harvested from the 9th to 12th week of gestation during routine chorionic villus sampling. Results indicate that human CV cytotrophoblasts contains a cell population expressing typical markers, able to differentiate *in vitro* into derivatives of all three germ layers and also able to populate depleted hematopoietic tissues. Moreover these cells, after injection into mouse blastocysts were incorporated into the inner cell mass and could be traced into several tissues of the adult chimeric mice. Finally no teratoma formation was reported after cell injection into SCID mice, demonstrating their usefulness in cell and gene therapy approach.

9. Adult Stem Cells: Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cell (MSCs)

Specialized connective tissues consist of blood, adipose tissue, cartilage, and bone. It has been generally believed that all cellular elements of connective tissue, including fibroblasts, adipocytes, chondrocytes, and bone cells, are generated solely by mesenchymal stem cells (MSCs) [Ashton BA, et al 1980; Prockop DJ.et al 1997; Pittenger MF, et al 999; Bianco P, et al 2008; Studeny M, et al 2002; Verfaillie CM, et al 2003; Gregory CA, et al 2005], while blood cells are produced by hematopoietic stem cells (HSCs).

Bone marrow (BM) is a complex tissue containing hematopoietic progenitor cells and a connective-tissue network of stromal cells.

The continued production of these cells depends directly on the presence of Hematopoietic Stem Cells (HSCs), the ultimate, and only, source of all these cells.

The term mesenchymal stem cells was coined by Caplan [Caplan AI.et al 1991] in 1991 to describe a population of cells present within the adult bone marrow that can be stimulated to differentiate into bone and cartilage, tendon, muscle, fat [Alhadlaq A., and Mao JJ. 2003; Alhadlaq A., et al 2004; Pittenger MF,et al 1999;], and marrow stromal connective tissue which supports hematopoietic cell differentiation [Dexter TM et al.1976;Friedrich C. et al. 1996]. In addition, controversial data suggest that MSCs may give rise to sarcomeric muscle (skeletal and cardiac) [Wakitani S, et al. 1995; Makino S, et al 1999; Planat-Bénard V, 2004;], endothelial cells [Oswald J,et al 2004] and even cells of non-mesodermal origin, such as hepatocytes [Chagraoui J, et al 2003], neural cells [Woodbury D, et al., 2000] and epithelial cells [Spees JL, et al. 2003; Ma Y, et al 2006] MSCs represent a very small fraction, 0.001–0.01% of the total population of nucleated cells in marrow [Pittenger MF et al., 1999].

Although Bone Marrow (BM) has been represented as the main available source of MSCs [Pittenger MF et al 1999 ; Haynesworth SE et al. 1992], the use of bone marrow-derived cells is not always acceptable because of potential viral exposure and a significant decrease in the cell number along with age. In addition, it requires a painful invasive procedure to obtain a BM sample. Therefore, the identification of alternative sources of MSCs may provide significant clinical benefits with respect to ease of accessibility and reduced morbidity.

The umbilical cord blood (UCB) has been used as an alternative source since 1988 [Gluckman E et al 1989]. The blood remaining in the umbilical vein following birth contains a rich source of hematopoietic stem and progenitor cells (HSCs/HPCs), and has been used successfully as an alternative allogeneic donor source to treat a variety of pediatric genetic, hematologic, immunologic, and oncologic disorders [Broxmeyer HE, et al1989; Gluckman E, et al 1997; Han IS, 2003; Kim SK, et al 2002].

9.1 MSCs and HSCs: Cell and gene therapy

Stem cell therapies utilizing adult mesenchymal stem cells (MSCs) are the focus of a multitude of clinical studies currently underway. Because large numbers of MSCs can be generated in culture, MSCs were thought to be useful for ''tissue-engineering'' purposes [Caplan AI, et al 2001], as exemplified by a number of clinical trials [Dazzi F, et al 2007; Prockop DJ, et al 2007].

MSCs are multipotent cells with the capacity to differentiate to produce multiple types of connective tissue and down-regulate an inflammatory response. MSC are being explored to regenerate damaged tissue and treat inflammation, resulting from cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, stroke, diabetes, cartilage and bone injury, Crohn's disease and graft versus host disease (GvHD) [Phinney DG et al. 2007].

Few years after multipotent MSCs were identified (1980), human trials were commenced to evaluate safety and efficacy of MSC therapy.

MSC transplantation is considered safe and has been widely tested in clinical trials of cardiovascular [Ripa RS et al 2005; Chen SL et al. 2004], neurological [Lee PH et al 2008; Bang OY et al 2005], and immunological disease [Lazarus et al 2005; Ringden O et al 2006] with encouraging results.

Widely described above, MSCs are an excellent candidate for cell therapy because (a) human MSCs are easily accessible; (b) the isolation of MSCs is straightforward and this stem cells can expand to clinical scales in a relatively short period of time [Colter DC et al 2000; Sekiya I et al 2002]; (c) MSCs can be bio-preserved with minimal loss of potency [Lee MW et al 2005; Ripa RS et al 2005]; and (d) human trials using MSCs thus far have shown no adverse reactions to allogeneic versus autologous MSC transplants.

More recently, a new study shows that umbilical cord mesenchymal stem cell transplant (UC-MSCt) may improve symptoms and biochemical values in patients with severe refractory systemic lupus erythematosus (SLE) [Sun L et al 2010]. Authors reported a clinical trials on 16 patients with severe SLE that did not respond to standard treatments [Sun L et al 2010]. After receiving umbilical mesenchymal stem cell transplants, 10 of them completed at least 6 months of follow- up. There was no treatment-related mortality or other adverse events. All patients achieved at least 3 months of clinical and serologic improvement, and for two of them this was achieved without any immunosuppressive drugs. For the first time allogenic UC-MSC transplanatation was shown to be safe and effective, at least short term, in treating patients with severe SLE.

HSCs were successfully employed in gene therapy protocols. An ADA-SCID (Adenosine Deaminase Severe Combined Immunodeficiency) clinical trial was performed on 10 affected childrens [Aiuti A et al 2009]. ADA-SCID is one of the most promising conditions for treatment with combine gene therapy and cell therapy and has been the source of early successes in the field. Autologous CD34+ bone marrow cells transduced with a retroviral vector containing the ADA gene were infused into 10 children with SCID due to ADA deficiency who lacked an HLA-identical sibling donor, after non-myeloablative conditioning with busulfan.

In vivo trials have showed a relevant restored immunity in patients treated by a combination of cell and gene therapy protocol, confirmed in the long-term outcome. After about 10 years, all patients are alive after a median follow-up of 4.0 years and transduced hematopoietic stem cells have stably engrafted and differentiated into myeloid cells containing ADA and lymphoid cells. Eight patients do not require enzyme-replacement therapy because their blood cells continue to express ADA. Nine patients had immune reconstitution with increases in T-

cell counts and normalization of T-cell function. In five patients in whom intravenous immune globulin replacement was discontinued, antigen-specific antibody responses were elicited after exposure to vaccines or viral antigens. Effective protection against infections and improvement in physical development made a normal lifestyle possible. Serious adverse events were reported including prolonged neutropenia (in two patients), hypertension (in one), central-venous-catheter-related infections (in two), Epstein-Barr virus reactivation (in one), and autoimmune hepatitis (in one).

Another clinical trial was reported reviewing long-term outcome nine patients with X-linked severe combined immunodeficiency (SCID-X1), which is characterized by the absence of the cytokine receptor common gamma chain. These patients, who lacked an HLA-identical donor, underwent *ex vivo* retrovirus-mediated transfer of gamma chain to autologous CD34+ bone marrow cells between 1999 and 2002. The immune function on long-term follow-up was also assessed [Salima Hacein-Bey-Abina et al 2010].

Gene therapy was initially successful at correcting immune dysfunction in eight of the nine patients. Transduced T cells were detected for up to 10.7 years after gene therapy but however, acute leukemia developed in four patients, and one died. Seven patients had sustained immune reconstitution and three patients required immunoglobulin-replacement therapy. Sustained thymopoiesis was established by the persistent presence of naive T cells and the correction of the immunodeficiency improved the patients' health.

So, after nearly 10 years of follow-up, gene therapy was shown to have corrected the immunodeficiency associated with SCID-X1.

Another recent study was published reporting the successful application of a gene therapy protocol by using lentiviral β-globin gene transfer in an adult patient with severe β(E)/β(0)-thalassaemia dependent on monthly transfusions since early childhood. About 33 months after the treatment, the patient has become transfusion independent for the past 21th months.

These results are not only important due to the tremendous medical need that exists for thalassemia patients around the world, but also represents a significant step forward for the field of autologous stem cell therapy as an emerging therapeutic modality [Cavazzana-Calvo et al., 2010].

Today, gene therapy may be an option for patients who do not have an HLA-identical donor for hematopoietic stem-cell transplantation and for whom the risks are deemed acceptable even if this treatments are associated associated with a risk of acute leukemia.

10. Induced Pluripotent Stem cells (iPS)

In 2006 researchers at Kyoto University identified conditions that would allow specialized adult murine cells, specifically fibroblasts, to be genetically "reprogrammed" to assume a stem cell-like state, by retrovirally transducting four important stem cell factors (OCT4, SOX2, KLF4 and c-MYC) into them.[Takahashi K et al 2006]. These cells, called "iPSCs" for induced pluripotent stem cells, were in this way genetically reprogrammed by being forced to express genes which themselves regulate the function of other genes important for early steps in embryonic development. These factors were involved in the maintenance of pluripotency, which is the capability to generate all other cell types of the body.

Mouse iPSCs demonstrated important characteristics of pluripotent stem cells: they express stem cell markers, form tumors containing cells from all three germ layers, and are also able to contribute to many different tissues, when injected into mouse embryos at a very early stage during development. After one year the same author, using similar experimental

design and the same four genetic factors, reprogrammed also adult human dermal fibroblasts to iPSCs [Takahashi K et al 2007]. Human iPSCs were similar to embryonic stem cells (ESCs) in numerous ways: morphology, proliferative capacity, expression of cell surface antigens, and gene expression. They could also differentiate into cell types from the three embryonic germ layers both *in vitro* and in teratoma assays. At the same time Thomson and coworkers published a separate manuscript that detailed the creation of human iPSCs through somatic cell reprogramming using four genetic factors, two of which were in common with those reported above[Yu J et al 2007]. These cells met all defining criteria for ES cells, with the exception that they were not derived from embryos.

Despite these common features, it is not known if iPSCs and ESCs differ in clinically significant ways.

First of all, it has to be considered that direct reprogramming was originally achieved by retroviral transduction of transcription factors. Retroviruses are highly efficient gene-transfer vehicles because they provide prolonged expression of the transgene after genomic integration and have low immunogenicity. Successively lentiviral vectors was successfully employed to generate hiPSCs from various cell types, including skin fibroblasts, keratinocytes [Maherali N et al 2008], and adipose stem cells [Wu X et al 2003]. Lentiviruses are a subclass of retroviruses capable of transducing a wide range of both dividing and non-dividing cells [Sun N et al 2009].

While for retroviruses, silencing in pluripotent cells is almost complete and provides a way to identify fully reprogrammed clones [Hotta A et al 2008], lentiviruses seem to escape silencing to varying degrees, depending in part on the species and the promoter sequence. In certain cases, probably due to the site of genomic integration, retroviral vector expression is maintained [Dimos T, et al 2008; Park IH et al. 2008]. Moreover some kind of promoter allowed a continued transgene expression that increases the efficiency of iPSC generation but on the other side severely impairs iPSCs differentiation both *in vivo* and *in vitro* [Sommer CA et al 2010]. Spontaneous transgene reactivation may also occur and lead to tumor formation [Okita K et al 2007]. Partial reprogramming may have arisen from cells that either did not receive all reprogramming factors or expressed the factors with stochiometries or expression levels that did not allow for complete reprogramming.

For the above reasons alternative gene delivery methods were experimented to generate transgene-free iPSCs that are suitable for basic research and clinical applications. Recent study reported the use of a single lentiviral 'stem cell cassette' vector flanked by loxP sites (hSTEMCCA-loxP) in order to accomplish efficient reprogramming of normal or diseased skin fibroblasts obtained from humans of virtually any age [Somers A et al 2010]. Human iPSCs obtained in this way contained a single excisable viral integration, that upon removal generates human iPSC free of integrated transgenes. More than 100 lung disease specific iPSC lines were generated from individuals with a variety of diseases affecting the epithelial, endothelial, or interstitial compartments of the lung, such as Cystic Fibrosis, Alpha-1 Antitrypsin Deficiency-related emphysema, Scleroderma, and Sickle Cell Disease. An high efficiency of reprogramming was obtained, using minute quantities of viral vector. Finally all clones generated with the hSTEMCCA-loxP vector expressed a broad complement of 'stem cell markers'.

Viruses are currently used to introduce the reprogramming factors into adult cells, but this process must be carefully controlled and tested before the technique can lead to useful treatments for humans, because sometimes this integration could causes cancers. The protocol efficiency by using retro/lentiviruses is low, with a reported reprogramming rates

of 0.001% to 1%. [Wernig M et al 2007; Maherali N et al 2007]. The differentiation stage of the starting cell appears to impact directly the reprogramming efficiency: mouse hematopoietic stem and progenitor cells give rise to iPSCs up to 300 times more efficiently than do their terminally-differentiated B- and T-cell counterparts [Emnli S et al 2009]. Also terminally differentiated human amniotic fluid (AF) skin cells were reprogrammed twice as fast and yielded nearly a two-hundred percent increase in number, compared to cultured adult skin cells, probably because these cells may have an embryonic like epigenetic background, which may facilitate and accelerate pluripotency [Galende E et al. 2010]. The ability to efficiently and rapidly reprogram terminally differentiated AF skin cells provides an abundant iPS cell source for various basic studies and a potential for future patient specific personalized therapies [Galende E et al 2010].

Significant progress has been made in improving the efficiency and safety of the reprogramming technique, such as investigating non-viral delivery strategies [Feng B et al 2009; Stadtfeld M, et al 2009; Stadtfeld M, et al 2008; Page RL,et al 2009]

Recent studies have reported on the generation of iPS cells using non viral systems, such as plasmids [Kaji K et al 2009], and transposons [Woltjen K et al 2009], all of which allow for subsequent transgene removal through the Crelox system or transposases. A feasible way is to combine the reprogramming factors into a single polycistronic vector [Utikal J et al 2009], transiently expressing the reprogramming factors required to induce pluripotency. Plasmid vectors [Ko K et al 2009] were successfully used to derivate miPSCs, demonstrating that proviral insertions are not necessary for iPSC generation. For non-integrating delivery systems, the reprogramming rates were very low (approximately 0.0005%). Another possible way to induce pluripotency in somatic cells while avoiding the risks of genomic modifications is through direct delivery of reprogramming proteins. Such a strategy has been reported by different groups [Deng J et al 2009; Doi A et al 2009]. A similar study have demonstrated the feasibility of generating iPSCs by applying recombinant OCT4, SOX2, KLF4 and c-MYC proteins which have been engineered to include a C-terminal poly-arginine sequence. This sequence is capable of mediating cell permeation of the reprogramming protein factors, which, upon entering the cells, could translocate into their nuclei. In combination with valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, these protein factors could induce the reprogramming of mouse embryonic fibroblasts (MEFs) to form iPSCs. [Zhou H et al 2009].

One group even reported that hypoxic treatment can enhance the efficiency of iPSC formation [Yoshida Y et al 2009]. These non-genetic strategies have the advantage of being more readily reversible, possibly facilitating downstream differentiation processes and minimizing any permanent deleterious effects on the cells.

It is widely accepted that the choice of the delivery method will impact the reprogramming efficiency, which is defined as the number of formed colonies divided by the number of cells that were effectively transduced with the reprogramming factors [Colman A et al 2009]. Besides to the delivery method, the overall efficiency of the protocol is subject to other sources of variation that include the transcription factors and target cell type employed, the age of the donor, the passage number of the cells (inversely correlated with efficiency), and whether the specific protocol includes splitting of cells after infection.

Researchers have also investigated whether all factors are absolutely necessary. c-Myc gene known to promote tumor growth in some cases, was eliminated. Three-factors were successfully tested, using the orphan nuclear receptor ESRRB with OCT4 and SOX2. [Feng B et al 2009; Wernig M et al 2008]. In subsequent studies the number of genes required for

reprogramming were further reduced [Huangfu D, et al 2008; Hester ME, et al 2009; Kim JB,et al 2008; Kim JB,et al. 2009; Kim JB, et al 2009] and researchers identified chemicals that can either substitute for or enhance the efficiency of transcription factors in this process [Feng B et al 2009].

Of the original four transcription factor-encoding genes, OCT4 is the only factor that cannot be replaced by other family members and the only one that has been required in every reprogramming strategy in either mouse or human cells. Different cell types have been reprogrammed, including hepatocytes [Scadcfeld M et al 2008], stomach cells [Aoi T, et al. 2008], B lymphocytes [Hannal, et al.2008], pancreatic cells [Stadfeld M et al 2008], and neural stem cells [Emnli S et al 2008] in the mouse; keratinocytes [Aasen T et al 2008], mesenchymal cells [Park H et al 2008], peripheral blood cells [Loh YH et al 2009], and adipose stem cells [Sun N et al 2009] in the human; and melanocytes in both species [Utikal J et al 2009] .

An extensive comparisons between iPSc and ESC to determine pluripotency, gene expression, and function of differentiated cell derivatives were made finding some differences whose clinical significance in the application to regenerative medicine has to be determined yet.

iPSCs appear to be truly pluripotent, although they are less efficient than ESCs regarding the differentiation capacity.

Moreover both iPSCs and ESCs appear to have similar defence mechanisms to counteract the production of DNA-damaging reactive oxygen species, thereby conferring the cells with comparable capabilities to maintain genomic integrity [Armstrong L et al 2010].

Comparative genomic analyses between hiPSCs and ESCs revealed differences in the expression of some genes due to detectable differences in epigenetic methylation status [Chin MH, et al 2009; Deng J, et al 2009; Doi A,et al 2009].

Recently gene-expression profiles performed comparing iPSCs and ESCs from the same species revealed that these cells differ no more than observed variability among individual ESC lines [Mikkelsen TS et al. 2008]. A more recent studies reported a detailed comparison of global chromatin structure and gene expression data for a panel of human ESCs and iPSCs, demonstrating that the transcriptional programs of ESCs and iPSCs show very few consistent differences [Guenther MG et al 2010].

An iPSC may carry a genetic "memory" of the cell type that it once was, and this "memory" will likely influence its ability to be reprogrammed. Understanding how this memory varies among different cell types and tissues will be necessary to reprogram them successfully.

Although much additional research is needed, investigators are beginning to focus on the potential utility of iPSCs which represent patient-specific stem cell lines, useful for drug development, modeling of disease, and transplantation medicine. It is now possible to derive immune-matched supply of pluripotent cells from patient's tissue, avoiding rejection by the immune system. Patients who receive ESC-derived cells or tissues may face the same complications that result from organ transplantation (for example, immunorejection, graft-versus-host disease, and need for immunosuppression). In case of iPSCs, the need for immunosuppressive drugs to accompany the cell transplant would be lessened and perhaps eliminated altogether. Reprogrammed cells could be directed to produce the cell types that are compromised or destroyed by the disease in question. Moreover induced pluripotent cells offer the obvious advantage that they are not derived from embryonic tissues, thereby circumventing the ethical issues that surround use of these materials.

iPSCs have the potential to become multipurpose research and clinical tools to understand and model diseases, develop and screen candidate drugs, and deliver cell-replacement therapy to support regenerative medicine.

10.1 Potential medical application of iPSCs

Easily-accessible cell types (such as skin fibroblasts) could be biopsied from a patient and reprogrammed, effectively recapitulating the patient's disease in a culture dish. The usefulness of iPS cells to model a disease in a culture dish is based on the unique capacity of these cells to continuously self-renew and their potential to give rise to all cell types in the human body [Murry CE and Keller G 2008; Friedrich Ben-Nun I, Benveniscy N 2006]. The potential use of iPSCs as treatments for various disorders has been proposed and tested on *in vitro* and/or *in vivo* animal models, with promising results. Direct injection of (non-autologous) iPSCs into the myocardium of immunocompetent mouse models of acute myocardial infarction led to stable engraftment and substantial improvement in cardiac function [Nelson TJ, et al 2009]. On the other hand, dopamine neurons differentiated from iPSCs have been grafted into the striatum of Parkinsonian rats, showing a motor function recovery [Wernig M et al 2008]. A mouse model of haemophilia A has also been successfully treated by iPSC-derived endothelial cells, which express wild-type Factor VIII, directly injected into the liver [Xu D et al 2009]. Furthermore, neural progenitors differentiated from iPSCs have shown further differentiation into neural and glial cells after transplantation into the cochlea, which suggests potential application in the treatment of hearing loss due to spiral ganglion neuron degeneration [Nishimura K et al 2009].

Thus, iPSCs such as ESCs could provide a limitless reservoir of cell types that in many cases were nor previously possible to obtain. Ideally, iPSC-based therapies in the future will rely on the isolation of skin fibroblasts or keratinocytes, their reprogramming into iPSCs, and the correction of the genetic defect followed by differentiation into the desired cell type and transplantation.

Several disease-specific iPSCs are being generated such as Adenosine Deaminase deficiency-related Severe Combined Immunodeficiency, Shwachman¬Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker Muscular Dystrophies, Parkinson's disease, Huntington's disease, type 1 Diabetes Mellitus, Down Syndrome/trisomy 21, and Spinal Muscular Atrophy [Ebert AD et al 2009; Park I-H et al 2008] in order to use them to model disease pathology. For example, iPSCs created from skin fibroblasts taken from a child with Spinal Muscular Atrophy were used to generate motor neurons that showed selective deficits compared to those derived from the child's unaffected mother [Ebert AD et al 2009]. Another study reported the potential of iPS cell technology to model disease pathogenesis and treatment by creating iPS cell lines from patients with familial dysauronomia (FD), a neuropathy caused by a point mutation in theB kinase complex-associated protein (IKBKAP) gene [Lee G et al 2009]. This mutation leads to a tissue-specific splicing defect that was recapitulated in iPS cell-derived tissues, by showing *in vitro* specific defects in neurogenesis and migration of neural crest precursors, tissues that were previously unobtainable.

Before any iPSC derivatives can be considered for applied cell therapy, the potential for tumor formation must also be addressed fully. Furthermore, in proposed autologous therapy applications, somatic DNA mutations (e.g., non-inherited mutations that have accumulated during the person's lifetime) retained in the iPSCs and their derivatives could

potentially impact downstream cellular function or promote tumour formation (an issue that may possibly be circumvented by creating iPSCs from a "youthful" cell source such as umbilical cord blood) [Haase A et al. 2009].

On the basis of the unlimited capacity to be propagated *in vitro*, iPSCs are good targets for genetic manipulation by gene therapy or gene correction by homologous recombination. Classical gene augmentation therapy has also been applied to iPSCs derived from Duchenne Muscular Dystrophy (DMD) [Kazuki Y et al 2009] and Fanconi Anaemia [Raya A et al 2009]patients. In the former case, a human artificial chromosome (HAC), carrying the full length, wild-type dystrophin genomic sequence [Kazuki Y et al 2009] was introduced into iPS cells generated using retroviral vectors. For Fanconi Anemia disease, gene therapy approach using lentiviral vectors, carrying FANCA or FANCD2 genes, were performed before iPS generation [Raya A et al 2009]. The authors demonstrated that gene augmentation was a pre-requisite for successful iPSC generation, as the genetic instability of the mutant fibroblasts made them non permissive for iPS cell generation. [Raya A et al 2009]. The resultant iPSCs were shown to be phenotypically disease-free, with a functional FA pathway, as well as haematopoietic progenitors derived from these iPSCs [Raya A et al 2009].

Gene targeting by spontaneous homologous recombination has similarly been demonstrated in iPSCs [Hanna et al 2007], by successfully treating the sickle cell anemia mouse model mouse with autologous iPSCs, whose β-globin gene has been corrected by homologous recombination [Hanna et al 2007]. Reprogrammed fibroblasts from an anemic mouse were corrected by homologous recombination, successfully differentiated into hematopoietic progenitors, and subsequently transplanted back into the mouse whose bone marrow has been destroyed by irradiation.

As result of the treatment, a substantial clinical improvement was observed in the various disease phenotypes, providing a paradigm for future preclinical and clinical studies regarding gene targeting in iPSCs. As demonstrated the potential of iPS cell technology is enormous for treating genetic diseases. However it is also mandatory to develop better methods of gene therapy, as genetic integration of lentiviral vectors used for expressing therapeutic transgene maybe oncogenic [Hacein-Bey-Abina S, et al 2008]. Regarding their use in gene therapy protocol, the efficiency of homologous recombination in ES and iPS cells remains extremely low [Zwaka TP et al 2003], in this direction recent advancements were reported with zinc finger nucleases [Zou et al 2009; Hockemeyer D et al 2009].

11. Predictive toxicology and drug discovery

The unique properties of pluripotent-stem cells-based models give them the potential to revolutionize the earliest steps of drug discovery and, in particular, the stages of pathological and toxicology modelling, by providing physiological models for any human cell type at the desired amount. In particular, hepatotoxicity and cardiotoxicity are the principal causes of drug failure during preclinical testing, while the variability in individual responses to potential therapeutic agents is also a major problem in effective drug development [Rubin LL 2004; Davila]C et al 2004]. Currently new drug development continues to suffer for the limited ability to predict the efficacy and toxicity of drugs developed and tested in animal models. As a result, several promising treatments in rodents and non human primates fail in human clinical trials. Differentiated cells and/or tissues derived from human iPS cells can address this issue by providing an unlimited source of

cells to screen drug efficacy and toxicity. The human cellular models used in this field are mainly of two types: primary cells coming from patients' samples, and transformed cell lines derived from tumours or resulting from genetic manipulations. Although these resources have widely demonstrated their utility, they present well-known limitations in terms of supply and relevance respectively. This is because primary human cells are difficult to standardize and to obtain in sufficient number for toxicity testing while human cell lines are often derived from carcinogenic origin and could have different properties than non-malignant cells.

Moreover specific ethnic and idiosyncratic differences in drug action and metabolism can also be evaluated with iPS cells derived from selected individuals thereby making possible customized treatments for individual conditions. Besides the possibility to give rise to high predictive phenotypic models, pluripotent stem cells offer the possibility to explore human polymorphisms associated with drug disposition. Several gene products, including drug-metabolizing enzymes and transporters or transcription factors, are known to be involved in drug disposition, and some of them display well-established associations between genotype and metabolism [Katz, DA, et al 2008]. The advantage of iPS cell technology is that it allows for the first time the generation of a library of cell lines that may represent the genetic and potentially epigenetic variations of a broad spectrum of the population.

Besides the common characteristics and properties that they share with hESCs, iPSCs present the additional advantage that they could be derived from any patient whose disease is to be studied. Therefore iPSCs allow the access both to diseases whose mutation is known and pathologies whose causal mutation is unknown. Pluripotent SCs can be an useful tool to study disease mechanisms, either at the undifferentiated stage or in specific cell types. Moreover, they enable the expression of the pathology in the specific cellular model to be correlated with the patient's symptoms.

They can theoretically provide relevant models for any pathology, including neurological disorders and rare diseases that are difficult to analyse *in vitro*. Moreover, as they are compatible with a miniaturized format, they open the way to screening techniques using genomic resources and chemical libraries.

The use of this tool in high-throughput screening assays could allow better prediction of the toxicology and the therapeutic responses induced by newly developed drugs offering insight into the underlying mechanisms. The net result of this approach would substantially decrease the risk and cost associated with early-stage c1inical trials and could lead toward a more personalized approach in drug administration.

Since the first description of iPS cell generation three years ago, there has been remarkable progress toward clinical implementation of reprogramming technologies. Before iPSCs can be used for clinical purposes, few issues need to be addressed. The recent successes in iPS cell derivation without viral vectors and genomic integration from human cells has brought the realization of the therapeutic potential of iPS cell technology closer than ever. Importantly, however, the suitability of individual iPS cell derivation methods for generating cell populations for cell replacement therapy, disease modeling, and drug discovery remains to be widely demonstrated, and studies assessing the equivalence of different types of iPS cells are ongoing.

Moreover the long term efficacy of iPSCs treatments has to be tested considering as fundamental both the survival and the functional integration of the iPSCs, after introduced them into the patient.

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Part 2

Tissue-specific Regeneration of the Heart

3

Chemical Biology of Pluripotent Stem Cells: Focus on Cardiomyogenesis

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1. Introduction

Heart disease is one of the leading killers in the world and accounts for 36% of all deaths in the United States. Unlike many other organs, the heart lacks the capacity to significantly regenerate new tissues to replace those lost to injury. Consequently, ischemia, viral infection, or other insults can lead to permanent loss of cardiac tissue and irreversible heart failure. Currently, the only definitive treatment for heart failure is heart transplantation, which is precluded from wider use due to the limited availability of donor organs. In the past several years, stem cell-based regenerative therapy has emerged as a potentially promising approach to treat heart failure. Its basic goal is to replace the damaged non-contractile scar tissue with functioning cardiomyocytes derived from pluripotent or multipotent stem cells. For many years, it was generally believed that heart is a terminally differentiated organ lacking capacity to replace cardiomyocytes lost to injury. Recent studies, however, have provided evidence for limited cardiomyocyte renewal in both mice and humans [1, 2]. Moreover, major inroads have been made towards understanding the nature of endogenous progenitor cells within the heart [3-5]. Although the definitive identification of the cardiac progenitor cells remains elusive, evidence of the heart's regenerative capacity, however minimal, gives hope that cell-based therapies could become a viable approach to treat human heart failure.

Among the various cell types considered for transplantation, the pluripotent embryonic stem cells (ESCs) are thought to be most suitable for the role of potentially unlimited reservoir of replacement cells given their ability to differentiate into any cell type in the body, including vascular and heart muscle cells. Following the isolation of the first human embryonic stem cell (hESC) line in 1998 [6], there has been intense interest in trying to direct differentiation of ES cells toward cardiovascular lineages, with the ultimate goal of using hESC-derived cardiomyocytes for transplantation. However the use of hESCs faces two

major obstacles: 1) the ethical issues concerning the use of human fertilized embryos, and 2) immune rejection following transplantation. In light of this, there has been tremendous excitement over the recent development of induced pluripotent stem cells (iPSCs). In 2006, Yamanaka and colleagues demonstrated that introduction of four transcription factors (Sox2, Oct4, Klf4 and c-Myc) into fibroblasts could reprogram these cells into pluripotent iPSCs that closely resemble ESCs [7]. Cellular reprogramming has potential to revolutionize the field of regenerative medicine, since iPSCs engineered from a patient's own cells could circumvent the core ethical and immune rejection problems associated with human ES cells. The discoveries of endogenous regenerative processes and iPSCs raise hope not only for replacement of damaged heart tissues with patient-derived cardiomyocytes but also for development of new cellular models to study the pathogenesis of human heart diseases [7-12].

Despite these advances, the future use of pluripotent stem cells for diagnosis and therapy faces a number of major obstacles. For instance, the stem cell field in general has been hampered by lack of simple and robust methodologies that give consistent results. Current methods typically utilize exogenous proteins and feeder cell layers to maintain the pluripotency of stem cells. While traditional culture conditions are amenable to typical laboratory use, the dependence on expensive biological reagents limits the scale to which these cells can be cultured. Furthermore, there is often considerable variability associated with the use of growth factors, cytokines and protein antagonists. In many respects, small molecules are superior to conventional protein effectors: they are far less expensive and often yield more reproducible results. Finally, from industrial and biotechnological perspectives, small molecules are attractive intellectual property assets that can provide protection of investments in regenerative medicine. These issues have helped to set the stage for the development of small molecular modulators for use in stem cell field. In this chapter, we will provide an overview of the use of small molecules for stem cell maintenance, cellular reprogramming and directed differentiation, with a focus on cardiomyocyte formation.

2. Role of small molecules in stem cell field.

A. Small molecules for stem cell maintenance.

As previously mentioned, an important challenge of using pluripotent stem cells involves devising culture conditions that ensure self renewal through the maintenance of both proliferative capacity and pluripotency. ES cells are traditionally maintained on an inactivated mouse embryonic fibroblast feeder layer in medium containing undefined animal serum and exogenous proteins. For example, mouse ES cells (mESCs) require LIF (Leukemia Inhibitory Factor) to inhibit differentiation and promote cell cycle progression. Human ES cells (hESCs), which are unresponsive to LIF, require Transforming growth factor-β (TGF-β)/Nodal and Basic Fibroblast Growth Factor (bFGF) for maintaining pluripotency [13].

An important issue with the traditional methods of maintaining pluripotent stem cells involves the use of undefined animal serum and feeder layers, which can produce variable and heterogeneous results. Moreover, the use of animal serum products precludes clinical applications given the potential for xenoantigen introduction [14]. To overcome these obstacles, a number of serum and feeder replacement media formulations have been developed. These include KnockOut Serum Replacement, a defined media, which provides

consistent growth conditions for maintaining ES and iPS cells [15], and mTeSR, a media specifically formulated to maintain human ESCs and iPSCs in serum-free and feeder-free conditions [16]. However, both KnockOut Serum Replacement and mTeSR require the use of animal-derived matrices and recombinant proteins, which increase costs and introduce variability [14, 17]. Consequently, there has been considerable interest in replacing exogenous proteins with chemically defined conditions for the long-term maintenance of human pluripotent stem cells.

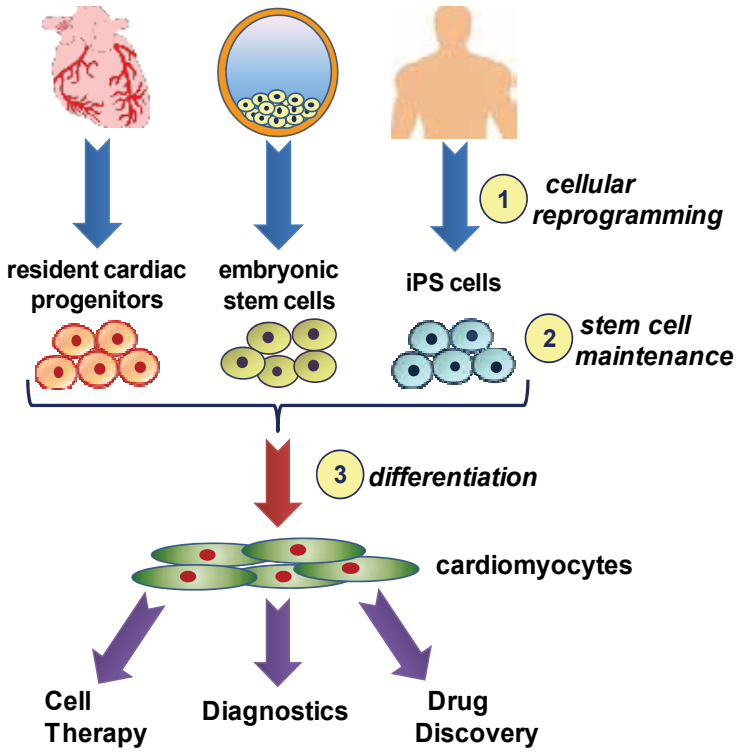


Fig. 1. Application of small molecules for stem cell-based efforts in cardiology.
Small molecules to 1) enhance reprogramming of adult somatic tissues into pluripotent stem cells, 2) maintain pluripotent stem cells in culture, and 3) promote cardiac differentiation of stem cells will be valuable for harnessing the full potential of stem cells in regenerative therapy, human cardiac tissue-based diagnostics, and drug discovery.

In a high-throughput chemical screen, Ding and colleagues identified a novel molecule, Pluripotin/SC1, which was useful for maintaining long-term self-renewal of mouse ES cells (mESCs) in the absence of a feeder layer, serum, leukemia inhibitory factor (LIF) or BMP4 [18]. The mechanism of action was found to be independent of known self-renewal pathways thought to be essential (BMP4/Smad-Id [19], Wnt/B-catenin [20], LIF/STAT3 [21]). Instead, pluripotin was characterized as a dual inhibitor of extracellular signal-regulated kinase-1 (ERK1) and RasGAP, two differentiation-inducing proteins. Blocking RasGAP improves the self-renewal capabilities of ES cells by enhancing the phophonositide-3 kinase (PI3K) pathway [22]. In contrast, ERK1 inhibition works by blocking ES cell differentiation [23, 24].

The pluripotin discovery has important implications for stem cell renewal. The mechanism of action of pluripotin suggests that pluripotency of ES cells can be maintained simply by inhibiting the endogenous activity of differentiation-inducing proteins, rather than also activating specific self-renewal pathways. Thus, inhibiting the effects of endogenously expressed inducers of cell death and differentiation may be sufficient for ES cell self-renewal. This hypothesis is supported by a study by Ying *et al* [25], in which a combination of glycogen synthase kinase-3 (GSK-3) inhibitor, CHIR99021 and mitogen-activated protein kinase (MEK) inhibitor, PD0325901, could maintain long-term self-renewal of mESCs in the absence of exogenous cytokines [25]. Other small molecules which has been used to maintain mouse ES cells in pluripotent state include PD98059, a MEK inhibitor, 6-Bromoindirubin-3′-oxime (BIO), another GSK-3 inhibitor/Wnt signaling activator, and IQ-1, a modulator of Wnt/CBP signalling (Table 1) [20].

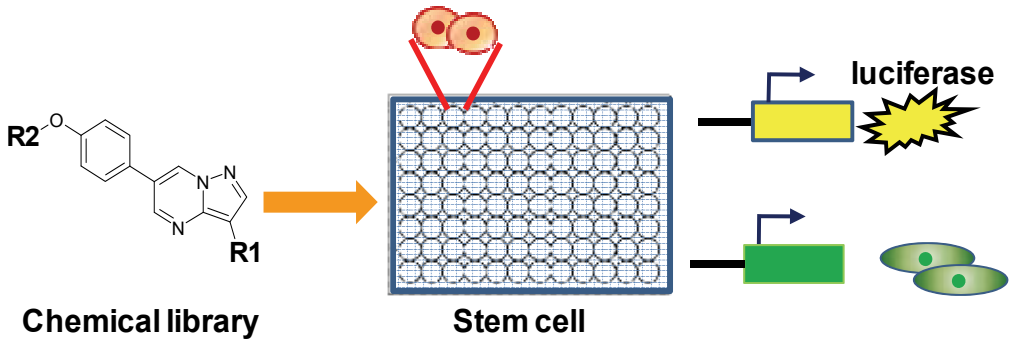


Fig. 2. Overview of the high-throughput screen to identify small molecules that promote cardiomyogenesis in pluripotent stem cells. Small molecules from a chemical library are added to the microtiter plates containing stem cells engineered to express either the luciferase or a fluorescent protein under a cardiac-specific promoter. After several days of differentiation, the potential procardiogenic hits are identified based on their ability to induce luciferase activity or fluorescence.

In addition to synthetic small molecules, certain physiological small molecules are capable of enhancing ES self-renewal. For example, Chen, *et al.* showed evidence that vitamin A (retinol) can promote self-renewal of mES cells in feeder-free conditions by modulating Nanog expression [26], a major transcription factor required for pluripotency. In addition, Garcia-Gonzaolo *et al* demonstrated that the positive effects of Knock-Out Serum Replacement (KOSR) on hESC self-renewal are mediated in part through albumin-associated lipids, although the direct mechanism remains unclear [27].

Thus far, a number of synthetic small molecules that promote self-renewal of mouse ES cells have been discovered, but none are known to support long-term maintenance of human ESCs or iPSCs in chemically defined, animal product-free conditions. Given increasing emphasis on human stem cells and the prospect for cell-based therapeutics, there is a substantial unmet demand for small molecules for maintenance of human pluripotent stem cells.

B. Small molecules for cellular reprogramming

In many respects, iPS cells are molecularly and functionally indistinguishable from ES cells. Like ES cells, they are pluripotent and capable of differentiating into every cell type in the

body [8-10, 12]. The early cellular reprogramming methodologies involved viral vector-mediated transduction of the combinations of either Sox2, Oct4, Klf4 and c-Myc 9, the so-called "Yamanaka Factors", or of Sox2, Oct4, Lin28 and Nanog (SOLN) [10]. It is now known that Oct4, Sox2 and Klf4 are sufficient for reprogramming [28], with the remaining three factors serving to increase reprogramming efficiency. Since the initial breakthrough in mouse embryonic fibroblasts, a number of cell types from variety of mammalian species have been successfully reprogrammed into iPS cells [10, 12].

Although revolutionary, the early efforts in cellular reprogramming had critical limitations. First, the use of lentiviral and retroviral vectors for gene transduction raised the specter of oncogenesis, particularly since these early reprogramming methods resulted in permanent genomic integration of known oncogenes (c-Myc and klf4). Second, the early cellular reprogramming efforts were extremely inefficient, with only .0006 to 0.02% of transduced cells becoming iPS cells, [9, 10, 29]. Subsequent refinements, such as the plasmid-based, protein-based and modified RNA-based strategies, have led to successful virus-free, integration-free methods for cellular reprogramming [30-33]. Despite these successes, reprogramming remains a largely mysterious and inefficient process.

There have been important breakthroughs in the use of small molecules to enhance the efficiency of cellular reprogramming (Table 2). For example, recognizing that cellular reprogramming involves wholesale epigenetic changes, Melton and colleagues used valproic acid (VPA), a small molecule histone deacetylase (HDAC) inhibitor, to dramatically improve the reprogramming efficiency by 100-fold without the use of c-Myc in the reprogramming cocktail [34]. In a related approach targeting the epigenetic machinery, Ding and colleagues used a G9a histone methyltransferase inhibitor, BIX01294, to substantially increase reprogramming efficiency of Sox2-expressing mouse neural progenitor cells (NPCs) transduced with just two factors, Oct4 and Klf4 (OK), to levels obtained with all four Yamanaka factors [35]. In a follow-up screen for small molecules that could synergize with BIX01294 to promote reprogramming in mouse embryonic fibroblasts transduced with Oct4 and Klf4, Ding and colleagues discovered that RG108, a DNA methyltransferase inhibitor, and BayK8644, an L-type calcium channel agonist, could each substantially enhance the reprogramming even in the absence of exogenous Sox2 [36]. In another approach, Eggan and colleagues sought to identify small molecules that could replace one or more of the reprogramming factors, reasoning that such an approach would not only reduce potential safety concerns but also enhance reprogramming efficiency. In a screen for small molecules that could replace Sox2 in the reprogramming cocktail, Eggan and colleagues identified a small molecule TGF-β signaling inhibitor, renamed RepSox, which promotes reprogramming via the induction of Nanog, a transcription factor known to be critical for self-renewal of ES cells [37]. The findings that an L-type calcium channel agonist and a TGF-β inhibitor could enhance reprogramming and bypass the need for Sox2 transduction suggests that a focused approach targeting specific cell signal transduction pathways could contribute to further improvements in reprogramming efficiency. Chemical biological approaches to enhance reprogramming efficiencies have been successfully applied to human somatic cells. For example, a combination of the small molecules SB431542 (an ALK5 inhibitor), PD0325901 (a MEK inhibitor), and thiazovivin (which improves the survival of hESCs upon trypsinization) was shown to improve reprogramming efficiency of human fibroblasts by 200-fold [38]. These chemical biological efforts, in conjunction with improvements in our understanding of the fundamental

mechanism of cellular reprogramming and epigenetic modifications, will lead to continual improvements in reprogramming methodologies, which will help move the iPSC field closer to the clinic.

C. Small molecules for directing stem cell differentiation toward cardiomyogenesis.

For the same reasons that small molecules are proving to be so useful for maintaining stem cell self-renewal and enhancing cellular reprogramming, small molecules will play an important role in directing differentiation of pluripotent stem cells toward the desired cell lineages as well. In principle, small molecules could be used for scaled-up production of stem cell-derived tissues for pharmaceutical testing, clinical diagnostics and even patient-specific cell replacement therapies. Importantly, small molecules can be developed for use as pharmacologicals to boost the heart repair and function following stem cell transplantations. Given the enormous potential of stem cells with respect to cardiovascular medicine, there has been significant interest in identifying small molecules that can promote differentiation of pluripotent stem cells toward cardiomyocytic development (Table 3).

A general approach to identify small molecules that promote cardiac development requires high-throughput screens for small molecules that induce cardiomyogenesis in various cultured stem cell models (Figure 2). In once such chemical screen, ascorbic acid (vitamin C) was found to significantly increase cardiomyogenesis of mouse ESCs. The precise mechanism by which ascorbic acid promotes cardiomyogenesis remains unknown, but it does not appear to involve ascorbic acid's well-known antioxidant activity, as other antioxidants like 4,5,-dihyroxyl-1,3,-benzene-disulfonic acid (Tiron) vitamin E, and N-acetylcysteine were not effective in inducing cardiomyogenesis under identical conditions [39]. A class of compounds named Cardiogenols is another example of procardiogenic small molecules identified in high-throughput screening (HTS) using ES cells. Treatment of mouse ES cells with Cardiogenol C during the initial 3 days of differentiation resulted in greater than 50% of cells expressing the a-MHC gene and greater than 90% of cells expressing GATA-4 at day 7 of differentiation [40]. While small molecules, like Cardiogenols, which are identified in HTS have great potential, one caveat is that procardiogenic effects of such compounds can vary depending on culturing conditions [41].

Nonetheless, Schneider and colleagues recently identified a class of small molecules, called Shz for sulfonyl-hydrazones, in a large-scale chemical screen using a Nkx2.5 promoter-dependent luciferase reporter in pluripotent P19CL6 mouse embryonal carcinoma cell lines [42]. The Shz compounds potently induced expression of cardiac markers Nkx-2.5, Myocardin, Troponin-I and sarcomeric α-Tropomyosin in a number of stem cells and progenitor cells. Interestingly, pretreatment of human mobilized peripheral blood mononuclear cells (M-PBMCs) with the Shz compounds followed by transplantation into injured rat heart lead to significant enhancement in myocardial repair. While the precise mechanism of action is unknown, the Shz compounds could prove to be clinically useful as an adjunct to cell-based therapies to repair damaged heart.

A second general approach to identifying small molecules that promote cardiomyogenesis involves testing known small molecules that selectively modulate key developmental signaling pathways. Since it is generally accepted that *in vitro* cardiomyogenesis in ES cells or iPS cells recapitulates many of the events during heart formation in the embryo, using small molecules to selectively modulate specific cell signaling events that govern embryonic development may be an effective approach to promote cardiomyogenesis in stem cells.

Studies in embryonic heart development highlighted the importance of signaling by Activins and bone morphogenetic proteins (BMPs), both members of the transforming growth factor β (TGF-β) superfamily, in the formation of mesoderm and in specification of myocardial lineages [43-49]. Indeed, the Activin and BMP signaling pathways play key roles in the formation of cardiac cells in both human and mouse ES cell models [47, 49]. Based on the procardiogenic effects of the natural extracellular BMP antagonist Noggin on ES cells, Hong and colleagues demonstrated that dorsomorphin, a selective small molecular inhibitor of BMP signaling, was one of the most robust chemical inducers of cardiomyogenesis in mouse ES cells, increasing the yield of cardiomyoctyes by up to 50-fold [50]. Surprisingly, dorsomorphin treatment limited to the first 24-hours of ES cell differentiation, fully 2 days prior to the robust expression of the early mesoderm marker BryT, was sufficient for massive cardiac induction. This, together with the fact that the robust cardiac induction occurs in the absence of similar induction of BryT or cardiac progenitor maker Mesp1, suggests that the procardiogenic effects of dorsomorphin is mediated by an still unknown mechanism.

Studies of *in vitro* cardiomyogenesis and *in vivo* embryonic heart development have revealed the central role of canonical Wnt/β-catenin signaling [51, 52]. As in many other contexts, Wnt signaling has complex stage-specific effects on cardiomyocyte formation [53, 54]. At the early stages of mouse ES cell differentiation, Wnt activation promotes cardiomyogenesis presumably by augmenting the formation of mesodermal cells committed to a cardiac lineage [55]. But, after the mesoderm formation, Wnt signaling prevents differentiation of committed cells into cardiomyocytes [53, 56]. Similar time-dependent biphasic effects of Wnt signaling on cardiomyogenesis are also noted in human ES cells [57].

Given the importance of Wnt// β-catenin signaling in cardiomyogenesis, it is not surprising then that selective modulators of this pathway have a strong impact on *in vitro* cardiomyogenesis. For example, 6-bromoindirubin-3'-oxime (BIO), a selective inhibitor of GSK-3 which activates Wnt/β-catenin signaling, significantly induced cardiomyocyte formation in mouse ES cells when treatment occurred early (day 0 to 3) in ES cell differentiation. In contrast, late addition of BIO (starting at day 5) completely abolished the expression of cardiac markers and the appearance of spontaneously contracting embryoid bodies [56]. Interestingly, the same compound BIO was identified in a HTS screen for small molecules that expand postnatal Isl1+ cardiovascular progenitor cells [58], and it was also shown to promote proliferation of neonatal and adult rat cardiomyocytes [59]. These intriguing results suggest that small molecule Wnt pathway activators like BIO may one day be used as pharmacological agents to restore function in the diseased heart.

Small molecule Wnt signaling inhibitors may also be useful for promoting cardiac development. For example, Hong and colleagues found that a selective small molecule inhibitor of Wnt/β-catenin signaling can also markedly induce cardiomyogenesis in mouse ES cells. When this inhibitor was administered at day 3, immediately after the formation of mesoderm, there was a nearly 30-fold induction of cardiomyocyte formation at the expense of other mesodermal lineages such as the endothelial and smooth muscle cell lineages (in press). These results hint that small molecule Wnt inhibitors could be useful for promoting cardiac differentiation of multipotent cardiovascular progenitor cell types.

While small molecules have been successful in directing differentiation of embryonic stem cells toward the cardiovascular lineage, accumulating evidence suggests that similar efforts using iPS cells face additional hurdles. For example, efficiency of cardiomyogenesis in

mouse and human iPS cells are significantly lower than for ES cells of respective species [60, 61]. The mechanism for this appears to be the recently discovered epigenetic memory, which stipulates reprogrammed cells tend to differentiate preferentially into tissue types from which they were originally derived [62]. Epigenetic memory is particularly germane to the cardiovascular field since human adult cardiomyocytes are not suitable substrates for reprogramming. Given the breathtakingly rapid advances in the field, however, we anticipate small molecules that can overcome epigenetic memory to facilitate differentiation of iPS cells into cardiovascular cell types will soon be found.

3. Summary

Recent breakthroughs in cellular reprogramming, coupled with the steady stream of advances in stem cell biology, have raised expectations for patient-derived stem cells. It is conceivable that they will someday be used for clinical diagnostic testing, drug discovery, and regenerative medicine to treat human heart failure, a common yet often fatal condition with no known cure. However, as outlined in this chapter, numerous hurdles must be overcome before the regenerative potential of stem cells can be fully harnessed. Increasingly, small molecules are being used to successfully overcome each of these challenges. Therefore, it seems reasonable to anticipate that small molecules will prove to be essential for translating basic stem cell discoveries into future regenerative therapies.

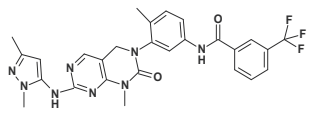
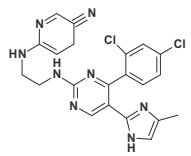
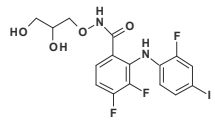
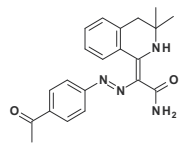
Molecule	Name	Target	reference
	Pluripotin/SC1	Dual inhibitor of RasGAP/ERK1	Chen, *et al*., PNAS 2006 Ref 18
	CHIR99021	GSK3 inhibitor	Ying *et al*., Nature 2008 Ref 19
	PD0325901	MEK inhibitor	Ying *et al*., Nature 2008 Ref 19
	IQ-1	Phosphatase PP2A Inhibitor (Wnt modulator)	Miyabayashi *et al*., PNAS 2007 Ref 20

Table 1. Small molecules for maintenance of pluripotent stem cells

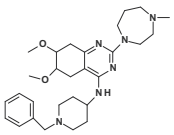
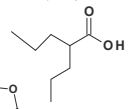
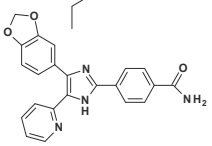
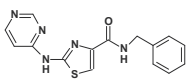
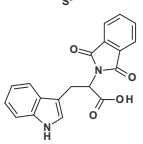
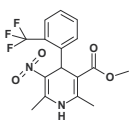
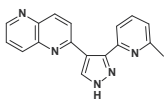
Molecule	Name	Target	reference
	BIX01294	G9a HMTase	Shi *et al*., Cell stem cell 2008 Ref 35
	Valproic Acid	Histone deacetylase (HDAC) inhibitor	Huangfu *et al*.,Nature Biotech , 2008 Ref 34
	SB431542	TGFβ inhibitor	Lin *et al*., Nature method, 2009 Ref 38
	Thiazovivin	unknown	Lin *et al*., Nature method, 2009 Ref 38
	RG108	DNA methyltransferase inhibitor	Shi *et al*., Cell stem cell 2008 Ref 35
	BayK8644	L-type calcium channel agonist	Shi *et al*., Cell stem cell 2008 Ref 35
	RepSox	TGFβ inhibitor	ichida, *et al*., Cell stem cell 2009 Ref 37

Table 2. Small molecules to enhance reprogramming efficiency

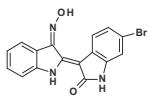
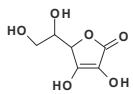
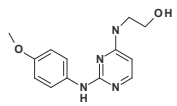
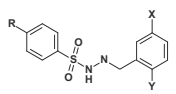
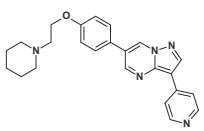
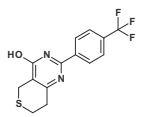
Molecule	Name	Target	reference
	6-Bromoindirubin -3'-oxime (BIO)	GSK-3 inhibitor (Wnt activator)	Naito *et al*., PNAS, 2006 Ref 56
	Ascorbic acid	unknown	Takahashi *et al*., Circulation, 2003 Ref 39
	Cardiogenol C	unknown	Wu *et al*., JACS, 2004 Ref 40
 **R= H, CH3 or Cl X= Br or Cl, Y=OH**	Sulfonyl-hydrazones	unknown	Sadek *et al*., PNAS 2008 Ref 42
	Dorsomorphin	BMP receptor, type-1 inhibitor	Hao *et al*., PLoS ONE, 2008 Ref 50
	XAV939	Tankyrase inhibitor (Wnt inhibitor)	Wang *et al*., ACS chem Bio (in press)

Table 3. Small molecules to promote cardiomyogenesis in pluripotent stem cells.

4. Reference

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4

Cardiac Differentiation of Embryonic Stem Cells by Patterning Culture

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1. Introduction

Although heart transplantation has been established as an ultimate therapy for severe heart failure, it is not a universal therapy because of the lack of donor hearts. As the alternative to heart transplantation, regenerative medicine based on cell transplantation attracts increasing attention. Recent studies revealed that bone marrow-derived cells (Messina et al., 2004; Wollert et al., 2004), endothelial progenitor cells (Kawamoto et al., 2001), adipose-derived cells (Wang et al., 2009), and myoblasts (Memon et al., 2005; Hata et al., 2006; Kondoh et al., 2006) have the potential to improve cardiac function when they are transplanted into a failing heart. Because these somatic cells can be harvested from the patients themselves, their clinical applications have already started. It is considered that the therapeutic potential of these cells depends on the paracrine effects such as the promotion of angiogenesis, suppression of fibrosis, suppression of apoptosis, and attraction of stem cells, and/or the direct contribution to angiogenesis by the differentiation into vascular cells. It was also reported that bone marrow-derived cells and adipose-derived cells have the potential to differentiate into cardiomyocytes (Makino et al., 1999; Planat-Benard et al., 2004). However, the efficiency is too low to directly contribute to the cardiac contractility. Therefore, although the above cells are significantly useful for cardiac regeneration, they cannot replace the dysfunctional cardiomyocytes in a failing heart, and thus their therapeutic potential is limited. Cardiac contractility mainly depends on the cardiomyocytes which account for one third of the total cell content of a heart (Brutsaert, 2003). Therefore, for the better recovery of cardiac contractility, supplementation of functional cardiomyocytes must be necessary.

The representative diseases requiring heart transplantation are dilated cardiomyopathy (DCM) and dilated form of hypertrophic cardiomyopathy (D-HCM). Actually, in Japan, approximately 90% of the patients undergoing heart transplantation so far were DCM or D-HCM. In both DCM and D-HCM, cardiac contractility is decreased by the dysfunction and loss of cardiomyocytes. In approximately 20% of DCM and 60% of HCM, the responsible gene mutations were identified. One of the destinations in cardiac regenerative therapy is to transplant enough amounts of functional cardiomyocytes to mechanically support the cardiac contractility, replacing the dysfunctional and lost cardiomyocytes in a failing heart of such cardiomyopathies. A human left ventricle contains several billions of cardiomyocytes (Olivetti et al., 1991). Therefore, more than a billion is the desired number of cardiomyocytes to be transplanted for the recovery of cardiac contractility. It is impossible

to prepare such a large number of cardiomyocytes from somatic stem cells, such as bone marrow-derived cells and adipose-derived cells, in the present technological situation. On the other hand, embryonic stem cells (ESCs) have substantial potential to generate such a large number of cardiomyocytes, because of their unlimited self-renewal capacity and pluripotency (Murry & Keller, 2008; Passier et al., 2008). Additionally, induced pluripotent stem cells (iPSCs), the equivalents of ESCs artificially created from somatic cells (Takahashi et al., 2007; Yu et al., 2007), are also candidates. At present, ESCs and iPSCs are the only and promising cell sources to generate enough amounts of cardiomyocytes for the direct compensation of lost contractility of a failing heart.

Here the characteristics of iPSCs are briefly mentioned. The major difference of iPSCs compared to ESCs is that they can be created from somatic cells of adults. Therefore, the ethical issue in the case of ESCs which must be extracted from embryos have nothing to do with iPSCs. Additionally, if the iPSCs created from the cells of patients themselves are used as the cell sources for regenerative therapies, immunological rejection after transplantation does not occur. On the other hand, as the drawback of iPSCs, it is considered that their tumorigenecity may be relatively higher than that of ESCs because of the artificial manipulation for the creation of iPSCs. It should be also discussed whether the iPSCs from patients themselves should be used or not when the diseases may be caused by gene mutation. Other biological properties of iPSCs can be considered basically equivalent to those of ESCs. Therefore, the research subjects in preparing differentiated cells for transplantation from iPSCs are almost same as those from ESCs described below.

When undifferentiated ESCs are transplanted into a body, they generate teratomas with high probability. Transplantation of unidentified ESC-derived cells also has the possibility to cause lethal problems. Therefore, for the clinical uses, ESCs must be differentiated into identified objective types of cells and the cells must be purified before transplantation. Accordingly, the major two subjects in ESC research have been, firstly, to enhance the differentiation efficiency into the objective cells such as cardiomyocytes, and secondly, to purify the differentiated objective ESC-derived cells. At an early stage of ESC research, the culture procedures for the induction of cardiac differentiation, such as the hanging drop method, were developed (Hescheler et al., 1997). Thereafter, a lot of biochemical substances, such as retinoic acid (Wobus et al., 1997), ascorbic acid (Takahashi et al., 2003), nitric oxide (Kanno et al., 2004), BMP inhibitors (Yuasa et al., 2005), Wnt inhibitors (Naito et al., 2006), and activin A (Laflamme et al., 2007), were found to promote cardiac differentiation of ESCs. Additionally, the coculture methods with specific cells, such as END2 cells (Mummery et al., 2003) and OP9 cells (Yamashita et al., 2005), were found to promote cardiac differentiation of ESCs. With respect to the purification of ESC-derived cardiomyocytes, Klug et al. developed a genetic purification method using antibiotic-resistant gene which was designed to express in differentiated cardiomyocytes, at an early stage of the research (Klug et al., 1996). Similar genetic purification methods were developed thereafter by the use of reporter genes such as EGFP (Anderson et al., 2007; Hidaka et al., 2003; Muller et al., 2000). Although these methods are significantly useful for the basic research of ESC-derived cardiomyocytes, the genetic modification of ESCs is a big hurdle for clinical uses. Therefore, nongenetic purification methods also started to be developed. For example, some research groups enriched ESC-derived cardiomyocytes by density gradient centrifugation methods (Laflamme et al., 2007; Xu et al., 2006), and other groups purified cardiac progenitor cells by labelling specific surface antigens (Hidaka et al., 2009; Hirata et al., 2007; Yamashita et al., 2005; Yang et al., 2008). Hattori et al. very recently reported a novel nongenetic purification

method using mitochondria as the marker of cardiomyocytes, and achieved more than 99% purity of ESC-derived cardiomyocyte (Hattori et al., 2010). As the nongenetic purification methods will be improved more and more hereafter, the subject of how the requisite number of cardiomyocytes can be prepared easily and at a low cost, that is, "cost-effectiveness" must become more important than mere the efficiency of cardiac differentiation. Because, even if high efficiency of cardiac differentiation is achieved, the culture methods requiring considerable labor and an extremely high cost are not suitable for the preparation of a large number of cardiomyocytes. In this chapter, we will introduce our newly developed culture method which achieves high cost-effectiveness in preparing ESC-derived cardiomyocytes.

2. Culture methods for cardiac differentiation of ESCs

It has been elucidated that the formation of ESC aggregates induces multicellular interactions and thus promotes their differentiation into the derivatives of all three germ layers (Wobus & Boheler, 2005). This property of ESC is utilized also for the induction of cardiac differentiation. ESC aggregates are generally prepared via the formation of suspended spherical ESC aggregates called embryoid bodies (EB). The prepared EBs are then usually plated on the cell-adhesive dishes and cultured for additional days for further differentiation. There are two major conventional methods for the preparation of EBs. One is a hanging drop method, and the other is a suspension culture method (Fig. 1). In the case of hanging drop method, the droplets of ESC suspension are hanged from the lid of culture dishes for several days, and then an EB is formed in each droplet. In this method, the size of EBs can be controlled by ESC concentration in the suspension. It has been elucidated that the direction and efficiency of ESC differentiation significantly depend on the size of EBs (Ng et al., 2005; Wobus et al., 1991). Therefore, the hanging drop method is advantageous to prepare size-controlled EBs for the efficient and reproducible ESC differentiation into a specific lineage. On the other hand, however, the hanging drop method is disadvantageous in the mass preparation of EBs, because of its time-consuming and labor-intensive procedure. In the case of suspension culture method, ESCs are cultured in non-cell-adhesive culture dishes for several days, and then EBs are formed randomly and spontaneously. In this method, a large number of EBs can be easily prepared. On the other hand, however, the size of EBs is not controlled, and thus the differentiation efficiency and reproducibility become much lower than the case of hanging drop method. As described above, a large number of cardiomyocytes, preferably more than a billion, are needed to be transplanted for the direct compensation of cardiac contractility of a failing heart. For this purpose, more cost-effective culture methods for the preparation of ESC-derived cardiomyocytes are needed. Driven by such a necessity, several research groups started to develop scalable culture methods for the mass preparation of size-controlled EBs, utilizing such as multiwell plates (Kim et al., 2007; Ng, et al., 2005), microwell substrates (Khademhosseini et al., 2006; Mohr et al., 2006; Ungrin et al., 2008), patterning culture (Bauwens et al., 2008), and rotary suspension culture (Carpenedo et al., 2007). These novel culture methods are certainly useful for the mass preparation of size-controlled EBs. Especially, the microwell substrates have been commercially available already (AggreWell™, STEMCELL TECHNOLOGIES INC). It should be noted, however, that the EBs prepared by these culture methods must be subsequently collected and plated onto cell-adhesive culture dishes for further promotion of cardiac differentiation. After plating EBs, ESCs proliferate and expand out of the EBs on the

surfaces of culture dishes. Accordingly, the size of resultant EB outgrowths cannot be regulated, which affect and possibly decrease the eventual efficiency and reproducibility of cardiac differentiation. To avoid this problem, EBs are often plated one by one into individual wells of multiwell plates. However, this procedure is of course time-consuming and labor-intensive, and thus not suitable for the mass preparation of ESC-derived cardiomyocytes.

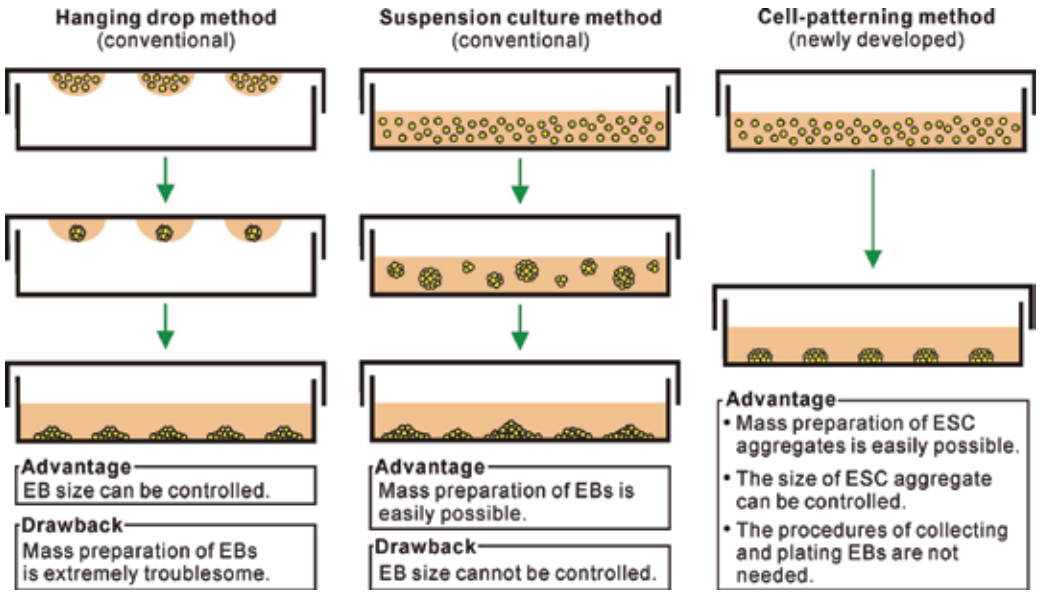

Fig. 1. Concept of the mass preparation of size-controlled EBs by patterning culture.

Here, we would like to introduce our newly developed culture method that enables easy mass preparation of size-controlled ESC aggregates, using cell-patterning technique (Fig. 1.). We fabricated the culture substrates, the surfaces of which comprised arrays of cell-adhesive circular micro-domains and the rest of non-cell-adhesive domains. Mouse ESCs seeded on this "cell-patterning substrates" successfully formed size-controlled aggregates on the circular micro-domains and differentiated into cardiomyocytes. In this method, ESC aggregates are prepared directly on the surface of culture substrates. Therefore, the preparation of suspended EBs is not necessary, and the overall procedure of preparing ESC-derived cardiomyocytes is further simplified than the other methods mentioned above. Moreover, the size of ESC aggregates is controlled throughout the culture period for differentiation, which certainly contributes to the reproducibility of final differentiation efficiency. The details of this "cell-patterning method" are described below.

3. Cardiac differentiation of ESCs by cell-patterning method

3.1 Fabrication of cell-patterning substrates

We fabricated the cell patterning substrates using photolithography-based technique. The details of fabrication process are described elesewhere (Sasaki et al., 2009). Figure 2 shows the schema of fabrication process.Glass coverslips were cleaned by oxygen plasma treatment. The

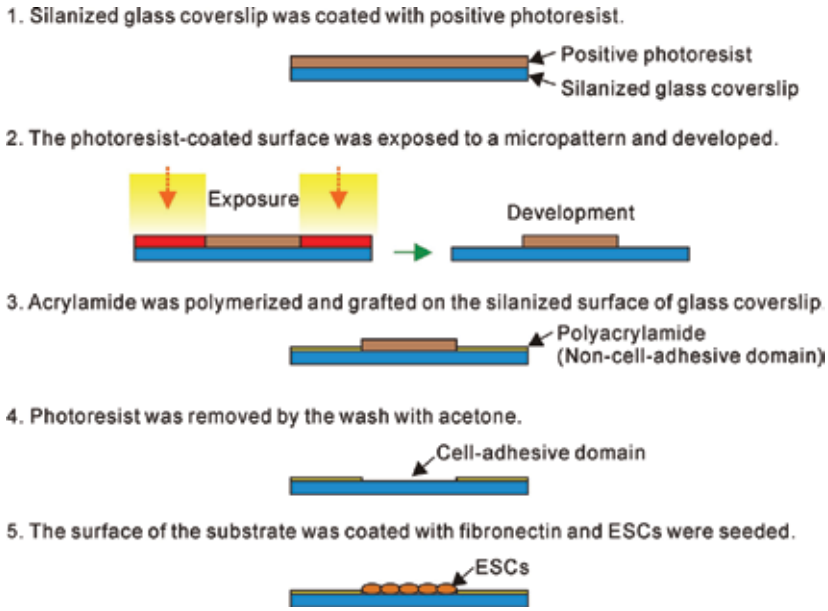

Fig. 2. Schema of the fabrication process of cell-patterning substrates.

surfaces of the coverslips were coupled with 3-methacryloxypropyltrimethoxysilane as described elsewhere (Okusa et al., 1994). The silanized coverslips were spin-coated with g-line positive photoresist (OFPR-800 LB, 34cP; Tokyo Ohka Kogyo) and pre-baked. The photoresist-coated surfaces were exposed with patterned visible light by a maskless photolithography device (Itoga et al., 2006) or through photomasks. The coverslips were washed with developer solvent so that the light-exposed portions of photoresist were removed. The coverslips were post-baked and immersed in acrylamide solution with *N,N'*-methylenebis(acrylamide), ammonium peroxodisulfate, and *N,N,N',N'*-tetramethylethylenediamine, so that the acrylamide was polymerized and grafted onto the silanized glass surface by redox reaction. The coverslips were thoroughly washed with water to remove the ungrafted polyacrylamide, and then washed with acetone to remove the remainig photoresist on the surface. The resultant surfaces comprised non-cell-adhesive hydrophilic polyacrylamide domains and cell-adhesive silanised glass domains. The cell-patterning substrates were thus fabricated. We fabricated the cell-patterning substrates, on the surfaces of which cell-adhesive circular domains with the diameter of 100 μm, 200 μm, 300 μm, 400 μm, or 1 mm were arrayed at constant intervals. The substrates were cut to appropriate size, put into culture dishes, sterilized with ethylene oxide gas, and used for the patterning culture of ESCs. Befor ESCs were seeded, the substrates were coated with fibronectin to promote cell attachment onto the silanized glass surfaces.

3.2 Formation of size-controlled ESC aggregates on the cell-patterning substrates and cardiac differentiation

We used a mouse ESC line EMG7 in which α-cardiac myosin heavy chain promoter-driven EGFP gene was introduced (Yamashita et al., 2005). EMG7 cells express EGFP when they differentiate into cardiomyocytes. Undifferentiated EMG7 cells were maintained on gelatine-coated dishes in the presence of leukaemia inhibitory factor as described elsewhere (Hirai et

al., 2003). For the induction of cardiac differentiation, we used α-MEM (Product # M0644; Sigma-Aldrich) supplemented with fetal bovine serum (FBS), and in some cases with L-ascorbic acid 2-phosphate. Undifferentiated EMG7 cells were seeded onto the cell-patterning substrates at the density of 2-3 × 10^4 cells/cm^2 and cultured for differentiation at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed every one or two days.

Figure 3 shows the time series of microscopic images of EMG7 cells cultured on the cell-patterning substrate with 200-μm-diameter cell-adhesive domains, in the presence of 5% FBS. EMG7 cells seeded on the substrate proliferated within the circular domains and reached confluency within the circular domains on day 3, forming circular cell colonies. As the cells further proliferated, the colonies gradually formed three-dimensional cell aggregates. Around day 9, EGFP fluorescence became obvious and some of the EGFP-positive aggregates were beating, which indicated the cardiac differentiation of EMG7 cells. We confirmed that over 50% of cell aggregates were EGFP-positive on day 10. Figure 4 shows the microscopic image of EMG7 cells cultured on the cell-patterning substrate with 1-mm-diameter cell-adhesive domains in the presence of 10% FBS, on day 13. This microscopic image reveals that the cell aggregate formation and EGFP expression (i.e., cardiac differentiation) occurs preferably near the edge of circular domain. Such a structure strongly indicates the mechanism of cell aggregate formation, as described below. Generally, when cultured cells become confluent on a culture surface and contact with each other, their proliferation is suppressed by the phenomenon which is known as "contact inhibition" (Takai et al., 2008). Consequently, the cells form monolayer structure in the case of ordinary adhesion culture. On the other hand, in the case of patterning culture, when the cells become confluent on the cell-adhesive circular domain, the cells at the edge of circular domain are still not surrounded by the cells. Therefore, their proliferation is not suppressed by contact inhibition. Because the proliferated cells at the edge of the circular domain cannot attach to the non-cell-adhesive surface outside the circular domain, they must fold toward the inside of the domain and form the aggregate. When the diameter of cell-adhesive circular domain was 200 μm, EMG7 cells formed aggregate over the whole region of the circular domain until day 6 (Fig. 3). Accordingly, eventual EGFP-expressing regions included not only the vicinity of edge but also the center part of the circular domain (Fig. 3).

We investigated the dependency of cardiac differentiation efficiency on the diameters of cell-adhesive circular domains. Undifferentiated EMG7 cells were cultured for differentiation on the cell-patterning substrates with the cell-adhesive circular domains with a diameter of 100 μm, 200 μm, 300 μm, or 400 μm, in the presence of 5% FBS. Figure 5 shows

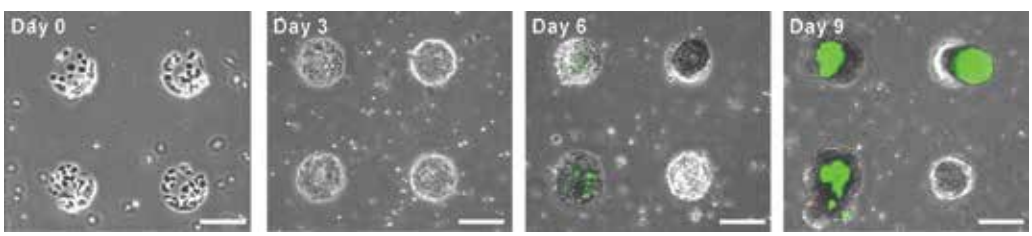

Fig. 3. Microscopic images of EMG7 cells cultured on the cell-patterning substrate with 200-μm-diameter cell-adhesive domains on day 0, 3, 6, and 9. EGFP fluorescence images are superimposed on phase contrast images. Scale bars represent 200 μm. This figure is taken from Sasaki et al., 2009 with modification.

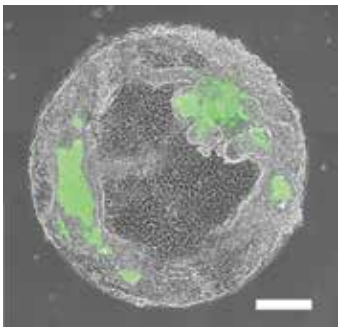

Fig. 4. Microscopic image of EMG7 cells cultured on the cell-patterning substrate with 1-mm-diameter cell-adhesive domains on day 13. EGFP fluorescence image is superimposed on phase contrast image. Scale bar represents 200 μm

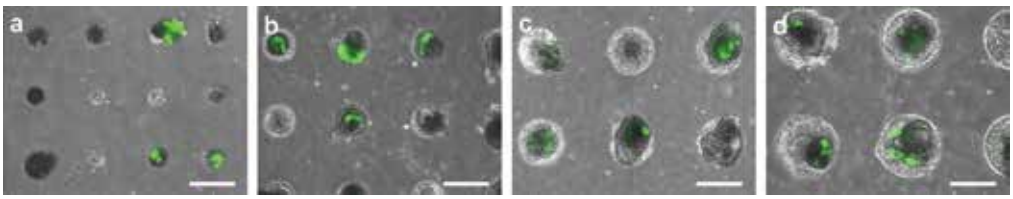

Fig. 5. Microscopic images of EMG7 cells cultured on the cell-patterning substrate with 100-μm-diameter domains (a), 200-μm-diameter domains (b), 300-μm-diameter domains (c), and 400-μm-diameter domains (d), on day 10. EGFP fluorescence images are superimposed on phase contrast images. Scale bars represent 300 μm. This figure is taken from Sasaki et al., 2009 with modification.

the microscopic images of cultured EMG7 cells on day 10. In all cases, the formation of cell aggregate on the circular domains and EGFP expression (i.e., cardiac differentiation) were confirmed. However, when the diameter of circular domains was 100 μm, the aggregates were easily detached from the substrates by medium changes, so that a number of the aggregates were lost. We prepared single cell suspension from these samples, and analyzed the percentages of EGFP-positive cells by flow cytometry. The details of single cell preparation are described elesewhere (Sasaki et al., 2009). Figure 6 shows the result of flow cytometry. The percentage of EGFP-positive cells was maximal when the diameter was 100 μm and 200 μm, reaching aproximately 1.5%. We consider the reason of this result as below. Because the aggregate formation occurs from the edge of circular domain, the aggregate formation on the center part of 300- and 400-μm-diameter domains might be insufficient at the initial stage of differentiation, which resulted in the lower efficiency of cardiac differentiation. When the diameter was 100 μm, a number of cell aggregates were detached, so that the yield of resultant differentiated cells was significantly reduced. From these results, we concluded that the optimal diameter of circular domains is 200 μm under this experimental condition.

As the next step of research, we are now investigating culture conditions to acquire ESC-derived cardiomyocytes more efficiently. We fabricated the cell-patterning substrates, on the

surface of which 200-μm-diameter cell-adhesive circular domains were arrayed triangularly at 100 μm intervals. Undifferentiated EMG7 cells were seeded onto this cell-patterning substrate and cultured for differentiation in the presence of 20% FBS and 0.5 mM L-ascorbic acid 2-phosphate for 7 days. The medium was changed everyday in this period. Consequently, cell aggregates were formed on the circular domains. Thereafter, the cells were cultured for further differentiation in the presence of 1% FBS and 0.5 mM L-ascorbic acid 2-phosphate for additional days. In this period, the medium was changed every other day. Figure 7 shows the microscopic image of differentiated EMG7 cells on day 14. We confirmed that over 80% of the cell aggregates were EGFP-positive. On day 15 the percentage of EGFP-positive cells was analyzed by flow cytometry (Fig. 8). The percentage reached over 5%, which is comparable to or rather higher than the reported values in the case of traditional hanging drop method.

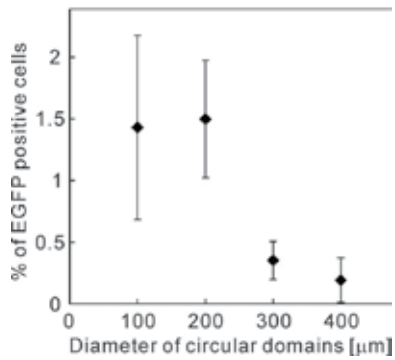

Fig. 6. Percentages of EGFP-positive cells in the differentiated EMG7 cells cultured on the cell-patterning substrates on day 10. Error bars represent the SD of 5 independent experiments.

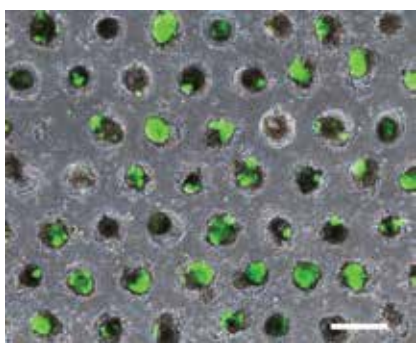

Fig. 7. Microscopic image of EMG7 cells cultured on the cell-patterning substrate with 200-μm-diameter cell-adhesive domains on day 14. EGFP fluorescence image is superimposed on phase contrast image. Scale bar represents 300 μm.

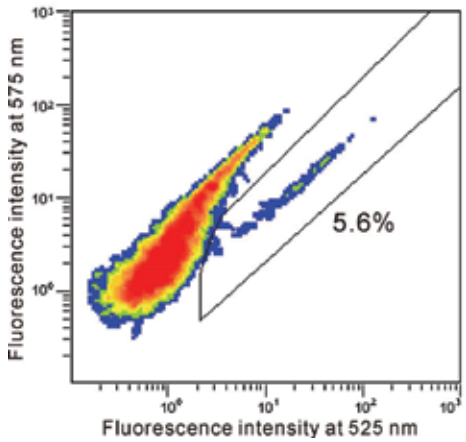

Fig. 8. Scatter diagram of differentiated EMG7 cells on day 15, obtained by flow cytometry

3.3 Future research in cell-patterning method

As described above, we have demonstrated that the cell-patterning method enables easy mass preparation of size-controlled mouse ESC aggregates and subsequent cardiac differentiation. As a next step of research, it is of course important to investigate more preferable culture conditions for efficient cardiac differentiation. However, the most important subject that should be done is to apply the cell-patterning method not only to mouse ESC differentiation but also to human ESC differentiation. As is well known, the difference between the properties of mouse ESCs and human ESCs is not so small. For example, undifferentiated human ESCs survive poorly when they are dissociated into single cells (Amit et al., 2000). Therefore, some kind of contrivance is needed, such as the use of Rho-associated kinase inhibitor (Watanabe et al., 2007), when human ESCs are seeded onto the cell-patterning substrates. It should be also investigated whether the cell-patterning method can be applied to the differentiation into the cells other than cardiomyocytes, such as neurons and hepatocytes. And finally, the cell-patterning substrates for ESC differentiation should be commercialized to promote the research of ESC-based regenerative therapies in the world.

4. Methods of cell transplantation

4.1 Cell Injection

For the establishment of cardiac regenerative therapy based on cell transplantation, the subject of how to transplant the cells is exceedingly important as well as how to create the cells for transplantation. The simplest method of cell transplantation is to inject cell suspension into the cardiac tissue of failing heart. A number of clinical studies and trials have been done by the cell injection method, and achieved some therapeutic gain (Mathur & Martin, 2004; Menasche et al., 2001; Wollert et al., 2004). However, it was reported that the engraftment of injected cells at the targeted region is very poor because of the outflow and death of the cells (Hofmann et al., 2005; Zhang et al., 2001). Moreover, it is pointed out that the injury of cardiac tissue due to the cell injection itself may disturb the intercellular electric connection and cause lethal arrhythmia. Therefore, if a large number of cells are injected into cardiac tissue to compensate the poor engraftment of cells, the probability of the occurrence of lethal arrhythmia must increase.

4.2 Tissue engineering

In order to improve the engraftment of transplanted cells, "tissue engineering" has been developed. Tissue engineering is a technology of creating three-dimensional tissues from cells *in vitro*. The engineered tissues generally consist of cells, signalling molecules such as growth factors, and scaffolds, which are the three major elements in tissue engineering (Langer & Vacanti, 1993). As the scaffolds of cells, biodegradable polymers such as collagen, gelatine, alginate, polylactic acid, and polyglycolic acid are generally used. After the transplantation of engineered tissues, these scaffolds are gradually degraded and finally replaced by native extracellular matrix. In the field of cardiovascular medicine, blood vessels and heart valves have been created by tissue engineering and clinically used (Poh et al., 2005; Shin'oka et al., 2001). The application of tissue engineering to the myocardial regeneration has been also investigated by a number of groups (Leor et al., 2000; Li et al., 1999; Radisic et al., 2004; Zimmermann et al., 2002). However, the engineered tissues with biodegradable scaffolds eventually form cell-sparse and fibrotic tissues, unlike the native cardiac tissues which are highly dense with cells for the close electrical connection and organized contraction. Additionally, inflammatory responses due to the degradation of scaffolds may result in the failure of engineered tissues (Mikos et al., 1998). Therefore, the engineered tissues without the scaffolds must be favorable for the regeneration of cell-dense tissues such as myocardium. Driven by such a necessity, we have developed a novel technology called "cell-sheet engineering" to create scaffold-free tissues. The summary of cell-sheet engineering and its application to cardiac regenerative therapies are described below.

4.3 Cell-sheet engineering

We developed temperature-responsive culture surfaces, on which the temperature-responsive polymer poly(N-isopropylacrylamide) is grafted at nanometer-level thickness (Okano et al., 1993). This surface is cell-adhesive at 37 °C, and becomes non-cell-adhesive below 32 °C. The cells cultured at 37 °C on this surface can proliferate and become confluent on the surface. By the decrease of temperature below 32 °C thereafter, the cells can be harvested noninvasively as an intact cell sheet. Now the culture dishes with the temperature-responsive surfaces are commercially available (UpCell™ Surface; Thermo Fisher Scientific). When the cultured cells are harvested from ordinary culture dishes by the treatment with an enzyme such as trypsin, extracellular matrix and membrane proteins on the surfaces of cells are disrupted, which certainly decreases the engraftment of these cells to the host tissue on transplantation (Fig. 9). On the other hand, when the cell sheet is harvested from the temperature-responsive surface by decreasing temperature, extracellular matrix and membrane proteins are retained on the surfaces of cells (Fig. 9). Accordingly, the cell sheet can engraft to the host tissue rapidly and stably on transplantation. It is also possible to create a thicker tissue by layering cell sheets *in vitro*.

Cell-sheet engineering enabled the creation of cell-dense tissues without noncellular scaffolds. As mentioned above, scaffold-free engineered tissues are favorable to be transplanted for myocardial regeneration. We have demonstrated the therapeutic effectiveness of the cell sheets created from cardiomyocytes (Miyagawa et al., 2005; Sekine et al., 2006), myoblasts (Hata et al., 2006; Kondoh et al., 2006; Memon et al., 2005), and cardiac progenitor cells (Matsuura et al., 2009). We also started the clinical study of myoblast-sheet transplantation for DCM patients. Although cell-sheet engineering is extremely useful to create scaffold-free tissues, the number of cell sheets which can be layered *in vitro* is limited to 3-4, because the deficiency of nutrient supply into the thick tissue causes necrosis. To

create

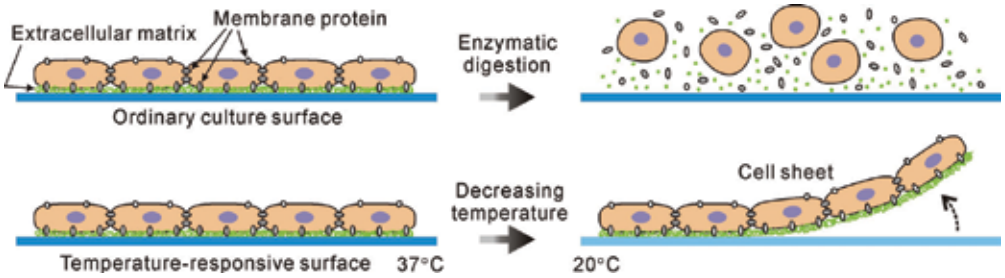

Fig. 9. Comparison of cell harvest between enzymatic digestion and decreasing temperature of temperature-responsive surface.

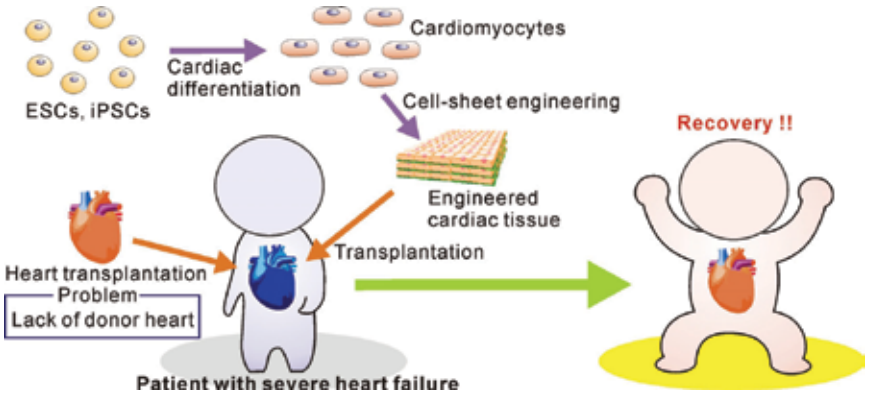

Fig. 10. Strategy for the establishment of cardiac regenerative therapy based on cell-sheet engineering, as the alternative to heart transplantation.

thicker tissues from cell sheets, vascularization in the engineered tissue to supply nutrients is necessary. We overcame this subject by utilizing the vascularization capacity of a living body. When the triple-layer cardiomyocyte sheet is transplanted into subcutaneous tissues, vascularization occurs within a day. Therefore the next triple-layer cardiomyocyte sheet can be transplanted on there without necrosis. By such a multistep transplantation, we succeeded to create 1-mm thick myocardium with a well organized microvascular network (Shimizu et al., 2006). We are now trying to simulate this *in vivo* situation *in vitro*. We also succeeded to create tubular pulsatile tissues by wrapping cardiomyocyte sheets around a blood vessel or tubular scaffold (Kubo et al., 2007; Sekine et al., 2006). We confirmed that the beating of thus prepared myocardial tube can contribute to blood pressure independently of intrinsic heartbeat. Our final goal is to create thick and powerfully contractile cardiac tissues from human ESCs or iPSCs, which can mechanically support the blood circulation of patient with heart failure. The schema of our strategy for the establishment of cardiac regenerative therapies as the alternative to heart transplantation is illustrated in Fig. 10.

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Part 3

Tissue-specific Regeneration of the Brain and Sensory Organs

5

Embryonic Stem Cell-Derived Multipotent Mesenchymal Stromal Cell Therapy Following Focal Ischemia in the Rat

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1. Introduction

Stroke is a major public health concern, with ~795,000 strokes occurring in the United States each year, resulting in over 130,000 deaths annually, and 4.8 million stroke survivors (CDC Risk Survey 2008). The estimated direct and indirect cost of stroke for 2010 is $73.7 billion (CDC Risk Survey 2008). Clinical treatment options for ischemic stroke (which accounts for ~87% of stroke cases) are limited to chemical or mechanical clot-busting interventions during a short time-window following stroke. Therefore, the development of post-ischemic therapies to reduce the mortality and disability associated with stroke would have clear public health benefits.

Multipotent mesenchymal stromal cell (MSC; also sometimes called mesenchymal stem cell) transplantation has shown protection against stroke in animal models, reducing infarct volumes and improving behavioral function (Chen et al. 2001; Li et al. 2001); transplanted MSCs, however, often do not show show long-term survival and integration into the brain. This has led many investigators to believe that paracrine mechanisms underlie the benefits of MSC transplantation. Indeed, neural stem cell derivatives, such as neuroepithelial cells, may be better suited than MSCs to promote neuronal replacement due to their neuroectodermal developmental origin (Kelly et al. 2004; Jiang et al. 2006; Darsalia et al. 2007; Fong et al. 2007; Daadi et al. 2008). Transplanted cells face a hostile, inflammatory environment in the near term post-ischemic cerebrum, which may contribute to the limited survival of the engrafted stem cells. MSC transplantation induces gene expression changes that suggest an altered inflammatory response following ischemia (Ohtaki et al. 2008). In addition to MSC promotion of endogenous repair and replacement processes, this inflammatory modulation (Aggarwal & Pittenger 2005) may provide for an ameliorated pro-survival and/or pro-differentiation milieu for subsequent transplantation of other stem cell types, such as neuroepithelial cells. Addressing the issues of potential allograft rejection and limited long-term cell survival/replacement will be important to the clinical application of stem cell therapies, regardless of which type of stem cell is used. The immunosuppressive attributes of MSCs put them under consideration for use in co-transplantation approaches to mitigate graft rejection concerns.

2. Differentiation of human ES cells into multipotent mesenchymal stromal cells

2.1 Origin and function of mesenchymal stromal cells

Mammalian bone marrow contains multiple cell types including a population of multipotent hematopoietic stem cells, which gives rise to erythroid, lymphoid and myeloid lineages of blood cells. Bone marrow stromal cells provide support to the hematopoietic components of the marrow through the production of extracellular matrix (ECM) components, cytokines and trophic factors (Yin & Li 2006; Méndez-Ferrer et al. 2010). They may also play a role in maintaining immunological memory by providing a survival niche in the marrow for plasma cells and memory T lymphocytes (Tokoyoda et al. 2010). Some of these marrow stromal cells are capable of giving rise to mesenchymal tissues such as bone, cartilage, connective tissue, muscle and fat, thus giving rise to the term "mesenchymal stem cell" (Caplan 1991). Such cells are not unique to the bone marrow, as cells with the characteristics of mesenchymal stem cells have been isolated from a variety of human and animal tissues, including umbilical cord blood (Bieback et al. 2004), adipose tissue (Zuk et al. 2001), skeletal muscle (Williams et al. 1999), and dental pulp (Gronthos et al. 2000). Indeed, MSCs may reside within the connective tissue of the majority of organs (Young et al. 1995).

Traditionally, MSCs have been obtained through gradient centrifugation of bone marrow aspirates, and subsequently plating the heterogeneous mononuclear cells on plastic dishes. Hematopoietic cells do not attach to the plastic substrate so that after approximately two weeks in culture, the adherent cells should be primarily MSCs. Flow cytometry is then used to either characterize or sort, depending upon the uniformity of the cells, the CD73+, CD34– population. Alternate cell surface markers are sometimes used in addition to or in place of CD73. The CD34 antigen is a marker of hematopoietic cells, so MSC preparations should be free of CD34+ cells. Differentiation in culture to osteoblasts, chrondrocytes and adipocytes is commonly assayed using staining with Alizaren Red, Alcian blue and Oil Red O, respectively (Pittenger et al. 1999).

Although "bone marrow stromal cell" and "mesenchymal stem cell" have been used somewhat interchangeably in the literature, neither name adequately reflects both the marrow stromal function and the multipotent differentiation capacity of the cells. Additionally, there has been question as to whether these cells fulfill the criteria for "stemness" at the individual cell level (Horwitz et al. 2005). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) promotes the use of "multipotent mesenchymal stromal cell" as a replacement moniker that better encompasses this range of function, yet still allows for the use of the widespread MSC acronym (Horwitz et al. 2005; Dominici et al. 2006). The diverse nature of MSC sources has also led to concerns that heterogeneity among different cell preparations may complicate comparisons of their functions in experimental systems. The ISCT has therefore further proposed (Dominici et al. 2006) minimal criteria to define human MSCs as the following:

a. Cells must be plastic-adherent under standard culture conditions.
b. Greater that 95% of the cell population must express CD73, CD90, and CD105 but lack expression (<2% positive) of CD34, CD45, CD11B or CD14, CD19 or CD79a, and should be unstimulated (lack HLA class II antigen expression).
c. Cells should be capable of in vitro differentiation into adipocytes, chondrocytes, and osteoblasts.

2.2 Overview of hES-MSC differentiation and comparison to BM-MSC

Using bone marrow as a source of MSCs has the advantage of a clinically relevant tissue source due to the history of bone marrow transplantation as a treatment for variety of blood disorders and cancers. Nevertheless, the expansion capacity of such bone marrow-derived MSCs (BM-MSCs), while significant, is not limitless (Fehrer & Lepperdinger 2005) and obtaining consistent homogeneous cultures among multiple individual donors can be challenging. In vitro differentiation of embryonic stem cells represents an alternate source of mesenchymal stem-like cells. Embryonic stem (ES) cells may be maintained in culture indefinitely (Amit et al. 2000) and should allow for the production of homogenous MSC preparations that vary little from batch to batch and in greater numbers than can be easily achieved with BM-MSCs.

One common method of differentiating ES cells begins with the transfer of undifferentiated colonies of ES cells into culture medium lacking basic fibroblast growth factor (bFGF). After approximately 4 days under these conditions, ES cells form a cluster of cells known as an embryoid body, in which the endodermal, mesodermal and ectodermal tissue layers are present (Itskovitz-Eldor et al. 2000). The embryoid body is dissociated enzymatically and the cells plated onto plastic for several days, after which the CD73+ population is selected by flow cytometry for further growth. The putative MSCs are further analyzed for appropriate marker expression and their differentiation capacity tested in vitro, as indicated above. Alternatively, the embryoid body stage may be skipped; undifferentiated ES cells may be plated directly onto plastic and the adherent cells sorted for CD73+ expression prior to further growth and characterization (Trivedi & Hematti 2008).

Human ES cell-derived MSCs (hES-MSCs) show very similar cell surface marker expression patterns (see Figure 1) and trilineage in vitro differentiation abilities compared to BM-MSCs (Trivedi & Hematti 2008; Liu et al. 2009; Seda Tigli et al. 2009). As will be discussed below, bone marrow-derived MSCs have been considered immunoprivileged and immunosuppressive, and can avoid or reduce an alloreactive immune response after transplant (Bartholomew et al. 2002; Di Nicola et al. 2002; Tse et al. 2003; Aggarwal & Pittenger 2005). Similarly to BM-MSCs, hES-MSCs do not express class II major histocompatibility complex antigens at their surface. MSCs of either origin do not induce proliferation of T lymphocytes in vitro and indeed, both types of MSCs can suppress activation in mixed lymphocyte reaction assays (Trivedi & Hematti 2008). Therefore, MSCs from these sources are quite comparable, phenotypically and immunologically.

3. Mesenchymal stromal cell therapy in preclinical stroke models

3.1 Rat middle cerebral artery occlusion model of stroke

Multiple animal species, including primates, cats, dogs and rodents (such as mice, gerbils and rats) have historically be used in stroke research. The rat has become a widely used animal model for stroke, however, due to its relatively low animal husbandry costs and the similarity of its cranial circulation to that of humans (Yamori et al. 1976; Lee 1995). In humans, the middle cerebral artery (MCA) is most commonly affected in stroke syndromes (Bogousslavsky et al. 1988) and multiple methods of MCA occlusion (MCAO) have been described to mimic this clinical syndrome in animal models (Macrae 1992). Because recanalization commonly occurs following an acute stroke in the human (Saito et al. 1987), reperfusion after a period of occlusion has been included in many of these models.

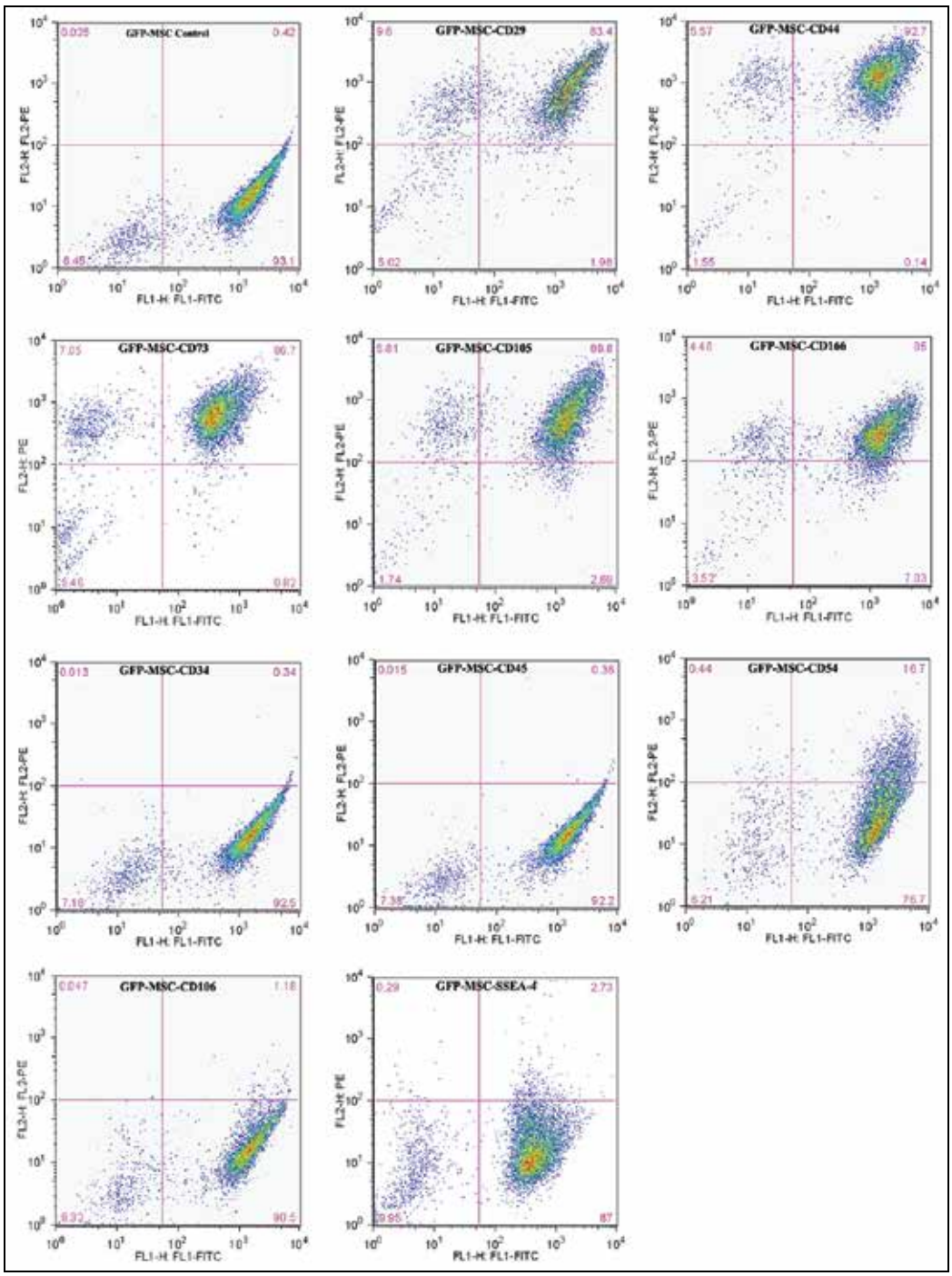

Fig. 1. Surface marker analysis of hES-MSCs expressing green fluorescent protein (GFP) using antibodies to the indicated antigens. GFP-MSC control cells represent unstained cells. The proportion of cells in each quadrant is indicated. Data taken from Liu et al. 2009.

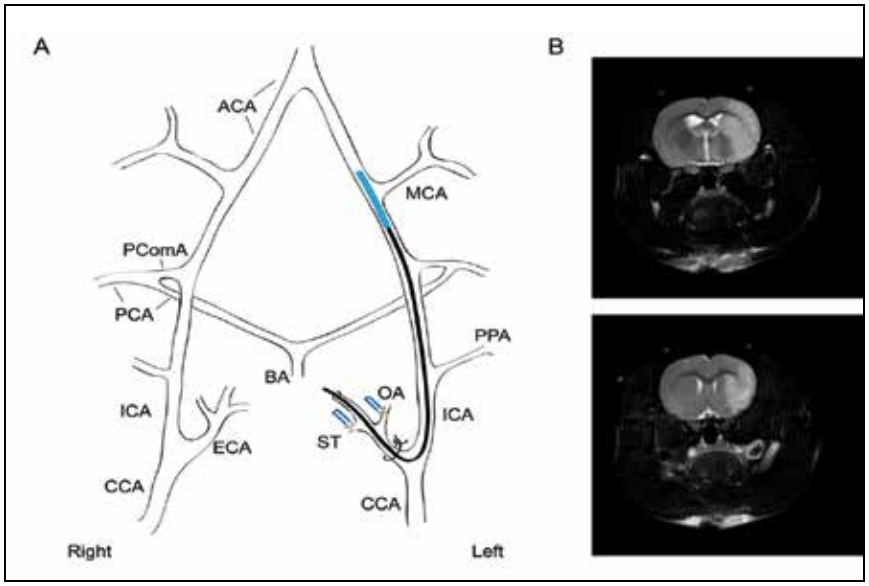

Fig. 2. Stroke induction in the rat. (A) Occlusion of the middle cerebral artery by endovascular silicon-coated suture in the rat. The OA and ST branches off of the left ECA are shown ligated and a suture tie around the ECA stump holds the intraluminal suture in place. ACA, anterior cerebral artery; BA, basilar artery; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; MCA, middle cerebral artery; OA, occipital artery; PCA, posterior cerebral artery; PComA, posterior communicating artery; PPA, pterygopalatine artery; ST, superior thyroid artery. (B) Representative T2-weighted magnetic resonance imaging of coronal rat brain 24 hours after 1 hour transient MCAO. The infarcted region appears hyperintense (bright).

One of the most widely used MCAO animal models (Figure 2) employs an intraluminal nylon suture, modified with a silicon-coated or flame-rounded end, which is advanced through the internal carotid artery to block the origin of the MCA (Koizumi et al. 1986; Longa et al. 1989). The period of occlusion may commonly range from 30 minutes to 2 hours, depending upon the rat strain utilized. Because the lenticulostriate arteries branching off of the MCA are end arteries that supply the basal ganglia without collateral branches, MCAO routinely causes a striatal infarct. The cortical territories of the MCA do receive collateral flow via leptomeningeal anastomoses and suffer a gradient of decreased blood flow from the periphery towards the center of the cortical MCA territory during MCAO. Severe reduction of cerebral blood flow disrupts both the functional and structural integrity of the brain, whereas more moderate blood flow impairment may result in loss of function without structural deterioration. This latter case underlies the concept of the "ischemic penumbra", as cells in such areas may recover function when reperfused. Variability in lesion size and location may occur due to surgical technique, physiological variables during surgery (including body temperature), rat weight, suture dimensions, occlusion period, etc.. The use of spontaneously hypertensive rat strains (which possess poor collateral circulation compared to other rat strains) in the hands of an experienced surgeon, however, generally provides reliable induction of cortical and subcortical infarction (Duverger & MacKenzie 1988; Macrae 1992; Dogan et al. 1998). Visualization of the infarct size in order to confirm a

similar extent of injury between study groups is, nevertheless, highly desireable. This is especially true in studies aimed at determining the efficacy of therapeutic interventions, e.g. stem cell transplantation. Although magnetic resonance imaging (MRI) of the infarct prior to stem cell delivery has not been universally employed, a growing number of studies are recognizing the importance of this approach for reducing the confounding effects of infarct induction variability when assessing potential neuroprotective regimens.

3.2 hES-MSC transplantation reduces infarct size and improves behavioral function

A number of investigators have evaluated the potential of MSCs to provide enhanced recovery from surgically-induced stroke in rodent animal models. Our laboratory has transplanted hES-MSCs intravenously into spontaneously hypertensive rats 24 hours after 1 hour MCAO (Liu et al. 2009). The cells were labelled with green fluorescent protein so that the survival, migration and differentiation patterns of the engrafted cells could be monitored. The hES-MSCs were found in the infarction region, ischemic penumbra and striatum of the ipsilateral hemisphere; hES-MSCs were not observed in the contralateral hemisphere, although transplanted cells have been reported to be present in both damaged and undamaged hemispheres in some other studies (Modo et al. 2002). Rats receiving hES-MSCs showed reduced sensory deficit during the first week following stroke, after which time the control animals had recovered to a similar extent (Figure 3); neither group returned

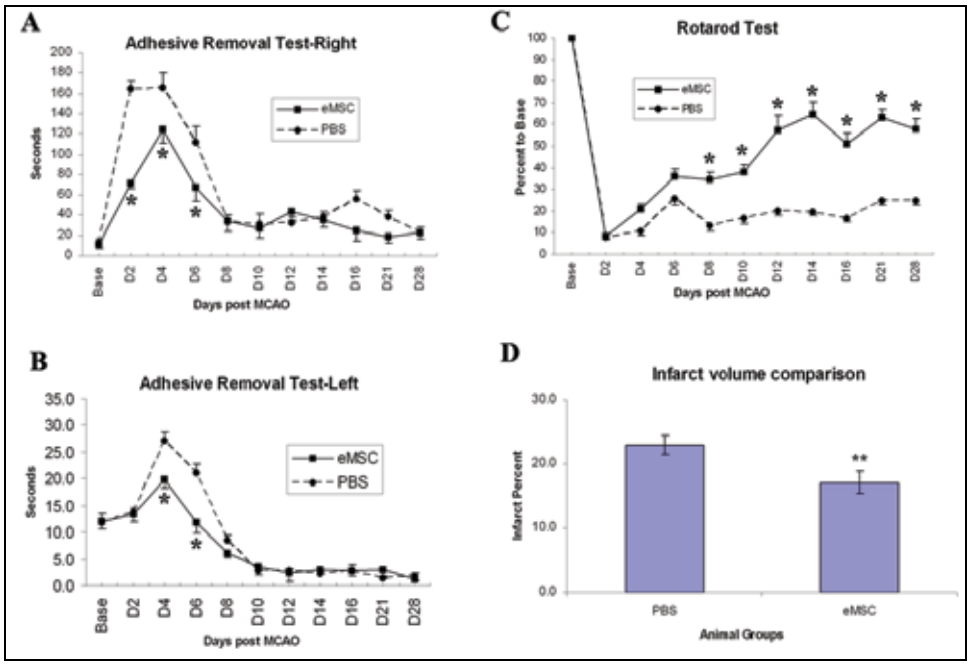

Fig. 3. Human ES cell-derived MSCs (eMSCs) improve sensory and motor function following MCAO. Rats received eMSC or PBS 24 hours after MCAO. Mean time to removal of a small adhesive label for the right (A) and left (B) forepaw. (C) Staying time on the rotarod is shown as a percentage of baseline pre-MCAO control. (D) Infarct volume calculated from cresyl violet-stained brain sections harvested 28 days after MCAO. * $P<0.05$, ** $P<0.01$. Data taken from Liu et al. 2009.

to baseline sensory performance, however. Transplant recipients also displayed improved motor function to approximately 60% of baseline performance by 8 days post-MCAO and this enhanced recovery was maintained through the study's end at 28 days. Infarct volume was ~25% smaller in the hES-MSC-recipient rats as determined by cresyl violet histological staining.

Many other reports have produced similar results using MSC-like cells from multiple sources, including bone marrow-derived stromal cells, human umbilical cord blood stem cells, adipocyte-derived stem cells, as well as other types of stem cells, such as neural progenitor cells [see (Locatelli et al. 2009) and (Bliss et al. 2007) for reviews]. Chopp's group, in particular, has performed a large number of studies with rat, mouse or human MSCs in young, adult or aged animals and observed significant improvement in neurological function following stroke (Li & Chopp 2009). The numbers of cells administered, the delivery route and the timing of cell administration vary with the investigating group and each method has its own advantages and disadvantages. For example, intracerebral injection can specifically target the peri-infarcted region but is invasive, risking further trauma to the already damaged brain. Intravenous injection is less invasive but may result in less discriminate delivery of stem cells to non-target organs. Certainly, these parameters will need to be optimized if this approached is ever to become broadly clinically applicable to stroke treatment.

3.3 Homing of MSCs to injured tissue

Several studies have internally labelled MSCs with radiolabelled, fluroescent or magnetic markers in order to follow the distribution of transplanted cells when administered intravenously. In the healthy animal, IV-infused MSCs home to the bone marrow with varying efficencies (Devine et al. 2001; Wynn et al. 2004) but are detected in multiple other tissues as well, including lung, liver and intestine (Gao et al. 2001; Devine et al. 2003). In the setting of injury or inflammation, however, MSC distribution is shifted preferentially to the site of inflammation (François et al. 2006; Jackson et al. 2010). The migration signals for stem cell homing include the stromal cell-derived factor 1 (SDF1)/chemokine CXC receptor 4 (CXCR4) pathway (Chapel et al. 2003; Dar et al. 2006; Shi et al. 2007; Ryu et al. 2010; Yu et al. 2010). SDF1/CXCR4 is the major signaling axis for homing of hematopoietic stem cells (Wright et al. 2002); MSCs, however, express receptors for and migrate in response to many growth factors and chemokines (Honczarenko et al. 2006; Ponte et al. 2007). In unstimulated human BM-MSCs, the growth factors IGF-1 and PDGF-AB had much stronger chemotactic activity than SDF1 in in vitro assays (Ponte et al. 2007). Upon pretreatment with the inflammatory cytokine, TNFα, however, migration of BM-MSCs in response to the chemokines RANTES, MDC and SDF1 increased dramatically (Ponte et al. 2007). In this study, the levels of CCR3 (one of the RANTES receptors) and CCR4 (receptor for MDC and RANTES) increased with TNFα pretreatment but CXCR4 (receptor for SDF1) levels did not change, leaving the authors to speculate that the downstream CXCR4 signal transduction pathway may have been modulated. Other studies reported that MSCs express low levels of CXCR4 at the cell surface (Rüster et al. 2006) but contain large intracellular pools of CXCR4 (Wynn et al. 2004). Fluid shear stress approximately doubled the number of MSCs with CXCR4 surface receptors (Rüster et al. 2006). Nitric oxide induced CXCR4 expression on mouse MSCs, and raised endogenous SDF1 levels in the ischemic brain (Cui et al. 2007). Co-treatment of mice with BM-MSCs and a nitric oxide donor following stroke improved homing to the ischemic brain, and enhanced functional recovery compared to BM-MSC

treatment alone (Cui et al. 2007). The added benefit of the combination therapy was not seen in the presence of a CXCR4 inhibitor. SDF1 can enhance nitric oxide synthase activity (Cherla & Ganju 2001) so SDF and NOS actions may be mutually reinforcing. The SDF1/CXCR4 signaling axis thus appears to be a major determinant of MSC homing to ischemic lesions in vivo.

At the cellular level, homing reflects the fluid shear stress-resistant interaction of cell surface homing receptors on the MSCs with surface receptors present on the vascular endothelial cells in the target organ. Following the model of leukocyte homing (Sackstein 2005), the initial tethering of the MSC to the vascular endothelium would be followed by further "rolling" and firm adhesion and, finally, extravasation (Rüster et al. 2006). BM-MSCs express multiple integrin proteins (De Ugarte et al. 2003; Rüster et al. 2006), including the integrin α4/β1 heterodimer involved in cell-cell and cell-ECM interactions with endothelial cell vascular cell adhesion molecule (VCAM)-1 and fibronectin, respectively (Guan & Hynes 1990). Blocking integrin β1 in the context of myocardial ischemia interfered with targeting of MSCs to the heart (Ip et al. 2007). MSC interaction with endothelial cells involves additional molecules, such as P-selectin, MMP-2 secretion, and cytokines (Rüster et al. 2006). Inflammation indirectly upregulates VCAM-1 expression in vascular endothelial cells in vitro (Stanimirovic et al. 1997) and in the intact ischemic brain (Frijns & Kappelle 2002; Hoyte et al. 2010). The binding of rat MSCs to vascular endothelial cells can be reduced in the presence of an anti-VCAM-1 antibody (Segers et al. 2006). Exit through the vasculature into the surrounding interstitia is reinforced via MSC integrin binding to ECM components, such as the V regions of fibronectin, which are increasingly exposed by fibronectin fragmentation occurring during tissue remodeling following injury (Valenick et al. 2005). Recent data from in vitro migration assays suggest that ECM collagens may also play a role in MSC chemotaxis (Mauney et al. 2010). Protease action on the ECM is an important component of cell migration. MSCs constitutively express a variety of matrix metalloproteases and a subset of these are induced by the proinflammatory cytokines TGF-β1, IL-1β and TNFα (Ponte et al. 2007; Ries et al. 2007) suggesting another means by which inflammation may direct MSC homing to the site of injury. Nitric oxide also induces expression of MMP9 and enhanced homing following stroke in mice (Cui et al. 2007).

3.4 Immunomodulation by MSCs

Allogeneic cells trigger an immune response by interaction of their cell surface MHC class I antigens in the presence of co-stimulatory molecules with the cognate receptors of host lymphocytes. MSCs express MHC class I, but not class II, markers on their cell surface. Intracellular pools of class II antigens can be brought to the surface by interferon-γ (IFN-γ) treatment of MSCs; IFN-γ fails to mobilize class II molecules, however, once MSCs are differentiated into adipocyte, chondrocyte or osteoblast lineages. Note that whether IFN-γ-stimulated or not, MSCs do not express co-stimulatory molecules such as CD40, CD80, or CD86. MSCs fail to invoke a proliferative response in allogeneic lymphocytes in vitro, including when antigen-presenting cells or costimulatory signals are provided. MSCs also fail to induce other indicators of lymphocyte activation, such as IFN-γ production or expression of activation-associated markers (e.g., CD25, CD38, or CD69). Furthermore, MSCs evade natural killer cell or cytotoxic lymphocyte-mediated cell lysis, despite lysis of other cell types from the same donor (Rasmusson et al. 2003). Not only do they avoid stimulating lymphocyte proliferation, MSCs can suppress T cell activation in mixed lymphocyte reaction assays (Aggarwal & Pittenger 2005; Trivedi & Hematti 2008)

Inflammation is a component of the acute phase response to ischemic injury. One means by which MSC transplantation might influence tissue injury and recovery is through the modulation of the extent of inflammation. A key mediator of inflammation is TNFα, which binds to the cell surface TNF receptor and initiates signaling through the NF-kB pathway. Activation of NF-kB reporter expression in rat hepatoma cells exposed to proinflammatory culture conditions (growth in serum from lipopolysaccharide-stimulated rats) was abrogated when the reporter cells were co-cultured with human BM-MSCs (Yagi et al. 2010). The MSCs in this study themselves showed upregulation of NF-kB signaling under proinflammatory conditions, which resulted in secretion of the soluble form of TNF receptor 1 (sTNFR1) as an anti-inflammatory measure. Production of sTNFR1 by transplanted MSCs in response to inflammation lowered the levels of inflammatory cytokines TNFα, IFN-γ and IL-6 and reduced organ injury in a rat endotoxemia model (Yagi et al. 2010); these effects were at least partly reduced in the presence of a neutralizing antibody to sTNFR1.

Administration of human MSCs into mouse hippocampus one day after transient global ischemia improved neurological function and reduced hippocampal neuronal degeneration (Ohtaki et al. 2008). Microarray gene expression analysis revealed that ~14% of the ischemia up-regulated mouse genes were reduced in mice receiving the MSCs, including many inflammatory and immune response genes. The MSCs, which showed altered gene expression patterns in response to the cerebral ischemic environment as well, elicited local expression of neuroprotective factors, such as insulin-like growth factor 1 and neuropeptide Ym, by microglial/macrophages. Furthermore, although only small scale changes in mouse TNFα, IFN-γ, and IL-4 occurred after MSC delivery, the investigators argued that the ratios of cytokines (e.g., increased IL-4/ IFN-γ and IL-4/ TNFα ratios) were altered in a manner suggestive of a shift from a proinflammatory type 1 helper T cell "Th1" directed immune response to a type 2 helper T cell "Th2" dominant response traditionally associated with lower inflammation and improved xenograft tolerance. This interpretation was supported by the increased presence of galectin-3-expressing microglia/macrophages, signifying alternate (i.e., Th2-directed) activation of these cells, in the MSC-recipient mice.

Transplantation of MSCs in non-human primate stroke models has also been performed. MSC administration was associated with increased IL-10 (an anti-inflammatory cytokine) levels, reduced neural apoptosis, and enhanced proliferation in the subventricular zone of the macaque hippocampus (Li et al. 2010). Such in vivo results are consistent with in vitro co-culture experiments in which human BM-MSCs decreased TNFα and increased IL-10 secretion in dendritic cells, reduced IFN-γ in Th1 cells, increased IL-4 secretion in Th2 cells, increased the proportion of immunosuppressive regulatory T cells and decreased IFN-γ in natural killer cells (Aggarwal & Pittenger 2005). The MSCs displayed enhanced secretion of IL-6, IL-8, vascular endothelial growth factor and prostaglandin E2 (PGE_2) in the presence of the immune cells or when MSCs were exposed to TNFα or IFN-γ. The alterations in cytokine production by the immune cells were mitigated by inhibition of MSC-mediated PGE_2 production (Aggarwal & Pittenger 2005). Thus, dampening of inflammatory responses by factors secreted by MSCs is likely to be one mechanism by which MSCs promote graft tolerance and reduce tissue injury.

3.5 Limited cell replacement

A common feature of many MSC transplant studies is limited term survival of the engrafted cells in the ischemic brain (Bliss et al. 2007). For example, we observed a dramatic reduction

in GFP-labelled cells between two and four weeks after post-stroke intravenous hES-MSC injection (Liu et al. 2009). Human MSCs transplanted into ischemic mouse hippocampus survived for fewer than seven days (Ohtaki et al. 2008). Interestingly, a report of survival of neural stem cells for as long as 540 days (Chu et al. 2004) may suggest a difference in the ability of this stem cell type relative to MSCs to integrate into the post-stroke cerebral environment (perhaps due to differing sensitivites to apoptosis, efficiencies of neural, glial, or oligodendritic differentiation, synaptogenic potential, detection by the immune system, etc.). Nevertheless, the bulk of the reports of stem cell transplantation, whether with MSCs or neural stem/progenitor cells, indicates more limited graft duration.

3.6 Do MSCs differentiate into neurons?

Controversy exists as to the potential for MSCs to differentiate into neuronal, glial or oligodendritic cell types. Because these latter cells are derived from a neuroectodermal embryonic origin, there would be no *prima facie* expectation that mesodermal MSCs would do so. Nevertheless, multiple groups reported neuronal-like or glial cells developing from bone marrow cells (studies used a mix of unfractionated marrow cells, HSCs or MSCs), either in culture (Sanchez-Ramos et al. 2000; Woodbury et al. 2000; Black & Woodbury 2001; Deng et al. 2001; Kohyama et al. 2001; Kabos et al. 2002; Kim et al. 2002; Jiang et al. 2003) or in vivo following cell transplantation (Eglitis & Mezey 1997; Eglitis et al. 1999; Kopen et al. 1999; Brazelton et al. 2000; Chopp et al. 2000; Mezey et al. 2000; Nakano et al. 2001; Hofstetter et al. 2002; Keene et al. 2003; Mezey et al. 2003; Weimann et al. 2003a). Some of these in vitro neural induction protocols were quite rapid and used chemicals such as β-mercaptoethanol, dimethylsulfoxide (DMSO), butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), either alone or in combination (Woodbury et al. 2000; Hung et al. 2002). Other neural induction protocols have employed cytokines, growth factors, retinoic acid, neurotrophins and Noggin, among other agents. These multistage processes take days or weeks to produce neural cells (Sanchez-Ramos et al. 2000; Kohyama et al. 2001; Kabos et al. 2002; Jiang et al. 2003), which is more akin to the time frame required for hES cell or neural stem cell differentiation into neurons.

Some later reports have failed to duplicate earlier studies (Castro et al. 2002), calling the "transdifferentiation" potential of MSCs into question (Krabbe et al. 2005; Prockop 2007). Because classification of MSC-derived neural cells has often relied upon expression of neural marker genes, criticisms of some studies have included: (i) the failure to use confocal microscopy to establish true three-dimensional colocalization of markers in the same cell, suggesting that the observed colocalization was artefactual due to signals from cells in overlapping focal planes; (ii) suggestion that cell fusion may account for apparent marker colocalization (Terada et al. 2002; Alvarez-Dolado et al. 2003; Weimann et al. 2003b); (iii) the observation of "neuron-like" morphology and neural marker expression in multiple cell types after simple rapid chemical "induction" protocols in vitro that may be the result of cell toxicity or shrinkage occurring as part of a stress response (Lu et al. 2004; Neuhuber et al. 2004); and (iv) the rapid reversibility of the neural phenotype after withdrawal of the inducing agent(s) (Rismanchi et al. 2003; Lu et al. 2004). It should be noted that BM-MSCs can express immature neural markers even before "differentiation" and at later passages, can express more mature neuronal markers (Tondreau et al. 2004).

Our laboratory has observed neuronal marker induction in transplanted hES-MSCs following MCAO in rats, although we cannot say whether the marker-positive cells

possessed any functional attributes of neurons, including electrophysiology. The development of induced pluripotent stem cells (Takahashi et al. 2007; Yu et al. 2007) clearly demonstrates that reprogramming cell development is feasible. Regardless of whether the purported MSC-derived "neurons" can be considered true neuronal cells, the number of any such cells generated following stroke is minimal. Therefore, engrafted cell integration and replacement of damaged neurons is unlikely to be a major mechanism driving the beneficial effects of MSC therapy.

3.7 Multiple processes underlie beneficial action of MSCs

If the long-term survival, neuronal differentiation and integration of engrafted MSCs is minimal, then by what potential mechanisms do MSCs evoke a beneficial response? There are multiple processes by which ischemia/reperfusion generates tissue injury; these include: energetic failure; acidosis-induced toxicity and neurotransmitter excitotoxicity, both of which can result in calcium dysregulation leading to endoplasmic reticulum stress and inhibition of protein synthesis; inflammation; free radical generation during reperfusion; mitochondrial perturbations leading to release of apoptogenic molecules; edema; and, spreading depolarization injury in non-infarcted regions (Hossmann 2009). Thus, multiple pathways are likely to be involved in stem cell-mediated repair.

We have already alluded to the potential for MSCs to reduce inflammatory damage. Enhancement of endogenous neurogenesis and/or migration of newly formed neurons, which occurs after brain injury, is another potential avenue for MSC-mediated tissue repair. Increased incorporation of 5-bromodeoxyuridine (BrdU) into endogenous neural cells in the subventricular zone and in the dentate gyrus has been observed in rats and mice receiving BM-MSCs following cerebral ischemia (Chen et al. 2003a; Munoz et al. 2005). Rats receiving MSCs after stroke exhibited significantly fewer apoptotic cells, especially along the ischemic boundary (Chen et al. 2003a; Wu et al. 2008; Deng et al. 2010). Protection from apoptosis of cerebellar granular neurons in culture could be achieved using conditioned medium from adipose-derived MSCs, and this was partially attributable to IGF-1 modulation of Akt signaling (Wei et al. 2009b). MSC-conditioned medium also conferred protection in vivo against hypoxia-ischemia in neonatal rats (Wei et al. 2009a).

Astrocyte function is important for neuronal survival and recovery after stroke (Chen & Swanson 2003), partly because astrocytes produce trophic factors such as VEGF, bFGF, and brain-derived neurotrophic factor (BDNF). Co-culture with rat MSCs enhanced astrocyte trophic factor production, reduced hypoxia-induced rat cortical astrocyte apoptosis and increased cell proliferation (Gao et al. 2005). These effects were accompanied by activation of the Akt/phosphoinositde 3-kinase and mitogen activated protein kinase kinase (MAPKK)/extracellular signal-regulated kinases 1/2 (Erk1/2) signaling pathways in the astrocytes (Gao et al. 2005).

Vascularization of the infarcted area is another important component of functional recovery. Patients with the highest microvascular density in the ischemic penumbra have the best survival (Krupinski et al. 1994). Angiogenesis in response to ischemia begins shortly after the onset of ischemia and pro-angiogenic gene expression changes can be observed within 1 hour (Hayashi et al. 2003). MSC administration has been associated with stimulation of angiogenesis (Zhang et al. 2002; Chopp et al. 2008) resulting in greater vascularization in the ischemic boundary zone (Chen et al. 2003b). This was accompanied by increased VEGF production by reactive astrocytes (Chen et al. 2003b; Gao et al. 2005).

4. MSCs in clinical trials

Bone marrow-derived cell transplation has been widely used in a clinical setting for the treatment of hematopoietic disorders and cancers, as well as for some autoimmune disorders and graft versus host disease. To date, however, just a handful of small scale Phase I and Phase II clinical trials (summarized in Locatelli et al. 2009) have been performed to test the safety of stem cell transplantation (of any type) in stroke patients, and of these, only one study used MSCs. This randomized Phase I/II study (Bang et al. 2005) consisted of 30 patients with cerebral infarcts within the middle cerebral artery territory (as assessed by diffusion-weighted MRI) and severe neurological deficits (e.g., hemiparesis and agnosia; NIH Stroke Scale score of 7 or higher at 7 days after admission). Autologous bone marrow was collected 1 week after the onset of symptoms, and adherent mononuclear cells were expanded in culture and assessed for surface marker expression (CD73+, CD105+, CD34–, CD45–, HLA I–, leukocyte antigen D–). The treatment group (5 individuals) received two intravenous infusions of 5 x 10^7 autologous MSCs each (1 x 10^8 cells total) at 4–5 weeks and 7–9 weeks post-stroke. This dose was chosen because, once corrected for mean body mass, it is within the range that has been effective in several studies using the rat stroke model. The control group (n = 25), which was similarly matched with respect to infarct size, stroke etiology, and risk factors but had a slightly younger median age, received no MSCs. A follow-up MRI at 52 weeks was performed on 5 control and on all MSC-treated patients.

No cell-related adverse events were reported immediately following MSC administration or within the one year follow-up period, suggesting that the intravenous delivery of the cells was well tolerated. The Barthel Index (assessing functional recovery) of the MSC recipients was higher at 3 months and 6 months, although the improvement did not achieve statistical significance at 12 months. Scores in the modified Rankin Scale, another index of functional recovery, were not statistically different between groups, although the MSC-treated group consistently trended lower (better recovery) at each timepoint. The MRI analysis revealed that the evolution of the infarct was no different among the two groups but MSC recipients were reported to have less prominent secondary ventricular dilation than controls after one year.

In two clinical trials employing either hematopoietic stem cells or an immortalized neuroteratocarcinoma cell line following stroke, no adverse effects were reported (Kondziolka et al. 2000; Suárez-Monteagudo et al. 2009), whereas a seizure and a hematoma (1 patient each) were reported in a third study, which classified these as non cell-related events (Kondziolka et al. 2005). The U.S. Food and Drug Administration terminated a study of fetal porcine cell transplantation due to the development of seizures in one patient and aggravation of motor deficits in another within 1 week and 3 weeks of transplantation, respectively (Savitz et al. 2005). Human fetal cell delivery into the subarachnoid space was performed in 10 patients after ischemic or hemorrhagic stroke and some patients exhibited fever and meningism within two days of transplantation (Rabinovich et al. 2005). There are many differences in cell type, delivery method, timing and study population among these trials that render comparisons difficult and only small numbers of patients were involved in these studies. Although these studies indicate that stem cell transplants may be generally well tolerated as a proof of concept, it is fair to say that safety concerns remain a high priority and that many of the details regarding stem cell therapy remain to be optimized.

Tumor development is also a safety concern with stem cell populations. There are reports of oncogenic transformation of human and murine BM-MSCs or adipose-derived MSCs after

long-term culture (Rubio et al. 2005; Wang et al. 2005; Miura et al. 2006; Tolar et al. 2007). Furthermore, co-mixing of murine BM-MSCs with melanoma or weakly tumorigenic breast carcinoma cell lines enhanced the tumorigenic or metastatic potential of the transformed cell lines in animal studies (Djouad et al. 2003; Karnoub et al. 2007). Therefore, it will be desireable to limit the time in culture and to screen any cell populations to be used in a clinical setting for normal karyotype.

At this early stage, it is premature to judge the safety, much less the efficacy of stem cell therapy. Much larger scale trials designed with sufficient statistical power to test efficacy will be required once safety issues have been further understood. In 2010, two additional clinical trials involving multipotent mesenchymal stromal cell interventions against stroke are in the recruiting or planning stages (www.clinicaltrials.gov, NCT00875654 and NCT01091701). In addition, at least six trials employing other stem cell preparations targeted to ischemic stroke are also recruiting participants. Thus, the next several years should provide emerging data on the potential of stem cell therapy to treat this major human health issue.

5. Conclusion

The relative ease of obtaining MSCs from patients, coupled with the reports of differentiation of MSCs into cells of all three germ layers, has generated enthusiasm for the use of these cells in autologous transplantation. While transdifferentiation has generated controversy, the immunomodulatory properties and tissue repair promotion via paracrine action keeps MSCs as viable candidates for therapeutic evaluation. The exciting development of induced pluripotent stem (iPS) cells (Takahashi et al. 2007; Yu et al. 2007) may eventually make the production of autologous nervous system cells practical. While iPS-derived neural cells might perhaps prove to be a more effective cell source for neural repair and replacement following stroke, MSC co-transplantation may theoretically be a useful adjuvant for neural stem cell therapy applications.

6. References

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Embryonic Stem Cell in the Therapy of Neurodegenerative Diseases

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1. Introduction

Neurodegenerative diseases comprise a heterogeneous spectrum of neural disorders in human and affect millions of individuals worldwide. Although these neurodegenerative diseases arise from unknown reasons, their common typical histological feature is the occurrence of disease-related cell death in specific regional subpopulations of neurons such as the loss of dopaminergic (DA) neurons in the substantia nigra in the Parkinson's disease (PD) and loss of medium spiny neurons in the striatum in the Huntington disease(HD), they can also possibly affect the diversity neurons and glial cells such as the multiple selective neurons loss throughout the basal forebrain, amygdala, hippocampus, and cortical area in Alzheimer`s disease (AD) and dysfunction of supportive glial cells around somatic motor neurons in amyotrophic lateral sclerosis (ALS) (Srivastava et al., 2008). Adult central nervous system (CNS), different from many other tissues, cannot regenerate and loss of mature neural cells is thought to be irreversible. As the increasing of life expectancy in this aging society, the neurodegenerative diseases usually cause morbidity and mortality as well as increased social and economic burdens of patients and caregivers. Current drugs used for treating neurodegenerative diseases provide only limited benefits by alleviating certain symptoms, but cannot halt progress of these diseases. In addition, their chronic use is often associated with serious side effects (Mangialasche et al., 2010; Kim et al.,2007). Fetal tissue and cell transplantation in animal models and patients has pointed to be an alternative treatment for these diseases, which can provide neurons to replace lost or degenerated neurons, but due to the lack of donor tissues, it will be limited in clinical trials. Promising donor cells need to be developed for treating these diseases (Schwarz & Schwarz, 2010;Freed et al., 2001; Nishimura et al., 2003).

Rapid advances in stem cell biology have opened an alternative, fascinating perspective treatment for neurodegenerative disease. Stem cells as donor cells used in neurodegenerative disease include three different sources: neural stem cells (NSCs) from the embryonic or adult brain, stem cells in other tissues, or embryonic stem cells (ESCs) from the fertilized egg (Shihabuddin & Aubert., 2010).

Adult progenitor cells from bone marrow or cord blood have been shown to generate neurons both *in vitro* and *in vivo* studies, although the number of neurons was very low (Chua et al., 2009; Greschat et al., 2008; Bicknese et al., 2002; Song & Sanchez-Romos.,2008). The results indicated that stem cells in these tissues would be promising candidates for cell-replacement therapy due to their accessibility. However, some experiments cannot replicate these results, no new neurons production has been observed in these studies, the neural differentiation of these stem cells supposed to be artifacts such as occasional cell fusion with host neurons appeared in original observations (Rizvanov et al., 2008; Luo et al.,2009). Trans-differentiation of adult progenitor cells from bone marrow or cord blood needs more evidence to confirm before they used as donor cells in treating neurodegenerative disease.

NSCs from the embryonic brain have the ability to renew themselves continuously and possess pluripotent ability to differentiate into three major CNS cell types, neurons, astrocytes, and oligodendrocytes (Reubinoff et al., 2001). Under certain conditions, NSCs can also be induced to differentiate into typical neurons such as dopaminergical neurons. When transplanted into animal models, embryonic brain-derived NSCs can survive and even replace the lost cells caused by brain injury(Goings et al., 2004; Arvidsson et al., 2002). But it has indicated that human or rat fetal NSCs or neural precursors(NPs) appeared to be more limited in terms of their differentiation potential, difficult to maintain long-term expansion without decline in proliferative and neurogenic capacity (Cao et al., 2002). The accessibility to get human fetal NPs also needs to be concerned. In the mammalian adult brain including human, NSCs isolated from subventricular zone (SVZ) and hippocampus dentate gyrus(DG) have the ability of self-renewing and pluripotency similar to those isolated from embryonic brain (Vandenbosch et al., 2009; Jin & Galvan, 2007; Consiglio et al., 2004; Tropepe, 2007; Okano, 2002). This prompts very promising new strategy to replace lost neurons through auto-transplantation, however, it is still stopped due to their uncertainty in long-term expansion and reliable differentiation, which will be partially determined by the stage of development when NSCs were isolated (Soares & Sotelo, 2004). Meanwhile, to increase proliferation rate and decrease the physiological senescence in culture, the immortalized cell lines of human NSCs usually were introduced with oncogenes which usually resulted in the high risk of transformation (De Filippis et al., 2008).

ESCs derived from the inner cell mass of blastocysts give rise to various organs and tissues. ESCs are able to differentiate into all cell types of ectoderm, endoderm and mesoderm (Evans & Kaufman, 1981;Thomson et al., 1998). It has been demonstrated that *in vitro* ESCs can differentiate effectively into NPCs which further differentiate into neurons and glia (Tang et al., 2008). The self-renewing and totipotent ability of ESCs have made it an intriguing and attractive donor source for cell-based therapies in neurodegenerative diseases and brain injury (Reubinoff et al., 2001). Human embryonic stem cells (hESCs) usually isolated from early human embryos which makes it encounter ethical risks(Dickens & Cook, 2007; Baylis, 2008). However, in 2006, researchers identified conditions of allowing some specialized adult somatic cells to be "reprogrammed" genetically to assume a stem cell-like state. This new type of stem cell, called induced pluripotent stem cells (iPSCs), make it possible to obtain human ESCs without destroying the human embryos (Takahashi & Yamanaka, 2006). On the contrary, some studies have suggested that the iPSCs might not be an alternative to ESCs, they have different pluripotent ability to turn into different types of tissues (Schwarz & Schwarz,2010). Moreover, the first-ever hESCs-based investigational new drug (IND) application to treat spinal cord injury has been approved by the U.S. Food

and Drug Administration (FDA) in January 2009. It seemed that hESCs could be expected as a potential future cell source for cell therapy in neurodegenerative disease.

AD and PD are the most prevalent kinds of neurodegenerative diseases in ageing people. Although not so prevalent, HD and ALS are devasting motor neuron degenerative diseases, the patients usually died of this disease shortly after diagnosis (Roze et al., 2010; Perry et al., 2010).These neurodegenerative diseases share many important commonalities, characterized by neuron loss as structural feature. Meanwhile, a different spectrum of cell types is affected in these diseases. Therefore, different types of neurons are required for replacement (Srivastava et al., 2008). When stem cell transplantation used in these diseases, the strategies will vary according to the primary defect of a disease. As above-mentioned, ESCs are promising cell source for cell therapy in neurodegenerative disease, recent progress of ESCs–based therapies in these diseases is discussed below.

2. Embryonic stem cell biology

2.1 Cellular and molecular characteristics of embryonic stem cell

ESCs were independently first derived from mouse embryos by Martin Evans and Mattew kaufman in 1981 (Evans & Kaufman, 1981). This is also the first time to reveal a new technique for culturing the mouse embryos in the uterus to allow for an increase in cell number, which has been further confirmed by Martin (Martin et al., 1987). In 1998, James Thomson firstly developed a technique to isolate and grow hESCs in cell culture (Thomson et al., 1998). Later, ESCs have been isolated from other species, including swine, simian, monkeys and human blastocysts, but only three species of mammals have yielded long-term cultures of self-renewing ESCs namely mice, monkeys and humans (Ohtsuka & Dalton, 2008).

Both mouse and human ESCs have the properties of self-renewal and the potential to be committed and differentiated *in vitro* into all three germ layers—namely, ectoderm, endoderm, and mesoderm (Murry & Keller, 2008). Both stem cell populations express the characteristic markers of undifferentiated cells, including *Oct-4*(octamer binding transcription factor-4), *nanog, Sox-2*(SRY- related HMG box 2) and *utf-1,* together with the lack of differentiation markers (Sauerzweig et al., 2009). They also express stage-specific embryonic antigen-3 and -4 (SSEA-3 and -4), tumor-rejection antigen-1-60 and -1-81, and high levels of telomerase activity (Murry & Keller, 2008). ESCs cultures from mouse and human are similar in that they grow as colonies of tightly packed cells on inactivated murine embryonic fibroblast(MEF) feeders or in conditioned medium(CM) derived from such MEFs (Li et al., 2005). The mechanisms by which ESCs from different species maintain self-renewal and pluripotency are still not yet fully understood. It seemed that they have different mechanisms. Maintenance of mouse ESCs pluripotency *in vitro* is achieved by co-culture on irradiated mouse fibroblasts or on gelatinized dishes with a differentiation inhibitory factor called leukemia inhibitory factor(LIF) (Cheng et al., 2003). In addition to LIF, bone morphogenetic protein 4(BMP4) and the secreted wnt proteins may also contribute to maintain ESCs pluripotency (Hiller et al., 2010). Other factors such as Sox2, FoxD3 (forkhead box D3) and Nanog can also maintain self-renewal of mouse ESCs.

Human ESCs pluripotency is regulated by a combination of extrinsic and intrinsic factors. Unlike mouse, extrinsic factors such as fibroblast growth factor(FGF) signaling and a balance between transforming growth factor-β(TGF-β)/activin and BMP signaling are central to the self-renewal of human ESCs (Xu et al., 2008). Intrinsic factors regulating

pluripotency in human ESCs include a battery of transcription factors such as Oct4, SOX2, SRY (sex determining region-Y), Box-2 and Nanog (Liber et al., 2010).

2.2 Embryonic stem cell based transplantation therapy

ESCs possess several characteristics that make them promising donor cells for transplantation therapy in neurodegenerative diseases. When ESCs transplanted into animal models which mimic the typical aspects of neurodegenerative diseases, structural and functional recovery were also confirmed, although the efficiency were variable. The safety of ESCs transplantation is always an important problem to be considered carefully. It has been demonstrated by many studies that ESCs transplantation in the brain tended to form teratomas, which is an unacceptable adverse effect for cell transplantation therapy (Wesselschmidt et al., 2007). The main strategy to enhance the safety of ESCs for potential clinical use is to differentiate the ESCs into neural precursors cells(NPCs) with different commitment or mature neurons before transplantation (Shihabuddin & Aubert, 2010). Thus, there are two principally different ways of using ESCs for grafting in neurodegenerative disease. First, ESCs are pre-differentiated *in vitro* to specific neurons such as DA neurons prior to transplantation in PD, thus, ESCs could become an almost unlimited source for the generation of specific neurons. The cell preparations could be standardized and quality-controlled with respect to viability and purity (Shihabuddin & Aubert, 2010). The second alternative is that the ESCs are induced effectively into NPCs with different commitment, which then differentiates *in vivo* to specific neurons after being implemented into specific brain regions.

In vivo studies have demonstrated that transplantation of NPCs and neurons derived from ESCs are safe and carry no risk of tumor formation, the immunogenic reactivity is also relatively low (Cai & Rao, 2007). We argue that long-term survival of new, functionally integrated neurons is the main goal to achieve maximum symptomatic relief through stem cell therapy. More evidence has confirmed that the observed functional recovery after ESCs-derived NPCs and neurons were transplanted into animal models of neurodegeneration and injury appeared to be related to structural reorganization including synapse formation and functional integration (Moghadam et al., 2009; Tang et al., 2008). In the developing brain, grafted ESCs-derived neurons developed functional properties of postmitotic neurons adopted excitatory and inhibitory neurotransmitter phenotypes and formed synapse with host cells (Wernig et al., 2004). In the injured brain regions, grafted ES cell-derived precursors differentiated into neurons and exhibited voltage-gated inward and outward currents, expressed functional neurotransmitter receptors, and formed synaptic contacts (Wernig et al., 2008). Thus, NPCs with different commitment and neurons from ESCs have been prospective in CNS repair.

2.3 NPCs and neurons differentiation potential of embryonic stem cell

2.3.1 NPCs induction from embryonic stem cell

NPCs derived from ESCs can be expanded in large numbers for significant periods of time and their plasticity potential allows them to differentiate according to the environmental cues of host brain (Sakaguchi et al., 2005; Takagi et al.,2005). Getting the NPCs of high purity from ESCs is an important step in transplantation research. Evidences from mouse ESCs *in vitro* culture studies have indicated that NPCs can be generated from ESCs and can be expanded and differentiated efficiently to neurons and glial cells by serum-free culture

(Okabe et al., 1996). The protocol of neural differentiation of ESCs usually involved the formation of embryoid bodies(EBs) and the subsequent culture of the attached EBs in a selective, serum-free medium to eliminate non-neural cells. Culture of the EBs-derived cell pool in this serum-free, selective medium results in a dramatic decrease in cell number, as the majority of the cells do not survive under these culture conditions (Lendahl et al., 1990). Insulin–Transferrin–Selenium–Fibronectin (ITSF) and N2 serum-free medium have been demonstrated to support the selective growth of NPCs (Xu et al., 2004). Considering fibronectin increases the neural differentiation of ESCs, offers a tool for growing NPCs as a monolayer without impairing their differentiation potential and to generate specific differentiated progeny for cell transplantation, we modified the culture medium by adding fibronectin (5 μg/ml) in the N2 serum-free selective culture and found that the number of surviving cells was increased significantly when EBs were plated. After ESCs were cultured in this modified NPC-selective medium for 5 days, more than 93% cells were nestin-positive. (Xu et al., 2005). The percentage of nestin-positive cells was increased compared with the classic method of Okabe, in which the ITSF media was used and the percentage of nestin-positive cells was 84.1%(Okabe et al.,1996).

The feasibility of hESCs based cell transplantation in the CNS depends on the generation of human neural precursors (hNPs) *in vitro*. There are several methods for induction of hESCs to hNPs, usually rely on EBs formation, stromal feeder co-culture or selective survival conditions similar to murine ESCs. Recently, rapid and simple generation of proliferating hNPs from feeder free cultures of undifferentiated hESCs has been described. In this protocol, hNPs typically expressed neuroectodermal and progenitor markers are derived by seeding undifferentiated hESCs on adherent surfaces of laminin or gelatine with normal hESCs culturing medium and with the addition of bFGF (Benzing et al., 2006). The process of induction of hESCs to hNPs is quite similar to neural induction during embryogenesis. Several lines of evidence demonstrate a crucial role for BMP inhibitors including chordin, follistatin and noggin as neural inducing factors during neural differentiation of hESCs. It seems that the synergistic action of two BMP inhibitors, Noggin and SB431542, are sufficient to induce rapid and complete neural conversion of >80% of hESCs under adherent culture conditions (Chambers et al., 2009; Pera et al., 2004). In another study, hNPs from hESCs produced with a feeder-free system, different media components were compared for the efficiency of hNPs generation. Media with N2 produced higher number of hNPs without proliferation, media with non-essential amino acids and knock-out serum replacement produced fewer number of hNPs with proliferation, and by five passages the culture consisted of >97% hNPs. This resulted in an efficient, robust, repeatable differentiation system suitable for generating large populations of hNP cells from hESCs (Dhara et al., 2008).

2.3.2 Neurons differentiation from embryonic stem cell

It has been demonstrated that relatively pure of neurons, astrocytes, and oligodendrocytes which constitute three major neural cell types of the CNS, can be generated from ESCs under appropriate culture conditions (Zhang &Zhang, 2010). Obviously, neuron production from ESCs *in vitro* is similar to neural induction occurred in embryo development, and many important factors or signaling pathways have been determined such as β-catenin and wnt signaling pathway (Cajánek et al., 2009). *In vitro* study has indicated that ESCs transfected with noggin or exposure to noggin-conditioned medium will enhance neuronal

differentiation, while addition of BMP4 strikingly inhibited neuronal differentiation (Zhang et al., 2010). Human ES cells can be directed to neurons when exposure to retinoic acid and mitogen FGF-2, although selected using cell-sorting methods, the cell population obtained after application of this differentiation strategy is still relatively heterogeneous (Guan et al., 2001).

Moreover, protocols have been developed for the generation of different subtypes of neurons including the sequential combination of regulators. Especially, fetal tissue transplantation trials provided important clues for factors inducing typical neurons differentiation. For instance, midbrain DA neurons have been generated in the EBs system by overexpression in the cells of the transcription factor nuclear-receptor-related factor1 (Nurr1), and the addition to the cultures of SHH and FGF8 (Lee et al., 2000). Using the co-culture approach together with the appropriate signaling molecules and selection steps, successfully generated cells that display many of the characteristics of motor neurons from hESCs (Wichterle & Peljto, 2008).

3. ESCs therapy for neurodegenerative diseases

3.1 Alzheimer's disease

AD is a most prevalent kind of dementia and clinically defined as progressive deficits of cognition, memory and the ability to live on oneself (Marlatt & Lucassen, 2010). It was diagnosed mostly in people over 65 years of age although the less-prevalent early-onset AD can occur much earlier (Brookmeyer et al., 1998; Marlatt & Lucassen, 2010). In 2006, there were 26.6 million AD sufferers worldwide, the number is predicted to be 1 in 85 people globally by 2050 (Brookmeyer et al., 2007). The neuropathology of AD is characterized by deposition of insoluble β-amyloid peptides/senile plaques, the intracellular neurofibrillary tangles(NFTs) and the diversity selected neurons loss throughout the basal forebrain, amygdala, hippocampus, and cortical area (Kim & de Vellis, 2009; Whitehouse et al., 1981).

The etiology and progression of AD are not well understood. It is multi-factorial and heterogeneous, which is related with the age, sex, the genotype for apolipoprotein E (Apo E), and the sequential proteolytic processing of the amyloid precursor protein(APP) (Blennow et al.,2006). Current treatment strategies for AD include targeting cholinergic and glutamatergic neurotransmission, moderating the γ-secretase to generate non-toxic β-amyloid(Aβ) fragment and delivering NGF into brain which can alleviate symptoms, while these treatments cannot delay or halt the loss and degeneration of neurons in AD brains (van Marum, 2008). Moreover, it has been recognized that the irreversible decline of cognitive functions was related to neuron loss in the forebrain cholinergic projection system: especially in nucleus basalis of Meynert(nbM) where a massive extracellular deposition of Aβ protein occurs in the later stages of the disease (Boncristiano et al.; 2002; Oliveira & Hodges, 2005).

The development of stem cell biology make it a promising treat approach for AD sufferers to restore the lost neurons. Currently stem cell-based therapy for AD is confined to preclinical studies on animal models (Sugaya & Merchant, 2008). The effectiveness of several stem cells transplantation has been explored and the results are quite similar, sustaining an improvement of the mouse's or rat's cognitive functions and a general improvement with reference to AD pathogenesis hallmarks (Shihabuddin & Aubert, 2010). When cholinergic-rich tissue and peripheral cholinergic neurons were transplanted into an AD rat model with nbM lesions, memory improvement which indicated a partial neuronal rescue has been

found in these animal models. However, it is difficult to get enough donor cells, no clinical trials in AD patients have been initiated with this method (Fine et al., 1985).

When ESCs as donor cells transplanted directly in AD animal model, it usually resulted in teratomas formation instead of producing neurons (Ringdén et al., 2003; Wang et al., 2006). However, ESCs-derived neurospheres, NPCs and neurons transplantation were evaluated in animal models and proved to be safe. Wang and colleagues has demonstrated that ESCs-derived neurospheres transplanted into frontal cortex of Meynert nucleus lesion mouse model survived and produced many ChAT-positive neurons and a few serotonin-positive neurons in and around the grafts, and the improved working memory was also observed after transplantation (Wang et al., 2006). Moghadam and colleagues further implanted primed and unprimed mouse ESCs–derived NPCs into the unilateral nbM of rat model of AD, Morris water maze and spatial probe test revealed a significant behavioral improvement in memory deficits following cells transplantation. Immunohistochemical analysis revealed that the majority (approximately 70%) of the NPCs retained neuronal phenotype and approximately 40% of them had a cholinergic cell phenotype following transplantation (Moghadam et al., 2009). These studies suggest transplantation of mouse ESCs-derived NPCs and/or following commitment to a cholinergic cell phenotype can promote behavioral recovery in a rodent model of AD.

Consistent with above studies, we also confirmed that therapy effects of NPCs derived from ESCs following transplantation into the rat model of AD. In our studies, we used a derivative of the MESPU35 ES cell line that constitutively expresses enhanced green fluorescent protein(EGFP), thus allowing us to distinguish between transplanted and host cells. Firstly, we investigated the *in vivo* characteristics of NPCs after differentiation from the ESCs following transplantation into the Aβ-injured dorsal hippocampus of rats. Aβ-injured rats receiving ESCs derived NPCs grafts revealed amelioration of the memory impairment at least 16 weeks. Obviously, only live transplanted NPCs can bring about functional recovery, the survival of transplanted NPCs in the hippocampus declined with time, while the differentiation rate was increased. We found that 9.41% of grafted EGFP-expressing NPCs differentiated into NF-200 positive cells and 11.31% into GFAP positive cells respectively 4 weeks after transplantation. These ratios gradually increased to 40.25% and 19.35% by 16 W. Meanwhile, we also noticed that most of the NPCs remained in a cluster and possessed a round morphology that kept unchanged over the experimental period, some NPCs were found dorsally along the needle tract, a few NPCs had migrated tangentially along the granule cell layer (GCL) in the DG region. Of particular note is the fact that these migrating cells within the hippocampus were clearly differentiating along a neuronal lineage indicating that the microenvironment can support neuronal differentiation, and should be, therefore, also suitable for transplanted NPCs. This may overcome the problem of low neuronal differentiation rate of the transplanted NSCs in the host brain (Tang et al., 2008). On the other hand, DG of the hippocampus is a few region neurogenesis occurred in the adult mammals including primates and humans, and the newborn granule cells contribute to processing of learning and memory. Using APPswe/PS1DeltaE9 mouse model of AD, we have demonstrated that the cell proliferation in the DG of AD was declined significantly (Li et al., 2008), meanwhile increased hippocampus neurogenesis contributes to memory function recovery has been reported in many studies (Duan et al., 2008; Mohapel et al., 2006). Therefore, NPCs transplantation in the DG of hippocampus, maybe a new promising therapeutic strategy of AD compared with dorsal hippocampus transplantation.

To confirm this hypothesis, in our recent study, injection of a toxic fragment of $A\beta_{1-40}$ into the upper leaf of DG was used to make an *in vivo*, AD-like rat model. Selective loss of granular neurons mainly observed in the upper leaf of the DG near the injection site, which is consistent with the results of Reyes(Reyes et al., 2004). Similar to our previous finding that NPCs derived from ESCs grafted into dorsal hippocampus, the spatial learning and memory were improved in animals with NPCs transplantation into DG of the hippocampus(Tang et al., 2008). After transplantation into $A\beta_{1-40}$-injured rats, ESCs-derived NPCs survived up to 12 weeks and migrated along the upper leaf of the DG, where granular cells were degenerated and lost. We further demonstrated that grafted cells differentiated well in the DG of the hippocampus as seen by checking neuronal and glial markers after transplantation. In addition, it showed that these incorporated donor neurons exhibited a broad spectrum of neuronal morphologies ranging from simple bipolar to complex multipolar phenotypes. Meanwhile, the abundant labeled glutamatergic and GABAergic neurons were seen in 8 and 12 weeks after grafting, donor neurons also expressed proteins essential for synaptic transmission, including the major subunits of ionotropic glutamate and GABA receptors (NR1, GluR1) and $GABA_A$ receptor β-chain, respectively(Li et al., 2010).

Numerous reports point out that the therapeutic effect mediated by the transplanted cells in various neurological disorders depends on the functional integration of transplanted cells into brain circuits upon *in vivo* differentiation into mature cells (Maroof et al., 2010; Aldskogius et al., 2009; Reubinoff et al., 2001). To testify the possibility of integration of ESCs derived neurons in the hippocampus of AD rats, the synapse formation between donor neurons and host neurons were demonstrated by PSD-95-positive puncta and synaptophysin positive puncta. The density of PSD-95 and synaptophysin positive puncta showed higher level at 12 weeks post transplantation compared with 8 weeks. This was further confirmed using Qtracker® Cell Labeling technique, that is, the engrafted cells were pre-labeled by Quantum dots (QDs) before cell transplantation which makes them visible under bright field, fluorescence field and electron microscopy(EM), they formed typical ultrastructure of post-synaptic or pre-synaptic connections with host neurons following transplantation when observed under EM. Our study directly showed that synapse formation between host and donor neurons, which provided morphological evidences for the reconstruction of damaged hippocampal neuronal networks in AD by cell transplantation (Li et al., 2010). Many studies have demonstrated that the grafted cells effectively signal to the host and vice versa by electrophysiological demonstration, for instance, paired recordings as the most stringent criteria for functional integration and crosstalk between host and graft. C-fos as another good candidate for the initial steps of learning inducing long term synaptic plasticity, we found that, after behavioral training, there was an increase in the percentage of co-localization of Fos and EGFP in hippocampal DG of $A\beta_{1-40}$-injured rats compared to that in rats with stationary stimulation. This indicates an involvement of the engrafted neurons in the circuitries contributing to learning and memory (Li et al., 2010).

Therefore, the effectiveness of these ESCs transplantation is quite similar, sustaining an improvement of the animal's cognitive functions and promising reconstruction of structural and function (Sugaya et al., 2007; Sugaya et al., 2006; Sugaya, 2005; Marlatt & Lucassen, 2010; Taupin, 2009). Since the neuropathological changes of AD is too widespread throughout the brain, it seems very difficult to decide the place for grafting and the dosage of cells, this makes the cell transplant therapy more difficult than that of single brain site

impact diseases like PD and HD (Imitola, 2007). Based on our study, DG is a promising site for grafting NPCs derived from ESCs, the long term recovery evaluation at structure and function level needs to be explored in the future.

As Aβ deposition is a hallmark of AD, the effect of Aβ on the survival, migration and differentiation of grafted cells is the major factor deserves to be taken into consideration. Sugaya reported that human NSCs transplanted into the brain of APP transgenic mouse exhibited less neurogenesis and active gliosis around the plaque like formations, while using a well-defined *in vitro* culture differentiation model of NSCs, it found that APP is necessary for the migration and differentiation of NSCs, suggest APP level can regulate NSCs biology, the changes of APP metabolism in AD will cause the deficiency of grafted stem cells (Sugaya & Brannen, 2001; Sugaya et al., 2006). It reported recently that the APP is expressed by human ESCs, and the differential processing of APP regulates the proliferation and differentiation of ESCs. The exogenously added soluble and fibrillar APP significantly reduces the proliferation of ESCs and increases the NPCs differentiation (Porayette et al., 2009). It was reported that human NSCs exposed to high concentrations of secreted amyloid-precursor protein (sAPP) *in vitro* differentiated into mainly astrocytes, suggesting that pathological alterations in APP processing in AD may prevent neuronal differentiation of NSCs (Marutle et al., 2007; Kwak et al., 2010). Thus, successful cell transplantation therapy for AD may require regulating APP expression to favorable levels to enhance neuronal differentiation of NSCs at the same time.

3.2 Parkinson's disease

PD is the second most common neurodegenerative disorder, clinically characterized initially by muscle rigidity, resting tremor, and slowing of movement. Over time, patients sustain a loss of mobility and dysautonomia, dystonic cramps and dementia (Jankovic, 2008). The common pathological features in PD patients are degeneration of DA neurons in the midbrain and diminished dopamine in the striatum, which normally receives substantia nigra (SN) dopamine inputs (Kish et al., 1988; Agid, 1991). The vast majority of cases are sporadic, and some of these are caused by mutations in α-synuclein, parkin, UCHL1, DJ1, PINK1, and LRRK2 , with a Mendelian pattern of inheritance (Hardy, 2003). All current PD therapies focus on restoring the dopamine levels by either the oral administration of the dopamine precursor levodopa (L-DOPA), which supplements the low level of endogenous dopamine, or inhibition of the breakdown of endogenous dopamine by treatment with the monoamine oxidase type B (MAO-B) inhibitor, selegiline (Hardy, 2003). Dopamine agonists are also used to directly stimulate the dopamine receptors. Unfortunately, these therapies are all symptomatic treatments, they do not prevent the progress of the DA neuronal degeneration and, more importantly, are not capable of curing PD. In the later stages of this treatment, L-DOPA becomes ineffective or, even worse, causes severe side effects, such as the development of dyskinesias (Fahn et al., 2004; Lang & Lozano, 1998 a,b). Surgical treatment by deep brain stimulation is practiced to further modify dyskinesias. Although advanced, this treatment does not stop the progression of the disease either, therefore there is a great need to develop new regenerative therapeutic strategies that directly target the degenerating DA neurons or their innervation areas (Walter & Vitek, 2004).

Cell transplantation from fetal tissues has offered some success in the treatment of Parkinson's disorder, but is limited by the difficulty of obtaining the tissues for transplantation (Lindval et al., 1990; Olanow et al., 1996; Kordower et al., 1997; Dunnett & Björklund, 1999). Extensive *in vivo* animal studies have shown that fetal ventral

mesencephalic (VM) grafted into rats striatum of PD models produced marked recovery from amphetamine induced rotational asymmetry. The grafts could survive in host striatum, restore the damaged nigrastriatal pathway and release dopamine (Perlow et al., 1979; Freund et al., 1985). Based on these experimental data, clinical trials with transplantation of fetal VM into patients were initiated in 1987, and transplantation efficacy has been shown in open-label trials without post-operative complications. Fetal VM transplantation seems to be promising in treating PD, and the clinical benefits have been reported in early clinical trials (Lindvall et al., 1989). However, outcomes following transplantation of fetal VM in PD patients were variable. In the best cases, DA neurons survived, released dopamine and the major clinical improvement could last for up to 6 to 12 years post-surgery (Piccini et al., 1999). On the other hand, two NIH-sponsored, double-blind clinical trials had found that some patients failed to show behavioral recoveries observed in the previous open-label trials and suffered graft-induced dyskinesias, probably caused by the grafts' excessive and uncontrolled proliferation and release of dopamine (Björklund, 2005; Winkler et al., 2005). Although the disparity in the outcome from clinical trials is not clear, probably is related to many factors, including the age of the donor tissue, the severity and stage of the disease and the difference in immunosuppressant treatment in patients. The variable results from clinical trials, together with the lack of donor tissues and standardization of protocols have led to re-evaluation of the use of such tissue for further transplantation into humans. In this regard, human ESCs could be a potential future alternative cell source for cell -replacement therapy for PD.

It is necessary for transplanted cells to synthesize dopamine and to control release, reuptake, and metabolizing dopamine, as the original DA neurons to treat PD. There are two principal different ways of using ESCs pre-differentiated into DA neurons and stem or progenitor cells with different commitment transplanted into the striatum or SN. Mouse ESCs transplanted into adult mouse brain and kidney capsule developed large numbers of cells exhibiting neuronal morphology including DA neurons. TH positive axons from intracerebral grafts grew into regions of the dopamine-lesioned host striatal gray matter (Kleppner et al., 1995; Morassutti et al., 1994; Wojcik et al., 1993; Miyazono et al., 1995; Isacson & Deacon 1996; Hemmati-Brivanlou & Melton, 1997). Then a further study in which with low numbers of undifferentiated mouse ESCs implanted into the rat DA-depleted striatum, the cells proliferated and differentiated into functional DA neurons that reduced Parkinsonism (Björklund et al., 2002). However, lethal teratomas developed in 20% of the animals. More studies have reported that transplantation with NPCs *in vitro* differentiated from ESCs could decrease the possibility of teratomas formation (Björklund et al., 2002). Highly enriched populations of NPCs were developed *in vitro* from mouse ESCs and then implanted into Parkinsonian rats. The engrafted cells led to the recovery from Parkinsonism, and teratoma formation was not observed (Geeta et al., 2008). We also demonstrated that NPCs derived from MESPU35 mouse ES cell line grafted into the striatum of PD rats produced therapeutic effect evaluated with rotation test. The number of survival NPCs was increased significantly by fibronectin and over 90% of the engrafted NPCs were TH-positive 6 weeks after transplantation into the striatum of PD rats (Xu et al., 2004). There are no signs of tumor growth or non-neural tissue formation in the transplant recipients. It seemed that uncommitted mESC-derived NPCs could differentiate into DA neurons in host brain after transplantation, thereby leading to functional recovery (Kim et al., 2002). On the contrary, Ben-Hur et al has noticed that transplantation of uncommitted hESC-derived NPCs induced partial behavioral recovery, while there is no detectable DA neurons from grafted NPCs.

The disparity may lie in the different biological characters of hNPCs compared to NPCs from mESCs when drived to DA differentiation (Ben-Hur et al., 2006). Ben-Hur et al has developed a simple protocol to harvest hNPCs from hESCs expressing transcripts of key regulatory genes of midbrain development, as well as markers of DA neurons, supporting their potential to differentiate into midbrain DA neurons. When these hNPCs grafted into the striatum of Parkinsonian rats, they survived for at least 12 weeks and differentiated *in vivo* into DA neurons. Transplanted Parkinsonian rats also exhibited significant behavioral recovery (Ben-Hur et al., 2006). Hence, induction of hNPCs differentiation toward a midbrain fate prior to transplantation is probably required for complete correction of behavioral deficit. In this regard, the host tissue probably could not direct the transplanted uncommitted hESC-derived NPCs to acquire a dopaminergic fate. The partial functional recovery induced by transplantation of uncommitted hESC-derived NPCs maybe due to the 'trophic' support provided by undifferentiated grafted cells within the damaged tissue (so called 'bystander effect').

Although NPCs might have greater developmental plasticity to respond to host environmental inductive cues that guide DA neuron differentiation, the adult striatum is probably unlikely to retain all instructive signals for DA neuron development from these. Therefore, pre-differentiation of ESCs into neural cells of a certain maturation stage is a prerequisite for obtaining a larger number of graft-derived DA neurons. It has been confirmed that ESCs-derived neurons carrying features of the ventral midbrain dopaminergic SN neurons will express En1, Pitx3, Nurr1, Lmx1b and Girk2, and will be labeled with dopaminergic markers, such as the dopamine transporter, vesicular monoamine transporter and the enzyme aromatic amino acid decarboxylase (Wernig et al., 2008). Signaling factors involved in CNS development have been used to control the differentiation of ESCs into mesencephalic dopamine (mesDA) neurons (Ye et al., 1998). Studies have used neurotrophic factors and overexpression of midbrain-specific genes to induce a dopaminergic phenotype in mouse ESC-derived neurons (McKay et al., 2006). It is well known that Shh, FGF8, Wnts affect the local environment in which mesDA neurons are generated during embryogenesis. However, these molecules underlie the regional specification of several cell types generated in midbrain (MB) and hindbrain during CNS development (Ling et al., 1998; Potter et al., 1999; Storch et al., 2001; Hynes & Rosenthal 1999; Wurst & Bally-Cuif,2001; Lee et al., 2000; Horiguchi et al., 2004) . As a consequence, in addition to mesDA neurons, cultures also include other regionally related neuronal subtypes such as serotonergic and GABAergic neurons (Deacon et al., 1998). Importantly, when used in cell therapy, some of these contaminating neuronal types may cause unwanted side effects after grafting. Thus, heterogeneity complicates development of a therapeutic application of stem cells and emphasizes the importance of developing methods that can generate highly enriched cultures of mesDA neurons. It is crucial to identify the fate-determining regulatory factors that influence dopamine cell fate decision and the underlying molecular machinery.

Neurotrophic factors and overexpression of midbrain-specific genes have been used to induce a dopaminergic phenotype in mouse ESCs-derived neurons. *In vitro* studies have shown that Shh, FGF-8, ascorbic acid, TGF-β and cyclic AMP all increase the differentiation of DA neuron in culture (Ye et al., 1998). One of the most effective inducers of a dopaminergic fate comes from stromal-derived inducing activity from co-culture with stromal cell lines such as PA6 and MS5 (Kawasaki et al., 2000). It will be important to define the specific developmental stage of such committed precursors that will result in optimal

survival, functional integration, and behavioral effects. A great deal of progress has been made in recent years in identifying transcription regulators that control the specification, migration and functional maintenance of mesDA neurons, providing insight into the generation of these cells *in vitro* and *in vivo*(Kim et al., 2002; Andersson et al., 2006). Researchers have over-expressed genes and transcription factors such as Nurr1, Pitx3 and Lmx1a known to promote the development of midbrain DA neurons resulting in much higher yields of DA neurons *in vitro* and *in vivo* (Kim et al., 2003; O'Keeffe et al., 2008; Chung et al. ,2005)

For DA neuron production from ESCs, genetic manipulation of transcription factors is by far the most effective approach. However, it is still in question how typical active ventral midbrain cell population contributes to functional reconstitution in PD brain. It can be determined by systematic transplantation studies using purified, molecularly defined cell populations from the developing midbrain. Studies on normal development of the midbrain DA lineage can help to analyze the molecular make up of cells at each step of DA lineage differentiation. It will be promising to determine the desired 'donor' cells for PD treatment from heterogeneous populations of differentiated ESCs progeny.

3.3 Huntington's disease

HD is a fatal hereditary and neurodegenerative disease characterized by involuntary choreiformic movements, cognitive impairment, and emotional disorder. In United States, there are nearly 30000 HD patients and 150000 are in high risk (Leegwater-Kim & Cha, 2004). HD is caused by mutation of a gene, which resulted in an abnormal expansion of CAG-encoded polyglutamine repeats in a protein called huntingtin (Walker, 2007). The presence of huntingtin leads to the loss of medium spiny neurons(GABAergic neurons) in the striatum, and to a lesser extent, in other extrapyramidal and cortical areas(Melone et al., 2005). Current pharmacotherapies of HD have been evaluated targeting the neurotransmitters such as GABA, dopamine, glutamate which are regarded as the predominant neurotransmitters affected in HD, however, few well conducted trials for symptomatic or neuroprotective interventions have yielded positive results. There is no current method to change the course of HD (Frank & Jankovic, 2010).

Since the hallmark of HD is the loss of some specific neurons in the striatum and other regions in the brain, cell transplantation may serve as a hopeful strategy for reducing neural damage and replacing the lost neurons in the HD brain (Bachoud-Lévi et al., 2000; Dunnett & Rosser, 2007; Kim et al., 2008; Clelland et al., 2008; Schwarz & Schwarz, 2010). As early as 1983, Deckel et al transplanted fetal rat striatal tissues fragments into the KA-injured striatum and behavioral improvement was reported (Deckel et al., 1983). In 1995 Isacson et al transplanted embryonic porcine neural cells into adult rat brains with neuronal and axonal loss typical of HD, and found donor axons in host white matter tracts sprouting a long distance from transplant sites, it demonstrated that adult host brain can orient growth of transplanted neurons in cellular repair of the mature CNS (Isacson et al., 1995). This directly proved that transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibers. Subsequently, numerous cellular therapy in excitotoxic striatal lesion HD animal models were reported, these transplantation experiments were carried out in HD animal models including mice , rats and monkeys (Bernreuther et al., 2006; Isacson et al., 1989). The transplanted cells derived from embryonic striatal tissues can survive and differentiate into the cell types of the normal striatum, the behavioral performance of the

HD models were also improved (Bernreuther et al., 2006; Emerich et al., 1998). However, in transgenic mice, the authentic HD animal model, striatal cell transplantation only produced a limited effect on behavioral function (Gutekunst et al.,2000).

The clinical study of cell-based therapy on HD was conducted in Mexico in 1990, after that several reports from several countries showed that the human fetal striatal transplants could survive in the striatum without displacing the surrounding tissue (Gallina et al.,2010; Capetian et al., 2009; Hauser et al., 2002). Furthermore, human fetuses from ganglionic eminences (GE) transplanted into patients had shown long-term success (Bachoud-Lévi et al., 2006; Bachoud-Lévi et al., 2000). The striatal tissues and GE used in these cell-based treatment were isolated from the spontaneously aborted fetuses or selective abortions. The ethical and social issues of using human fetal tissues are still the major obstacles in the cell based replacement in HD.

ESCs have the potential to divide indefinitely in culture, producing unlimited cell source for cell-based therapy, which is considered as a major advantage. The production of NPCs from ESCs is the practical strategy of ESCs based cell transplantation for HD (Song et al., 2007; Bosch et .,2004; Bernreuther et al., 2006). Song and colleagues differentiated hESC into hNPs by co-culturing with PA6 stromal cells, and subsequently transplanted them into the striatum of quinolinic acid(QA)-induced HD model (Song et al., 2007). The transplanted animals exhibited a behavioral recovery for 3 weeks after transplantation (Song et al., 2007). It suggested that hESC-derived hNPCs can lead to a behavioral recovery, as well as neuronal differentiation in HD model. However, hESC-derived hNPCs differentiated into limited number of GABAergic neurons in this way, which is selected death in HD. One research group has generated a homogenous population of functional GABAergic neurons from a NSCs line, using retinoic acid and potassium chloride depolarization (Bosch et al., 2004). When transplanted in the QA model, these cells survived and improved functional deficits *in vivo* (Bosch et al., 2004). Restoring the lost brain functions by replacing damaged striatal neurons will be a crucial step to develop efficient cell replacement therapy in HD.

Recently, several protocols have been developed to produce striatal medium-spiny GABAergic neurons. L1-transfected mESCs cell line generated by Bernreuther et al showed decreased cell proliferation *in vitro*, enhanced neuronal differentiation *in vitro* and *in vivo*. Mice grafted with NPCs derived from L1-transfected ESCs showed functional recovery as decreasing rotation behavior 1 and 4 weeks post-transplantation and increased yield of GABAergic neurons and enhanced NPCs derived from ESCs migration into the lesioned striatum (Bernreuther et al., 2006). Thus L1 overexpression in the ESCs provides an efficient way to get striatal medium-spiny GABAergic neurons to treat HD. In one recent study, mESC line transfected with the extracellular matrix molecule tenascin-R (TNR), which is expressed by striatal GABAergic neurons, showed enhanced differentiation into neurons *in vitro*, reduced migration *in vitro* and *in vivo* (Hargus et al., 2008). Mice grafted with NPCs generated from TNR-transfected ESCs produced more GABAergic neurons and less astrocytes in grafts 1 month and 2 months after transplantation into the QA-treated striatum. Moreover, TNR-overexpressing ESCs transplanted into the striatum attracted host-derived neuroblasts from the rostral migratory stream and promoted recruitment of host-derived newborn neurons within the grafted area (Hargus et al., 2008). These indicated that both L1 and TNR guide ESCs to differentiate to GABAergic neurons, although they share different mechanism. It is likely that a combination of different molecular cues including recognition molecules, neurotrophic factors, and extracellular matrix molecules with cell transplantation will lead to at least additive effects on functional recovery in HD treatment.

In another study, Aubry et al has designed an *in vitro* protocol to push hESCs along the neural lineage, up to postmitotic neurons that exhibit phenotypic features of the medium-spiny GABAergic neurons (MSNs) of the striatum (Aubry et al., 2008). hESCs-derived striatal progenitors have been shown efficiency differentiation into striatal GABAergic neurons following xenotransplantation into adult rats. Such these hESCs-derived striatal progeny may provide a potential cell therapy product to substitute for fetal neural precursors that are currently used in clinical trials to treat patients with HD. On the other hand, apparent overgrowth of human xenograft in rat brain was observed, the hyperplasia of grafted ESCs derived NPCs in the brain should take into account. Vazey et al reported that excessive proliferation was observed in the brain of HD rats in the spontaneously-induced hNPCs group 8 weeks post-transplantation (Vazey et al., 2006). Similar result has also been reported in a recent study when using MS5 stromal-induced hNP to graft into the QA lesioned striatum, significant numbers of transplant-derived overgrowths could not be identified until eight weeks post-transplantation. Vazey et al further demonstrated that noggin-primed hNPs have a greater capacity to survive long-term and differentiate into neurons than spontaneously-derived hNPs after transplanted into the striatum of QA-induced HD model (Vazey et al., 2010). In addition, hyperplasic growths and aberrant differentiation were efficiently controlled by noggin-primed hNPs transplantation even up to 8 weeks post transplantation, which suggested that noggin-primed hNPs transplantation provides an effective and safe treatment for HD.

3.4 Amyotrophic lateral sclerosis

ALS, also known as Lou Gehrig`s disease, is an adult-onset neurodegenerative disorder characterized by the gradual degeneration and death of motor neurons in the cerebral cortex, brain stem and spinal cord. The loss of motor neurons in the long run resulted in progressive paralysis and death within two to five years after diagnosis. The vast majority of ALS cases are sporadic (sALS), while approximately 5-10% of cases are inherited (known as familial; fALS) (Gros-Louis et al., 2006). Mutation in genes including cytosolic Cu/Zn superoxide dismutase (SOD1), alsin, senataxin, synaptobrevin/VAMP (vesicle-associated membrane protein-associated protein B) and dynactin have been reported to cause fALS (Rosen et al., 1993; Yang et al., 2001; Hadano et al., 2001; Chance, 1998; Chen et al., 2004; Nishimura et al., 2004). Transgenic rodents carrying mutant human SOD1 genes appear many of the clinical and neuropathological features of familial as well as sporadic ALS, seem to be one useful model of the disease for preclinical studies. Several mechanisms are possibly involved in the etiology of motor neuron degeneration in ALS such as glutamate excitotoxicity, impaired axonal transportation, protein misfolding, cytoskeletal abnormalities and exposure to toxic or infectious agents. Currently, many evidences have shown that the activation and proliferation of astrocytes and microglia appeared common in ALS are regarded as another possible cause for motor neuron degeneration in ALS (Barbeito LH et al., 2004). Riluzole (Rilutek) is the first FDA-approved medicine for ALS, used to reduce motor neurons damage by decreasing the release of glutamate, which only prolongs patient survival by several months (Gurney et al., 1998). Other potential therapies for ALS, which partially alleviated symptoms has been shown in human clinical trials. So far, there is no effective treatment to cure the patients suffering from ALS.

As the lack of effective drug treatments and the typical pathological changes of ALS are the degeneration and loss of motor neurons in the CNS, cell replacement is considered as a

promising treatment strategy. Xu and colleagues used NSCs from human fetal spinal cord in the transplantation experiments of SOD1 transgenic ALS rats (Xu et al., 2006). The grafted hNSCs were found to differentiate into neurons and formed synapse, secreting several neurotrophic factors, and the degeneration progression of motor neurons in this ALS model was delayed (Xu et al., 2006). It suggested that NSCs grafts can survive well in a ALS environment and the functional improvement was partly caused by the motor neuron growth factors released by grafted NSCs. Intrathecal transplantation of immortalized human NSCs overexpressing vascular endothelial growth factor(VEGF) gene was also reported to significantly delay the onset and prolonged the survival of the SOD1G93A mouse model of ALS. By concomitant downregulation of proapoptotic proteins and upregulation of antiapoptotic proteins, the transplanted human NSCs provided neuroprotective effect in the spinal cord of ALS (Hwang et al., 2009). Recently Mitrecić and colleagues compared the efficiency of NSCs delivery to the CNS in ALS at different stages and found that NSCs via intravenous tail vein injections enter the CNS affected by inflammation/degeneration, the highest delivery efficiency was found in symptomatic ALS and moderate in presymptomatic ALS, while the lowest in wild-type group (Mitrecić et al., 2010). The grafted NSCs preferentially localize in the motor cortex, hippocampus, and spinal cord.

Considering the human NSCs usually isolated from fetal spinal cord or brains, the ethics obstacles make it unsuitable in clinical application. ESCs as totipotent cell are able to differentiate into motor neurons under defined conditions (Nizzardo et al., 2010 ; Li et al., 2008; Soundararajan et al.,2007). Evidences have shown that developmentally relevant signaling factors such as retinoid acid (RA) and sonic hedgehog (Shh) can induce mouse ESCs to differentiate into spinal progenitor cells, and subsequently into motor neurons (Hu & Zhang, 2010; Kim et al., 2009; Li et al., 2008). When transplanted into embryonic chick or adult rat spinal cords, ESCs cell-derived motor neurons can populate the embryonic spinal cord, extend axons, and form synapses with target muscles (Goldstein, 2010). In the similar approaches used for mouse ESCs with small modification, it has been successful in generation of spinal motor neurons from human ESCs in an adherent culture. In terms of the initial neural induction, human ESCs, require FGF2 or those generated from co-cultured feeder cells, then treated with a combination of Shh and RA to generate spinal motor neurons efficiently (Hu & Zhang, 2010). The *in vitro*–generated motor neurons from human ESCs expressed HB9, HoxC8, choline acetyltransferase and vesicular acetylcholine transporter, form synapses on co-cultured myoblasts/myotubes and are electrophysiologically active(Li et al., 2005). These indicate that ESCs are promising donor cells for supplying motor neurons. High purified generation of motor neurons can improve safety, due to decreased number of cells for transplantation. Most recently, Li has described a simple chemically defined suspension culture generated 50% efficiency of motor neuron differentiation from human and primate ESCs treated with purmorphamine, a small molecule that activates the Shh pathway (Li et al., 2008). Furthermore, stable transfection of human and mouse ESCs with Hb9 promoter-driven green fluorescent protein genes, followed by a further isolation of the green cells by fluorescence-activated cell sorting (FACS), could generate enrichment of the motor neuron population from ESCs (Giudice & Trounson, 2008).

In vivo studies, ESCs are seemed to be promising cell sources of cell based treatment of ALS. The study reported that the expression of Hb9 could directly induce mouse ESCs into cholinergic motor neurons. When transplanted into the spinal cord, these ESCs-derived

motor neurons were observed to extend the axons and form synapses with muscles (Giudice & Trounson, 2008). Domos and colleagues had generated iPSCs from an 82-year-old with familial ALS woman and successfully directed to differentiate into motor neurons. These patient-specific iPSCs possess properties of ESCs and supposed to be used in the cell transplantation of ALS (Dimos et al., 2008). Recently, López-González et al differentiated mouse ESCs into motor neurons and grafted into the lumbar spinal cord of hSOD1(G93A) ALS rats. They found that grafted cells with motor neuron phenotype can survive for at least 1 week in hSOD1(G93A) animals and the significant improvement in movement was transiently recovered, However, the long-term effect of cell transplantation was not guaranteed because the transgenic hSOD1(G93A) environment is detrimental to grafted motor neurons (Lopez-Gonzalez et al., 2009).

Glial cells such as astrocytes and microglias create a hostile environment that contributes to motor neuron toxicity in transgenic ALS animal models and then may also adversely affect the survival and maturation of transplanted stem cell derived-motor neurons. Makiko has demonstrated that rodent astrocytes expressing mutated SOD1 kill spinal primary and mouse ESCs–derived motor neurons without leading to the death of spinal GABAergic or dorsal root ganglion neurons or ESCs–derived interneurons (Nagai et al., 2007). Conversely, expression of mutated human SOD1 in primary mouse spinal motor neurons does not provoke motor neuron degeneration (Nagai et al., 2007). These findings suggest that astrocytes may play a role in the specific degeneration of spinal motor neurons in ALS. Marchetto et al co-cultured human ESCs-derived motor neurons with human primary astrocytes expressing mutated SOD1 and found that a selective motor neuron toxicity was correlated with increased inflammatory response in SOD1-mutated astrocytes (Marchetto et al., 2008). Consistent with this study, it has been confirmed that human ESCs-derived motor neurons are selectively sensitive to toxic effect caused by astrocytes carrying an ALS-causing mutation in the SOD1, while interneuron populations produced from ESCs are unaffected (Di Giorgio et al., 2008). Taken together, these studies suggest a scenario in which mutation of SOD1 activates inflammatory pathways in astrocytes, including activation of prostaglandin D2(PGD2) signaling and nitric oxide release. Furthermore, primary motor neurons co-cultured in direct contact, particularly with primary mutant SOD1-expressing microglia, showed decreased survival and shortened neurite length (Zhao et al., 2010). As further evidence for microglial toxicity to motor neurons in ALS, several studies have indicated that isolated primary microglia become toxic to primary motor neurons when activated with either proinflammatory LPS or IgG immune complexes isolated from human ALS patients (Thonhoff et al., 2009; Weydt et al., 2004; Dewil et al., 2007).

Different from other neurodegenerative diseases, both the motor neurons and non-neuronal cells (mainly astrocyte or microglial) should be taken into account in the stem cell replacement of ALS. When using ESCs transplantation treating ALS, the replacement of dysfunctional non-neuronal glial cells such as astrocytes and microglia will be helpful to protect motor neurons.

4. Prospects and conclusions

Although ESCs transplantation has been proved to be a promising tool and potential therapies for treating incurable neurodegenerative diseases such as AD, PD, HD, and ALS, it is too early to be optimistic that ESCs will be used immediately clinically to cure the neurodegenerative diseases. Several issues about safety, effectiveness, ethical and feasibility

must be considered before the adoption of stem cell replacement is widely accepted in clinical medicine. The risk of tumorigenesis is primary consideration of ESCs replacement in patients suffered from neurodegenerative diseases. The small number of ESCs escape differentiation and selection procedure may over proliferate and bring the risk of teratoma formation (Rao, 2007).With development of procedures to produce high purified NPCs with commitment and mature neurons derived from human ESCs is safe in cell transplantation of neurological diseases. It has been achieved to generate enrichment neuron or NPC populations from ESCs, while the efficiency to produce more specific neuron such as DA neuron is very low. It maybe overcomed by uncovering the developmental mechanism and improved techniques (Cho et al., 2008). It seemed effective when ESCs were used in the transplantation experiments in the animal models of neurodegenerative diseases, but the long term effects related to security and recovery of structure and function need to be evaluated carefully before clinical practice.

The ethical and feasibility problems of ESCs based treatment in neurodegenerative disease are also expected to be solved in near future. The success in iPSCs seems to offer the advantages of hESCs without the ethical and rejection problems, although the iPSCs is not as versatile and plastic as ESCs. Revazova and collegues have established four unique HLA-homozygous human ESCs lines which have a simple genetic profile in the critical areas of the DNA that code for immune rejection, these ES cell lines will not provoke an immune reaction in large segments of the population and could serve to create a stem cell bank as a renewable source of transplantable cells for use in cell therapy to treat degenerative diseases (Revazova et al., 2008).

In conclusion, although there is still a long way to go in ESCs replacement therapy to become a clinical reality for AD, PD, HD and ALS, this therapeutic approach is promising for neurodegenerative diseases.

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6. References

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7

Engineering Therapeutic Neural Stem Cell Lines for Parkinson's Disease

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1. Introduction

The isolation of tissue-specific self-renewable stem cells from human embryonic or adult stem cells is one of the most promising applications for regenerative medicine. Ongoing research suggests that multipotent stem cells are a viable source of specialized cells for tissue repair. Undifferentiated stem cells are better suited when multiple cell types are lost to injury or disease. However, when a single lineage species is associated with an injury or disease, multipotent stem cells can be instructed to terminally differentiate into specific cell types. The terminal differentiation is induced by culturing the multipotent stem cells in media containing specific instructive molecules or by over-expressing lineage-specific gene(s). Further studies are needed to generate cellular phenotypes with stable expression in vitro and after grafting into diseased or injured tissue. Harnessing mechanisms governing tissue histogenesis that take place during early embryogenesis is a promising strategy for engineering specific cell types or tissues. Likewise, the same developmental pathways could also be induced in vitro using a set of instructive cues different from those normally involved during embryonic development.
Within the framework of developing therapeutic products, this chapter will discuss the cellular and molecular control of neural stem cell derivation from adult and pluripotent stem cells and their differentiation into dopaminergic lineage.

2. Derivation and properties of neural stem cells

Neural stem cells (NSCs) are defined by their ability to self-renew and generate a large number of progeny able to differentiate into the principal central nervous system (CNS) cell types: neurons, astrocytes and oligodendrocytes. NSCs have the ability to maintain themselves in culture under genetic or epigenetic stimulation and to generate a large number of progeny. Contrary to the hematopoietic system where the hematopoietic stem cells are defined by a set of cell markers and thus can be purified by fluorescence-activated cell sorting (FACS), there are no specific cellular markers, necessary and sufficient to identify NSCs. The cell surface marker CD133 (prominin-1/2) epitope has been used to isolate the neurosphere-forming neural precursors from human fetal brain (Uchida et al.,

2000). However, this cell surface marker is also expressed by immature hematopoietic stem cells, epithelial, tumor cells and endothelial progenitors characterized by the expression of other cell surface markers including CD34 and CD45 (Miraglia et al., 1998; Corbeil et al., 2000; Peichev et al., 2000; Florek et al., 2005). Thus, to purify the neural precursor population it has to be sorted out from the CD34 and CD45 negative population. Using a combinatorial surface antigen code, specific populations of neural precursors are isolated from adult, fetal brain or pluripotent human embryonic stem cells. A recent study used the same combination of cell surface markers to isolate neural stem cells from hESCs (Golebiewska et al., 2009). This population of neural precursors expressed low levels of the pluripotency markers Oct4 and Nanog (Johansson et al., 1999) CD133+ cells expressed the neural specific marker Pax6, nestin, vimentin, Olig2, Sox1, sox3, Gli3, beta-tubulin3 and myelin basic protein (MBP). Interestingly these cells also expressed the transcription factor Pax7 that is characteristic of muscle precursor cells (Golebiewska et al., 2009). This mixed expression pattern suggests that cells do not correlate with a specific regional identity and that CD133+ cells perhaps retain the potential to differentiate into a wide range of cells.

A second cell surface marker expressed by neural precursors in vivo in the subventricular zone (SCZ) is CD15 (stage-specific embryonic antigen-1, lewis –X antigen) (Capela and Temple, 2002). CD15+ SVZ-derived precursors give rise to neurospheres capable of differentiating into all major CNS cell types. In this study, the CD15- / CD24+ ependymal cells lining the 3rd ventricle, previously thought to contribute to the NSC compartment (Johansson et al., 1999), were incapable of forming neurospheres in vitro (Capela and Temple, 2002).

In an effort to isolate homogenous populations of neural stem cells from hESCs, a combination of markers was used simultaneously. This combinatorial flow cytometry included the cell surface markers CD15+, CD29 high (small cell lung carcinoma cluster 4 antigen) and CD24 low (beta1-integrin) (Pruszak et al., 2009). CD15 is strongly expressed in Sox1+ and Sox2+ neurepithelial rosette structures derived from hESCs, as well in the CD133+ cells. The CD15+/$CD24_{LO}$/$CD29_{HI}$ subset was enriched for the neurosphere forming colonies. Interestingly, transplantation of this cell type showed neuroepithelial tumors that displayed characteristic neural rosettes expressing neural precursor markers: Sox2, nestin, vimentin and radial glial markers 3CB2 and RC2. The expression profile of the CD15–/$CD24_{LO}$/$CD29_{HI}$ fraction was characteristic of the neural crest/mesenchymal stem cells and expressed the cell surface markers CD271, CD57 and CD73. This subpopulation was also tumorigenic after injection in animals. The third subpopulation with the CD15–/$CD24_{HI}$/$CD29_{LO}$ surface antigen signature defined a neuronal/neuroblast population that was highly enriched for neuronal markers, such as doublecortin and microtubule-associated protein (MAP)-2. In contrast, CD15–/$CD24_{HI}$/$CD29_{LO}$ grafts did not form tumors, differentiated into NCAM-positive cells and extended neuronal processes into the host brain (Pruszak et al., 2009).

Forse1 (forebrain surface embryonic antigen-1) is another cell surface marker expressed by neural precursors (Tole et al., 1995) and used to identify multipotent neural stem cells (Tole et al., 1995; Pruszak et al., 2007; Elkabetz et al., 2008). Cells expressing Forse1 within the hESC-derived neural rosettes exhibited anterior neural fate as assessed by the expression of the forebrain transcription factor BF1. Forse1- cells gave rise to neural crest stem cells and were enriched for posterior CNS markers. Interestingly, both Force1+ and Force1- retain the ability to form rosettes and Force1+ have the potential to revert to caudal fates, including

spinal cord motor neurons and midbrain dopamine neurons and to generate neural crest cells (Elkabetz et al., 2008). Transplantation of the Force1+ neural precursors into the rat striatum led to graft overgrowth and formation of rosette in vivo. This overgrowth was observed even when Force1+ cells were sorted before transplantation suggesting that cell contamination is not the cause of the tumorigenicity.

A recent study, however, demonstrated that neither CD133 nor CD15 are necessary markers to define a neural stem cell. Human NSCs positive or negative for either CD133 or CD15 have exhibited multipotency and the ability to differentiate into neurons, astrocytes and oligodendrocytes (Sun et al., 2009). Of interest, the CD133 marker appears to be down regulated as the cells enter the S phase and during the G2 or M phases. This observation renders the neural stem cell identity even more elusive.

In addition to cell surface markers, specific populations of NSCs may be isolated based on their responsiveness to mitogenic growth factors. The fundamental three properties necessary for cells to be defined as NSC are: 1) self-renewal ability and maintenance of long-term cultures through multiple passages under clonogenic conditions, 2) generation of a large number of progenies and 3) differentiation into the three principal neural lineages i.e. neurons, astrocytes and oligodendrocytes.

Conti et al. isolated homogenous and clonogenic populations of NSCs from mouse and human ESC-derived rosettes (Conti et al., 2005). The rosettes were mechanically transferred into serum free media in the presence of EGF and bFGF and propagated for up to 5 months. When exposed to appropriate differentiation factors, these NSCs expressed nestin, vimentin and the radial glial marker 3CB2 and differentiated into neurons, astrocytes and oligodendrocytes, (Sun et al., 2008). After transplantation into adult mouse hippocampus and striatum, these NSCs engrafted and differentiated into neurons and astrocytes without forming tumors.

Daadi et al. recently reported the isolation and perpetuation of a homogenous population of hNSCs, from hECSs based on their proliferative response to the exposure to EGF, bFGF and LIF (Daadi et al., 2008). The cumulative cell number and population doubling analysis demonstrated the continuous and stable growth of the hNSCs. These hNSCs were clonogenic and expressed the neural precursor cell markers nestin, vimentin and the radial glial cell marker 3CB2. Under differentiation conditions, the hNSCs gave rise to neurons, astrocytes and oligodendrocytes, expressed transcripts for the neural-specific genes nestin, Notch1 and neural cell adhesion molecule (N-CAM), Sox2 and for the lineage specific markers β-tubulin class III, medium-size neurofilament (NF-M) and microtubule-associated protein 2 (MAP-2) for neurons, GFAP for astrocytes and myelin basic protein (MBP) for oligodendrocytes.

Koch et al. recently reported the isolation, perpetuation and characterization of the rosette-derived EGF+FGF2 responsive hNSCs (Koch et al., 2009). These neural precursors were isolated from rosettes dissected out of the culture plate and grown in suspension as spheres. In this study the self-renewable NSCs were maintained for up to 75 passages without apparent changes in proportions of the neural lineages and a pronounced differentiation toward neuronal lineage (40 to 70%). These NSCs developed an anterior hindbrain identity with predominant generation of GABAergic neurons. They retained the ability to convert to a ventral midbrain identity in response to sonic hedgehog (SHH) and FGF8 treatment with 31% of beta-tubulin+ neurons expressing tyrosine hydroxylase.

Another approach used to generate a homogeneous and specific NSC population at the clonal level is the genetic immortalization of neural precursors with propagating genes, such as v-myc, large T-antigen and telomerase reverse transcriptase (hTER) (Snyder et al., 1992; Whittemore and Synder, 1996; Lundberg et al., 1997; Roy et al., 2004). Over expression of human telomerase reverse transcriptase (hTERT) was used to immortalize neural progenitors from the human fetal spinal cord. These cells have been shown to yield multiple cell lines with different lineage proportions including some restricted to a neuronal lineage both *in vitro* and *in vivo*. The cell line expressed markers consistent with a ventral spinal neuronal (interneurons and motor neurons) phenotype. The functional property of the neurons was demonstrated electrophysiologically by using calcium influx in response to depolarizing stimuli. The cells were passaged without evidence of senescence, karyotypic abnormality or loss of normal growth control. The cells did not form tumors or overgrow after transplantation into developing rat fetal telencephalon or spinal cord. Human NSC clones were genetically propagated using v-myc (Flax et al., 1998; Villa et al., 2009). Transplantation of these hNSCs demonstrated their ability to migrate throughout the CNS and differentiate into multiple developmentally and regionally appropriate cell types. The gene product of v-myc was undetectable in grafted hNSCs 24-48 hours following transplantation, which suggests the lack of graft overgrowth in vivo. However, there is possibility of clonal variations in v-myc expression or re-activation of v-myc in vivo and tumor formation. Thus, regulated expression of immortalizing genes would be a safer approach for exploring therapeutic application of the immortalized cell lines (Hoshimaru et al., 1996).

The prospective isolation and perpetuation of homogenous populations of neural stem cells have also been carried out using reporter genes placed under the regulatory control of cell-specific promoters. Using human cells, this technique requires the transfection and FACS isolation of the cell population expressing the reporter gene, such as green fluorescent protein (GFP). Among the cell-specific gene promoters used to isolate neural stem cells are nestin, musashi, Sox1 and Sox2. Nestin is an intermediate filament expressed by neuroepithelial stem cells. The second intronic enhancer of nestin directs its transcription to neural stem and progenitor cells. This strategy was used to isolate homogenous nestin+ neural stem cells and to differentiate them into specific lineages (Keyoung et al., 2001). Musashi1 is an RNA-binding protein expressed by neural progenitors of the fetal brain (Kaneko et al., 2000). Neural cells expressing the musashi1/hGFP co-expressed nestin in 96% of the progeny. The majority of cells (93%) are undergoing cell division as monitored by BrdU incorporation (Keyoung et al., 2001). Sox1 gene is one of the earliest genes that mark the neuroectoderm specification in the developing mouse embryo. It is expressed in neuroepithelial precursors but down-regulated during neuronal and glial differentiation. Using a Sox1-GFP knock-in line, purified populations of neural stem cells were isolated, perpetuated and differentiated into specialized neuronal populations. (Ying et al., 2003; Barraud et al., 2005; Chung et al., 2006). Similarly, Zappone et al defined the regulatory element of Sox2 gene expression in both stem and progenitor cells (Zappone et al., 2000). Using adenoviral vector expressing Sox2/EGFP Wang et al transduced and FACS purified Sox2 expressing neural stem cells from the developing human fetal brain (Wang et al.). The Sox2+ neural precursors were self-renewable, multipotent and displayed higher telomerase enzymatic activity, in comparison to the Sox2-depleted population.

3. Therapeutic application in Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopamine (DA) neurons in the substantia nigra pars compacta, resulting in decreased dopaminergic input to the striatum. Symptoms include tremors, rigidity, bradykinesia and instability. Existing therapies for PD are only palliative and treat the symptoms but do not address the underlying cause or prevent the progression of the disease. Levodopa (L-dopa), the gold standard pharmacological treatment to restore dopamine, is compromised over time by decreased efficacy and by increased side effects. Neurosurgical treatments, such as pallidotomy, thalamotomy and deep electrical stimulation are only considered after the failure of pharmacological treatment. A reliable long-term treatment to halt the progression of the disease and restore function remains elusive.

Neural transplantation is a promising strategy for improving dopaminergic dysfunction in PD. Over 20 years of research using fetal mesencephalic tissue as a source of DA neurons has demonstrated the therapeutic potential of cell transplantation therapy in rodents and non-human primate animal models and in human patients (Mendez et al., 2008). In many patients grafts have survived, formed synaptic connections and improved motor function (Olanow et al., 1997; Barker and Dunnett, 1999). However, there are limitations associated with human fetal tissue transplantation, including high tissue variability, lack of scalability, ethical concerns and inability to obtain an epidemiologically meaningful quantity of tissue. Thus, the control of the identity, purity and potency of these cells becomes exceedingly difficult and jeopardizes both the safety of the patient and the efficacy of the therapy. With a reliance on fetal tissue as a source of neurons, cell replacement therapy cannot develop into a widely available treatment option for patients with neurodegenerative diseases. These critical issues render the search and development of alternative sources of cells a very worthwhile goal with societal importance and commercial application.

4. Differentiation of neural stem cells into dopaminergic neurons

Alternative sources of natural dopamine expressing cells explored have been the adrenal medulla cells (Schueler et al., 1993), PC12 cells (Ono et al., 1997), the glomus cells of the carotid bodies (Espejo et al., 1998) and the porcine fetal tissue (Deacon et al., 1997). Most of these sources have been abandoned due to poor cell survival, inefficiency or health risks for the patient (Yurek and Sladek, 1990; Isacson and Breakefield, 1997). The current most promising strategy in generating an unlimited supply of cells for neural transplantation is the generation of dopaminergic neurons from NSCs.

Cellular differentiation may be defined as a multistep process driving a given cell from a precursor stage to functional competence. These steps often are manifested by changes in cellular morphology and by the appearance of new gene products. Each differentiation step is timely orchestrated and often depends on the interplay between the cell's intrinsic and extrinsic programs. Knowledge of both extrinsic differentiation signals and the molecular machinery underlying the intrinsic events is rapidly progressing.

Extrinsic cues may regulate neuronal diversity by selectively rescuing a specific subpopulation of neuronal precursors committed to expressing a specific neurotransmitter phenotype or by instructing the neuronal precursors during a narrow developmental window to adopt a specific fate.

The relative distribution of the *in vivo* environmental cues is thought to play a critical role in directing fate choices of stem cell neuronal progeny. For instance, in the peripheral nervous

system (PNS), neural crest stem cells (NCSCs) derived from the E10.5 neural tube behave differently from the E14.5 sciatic nerve-derived NCSC. The latter became less sensitive to the autonomic instructive action of bone morphogenetic protein-2 (BMP-2) and consequently their potential is limited to a cholinergic fate. This time-dependant decrease in the BMP-2 sensitivity may have resulted from a combination of an *in vivo* selection and developmental change in the NCSCs mode to respond to BMP2.

A promising stem cell source for DA neurons is embryonic stem (ES) cells. Early studies demonstrated that these cells have the potential to generate DA neurons (Kawasaki et al., 2000; Lee et al., 2000a). In presence of serum, ES cells form clusters of floating cells or embryoid bodies (EBs) containing ectodermal, mesodermal and endodermal derivatives. When these EBs are treated with FGF2, FGF8 and Shh, 71% of the cells differentiated into neurons as identified with the neuronal marker class III β-tubulin and 33% of these neurons displayed characteristics of the midbrain DA neurons (Lee et al., 2000a). A second group of investigators proceeded first to generate a homogenous neural lineage from the ES cells (Kawasaki et al., 2000) before inducing DA phenotype. This was achieved by co-culturing ES cells with the stromal cells PA6 that induced the pan-neural marker NCAM in 92% of the ES cells colonies. PA6-derived conditioned media was inefficient in inducing neural differentiation, but was not blocked by a 0.4 μm membrane barrier. Paradoxically, paraformaldehyde fixed PA6 cells retained the inductive activity. Under these culture conditions, 52% of differentiated cells expressed neuronal markers and 30% of these neurons assumed midbrain DA phenotype. These DA induced neurons appear to engraft after implantation and to improve behavioral deficits of 6-OHDA lesioned mice. Mesencephalic explant cultures studies (Baizabal and Covarrubias, 2009) showed that ES-derived neural precursors exhibit a limited developmental window to respond to the midbrain DA cues and that FGF8 +SHH treatment promotes commitment to DA lineage.

There has been a concerted effort to isolate a stable, expandable stem cell from the midbrain, based on the hypothesis that the progeny will be destined or at least inducible to become the A9 class of the midbrain projecting DA neurons and differentiate exclusively into nigro-striatal-like DA neurons. Early studies have demonstrated that EGF responsive precursor cells do exist within the midbrain, however, these progeny did not consistently or robustly, differentiate into DA neurons neither *in vitro* (Mytilineou et al., 1992; Svendsen et al., 1995; Potter et al., 1999) nor after implantation into the rat striatum (Svendsen et al., 1996; Svendsen et al., 1997). Interestingly, IL-1 induced TH expression in the midbrain-derived progenitors (Potter et al., 1999). In addition, membrane-bound factors potentiated the TH induction and stimulated morphological maturation in these progenitors (Ptak et al., 1995; Ling et al., 1998). The continuous generation of DA neurons from a long-term expandable midbrain-derived stem cell will require the development of processes for proliferation and maintenance of DA-specific precursors. Noteworthy, radial glia of the floor plate can give rise to the midbrain DA neurons *in vivo* (Bonilla et al., 2008). In addition, ascorbic acid and lowered oxygen concentration appear to support survival and proliferation of DA neurons, respectively (Studer et al., 2000; Yan et al., 2001). The effect of a low oxygen level (3±2%) was partially mimicked by erythropoietin (Epo). A different approach (Sawamoto et al., 2001) consisted of FACS sorting mesencephalic precursors according to their expression of GFP driven by nestin enhancer. Nestin is a neurofilament, expressed by neuroepithelial stem cells (Lendahl et al., 1990). The nestin-GFP+ precursors were clonally analyzed and shown to have the ability to self renew and generate clusters of progeny able to differentiate into neurons, astrocytes and oligodendrocytes. Among this neuronal population TH+ neurons

were identified with no particular treatment. Importantly, five weeks after implantation of the sorted GFP+ cells into the striatum of 6-OHDA hemiparkinsonian rats, the animals showed reduction in amphetamine induced rotation. Using this FACS approach, midbrain DA radial glial-like precursors were isolated at the embryonic age E10.5, based on the expression of Lmx1a and the floor plate marker Corin (Jonsson et al., 2009). This study and others highlight the therapeutic efficacy of the A9 DA progenitors in cell transplantation therapy for PD. As follow up to the 2 early studies described above (Kawasaki et al., 2000; Lee et al., 2000b) numerous reports have described techniques of generating dopaminergic neurons from hESCs (Schulz et al., 2004; Zeng et al., 2004; Park et al., 2005; Yan et al., 2005; Sonntag et al., 2007; Cho et al., 2008). Some approaches require co-culturing with stromal cells, human astrocytes, meningeal, sertoli cells or others (Kawasaki et al., 2000; Buytaert-Hoefen et al., 2004; Perrier et al., 2004; Takagi et al., 2005; Roy et al., 2006; Yue et al., 2006; Chiba et al., 2008; Hayashi et al., 2008). Studies have now begun to decipher the active components responsible for the DA phenotype induction. For instance, a recent study demonstrated that the DA-inductive signals of the stromal cell line was mimicked by the combination of a defined set of factors, including stromal cell-derived factor 1, pleiotrophin, insulin-like growth factor 2 and ephrin B1 (Vazin et al., 2009). The inducing factors in the other cell lines and signaling pathways involved in the DA specification remain to be determined.

5. Conclusions

Neural stem cells offer us a great tool for understanding the basic biology of cell fate choices and allow us to explore novel inducing factors and new developmental networks of gene cascades that may not necessarily occur under *in vivo* physiological conditions. A deeper and broader understanding of the molecular and cellular functioning in the development of specialized neural cells, strengthens our ability to efficiently produce stable, pure and viable sources of DA neurons. Ideally this knowledge will also enlighten the next step when cellular products are tested *in vivo* and pre-clinical efficacy is determined. Among challenging issues in product development for PD are the cell line stability, scalability, composition, efficiency in DA neurons generation, viability, cryo-preservation, recovery, identity, purity, potency and the *in vivo* fate of the implanted cell. Thus, given the complexity of neural system, long-term translational research will play an important and critical role in developing safe and efficacious cellular products for treating PD patients.

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8

Embryonic Stem Cells Overexpressing the Recognition Molecules L1 and Tenascin-R Enhance Regeneration in Mouse Models of Acute and Chronic Neurological Disorders

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1. Introduction

Important issues in transplantation of stem cells into the central nervous system that need to be solved to achieve restoration of function are adequate differentiation, survival, migration, and integration of transplanted cells. Furthermore, a major obstacle to transplantation of embryonic stem (ES) cells into the human brain is the formation of teratomas. In this chapter, we provide an overview on how cell adhesion molecules and extracellular matrix molecules can be applied to successfully modify ES cells for cell therapy approaches in animal models of neurological diseases, as both groups of recognition molecules provide important support to cells, participate in the control of cell development, and mediate cell survival both *in vitro* and *in vivo*. As an example from our own work, we describe how mouse ES cells that had been genetically modified to overexpress the neural cell adhesion molecule L1 or the extracellular matrix protein tenascin-R (TNR) promote several aspects of ES cell-mediated regeneration in animal models of neurological diseases. As a surface molecule on postmitotic neurons, L1 is expressed in the developing and adult central nervous system and has been shown to promote neuronal survival, neurite outgrowth, synapse formation, and cell migration. The extracellular matrix molecule TNR, on the other hand, is secreted by both subsets of neurons and myelinating oligodendrocytes in the postnatal brain, is a constituent of perineuronal nets, which promote cellular integrity and synaptic excitability of neurons, and can act as an attracting guidance molecule for migrating endogenous newborn neurons when ectopically expressed *in vivo*.
Both L1 and TNR promote neuronal differentiation of ES cells *in vitro* and increase survival of ES cell-derived neurons after transplantation in the adult rodent brain. L1-overexpressing

ES cell-derived cells migrate over a longer distance after transplantation into the host brain and spinal cord in comparison to non-transfected control cells and mediate functional improvement in animal models of Parkinson´s and Huntington´s disease. In contrast, TNR does not support the migration of engrafted ES cell-derived cells, but attracts host-derived migrating neuroblasts from the rostral migratory stream in an animal model of Huntington´s and promotes the recruitment of host-derived newborn neurons within the grafted area, thereby positively influencing the response of the host to engrafted ES cell-derived cells.

Furthermore, we discuss different aspects of ES cell-mediated regeneration. We describe how genetic modifications have been applied to improve the ability of ES cells to differentiate into specific cellular subtypes *in vitro.* We review how fluorescent activated cell sorting for cell adhesion molecules has been applied on differentiating ES cells to prevent teratoma formation by cell purification, a necessary safety requirement in any potential clinical application of ES cells. These strategies are first steps in the validation of such procedures for therapy in humans. In summary, we provide an overview on how ES cells can be successfully modified for cell therapy approaches in animal models of neurological diseases highlighting the importance of neural cell adhesion molecules and extracellular matrix molecules.

1.1 Characteristics and importance of embryonic stem cells

About three decades ago, the first ES cell lines were established from mouse blastocysts and the isolation of human ES cells has been accomplished thereafter (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Under optimal conditions, ES cells have the ability to divide indefinitely and, as pluripotent stem cells, can differentiate into cells of the three germ layers mesoderm, endoderm and ectoderm. Therefore, ES cells have been widely used to study developmental processes *in vitro* and have been applied to generate gene knockout animals to study gene function *in vivo.* Furthermore, ES cells provide a useful tool for biomedical research and regenerative medicine, as ES cell-derived cells of interest (e.g. cardiomyocytes or neurons) can be used in toxicity assays or drug screens and, importantly, comprise a source for cell therapy in animal models of diseases to rescue or replace imperilled host-derived cells.

1.2 Neuroectodermal differentiation of ES cells

Since ES cells differentiate spontaneously into various cell types *in vitro*, while only certain ES cell-derived cell types are needed for cell-replacement therapy (e.g. neuroectodermal cells for the treatment of neurodegenerative diseases), several protocols have been established to direct the differentiation of ES cells into cells of a specific lineage. Neuroectodermal differentiation of ES cells can be induced by culturing ES cells at low density without the support of inactivated embryonic mouse fibroblasts (Tropepe et al., 2001). In this protocol ES cells follow a default pathway of neural differentiation. Other protocols apply a co-culture system to differentiating ES cells including MS5 feeder cells that express the signalling molecule Wnt-1 (Perrier et al., 2004) or PA6 feeder cells, which provide a stromal cell-derived inducing activity (SDIA) to differentiating ES cells (Kawasaki et al., 2002). Furthermore, the application of recombinant proteins to the culture medium has been shown to significantly promote neuroectodermal or even neural subtype specification of differentiating ES cells. Examples include noggin, an antagonist of the transforming growth factor β family, which enhances neuroectodermal differentiation of ES cells (Pera et

al., 2004; Itsykson et al., 2005; Sonntag et al., 2007), fibroblast factor 4 (FGF-4) to promote serotoninergic neuronal differentiation (Barberi et al., 2003), a combination of retinoic acid (RA) and sonic hedgehog (Shh) to improve cholinergic motor neuron differentiation (Wichterle et al., 2002) or a combination of Shh and FGF-8, which has been shown to direct differentiation of ES cells from various species into dopaminergic neurons (Cooper et al. 2010; Lee et al., 2000; Perrier et al., 2004; Sanchez-Pernaute et al., 2008). Finally, a lineage selection protocol has been established to generate a high number of FGF-2-responsive nestin-positive neural precursor cells from ES cells via so-called embryoid bodies by applying culture conditions, that favor the survival and proliferation of neural precursor cells but not of mesodermal and endodermal cell types (Okabe et al., 1996). Embryoid bodies represent aggregates of differentiating ES cells that consist of a core of ectoderm, mesoderm and endoderm surrounded by visceral and parietal endodermal cells (Maye et al., 2000) and have been applied as model system to study early cell differentiation *in vitro* (Rohwedel et al., 1994; Wobus et al., 1997; Guan et al., 1999; Hegert et al., 2002; Hargus et al., 2008b). The lineage selection protocol comprises 5 different stages and generates a high number of postmitotic neurons at the end of differentiation (Fig. 1). While this protocol has been optimized to enhance overall dopaminergic neuronal differentiation by the application of Shh and FGF-8 during stage 4 (Lee et al., 2000), we have slightly modified this protocol to promote GABAergic differentiation of ES cells *in vitro* (Bernreuther et al., 2006).

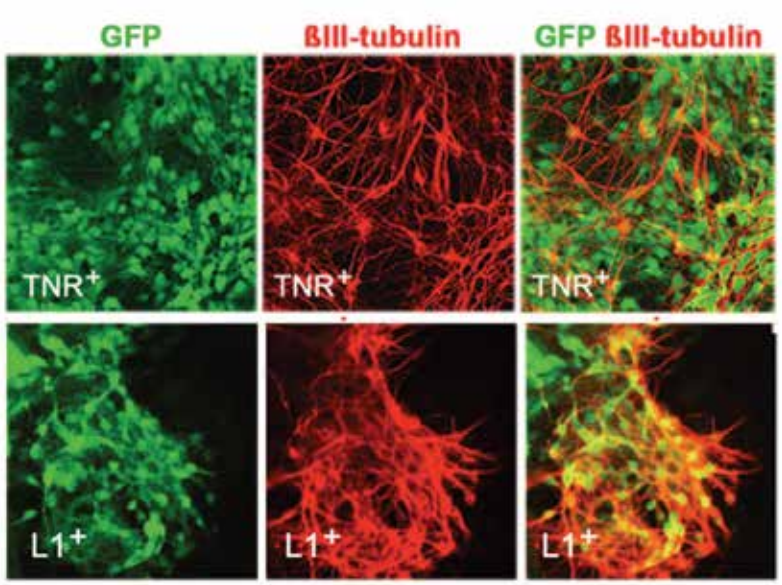

Fig. 1. Tenascin-R (TNR+) and L1 (L1+) overexpressing mouse ES cells differentiate into βIII-tubulin-positive neurons (red) *in vitro*. The images in the upper row show cells at the end of differentiation using a 5-stage differentiation protocol, whereas images in the lower row show differentiated cells after prolonged cultivation *in vitro*, that form so-called substrate-adherent embryonic stem cell-derived neural aggregates (SENAs). Both ES cell lines constitutively express green fluorescent protein (GFP; green) for better visualization after transplantation into animal models of disease. Images from Hargus et al., 2008 and Cui et al., 2010 with permission from *Stem Cells* and *Brain*

Furthermore, by extending the time of culture at stage 4 for several weeks without passaging, we were able to derive three-dimensional neural aggregates that predominantly consist of postmitotic neurons and radial glial cells (Dihne et al., 2006). These substrate-adherent embryonic stem cell-derived neural aggregates (SENAs) can readily be isolated for transplantation and survive well after engraftment into the adult rodent brain (Dihne et al, 2006; Cui et al., 2010).
ES cells can be easily modified to overexpress genes of interest, which could have beneficial effects on the survival of ES cells and the differentiation of ES cells into certain cell lineages. Therefore, besides applying optimized differentiation protocols, several groups have overexpressed transcription factors in mouse and human ES cells in order to promote the differentiation of ES cells into particular neuronal subtypes. For example, overexpression of the transcription factor pitx-3 (Chung et al., 2005) or the LIM homeodomain transcription factor lmx1a (Friling et al., 2009) in mouse ES cells caused enhanced differentiation into midbrain dopaminergic neurons, which are the cell population at risk in Parkinson's disease. Similarly, mouse ES cells overexpressing the nuclear-receptor-related-factor-1 (nurr-1) showed enhanced differentiation into midbrain dopaminergic neurons *in vitro* and improved functional impairment after transplantation in an animal model of Parkinson`s disease (Kim et al., 2002).

2. The role of the cell adhesion molecule L1 and the extracellular matrix protein TNR during neuroectodermal differentiation of ES cells *in vitro*

Little is known about the effects of an overexpression of neural cell adhesion molecules or extracellular matrix (ECM) proteins in ES cells on their differentiation into postmitotic neurons and about their role in ES cell-mediated regeneration in animal models of neurological diseases. This is quite surprising, given that both groups of recognition molecules have important functions on cellular development and survival both *in vitro* and *in vivo*. Indeed, during embryogenesis but also during the entire postnatal life, the specification and integrity of cells is highly dependent on the communication of cells with their surrounding environment through transmembrane glycoproteins on neighboring cells (cell-cell interactions) or through soluble and structural components of the extracellular matrix (cell-matrix interactions). These properties of recognition molecules could have significant implications for cell replacement therapy approaches, since they may help to improve differentiation of ES cells *in vitro* and may support integration, survival and function of ES cell-derived cells after transplantation into animal models of disease.
We have generated mouse ES cells that overexpress the recognition molecules L1 or TNR and have analyzed several effects of these molecules on ES cell-mediated regeneration in animal models of acute and chronic neurological disorders.

2.1 The effects of L1 on neuroectodermal differentiation of ES cells *in vitro*

L1 is a transmembrane cell surface molecule, which is expressed on postmitotic neurons in the central nervous system (CNS) and is found on Schwann cells in peripheral nerves (Lindner et al., 1983; Rathjen and Schachner, 1984; Moos et al., 1988; Kamiguchi and Yoshihara, 2001). L1 is essential for the development of the central nervous system, as L1 promotes neuronal survival, neuronal migration and neurite outgrowth (Lemmon et al., 1989; Appel et al., 1993; Brummendorf et al., 1998; Kamiguchi and Yoshihara, 2001; Kleene et al., 2001). A lack of L1 results in severe malformations within the CNS such as

hydrocephalus or hypoplasia or even aplasia of fiber tracts including the corticospinal tract and the corpus callosum (Jouet et al., 1994). Furthermore, L1-deficient mice have a reduced number of hippocampal neurons and anatomical malformations similar to those seen in patients have been described in these animals (Dahme et al., 1997; Demyanenko et al., 1999). Besides its role during development, L1 has important functions in memory acquisition and is known to increase axon guidance and axon myelination after traumatic injury of the CNS and peripheral nervous system (Martini and Schachner, 1988; Zhang et al., 2000; Becker et al., 2004b).

When L1-overexpressing mouse ES cells were differentiated into neuroectodermal cells following the five stage differentiation protocol, the proportion of postmitotic neurons was significantly increased *in vitro* when compared to cultures consisting of differentiated mouse ES cells that had been transfected with an empty vector (WT ES cells; Bernreuther et al., 2006). This pro-neuronal effect of L1 on cell differentiation, which happened at the expense of differentiation into astrocytic cells, is consistent with previous studies that showed increased neuronal differentiation of somatic neural stem cells when cultured on a surface coated with recombinant L1 (Dihne et al., 2003) or when genetically modified to overexpress L1 protein (own unpublished observations). This pro-neuronal effect could be attributed to homophilic interactions of L1. Furthermore, heterophilic cell-cell interactions through integrins, F3/contactin, NCAM, CD9, and CD24 on other neurons have been described for L1 as well as cell-matrix interactions (Silletti et al., 2000), which could additionally account for the beneficial effects of L1 on neuroectodermal differentiation of ES cells *in vitro*.

Notably, other neuronal surface molecules besides L1 have been shown to promote neuronal differentiation *in vitro*, which further supports the hypothesis that an overexpression of neuronal cell adhesion molecules in ES cells is a suitable approach towards their application *in vivo*. ES cells overexpressing the glycoprotein M6A, which is a cell adhesion molecule expressed on neurons in the CNS, differentiated more efficiently into neurons when compared to non-modified control ES cells (Michibata et al., 2009) and the L1-binding partner molecule NCAM significantly increased neuronal differentiation of embryonic neural precursor cells into mature neurons *in vitro* when added into medium of cultured cells (Shin et al., 2002). However, in contrast to L1, the beneficial effects of ES cells overexpressing these molecules have not been tested in animal models of disease to date.

2.2 The effects of TNR on neuroectodermal differentiation of ES cells *in vitro*

TNR is an ECM protein and is almost exclusively expressed by oligodendrocytes and subpopulations of neurons in the CNS after birth. In white matter, TNR is located at nodes of Ranvier and internodes (ffrench-Constant et al., 1986; Bartsch et al., 1993). In grey matter, TNR is detectable in perineuronal nets that surround inhibitory interneurons and motorneurons and which provide neuroprotective cues to these cells (Angelov et al., 1998; Bruckner et al., 2000; Dityatev et al., 2010). Several, in part opposing functions have been described for TNR and therefore, this molecule was also named *janusin* adopted from the name of the Latin god Janus, the god with the two faces symbolizing ambivalence (for review see Schachner et al., 1994). For instance, TNR acts as a repellent guidance molecule in the optic nerve of zebrafish (Becker et al., 2003), but mediates the detachment of migrating cells from the RMS within the olfactory bulb in mice, establishing TNR also as an attracting guidance molecule (Saghatelyan et al., 2004). Furthermore, TNR promotes neurite outgrowth *in vitro* when presented as a smooth substrate (Husmann et al., 1992; Norenberg et al., 1995), but inhibits neurite outgrowth when presented as sharp substrate border (Becker et al., 2004a).

Similar to L1-overexpressing ES cells, TNR-overexpressing ES cells showed an enhanced neuronal differentiation into postmitotic neurons at the end of differentiation when compared to differentiated mouse ES cells that had been transfected with an empty vector (WT ES cells; Hargus et al., 2008a). It is currently not known why TNR promotes neuronal differentiation of ES cells *in vitro* but similarly to TNR, a pro-neuronal effect has also been described for tenascin-C, another member of the tenascin family of ECM molecules, which significantly increased neuronal differentiation of embryonic mesencephalic explant cultures when added to the cell culture medium (Marchionini et al., 2003). Interestingly, TNR-deficient mice have reduced numbers of inhibitory interneurons in the motor and sensory cortex, which illustrates a pro-neuronal effect of TNR also *in vivo* (own unpublished observations).

Notably, extracellular matrix molecules are widely used to promote the differentiation of ES cells into neuroectodermal cells. For example, current differentiation protocols recommend the application of fibronectin and laminin to ES cell-derived neural precursor cells (Lee et al., 2000). Furthermore, culture of differentiating ES cells on substrate-bound poly-L-ornithine or soluble Matrigel - a basement membrane extract consisting of collagen IV, heparin sulphate proteoglycans, entactin, and nidogen (Kleinman et al., 1986) - increases neuronal differentiation of ES cells *in vitro* (Goetz et al., 2006; Ma et al., 2008). It should be mentioned, however, that also inhibiting effects of some extracellular matrix proteins on neuronal differentiation of ES cells have been described. Gelatine - a mixture of collagen components - increases astrocytic but significantly decreases neuronal differentiation of ES cells *in vitro* (Goetz et al., 2006).

3. Application of differentiated ES cells and fetal cells in animal models of neurological diseases

Several studies have shown that the transplantation of differentiated and specialized neurons can lead to functional improvement in animal models of neurological diseases. For instance, neurons isolated from mouse or human embryonic mesencephalon have been widely used for transplantation in the 6-OHDA-lesion rodent animal model of Parkinson´s disease (Grealish et al. 2010; Brundin et al., 1986), and human fetal cells from the ventral mesencephalon have also been used in several clinical trials in Parkinson patients, some of which showed significant clinical improvement (Mendez et al., 2005; Astradsson et al., 2008; Mendez et al., 2008; Lindvall and Kokaia, 2009). Similarly, fetal striatal neurons have been successfully transplanted into animal models of Huntington´s disease to replace damaged GABAergic medium-sized spiny projection neurons in the host striatum (Isacson et al., 1986; Dunnett and Rosser, 2007), and clinical trials have shown improvement in some of the transplanted patients suffering from Huntington's disease (Philpott et al., 1997; Dunnett and Rosser, 2004).

However, alternative cellular sources are required because of the limited availability of fetal tissue. Due to their ability to generate functional neurons at high numbers *in vitro*, ES cells constitute a promising cell population for such therapeutic approaches and have been applied in several animal models of neurological diseases after pre-differentiation into desired neuronal phenotypes *in vitro*. Several studies have shown that transplantation of mouse, primate or human ES cell-derived neural precursor cells or neurons can lead to functional improvement in animal models of Parkinson´s disease (Bjorklund et al., 2002; Kim et al., 2002; Ben-Hur et al., 2004; Roy et al., 2006; Sanchez-Pernaute et al., 2008; Yang et

al., 2008; Cui et al., 2010). Furthermore, differentiated mouse and human ES cells have been successfully applied in animal models of Huntington`s disease (Bernreuther et al., 2006; Dihne et al., 2006; Aubry et al., 2008; Hargus et al., 2008a), stroke (Wei et al., 2005; Buhnemann et al., 2006; Oyamada et al., 2008), and spinal cord injury (Chen et al., 2005; Keirstead et al., 2005; Sharp et al., 2010) to improve different aspects of regeneration. Notably, the American Food and Drug Administration (FDA) has recently for the first time approved a clinical trial on the transplantation of human ES cell-derived oligodendrocyte progenitor cells in patients with acute spinal cord injury conducted by the Geron Corporation. In this context, however, it should be emphasized that the transplantion ES-cell derived cells is associated with a specific risk of teratoma formation due to the presence of undifferentiated ES cells in the cell suspension for transplantation, emphasizing the requirement for efficient cell differentiation *in vitro* and for thorough cell purification before engraftment. We will focus on this topic at the end of this chapter.

4. Transplantation of differentiated L1-overexpressing and TNR-overexpressing ES cells in animal models of acute and chronic neurological disorders

Several challenges are associated with transplantation of ES cell-derived neurons, which determine functional outcomes of a cell replacement therapy. Such critical aspects include an efficient differentiation of ES cells into desired neuronal phenotypes *in vitro* as described above, sufficient survival of donor cells after transplantation, and efficient integration of transplanted neurons within the host tissue in order to mediate functional graft-host communication. Several studies have described poor survival of ES cell-derived neurons and limited graft-host interactions after transplantation into the adult rodent striatum (Schulz et al., 2004; Yurek and Fletcher-Turner, 2004; Sonntag et al., 2007) and that survival of cells depends on time of injection after injury (Johann et al., 2007; Darsalia et al., 2010).
By overexpressing the recognition molecule L1 in ES cells, we found that *in vitro*-generated L1-overexpressing SENAs showed two-fold improved survival after transplantation into MPTP-treated Parkinsonian mice when compared to engrafted WT SENAs (Cui et al. 2010). Furthermore, the L1-overexpressing SENAs contained an approximately two-fold increased number of dopaminergic neurons, and engrafted L1-overexpressing cells migrated 2.5× longer distances within the host striatum than wt cells. Also, transplanted L1-overexpressing SENAs rescued a higher number of endogenous imperilled midbrain dopaminergic neurons and improved functional recovery when compared to engrafted differentiated WT SENAs (Cui et al. 2010). In two other studies, we applied the 5-stage differentiation protocol to L1-overexpressing and WT ES cells, which were transplanted into the quinolinic-acid mouse model of Huntington´s disease (Bernreuther et al., 2006) and into an animal model of acute spinal cord injury (Chen et al., 2005). In the former study, the L1-overexpressing grafts contained a higher number of surviving GABAergic neurons and L1-overexpressing cells migrated 3× longer distances within the host striatum when compared to WT cells. Importantly, L1-overexpressing ES cell-derived cells showed functional effects on apomorphine-induced rotational asymmetry in these quinolinic acid-lesioned animals in contrast to engrafted WT control cells (Bernreuther et al., 2006). In line with these findings, differentiated L1-overexpressing ES cells showed robust survival and migrated up to 700 µm in an animal model of acute spinal cord injury, while only few differentiated WT ES cells survived the first few weeks after transplantation into the spinal cord (Chen et al., 2005).

Similar to L1-overexpressing ES cell grafts, transplants consisting of TNR-overexpressing ES cells, differentiated according to the 5-stage differentiation protocol, contained a two- to three-fold higher number of surviving GABAergic neurons in the quinolinic acid-lesioned mouse striatum when compared to WT ES cells (Hargus et al., 2008a). However, in contrast to engrafted L1-overexpressing cells, TNR-overexpressing ES cells showed slightly decreased migration into the host striatum when compared to WT ES cells, but attracted host-derived neuroblasts from the subventricular zone (SVZ) and the rostral migratory stream (RMS) leading to the recruitment of host-derived newborn neurons within the grafted area (Hargus et al., 2008a).

4.1 The influence of L1 and TNR on survival of transplanted cells

Most cells die shortly after transplantation into the adult brain and spinal cord probably due to hypoxia, reduced supply of trophic factors and immune responses. It has been proposed that also limited cell-cell and cell-matrix interactions account for cell death after transplantation into the CNS (Marchionini et al., 2003). Indeed, cell apoptosis can be induced by lack of structural support from surrounding neighboring cells and from the extracellular environment (Raff, 1992; Meredith et al., 1993; Frisch and Francis, 1994). This kind of apoptosis has been shown as early as during the trituration of neural stem cells *in vitro* prior to transplantation (Schierle et al., 1999). Therefore, stable expression of surface or matrix molecules in engrafted cells might help to increase cell-cell contacts and cellular survival. Candidate molecules include NCAM and L1, since both recognition molecules have neuroprotective effects on dopaminergic neurons *in vitro* (Hulley et al., 1998; Ditlevsen et al., 2007), and mechanisms for L1-mediated neuroprotection have been described, which include inhibition of caspase-3 and increased phosphorylation of extracellular signal-related kinases 1/2, Akt and Bad (Loers et al., 2005). Increased L1-mediated cell-cell interactions in grafts could explain why L1-overexpressing cells contained a reduced number of caspase-positive apoptotic cells and an increased number of surviving dopaminergic neurons after engraftment into Parkinsonian mice (Cui et al. 2010), and why L1-overexpressing ES cells survived after transplantation into the injured spinal cord while only few WT ES cell-derived cells were detectable (Chen et al., 2005). Therefore, the microenvironment around grafted cells seems to further influence those mechanisms of cell survival, which are mediated by cell surface molecules.

Since ECM proteins provide structural support to cells and may help to trap and store growth factors, several groups have analyzed the effect of co-delivery of cells and matrix proteins on the survival and function of these cells in the brain but also outside the CNS. When rat cardiomyoblasts were engrafted in collagen matrices into a rat model of myocardial infarction, larger grafts and an improved ventricular heart function were observed in these animals (Kutschka et al., 2006). Similarly, human ES-cell derived cardiomyocytes survived better in infarcted rat hearts when co-delivered with a factor-enriched Matrigel matrix (Laflamme et al., 2007). The addition of the ECM protein tenascin-C to a single cell suspension of fetal mesencephalic neurons before transplantation significantly increased the survival of graft-derived dopaminergic neurons, when engrafted at low density in a rat model of Parkinson´s disease (Marchionini et al., 2003). This study also showed that the cell density of engrafted cells is a critical parameter for the impact of co-delivered ECM molecules on the survival of implanted cells, as tenascin-C did not

influence cell survival in grafts with high cell density. Using a similar high-cell-density approach, we could show that grafts consisting of differentiated TNR-overexpressing ES cells contained increased numbers of surviving GABAergic neurons in a mouse model of Huntington´s disease (Hargus et al., 2008a) when compared to WT ES cells. However, this effect is most likely a result of the positive effect of TNR on the *in vitro*-differentiation of ES cells into postmitotic neurons rather than a result of increased cell survival (as similarly seen for $L1^+$ grafts in the same animal model), since the graft sizes were not altered by the presence of TNR. It will be very interesting to determine how different densities of TNR-overexpressing ES cells for transplantation influence cell survival and function in this and other animal models of neurological diseases.

4.2 The role of the recognition molecules L1 and TNR on cell migration in animal models of neurological disorders

Successful outcomes of a cell therapy in neurological diseases depend on sufficient interaction of engrafted neurons with host-derived cells. Such interaction could lead to functional integration of graft-derived neurons into endogenous neuronal circuitries, mediate important structural and trophic support to imperilled host-derived neurons and result in the mobilization of endogenous host-derived neural progenitor cells, which in turn might support graft-mediated regeneration within the host brain.

Enhanced migration of implanted cells into the host tissue could be beneficial for the integration of engrafted cells, since this process favors a higher degree of functional connectivity to host circuitries (Dunnett and Rosser, 2007). It is well known that engrafted differentiated ES cells show only poor migration in the recipient brain in contrast to implanted fetal neural progenitor cells (Englund et al., 2002; Dunnett and Rosser, 2007). By transplanting differentiated ES cells as SENAs instead of single cells, we could show that the migration of engrafted ES cells into the rodent striatum was significantly enhanced possibly due to the altered microenvironment provided by different cell-cell and cell-matrix interactions (Dihne et al., 2006). Furthermore, an overexpression of L1 in engrafted ES cell-derived cells resulted in significantly enhanced migration into the host striatum in both, MPTP- and quinolinic acid-lesioned Parkinsonian and Huntington mice (Bernreuther et al., 2006; Cui et al., 2010). In addition, differentiated L1-overexpressing ES cells migrated rostrally and caudally from the lesion site when transplanted in an animal model of acute spinal cord injury, while WT ES cells remained at the injection site (Chen et al., 2005). Importantly, the engrafted L1-overexpressing cells showed close proximity to re-growing corticospinal tract axons, which were guided into and also beyond the lesion site in the injured spinal cord. Similar beneficial effects of L1 on axonal outgrowth of corticospinal neurons have been decribed after infusion of soluble Fc-tagged L1 into the lesioned spinal cord, which resulted in behavioral recovery in most of the L1-Fc-treated animals (Roonprapunt et al., 2003).

Overexpression of polysialic acid (PSA), a carbohydrate polymer attached to the neural cell adhesion molecule NCAM, which was achieved by transduction of ES cell-derived cells with retroviruses encoding the polysialyltransferase STX, modified the susceptibility of differentiated ES cells to cytokines after transplantation into the rodent brain thereby influencing migration (Glaser et al., 2007). Since these PSA-expressing ES cells were transplanted into the striatum of healthy unlesioned rats, it is not known how these cells influence function in animal models of neurological diseases. However, such transplantation

studies could be very promising given that PSA glycomimetica promote functional recovery in mice after peripheral nerve injury (Mehanna et al., 2009) and spinal cord compression (Mehanna et al. 2010).

ECM molecules can act as attractant or repellent guidance molecules and both functions have been described for TNR *in vitro* and *in vivo* (Schachner et al., 1994; Jones and Jones, 2000). Differentiated TNR-overexpressing ES cells migrated shorter distances *in vitro* and after transplantation into the striatum of quinolinic acid-lesioned mice *in vivo* when compared to WT cells (Hargus et al., 2008a). However, despite reduced migration of engrafted cells, TNR-overexpressing ES cells showed a tendency towards increased coverage with host-derived synaptic boutons (Hargus et al., 2008a), reflecting increased synaptic input from host-derived neurons towards engrafted cells. This finding goes in line with the reduced density and abnormal structure of symmetrical synapses in TNR-deficient mice (Nikonenko et al., 2003; Apostolova et al., 2006). The generally low degree of synaptic coverage of engrafted ES cell-derived neurons (less than 6% of all engrafted cells for both TNR-overexpressing and WT cells), could explain, however, why engrafted rats did not show reduction in apomorphine-induced rotational asymmetry (Hargus et al., 2008a).

4.3 The role of the recognition molecules L1 and TNR on endogenous neurogenesis and neuroprotection in animal models of neurological disorders

The TNR protein secreted by implanted TNR-overexpressing cells had interesting positive effects on graft-host interactions, as host-derived doublecortin-positive neuroblasts were attracted by engrafted TNR-overexpressing ES cell-derived cells and migrated from the SVZ and the RMS towards and into the grafted area. This effect was sustained for at least 2 months after transplantation (Hargus et al., 2008a). This attractant effect of ectopically presented TNR on endogenous migrating neuroblasts from the SVZ, that migrate toward the olfactory bulb but no other brain regions under physiological conditions (Luskin, 1993; Lois and Alvarez-Buylla, 1994), has been previously described after transplantation of non-neuronal TNR-overexpressing fibroblast-like cells into the adult forebrain in close proximity to the RMS (Saghatelyan et al., 2004) and is in line with the observations that TNR serves as a detachment signal for migrating cells in the adult olfactory bulb (Saghatelyan et al., 2004) and developing cerebellar cortex (Husmann et al., 1992). Furthermore and in line with our observations on enhanced TNR-mediated neuronal differentiation of ES cells *in vitro*, we found that TNR-overexpressing ES cell-derived cells promoted the generation of newborn host-derived neurons in the grafted area, and the degree of this recruitment of endogenous neurons was three-fold higher than in grafts consisting of WT ES cell-derived cells (Hargus et al., 2008a).

It remains open to which extent a recruitment of migrating or *in situ*-generated host-derived neural precursor cells or newborn neurons supports regeneration in the adult lesioned brain. However, a recruitment of endogenous neural progenitor cells from the SVZ into lesioned areas has been described in several animal models after ischemic (Arvidsson et al., 2002; Nakatomi et al., 2002; Parent et al., 2002), physical (Magavi et al., 2000) or excitotoxic (Tattersfield et al., 2004) brain lesions, and differentiation of these recruited precursor cells into neurons with adequate phenotypes has been shown in many of these studies (Magavi et al., 2000; Arvidsson et al., 2002; Nakatomi et al.; Parent et al., 2002).

In this context it should be noted, that other beneficial molecules including glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) could

also be considered for overexpression in ES cells to improve regeneration in animal models of neurological diseases. Indeed, intraventricular application of BDNF enhanced neurogenesis in the SVZ (Zigova et al., 1998) and induced migration of neural progenitor cells from the SVZ into adjacent non-neurogenic areas in the adult brain (Benraiss et al., 2001; Pencea et al., 2001). Furthermore, both BDNF and GDNF are used in established protocols for the differentiation of human ES cells into dopaminergic neurons *in vitro* (Perrier et al., 2004; Sonntag et al., 2007; Cooper et al., 2010) and have neuroprotective effects on endogenous neurons in animal models of neurodegenerative diseases (Grondin and Gash, 1998; Zuccato and Cattaneo, 2009). Interestingly, BDNF (Cassens et al. 2010) and GDNF (Nielsen et al., 2009) have been shown to be functionally connected to neural cell adhesion molecules and thus, beneficial effects of overexpression of these neurotrophins might be mediated by neural cell adhesion molecules and therefore, overexpression of cell adhesion molecules instead of neurotrophins might prevent potential adverse effects of neurotrophin overexpression such as induction of neuropathic pain (Geng et al., 2010).
Similar neuroprotective effects on endogenous host-derived neurons are mediated by the transplantation of neural stem cells into rodent animal models of Parkinson´s disease (Ourednik et al., 2002; Yasuhara et al., 2006) or spinal cord injury (Teng et al., 2002), probably due to neuroprotective factors secreted by engrafted cells. Interestingly, an overexpression of L1 in neural stem cells improved their distribution within the host midbrain and rescued about 1.5 x more host-derived imperilled dopaminergic neurons after transplantation into MPTP-lesioned transgenic L1-overexpressing Parkinsonian mice, when compared with engrafted WT neural stem cells (Ourednik et al., 2009). This finding demonstrates that a recognition molecule can positively influence survival of endogenous neurons and led us to analyze the effects of overexpression of L1 in engrafted differentiated ES cells on host-derived dopaminergic neurons in the MPTP-lesion mouse model of Parkinson´s disease (Cui et al. 2010). L1-overexpressing SENAs transplanted in close proximity to the substantia nigra increased the number of host-derived dopaminergic neurons and enhanced striatal dopamine levels after intrastriatal transplantation, demonstrating neuroprotective effects of L1-overexpressing SENAs, which were not found after transplantation of WT SENAs.

5. Methods to purify ES cell-derived cells for transplantation into animal models of neurological diseases

Before differentiated ES cells can be considered for any clinical application, a purification of ES cell-derived cells is required in order to enrich the cellular phenotypes of interest and to remove residual undifferentiated cells.
Although ES cells can be efficiently differentiated into a variety of desired cell types *in vitro*, current differentiation protocols do not generate a homogenous population of cells. As described above, a directed differentiation of ES cells into neuroectodermal cells can significantly enhance the number of functional neurons with specific neurotransmitter profiles *in vitro* but other neural phenotypes and even cells of other germ layer origins commonly contaminate the final cell population. This finding has an important impact on ES cell-based replacement therapies, since unwanted cellular phenotypes could significantly reduce the efficiency of such approaches. For instance, fetal mesencephalic tissue

transplanted into Parkinsonian animals or Parkinson patients can cause side effects such as graft-induced dyskinesia, which has been discussed to be a result of the heterogeneity of engrafted cells and the presence of donor-derived serotoninergic neurons in grafts (Carlsson et al., 2007; Allan et al., 2010). Most importantly, undifferentiated ES cells can lead to the formation of teratomas consisting of cells of all three germ layers after transplantation.

Cell separation methods include immunopanning, magnetic-associated cell sorting (MACS) or fluorescence-activated cell sorting (FACS), which have been applied on differentiated ES cells.

Immunopanning of cells is achieved by plating cells on a surface coated with an antibody directed against specific epitopes of interest. By applying this method involving L1 antibody-coated surfaces, mouse ES cell-derived neurons have been isolated at high purity, which formed excitatory and inhibitory synapses and were electrically excitable after re-plating (Jungling et al., 2003). Similarly, ES cell-derived neural precursor cells have been efficiently purified after immunopanning for PSA-NCAM (Schmandt et al., 2005).

MACS purification for cell surface molecules has been applied on both, mouse (David et al., 2005) and human (Pruszak et al., 2007) ES cell-derived cells and an enrichment for labelled cells was described in these studies. However, the purity of MACS-sorted cells was lower compared to FACS-sorting procedures on the same cell population (Pruszak et al., 2007). Furthermore, a significant enrichment of neural cells was achieved by FACS-sorting differentiated ES cell cultures for single neural cell adhesion molecules such as NCAM (CD56) or CD146 (Pruszak et al., 2007), or for a combination of cell surface antigens including CD15, CD24 and CD29 (Pruszak et al., 2009). To determine safety and efficiency of a FACS-sorting procedure for a neural cell adhesion molecule, NCAM-FACS-purified human pluripotent stem cell-derived neural cells were transplanted in an animal model of Parkinson´s disease (Hargus et al. 2010). The FACS-purified cells survived and showed functional effects on rotational asymmetry in these animals, while formation of teratomas was not observed. The same study demonstrated that human pluripotent stem cell-derived neurons express the recognition molecule L1 at high levels (Hargus et al., 2010). Therefore, L1 could also be a suitable candidate molecule for FACS purification experiments with the advantage that postmitotic neurons could also be separated from immature L1-negative but NCAM-positive neural precursor cells and astrocytic cells for transplantation.

6. Conclusion

In this chapter, we provided examples that an overexpression of recognition molecules in ES cells can influence different aspects of stem cell-mediated regeneration in animal models of acute and chronic neurological disorders including cellular differentiation, migration, recruitment of endogenous neural cells, neuroprotection, and replacement of imperilled host-derived neurons. These findings encourage further investigation of supporting functions of recognition molecules for stem cell-based therapeutic approaches in human diseases. Furthermore, several studies on cell separation of ES cell-derived neurons preventing the formation of teratomas show important progress towards an application of ES cell-derived cells in patients with neurological disorders, and encourage further refinements of these separation techniques for a potential standardized ES cell-based cell therapy.

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9

Perspectives of Stem Cell-Derived Microglia for Medicine

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1. Introduction

Microglia, the resident immune cells of the central nervous system (CNS), are responsible for the innate brain immune defence. The exact origin of microglia is still unclear, but several reports suggest that microglia are of myeloid origin (Chan et al., 2007). They appear for the first time at an early embryonic state in the neuroepithelium and populate the brain from the blood in a second perinatal phase. Under pathological conditions or infections, microglia migrate to the affected tissue. On site, they change their phenotype to a pro-inflammatory cell type, release cytotoxic molecules such as reactive oxygen species to fight against microbes or clear tissue debris after sterile injury. Microglia also phagocyte oncogenically transformed brain cells and are able to activate additional immune cells. Pro-inflammatory microglial cells are also involved in the progression of neurodegenerative diseases.
Research on microglia is mainly performed on primary cells. However, only a limited amount of murine or human microglial cells can be obtained from the brain tissue, thus complicating investigations of drug screenings or new cell therapy approaches that are requiring a high number of cells. For this reason, stem cell-derived microglia represent a useful tool for further studies to elucidate the role of microglia in diseases and therapeutical approaches. Data from others and our laboratory show that microglial precursor cell lines can be obtained from mouse embryonic stem cells or human induced pluripotent stem cells via a neural differentiation protocol. Human microglial cell lines open new alternatives for drug screening, combating cancer and regenerative approaches for neurological diseases.

2. Microglia in neuroinflammation and neurodegeneration

The CNS contains two major cell types: neurons and glial cells. Neurons build a network throughout the nervous system and forward information through electrical conduction. Glial cells are only indirectly involved in the conduction process, but play an essential supportive role to neurons. Glial cells of the CNS can be subdivided into two main classes: macroglia consisting of astrocytes and oligodendrocytes and microglia. Microglia are the only immune competent cells in the intact CNS. They were defined by Ramon Y Cajal in 1913 as a third group of cells in the CNS and termed mesoglia (Rezaie and Male, 2002). Microglial cells were further characterized by Rio del Hortega using silver carbonate staining (del Rio-Hortega, 1933). He defined the term microglia and proposed that they can

change their morphology dependent on their activation state. Microglial distribution varies by brain region, but they predominate in the grey matter with highest concentrations in the hippocampus, the substantia nigra, the olfactory telencephalon and the basal ganglia (Block et al., 2007). In the healthy CNS the so called 'resting' microglia display a ramified morphology. However, microglial cells are not resting at all, but continuously survey their microenvironment with their processes. Time-laps imaging using *in vivo* two-photon microscopy revealed that the somata of microglial cells remain stably localized whereas the processes are remarkable motile (Nimmerjahn et al., 2005). The microglial processes show continuous cycles of withdrawal and new formation, thereby scanning the whole brain parenchyma every few hours. Upon immunological stimuli or in response to brain injuries microglia become activated and migrate towards the stimuli. At the lesion site they change their phenotype from a ramified to an amoeboid morphology, clear debris and apoptotic cells and promote tissue repair. It was reported that microglia increase neuronal survival through the release of trophic and anti-inflammatory factors (Block et al., 2007; Streit, 2002). In their activated state microglia up-regulate several surface molecules such as CD14, major histocompatibility complex (MHC) molecules, complement receptors and chemokine receptors (Rock et al., 2004). Furthermore, pro-inflammatory activated microglia release a wide range of soluble factors like superoxide, nitric oxide and tumor necrosis factor-α (TNFα), which have neurotoxic effects on neurons. It seems that microglia have both, neuroprotective and neurodestructive functions. In contrast to acute CNS injuries, chronic diseases are characterized by slow progressive neurodegeneration that takes years to develop (Streit, 2002). There is strong evidence that microglial cells are involved in a wide range of neurodegenerative diseases (Block and Hong, 2005) like Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). Actually, AD was one of the first neurodegenerative diseases associated with neurotoxic microglia (Block and Hong, 2005). AD is a neurodegenerative disorder leading to cognitive, memory and behavioral impairments. The pathological hallmark of AD consists of cortical atrophy with accumulation of extracellular deposits of amyloid β (Aβ) in senile plaques and intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau protein in the cerebral cortex (De Strooper and Annaert, 2000). Senile Aβ plaques recruit and activate microglia, which in turn produce pro-inflammatory factors that act neurotoxic on neurons. Damaged neurons activate microglia independent of the mode of damage. Finally, this leads to neurotoxin producing microglia resulting in a perpetuating toxicity.

3. Origin and sources of microglia

During embryonic development gastrulation conduces to the creation of the three germ layers: ectoderm, mesoderm and endoderm (Arnold and Robertson, 2009). The ectoderm gives raise to the peripheral and central nervous system as well as to the epidermis structures. The mesoderm which is located between the endoderm and ectoderm develops into blood, muscles, bone, cartilage, notochord and the connective tissue. Organs associated with the digestive system such as pancreas, liver and the epithelium of the digestive and respiratory system are of endodermal origin.

The origin of microglia is still under debate due to the lack of a specific microglial marker. There are different hypotheses about the origin of microglia. In previous times some scientists held the view that microglia are of neuroectodermal origin and are derived from either the ventricular zone of the lateral ventricle or glioblasts (Kitamura et al., 1984;

Paterson et al., 1973). Kitamura and colleagues (Kitamura et al., 1984) used 3H-thymidine autoradiographic analysis to investigate the genesis of microglia within the mouse hippocampus. They concluded that resting microglia originate from glioblasts, direct derivatives of matrix cells, that are of neuroectodermal origin. Interestingly, the possibility that microglia might be derived from the neuroectoderm via a mesenchymal stem cell type was supported by a recent publication (Takashima et al., 2007). Embryonic stem cells were cultivated and differentiated under two different conditions, one gave rise to mesodermal cells and the other one generated cells from the neuroectodermal lineage. Takashima and colleagues could show that mesenchymal stem cells (MSC) do not develop from mesodermal cells but from cells positive for Sox1, a molecular marker specifically expressed on neuroepithelial cells. They concluded that this way is representing the earliest recruitment of MSCs, whereas the origin of the later waves remains unsolved.

However, in 2002 it was shown that microglia express the transcription factor PU.1, which is myeloid specific. Therefore, it was suggested that prenatal microglia are derived from mesenchymal cells from the myeloid lineage (Rezaie and Male, 2002). Various monoclonal antibodies that are used to identify monocytes and macrophages like CD11b, CD45, CD68 and MHC class II also label microglia which supports the notion that microglia are derived from blood monocytes or another monocyte-related myeloid cell type. This view was further supported by the discovery that the first appearance of microglial cells coincide with the vascularization of the brain. It was suggested that circulating monocytes enter the nervous system and transform to microglia (Imamoto and Leblond, 1978; Ling, 1979) before the formation of the blood-brain-barrier has been completed (Perry et al., 1985). Infiltration of monocytes could be confirmed by carbon-labelling (Ling et al., 1980) and histochemical studies using marker enzymes for monocytes (Ferrer and Sarmiento, 1980; Ling et al., 1982).

Andjelkovic and colleagues labelled microglia in the developing and adult human brain using lectin-histochemistry (Andjelkovic et al., 1998). Lectin positive cells can be subdivided into two groups which both exist in the adult brain, but show different distribution and time schedule of morphological changes. Their results support the consideration that there could be different origins of microglia.

Two questions remain to be answered: when do the first microglia appear and what is their origin. It has been established that microglial progenitors populate the nervous system during embryonic and fetal development (Rezaie and Male, 1999). Some authors have suggested the yolk sac as the tissue of origin for all tissue macrophages during embryonic development (Alliot et al., 1999; Kaur et al., 2001). Alliot and colleagues (1999) reported that cells with properties of microglial precursors can be first detected at embryonic day E8 in mice and that the majority of these progenitors originate from the yolk sac.

Nowadays, it is suggested that microglia populate the brain in two waves. The first one takes place in the neuroepithelium during embryonic development around E8-9 in rodents with unknown origin (Alliot et al., 1999). The second wave occurs in the brain during fetal development at E17-18. These cells are derived from a pool of circulating myeloid precursor cells of mesodermal origin (Chan et al., 2007).

In the adulthood, it is widely accepted that microglia invading the diseased CNS are derived from circulating monocytes originating in the bone marrow (Perry et al., 1985; Rezaie and Male, 2002). It was described that in bone marrow chimeric mice circulating monocytes are recruited to the lesion site of the brain and differentiate into microglia (Mildner et al., 2007).

However, it seems that the inappropriate stimulus of irradiation is required for the recruitment (Mildner et al., 2007).
Microglial function is often studied on primary microglial cells, which are isolated and enriched from mixed glial cultures derived from the brains of postnatal mice or rats. But the cell number obtained by a shaking procedure from mixed glial culture flasks is rather low (Giulian and Baker, 1986). Optionally, a pure population of microglial cells can be obtained using a combination of density gradients and flow cytometric sorting (Ford et al., 1995). This methodology is applied to isolate both mouse and human microglia. However, the obtained number of primary microglia is very limited in rodents and humans. This aspect complicates classical biochemistry studies, systematic screening tests or cell therapy approaches. Therefore, the BV2 cell line which is originated from primary mouse microglia by oncogenetic transformation (Blasi et al., 1990; Bocchini et al., 1992) is used as a substitute for primary microglia. Murine cultured microglia are immortalized via injection with v-raf/v-myc recombinant retroviruses. The obtained BV2 cells show properties like macrophages in terms of antigen profile and phagocytic capacity. However, due to the transformation process these cells show an altered cytokine profile and changes in their migratory capacity (Horvath et al., 2008; Napoli et al., 2009). Later on, Nagai and colleagues established an immortalized cell line of human microglia termed HMO6 (Nagai et al., 2005; Nagai et al., 2001). This cell line is developed from human embryonic telencephalon tissue by retroviral transformation with v-myc. Certainly, these cells can not be used in therapeutical approaches due to the usage of retroviruses for transformation. Therefore, microglia derived from embryonic stem (ES) cells might provide a new tool to study microglial function and to apply them for cellular therapies.

4. Embryonic and induced pluripotent stem cell-derived microglia

During the last decades a major breakthrough was the development of mouse pluripotent embryonic stem cell lines (Evans and Kaufman, 1981). These cells are isolated from the inner cell mass of embryos and can be cultivated *in vitro* on a feeder layer of mouse embryonic fibroblasts (MEF) in the presence of the leukemia inhibitory factor (LIF). In addition to the MEF, which are supposed to secrete crucial factors to promote self-renewal, cultivated murine ES (mES) cell lines also require the presence of LIF to maintain their undifferentiated stage (Smith et al., 1988; Williams et al., 1988). In culture, mES cell lines show an almost unlimited proliferation capacity and maintain nearly a normal and stable karyotype (Smith, 2001). During cultivation ES cell lines do not loose their pluripotency and are able to generate all cell types of an embryo (Wobus and Boheler, 2005).
Due to their crucial role in neurodegenerative diseases microglia could be used as a cellular vehicle for gene or regenerative therapies in the CNS (Neumann, 2006). The CNS is delimited from the circulating blood by the blood-brain-barrier which controls the entry of substances and molecules. Theoretically, only 2 % of small molecules are able to enter the CNS, whereas large molecules are unable to pass the blood-brain-barrier. Therefore, only a limited number of drugs can be introduced in CNS diseases. Microglial cells are a migratory cell type that is able to pass the blood-brain-barrier and could therefore be used as a vehicle to introduce therapeutical proteins into the CNS to the affected tissue.
As already discussed, the obtained number of primary microglia is very limited in rodents and humans using the before mentioned protocols. Contrary to these methods, the generation of microglia derived from pluripotent stem cells constitute a source of unlimited

numbers of microglia which can be used for biochemical studies, systemic screening tests and cell therapy approaches. Recently, a protocol for the generation of microglia derived from mES cells was developed (Beutner et al., 2010; Napoli et al., 2009). This effort was initiated by a work from Tsuchiya and colleagues who succeeded in the differentiation of microglial-like cells derived from mES cells (Tsuchiya et al., 2005). During their neuronal differentiation based on a protocol for the generation of dopaminergic neurons (Lee et al., 2000) they found a population positive for Iba1 and CD45. Using a density gradient, these cells with morphological characteristics of primary microglia could be isolated. However, these isolated cells were not described to survive and proliferate. Thus, we generated a protocol to obtain microglial precursors from mES cells (Beutner et al., 2010; Napoli et al., 2009). These ES cell derived microglial precursors (ESdM) are stable proliferating in culture and have substantially most characteristics of primary microglia. The differentiation of mES cells to microglial precursors requires a five step protocol (Beutner et al., 2010). ES cells are cultivated on an irradiated MEF layer in defined medium in the presence of LIF. To induce spontaneous differentiation, ES cell clusters are detached and cultured on non-adherent culture dishes without LIF. So called embryoid bodies (EBs) are generated and plated on gelatine-coated cell culture dishes in ITS-medium supplemented with fibronectin. Under these conditions cells start to proliferate and different cell types develop. Most of these cells can be immunostained against the intermediate filament protein nestin which is mostly expressed by neuronal precursors. After 6 days in this selection stage medium is changed to N2-medium to expand cells in the presence of FGF2 and laminin for 6 more days. Withdrawal of FGF2 induces differentiation of neurons, astrocytes and oligodendrocytes (Beutner et al., 2010). Within this mixed cell culture cells with microglial morphology can be detected around three weeks after removal of FGF2. The proliferation of these cells can be enhanced through addition of granulocyte macrophage colony stimulating factor (GM-CSF). Microglial precursors are manually isolated and cultivated in N2-medium. They attach to the cell culture dish and typically follow an exponential growth rate. Expression of the marker Iba1 and CD68 indicate that the isolated cells are microglial precursor cells derived from mouse embryonic stem cells. Comparable to primary microglia ESdM show high expression of CD11b and CD45 (Beutner et al., 2010). Analysis of α4 integrin and β1 integrin show high expression levels of these two integrins by ESdM and primary microglia. In contrast, the expression of CD34 and cKit which are both expressed on certain stem cell populations can not be confirmed indicating that ESdM cell lines lost their stem cell properties. Besides, ESdM show expression of B7.2, a costimulatory ligand that is expressed on antigen-presenting cells and that together with its receptor is responsible for T and B cell activation. In addition, a weak expression of major histocompatibility complex (MHC) class II which is also expressed on antigen-presenting cells can be confirmed. Up to passage 20 no relevant changes of the surface marker profile can be observed. However, from passage 25 onwards ESdM lines show a decreased expression of the analyzed markers indicating that the phenotype is not stable over very long proliferation periods.

A typical hallmark of microglia is the inducibility of pro-inflammatory cytokines and reactive oxygen species after stimulation. ESdM cell lines and primary microglia stimulated with lipopolysaccharide (LPS) show an upregulation of inducible nitric oxide synthase (iNOS) and interleukin 1β (IL-1β) transcript levels (Beutner et al., 2010; Napoli et al., 2009). Interferon-γ (IFNγ) stimulation results in an increase in iNOS transcripts in ESdM comparable to primary microglia whereas IL-1β gene transcription is almost not affected in both cell types. Furthermore, a chemokine assay determines that ESdM migrate towards the chemokine

CX3CL1 in a dose-dependent manner (Beutner et al., 2010). Comparable to primary microglia, migration is significantly increased following a CX3CL1 gradient. The phagocytic capacity is an important aspect of microglia. After LPS stimulation ESdM show an increase in phagocytosis of microsphere beads in comparison to unstimulated cells (Beutner et al., 2010).
In summary, ESdM show most characteristics of primary microglia and therefore can be seen as a new promising tool to study microglial function *in vitro* and *in vivo*.
In 2006, Takahashi and Yamanaka identified 24 potential candidate genes that were thought to be involved in maintaining pluripotency and self renewal of ES cells (Takahashi and Yamanaka, 2006). They showed that reprogramming of mouse embryonic fibroblasts to an embryonic-like state is possible after retroviral transduction with the four factors Oct4, Sox2, Klf4 and c-Myc. These so called induced pluripotent stem (iPS) cells showed similarities to ES cells in terms of morphology, proliferation and expression of pluripotency markers. Furthermore, iPS cells are able to differentiate into all three germ layers and therefore can be used to generate every cell type of the body. Some month later different groups succeeded in the generation of iPS cells from human adult fibroblasts (Takahashi et al., 2007b; Yu et al., 2007). Fibroblasts are obtained from human skin tissue biopsies and cultured under defined conditions. For reprogramming, human fibroblasts are retrovirally co-transduced with Oct4, Sox2, c-Myc and Klf4 genes. The obtained iPS cells are cultured according to protocols established for human embryonic stem cells. The analysis of iPS cells shows that they display a normal morphology, a normal karyotype and the expression of several pluripotency markers like Oct4, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81. Furthermore, multilineage differentiation of iPS cells is confirmed by embryoid body and teratoma formation. However, the generation of iPS cells using retroviruses includes the integration of genetic material and thereby the potential risk of genetic modifications of the target cells. To overcome this issue modified methods were developed to generate iPS cells (Yamanaka, 2009). Recently, it was shown that proteins conjugated with a short peptide mediating protein transduction can be directly delivered into the target cell. Using recombinant cell-penetrating reprogramming proteins, iPS cells are obtained from mouse fibroblasts (Zhou et al., 2009).
In a next step, human iPS cells are used for differentiation into microglial precursors (unpublished, pending European patent EP2010_055731). A differentiation protocol is applied with nearly the same five steps described for the generation of ESdM. Human iPS cells are cultured on a layer of irradiated MEF in the presence of FGF2. After some days in culture, iPS colonies can be detached and cultivated on non-adherent dishes without any growth factors to induce spontaneous differentiation. The resulting EBs are allowed to attach to the culture dish and grow out in defined medium supplemented with FGF2 and fibronectin. For expansion of the cells medium is changed to N2-medium in the presence of FGF2 and laminin. Withdrawal of the growth factor after some days induces the differentiation of microglial precursors identified by morphology. Microglial identity is confirmed using immunocytochemical analysis with antibodies against the microglial marker Iba1 and CD68. The iPS-derived microglial precursor (iPSdM) cells can be isolated and cultivated. Because disease-specific iPS cells (Dimos et al., 2008; Ebert et al., 2009; Soldner et al., 2009) can be generated, this method might represent a new tool for regenerative medicine for study and treatment of disease models.

5. Target pathway identification and drug screening

Drug discovery processes are costly in terms of time and expenses. High throughput rate screening technologies allow rapid testing of compounds, but might lack physiological

conditions. Classically two distinct sources of human cell types, primary or immortalized cell lines, are available for drug screening technologies (Ebert and Svendsen, 2010). Primary cell lines are fully differentiated cell types with close approximation of native function, but often are inaccessible, require fresh preparation and have questionable reproducibility. In principal, primary microglial cell lines are not available due to the inaccessibility of the human brain tissue and difficulty to maintain the cells in culture.

In contrast, immortalized cell lines have low costs for maintenance and are homogenous cell populations, but lack important aspects of native cells such as certain metabolic or migratory functions. Furthermore, many cell types are not available as immortalized cell lines. In literature, a human immortalized microglia cell line was described (Nagai et al., 2005; Nagai et al., 2001). This cell line (HMO6) is developed from human embryonic telencephalon tissue by retroviral transformation with v-myc and is reported to possess most immunological and functional characteristics of primary microglia. However, in comparison to primary microglia, HMO6 cells display an altered cytokine profile (Nagai et al., 2001). Therefore, they probably do not fully reflect the properties of primary microglia.

Now, a new technology to obtain human microglial cells becomes available for drug screening technologies. As described above for microglia, embryonic stem cells or induced pluripotent stem cells can be differentiated into stably proliferating cell types. Most adult tissue cell types including microglia can be obtained at high quantities using this technology. The tissue cell types differentiated from stem cells show functional properties very close to the native cell types. Most interestingly, cell lines can be generated from diseased patients, too. However, reproducible methods to obtain fully differentiated cells and to achieve purified populations are still under development. As described above, microglial lines now can be generated from human induced pluripotent stem cells, which reflect very much the native function of primary microglia. The microglial lines can then be used for screening technologies.

One possible use of human microglial cell lines could be screening tests to evaluate the effect of new possible drugs or substances that might act toxic. Nanoparticles which are sized between 1 and 100 nm are basically of interest in pharmacy and medicine as the number of products containing synthesized nanoparticles steadily increases. Orally absorbed nanoparticles via drugs or food can pass the blood system via the gut. It was reported that silica crystals can activate immune cells, are phagocytosed and transported into the lysosome (Hornung et al., 2008). Given that these nanoparticles can not be digested, lysosomal swelling and damage might be induced in phagocytes including microglia. In addition, lysosomal damage activates the inflammasome and the release of the pro-inflammatory cytokine IL-1β. It is expected that the size, structure and type of nanoparticles might influence this lysosomal toxicity. Interestingly, aggregated amyloid β (Aβ) which is involved in the pathology of AD and exhibit a size of 10 – 200 nm can activate microglia in a similar way (Halle et al., 2008). Halle and colleagues demonstrated that microglia phagocytose fibrillar Aβ and transport it into the lysosome, where it induces swelling and damage of this organelle. The inflammasome of microglia is activated which leads to the release of inflammatory mediators like IL-1β. Furthermore, diesel exhaust particles (DEP) mediate the neurotoxicity on dopaminergic neurons caused by microglia, possibly by a similar mechanism (Block et al., 2004). Diesel exhaust particles uptake leads to the activation of microglia and their production of reactive oxygen species and nitric oxide, which induces toxicity of dopaminergic neurons. In summary, nanoparticles and other small particles principally have the risk to activate microglia leading to the release of pro-inflammatory

cytokines and radicals which can promote the pathology of neurodegenerative diseases. Thus, microglial lines could help to evaluate the possible brain toxicity of very small particles.

Another possibility to use human microglial cell lines for screening is the development of novel drugs for neurodegenerative diseases. Activated microglia are reported to be involved in the pathology of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS). Approximately, 10 – 20 % of patients with familiar ALS show a mutation in the superoxide dismutase-1 (SOD1) gene whose product is involved in the conversion of free superoxide radicals. In an animal model of ALS it was demonstrated that microglia with mutant SOD1 drive the disease onset and progression in particular via the release of pro-inflammatory molecules and radicals (Boillee and Cleveland, 2008). Mutant SOD1 stimulates the NADPH oxidase resulting in an overproduction of reactive oxygen species (ROS). Therefore, it is proposed that ROS produced by microglia mediate the pathogenesis of familiar ALS associated with a mutation of SOD1.

Human iPS-derived microglia cell lines would provide a new possibility to investigate the effect of different SOD1 mutations on the toxicity of microglia. Therefore, iPS-derived microglia could be transduced with mutant SOD1 or obtained from patients with a SOD1 mutation and analyzed for abnormal radical and pro-inflammatory cytokine production. Furthermore, co-culture of those genetically modified or mutated microglia and motor neurons could give more insight in the pathology of the disease.

Since iPS cells can be generated from diseased patients (Dimos et al., 2008; Ebert et al., 2009; Park et al., 2008) they represent a new tool for disease modelling screens of several neurodegenerative diseases. Ideally, iPS cells are expanded and differentiated in the disease relevant cell type such as microglia. The differentiated cell type would allow drug screening tests and investigations of genes differently regulated or mutated in this cell type.

In summary, iPS-derived microglia represent human conditions better than other cell lines, thus allowing drug screening and identification of pathways affected in neurodegenerative diseases.

6. Combating cancer

The most common and aggressive type of primary brain tumors in humans is the glioma, which most probably arises from glial cells or their progenitor cells. As primary tumors, gliomas occur rather rarely with an estimated prevalence of 15 cases for 100 000 people (American Cancer Society, 2006). These anaplastic and malignant tumors result in a median of survival after diagnosis from 2 to 8 months, depending on the age of the patient (Curran et al., 1993; Scott and Mickle, 1987). One of the major problems in combating tumors is their ability to override the immune system. They are known to express a variety of immunosuppressive molecules like interleukin-10 (IL-10), prostaglandin E2 or transforming growth factor-β (TGFβ) (Wiendl et al., 2002; Wischhusen et al., 2002; Wrann et al., 1987). The secretion of these molecules leads to a local immunosuppression as well as to a systemic immunodeficiency (Platten et al., 2001).

The main treatment of glioma is surgical therapy, whereas the extent of the tumor resection closely correlates with the survival time of the patient (Jeremic et al., 2003; Keles and Berger, 2004; Soffietti et al., 1989). Nevertheless, brain surgery is not capable of removing all tumor cells. Thus, infiltrating tumor cells still reside in the healthy brain tissue and cause reformation of gliomas. Radiotherapy is another standard therapy for treatment of glioma.

But, in respect to long term survival, no effect of radiation alone is found in case of grade II gliomas (Karim et al., 2002).
In addition to these treatments, chemotherapy is commonly used. Chemotherapy aims at the impairment of cell division, which mostly affects fast-dividing cells including tumor cells. One of the biggest disadvantages of this therapy is the side effect caused by the effects on healthy dividing cells, which leads to hair loss and damage of the intestinal epithelium (Krauseneck and Muller, 1994).
Still, those therapies do not result in a satisfactory therapy of gliomas and there is need for new therapeutical approaches.
Experimental therapies are aiming at several mechanisms in tumor progression. On a molecular level, therapies aim at the cancer specific signal cascades and molecules to influence proliferation, invasion and angiogenesis (Adjei and Hidalgo, 2005; Krause et al., 2005). One of the most promising targets is TGFβ. Encouraging data of longer survival rates and slower tumor growth was obtained using a TGFβ antisense oligonucleotide for the treatment of human glioblastoma (Schlingensiepen et al., 2006). These findings were endowed by animal experiments and *in vitro* data (Jachimczak et al., 1996). The platelet derived growth factor receptor (PDGFR) is another possible candidate in molecular approaches of combating cancer. Upon inhibition of this receptor with imatinib, the tumor growth rate decreases and even complete remission can be observed in some patients (Dresemann, 2005).
Another approach in this field is the active specific immunotherapy which includes the *in vivo* induction of tumor-specific cytotoxic T lymphocytes. Upon induction, those cells are likely to be able to trigger apoptosis of tumor cells (Kagi et al., 1994).
During the recent years, focus has been cast on gene-based therapy in regard to malign gliomas. Mostly, adenoviral and retroviral vectors are used to deliver genes with therapeutic effects into the glioma site. The most promising candidates are oncolytic gene therapy and gene transfer of p53 or interferon-β (IFNβ) (Lang et al., 2003; Mineta et al., 1995; Yoshida and Tanaka, 2004). It was demonstrated that bone marrow derived cells express thymidine kinase of herpes simplex virus have a therapeutic effect on gliomas through bystander-mediated glioma cell killing after gancyclovir application (Miletic et al., 2007).
Microglia are known to be attracted by and to infiltrate the glioma site (Umemura et al., 2008). However, their phagocytic ability, antigen presentation and secretion of pro-inflammatory cytokines is suppressed by the tumor cells (Hanisch and Kettenmann, 2007; Komohara et al., 2008; Yang et al., 2010). These tumor-infiltrating microglial cells obtain an immunosuppressive phenotype and promote TGFβ production via an autocrine loop (Umemura et al., 2008). In addition to the immunosuppressive cytokine milieu provided by the glioma cells, this leads to T cell tolerance and inactivation of tumor targeted immune responses (Carpentier and Meng, 2006). Nevertheless, microglia are potent immune effector cells and are able to mediate the innate and boost adaptive immune responses. It is known that the classical activated microglial cell, in contrast to the alternatively activated microglia with an immunosuppressive phenotype, could phagocytose glioma cells and furthermore create a pro-inflammatory milieu (Mantovani et al., 2004). Additionally, chemokines from microglia are known to attract cytotoxic cells such as natural killer cells (Carter et al., 2007; Hughes et al., 2002; Napoli and Neumann, 2009), they yield a high potential for immunostimulatory therapy approach. Clearly, microglia are situated ideally within the CNS to confront migrating and resident tumor cells. Another feature of microglia is their ability to migrate from the blood stream into the CNS, which makes them an interesting

vehicle for therapeutic gene transfer into mature CNS tissue and the tumor residuum (Flugel et al., 2001).
Thus, the resolution of the immunosuppressive milieu by triggering a cytotoxic phenotype of microglia in the glioma site in combination with recruitment of cytotoxic leukocytes yields a promising approach. Especially microglial cells derived from embryonic stem cells appear suitable for therapy approaches, as they have unlimited proliferative potential and differentiated cells bear the potential to stimulate cytotoxicity towards glioma cells. Thus, iPSdM might therefore be a suitable and powerful tool for immune system-based or gene-transfer-based cell therapy of human glioma.

7. Regenerative approaches

Microglial cells are the immune effector cells which mediate immune responses in the CNS. They are known to play crucial roles in several neurodegenerative diseases such as multiple sclerosis (MS), AD or PD. Thus, they are an interesting target for regenerative therapies.
In MS, the immune system reacts with a destructive immune response against antigens of the CNS like myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG), which leads to demyelination and axonal injury (Mattson and Taub, 2004). During disease course, scar-like plaques appear around the damaged axons, called lesions (Chari, 2007). Additional to the process of demyelination, inflammation is a hallmark of MS. This inflammation is mainly mediated by activated T cells, which invade the brain via the blood-brain-barrier and attack the myelin sheath of axons (Compston and Coles, 2002; Wucherpfennig and Strominger, 1995). This subsequently leads to stimulation of other immune cells like microglia and macrophages, which then secrete inflammatory cytokines (Cannella and Raine, 1995). This in turn leads to further recruitment of T cells, B cells and macrophages to the inflammation site (Steinman et al., 2002).
Until now, there is no known cure for MS, though some improvement in prevention of attacks and disability could be achieved through administration of immunosuppressants like interferons (Comi, 2009). Nevertheless, there is still need for other therapeutical approaches, especially in regard to neuroprotection and regeneration. Stem cell research has lead to new approaches in addressing neuroinflammatory disorders. Several cell types have been used for experimental therapies. Neural stem cells have been reported to migrate to inflammation sites in the CNS and to contribute to functional recovery and tissue repair (Pluchino et al., 2003). A similar beneficial effect was shown using bone marrow cells in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS (Zappia et al., 2005).
It has been shown that microglia are able to pass the blood-brain-barrier and migrate into the brain under pathological conditions (Imai et al., 1997). In our group, we could show that ESdM like other bone marrow derived myeloid precursors migrate to lesion sites in EAE afflicted mice. Bone marrow myeloid precursors as putative progenitors of microglia have a beneficial impact after transduction with the microglial receptor triggering receptor expressed on myeloid cells-2 (TREM2) on the clinical course of EAE, on demyelination and axonal damage (Takahashi et al., 2007a). Furthermore, an anti-inflammatory milieu is established in the lesion sites due to the treatment (Takahashi et al., 2007a). Similar effects might be expected after transplantation of microglial precursor cells that are derived from ES cells and constitutively expressing TREM2.
As iPS-derived microglia principally can be generated from human iPS cells without oncogenic transformation and in high numbers, they might be a suitable and save vehicle

for neurotrophic factors to be released at inflammatory lesions in MS. In regard to AD, microglia can be seen as a possible therapeutic target, too. The pathological hallmarks of AD are cortical atrophy with accumulation of extracellular deposits of amyloid β (Aβ) in senile plaques and intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau in the cerebral cortex (De Strooper and Annaert, 2000). It is known that Aβ serves as a microglial activator and leads to production of various inflammatory mediators (Benveniste et al., 2001). On the other hand, a neuroprotective role of microglia was found in AD. Activated microglia migrate to Aβ plaques, where they release proteolytic enzymes like metalloproteinases that degrade Aβ (Qiu et al., 1997). Although it is unclear why microglia fail to completely clear the Aβ plaques by phagocytosis, at least they appear to restrict plaque growth (Bolmont et al., 2008). Nevertheless, it was reported that administration of exogenously applied microglia in a rat animal model of AD increases Aβ clearance (Takata et al., 2007). It has been shown by several groups that bone marrow-derived cells are capable of becoming brain macrophages, which then might play a major role in Aβ clearance (Malm et al., 2005; Simard and Rivest, 2004). Therefore, microglial cells may be a suitable tool for clinical application, if they are able to overcome the blood-brain-barrier. This problem could be addressed by irradiation (Ajami et al., 2007) or by intra-arterial injection (Imai et al., 1997), which both facilitates the entry of microglia into the brain. Otherwise, it would be possible to directly apply the iPS-derived microglia into the CNS by local transplantation. Thus, iPS-derived microglia might broaden the therapeutical approaches in AD, as they might be able to clear Aβ plaques and attenuate disease course.

8. Conclusion

Microglia are involved in a variety of neurodegenerative diseases. They are a promising target for new therapies. Their role as immune effector cells in the CNS might yield interesting perspectives for locally modulating the cytokine milieu or phagocytic properties to influence the disease course in a beneficial way. Glioma is the most common brain tumor which is not curable so far. Microglia were demonstrated to phagocytose glioma cells and attract cytotoxic cells to the glioma site. Therefore, they display a high potential for usage in therapy. In addition, microglia were shown to migrate from the blood stream into the CNS which makes them a suitable cellular vehicle for therapeutic gene transfer. Microglia overexpressing neurotrophic factors could contribute to the regeneration of lesioned brain tissue. Therefore, the generation of microglia from embryonic stem cells and iPS cells provides a new technology for combating cancer and for usage in regenerative medicine. Since iPS-derived microglia resemble human primary cells they can be further used in drug screening tests. It has been shown that very small sized particles such as nanoparticles could principally activate microglia. This leads to the production of pro-inflammatory cytokines and radicals by microglia which in turns promotes the pathology of neurodegenerative diseases. Therefore, iPS-derived microglia provide a new tool to evaluate the possible toxicity of very small-sized particles to the CNS.

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10. Conflict of interest

K.R. and H.N. have interest in royalty rights on a pending European patent EP2010_055731, Method for obtaining human microglial precursor cells from pluripotent stem cells'. H.N has interest in royalty rights on a pending European patent EP2010_055725 ,Microglial precursor cells for the treatment of malignant neoplasms of the central nervous system'.

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10

Embryonic Stem Cell-Derived Neurons for Inner Ear Therapy

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1. Introduction

Sensorineural hearing loss is a major public health problem affecting more than 278 million people worldwide. The primary cause of sensorineural hearing loss is loss or damage of sensory hair cells in the organ of Corti. However, approximately 10-15% of cases with profound hearing loss in children are caused by degeneration of the spiral ganglion neurons (SGNs) or neurons in the auditory brainstem. Moreover, SGNs gradually degenerate after the loss of hair cells due to a lack of excitatory stimulation. Since SGNs do not regenerate to any clinically significant extent, novel therapies for their preservation, regeneration or replacement are being heavily sought. Currently, no treatment option is available for peripheral auditory neuropathy.

Cell-based therapies offer a strategy to enhance auditory functions in the deaf patient and improve the benefits of cochlear implantation. There are three major areas for potential clinical applications relevant to this approach. First, for patients who have received cochlear implants, generation or preservation of SGNs via cell replacement therapy could significantly improve the quality of their sound perception. Another group of potential recipients who would benefit from cell replacement therapy are patients suffering from acoustic neuroma or neurofibromatosis. These patients generally exhibit significant loss of auditory or vestibular primary neurons with relatively intact sensory hair cells (Kaga et al., 1997; Evans et al., 2000; Sperfeld et al., 2002). Thus, replacing dead or damaged neurons with stem cells could be critical in restoring their hearing or balance sensation. Finally, auditory neurons generated from stem cells could be used in *in vitro* assays to test the effectiveness and safety of newly developed drugs before clinical trials.

Type I SGNs, comprising 95% of all neural populations in the SG, innervate inner hair cells in the organ of Corti and function as the primary auditory afferent neurons (Berglund and Ryugo, 1987; Liberman et al., 1990; Rusznak and Szucs, 2009). These SGNs predominantly express AMPA receptors (mainly GluR2-4) (Niedzielski and Wenthold, 1995; Parks, 2000; Dulon et al., 2006; Chen et al., 2007; Flores-Otero et al., 2007), which bind the neurotransmitter glutamate released from inner hair cells (Fig. 1). This subsequently triggers action potentials that propagate along the nerve fibers to the cochlear nucleus. The majority, if not all, of type I SGNs are glutamatergic and release glutamate from their pre-synaptic membrane in the cochlear nucleus (Rebillard et al., 2003; Reyes et al., 2008). Expression of AMPA receptors and glutamate transporters thus is the hallmark of mature type I SGNs. During embryonic development, SGNs arise from the otic placode and

transiently express several key transcription factors, including Neurogenin 1 (Ngn1), NeuroD and Brn3a (Fig. 3). Inactivation of these transcription factors results in a significant reduction in the number of SGNs or severe retardation in the axon projections to the cochlea (Ma et al., 1998; Huang et al., 2001; Kim et al., 2001). Thus, Ngn1, NeuroD and Brn3a represent phenotypic markers for immature SGNs.

Over the past several years, progress has been made to generate *in vitro* functional neurons bearing a SGN phenotype from embryonic stem cells and different types of somatic stem cells (Martinez-Monedero et al., 2008; Reyes et al., 2008; Chen et al., 2009). Some of these stem cell-derived neurons were shown to establish synaptic contacts with sensory hair cells, the peripheral target for SGNs, *in vitro* (Matsumoto et al., 2008) and to survive in animals with selective loss of SGNs (Corrales et al., 2006; Matsuoka et al., 2007). However, little information is currently available how to promote pluripotent stem cells competent to give rise exclusively to glutamatergic sensory neurons.

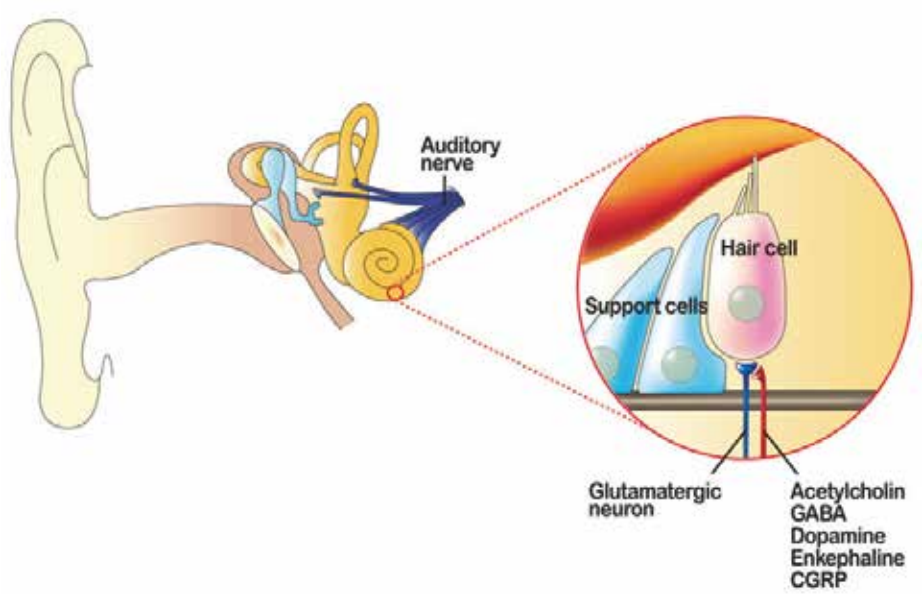

Fig. 1. Glutamate is a primary afferent neurotransmitter in the cochlea

2. Embryonic stem cells can be efficiently directed to differentiate into neurons *in vitro*

We have established an efficient feeder-cell free neural induction protocol based on a previous study (Watanabe et al., 2005) and, using this protocol, extensively characterized temporal changes in gene expression in ESCs undergoing neural differentiation. Briefly, undifferentiated mouse R1 ESCs were dissociated and cultured in bacteria plates to allow embryoid body (EB) formation, after which EBs were plated on tissue culture plates and cultured in a pre-induction medium (Step 1). After 2 days, the medium was changed to a neural induction medium and maintained for an additional 1-15 days (Step 2). During the first differentiation, EBs became larger in size, but there was no indication of neurite-like processes. Only one day after the start of the second step, cells in the outer edge emigrated

from the EB (Fig. 2A). Three days after the start of neural induction, numerous neurite processes emerged from the outer edge of EBs. At 7 days after the start of neural induction, much denser and longer processes than those seen at 3 days of neural induction were observed in EBs. At 15 days after the start of neural induction, processes remained dense and they appeared to extend to other EBs to make contacts. Immunohistochemical staining for HuC and TuJ1, specific neuronal markers, indicated that the cells bearing neurite-like processes were indeed neurons. Morphological changes in ES cells during neural induction, characterized by growth of neurite processes, were progressive in the first 7 days, after which they remained largely unchanged until the last day of 15 day neural induction.

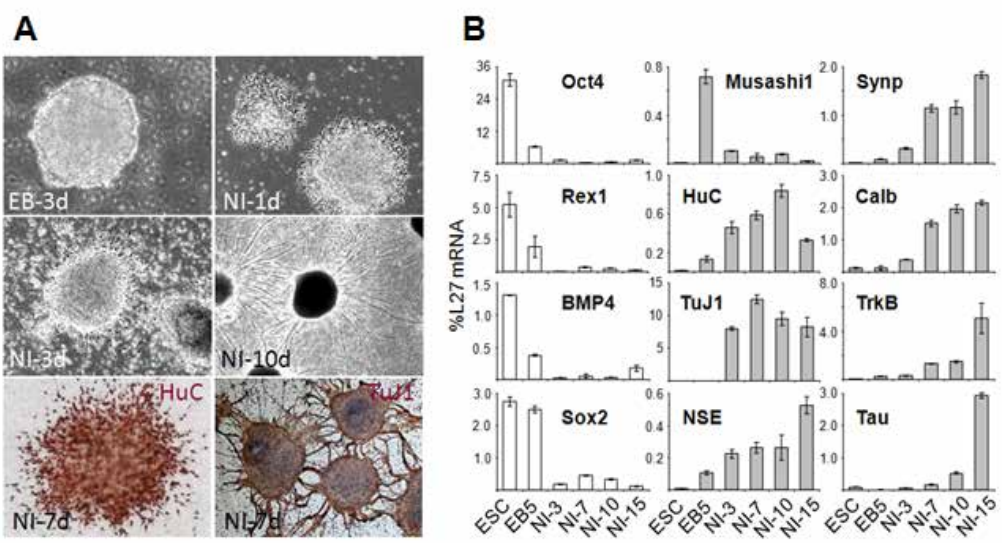

Fig. 2. (A) Morphological changes in ESCs undergoing neural differentiation. TUJ1 and HuC, neuron-specific proteins, are detected in a subpopulation of cells suggesting a neuronal fate. TUJ1 accumulates in the axon-like processes, while HuC is restricted to the cell body consistent with its function as a translation factor. (B) RT-PCR analysis for ESC- and neural-marker genes in undifferentiated ESCs (ESC), ESCs at EB stage (day 5), 3d, 7d, 10d, and 15d after start of neural induction (NI-3, -7, -10 and -15, respectively)

To evaluate temporal changes in gene expression, qRT-PCR analyses were performed with RNAs collected from ESCs or ESC-derived cells at various time points during differentiation. The results demonstrated steep reductions of ESC-specific genes, including *Oct4*, *Rex1* and *Sox2*, in contrast with rapid (early neural genes: *Musashi1* and *HuC*) or gradual (mature neural genes: *TUJ1*, *NSE*, *Synaptophysin*, *Calretinin*, *TrkB* and *Tau*) increases of neural marker genes (Fig. 2B). Results from our Western blot analysis for the corresponding proteins were consistent with the qRT-PCR results with delayed peaks of about 3 days (Kondo et al., 2008). Down-regulation of Oct4 and Sox2 was accompanied by transient up-regulation of early neural marker proteins, including Musashi1 and HuC, which was followed by stable induction of mature neural proteins, including TUJ1, Calretinin, Synaptophysin, NSE and Tau. To evaluate the percentage of ESC-derived cells exhibiting neural cell surface markers during neural differentiation, we performed flow cytometric analyses for CD24 and Tau. Virtually none of undifferentiated ESCs express CD

24 or Tau, while over 90% of those are positive for both neural markers at neural induction day 7 (Kondo et al., 2008). To assess electrophysiological properties of induced ESC-derived cells, we performed single-cell current-clamp recordings. Robust action potentials and voltage-dependent inward sodium currents were recorded from ≈70% of ESC-derived cells at neural induction day 7 (Kondo et al., 2008).

3. Tlx3 promotes glutamatergic neuronal specification and is a direct target for Wnt signaling

We have previously demonstrated that T cell leukemia 3 (Tlx3; also known as Hox11-L2/Rnx) can be used as a potent intrinsic factor to generate, from mouse ESCs, excitatory neurons with a phenotype that resembles type I SGNs (Kondo et al., 2008). Tlx3 is a member of the Tlx family of homeobox transcription factors and is selectively expressed in cranial and dorsal root sensory ganglia, including SGNs, during early embryogenesis (Logan et al., 1998; Cheng et al., 2004; Cheng et al., 2005; Kondo et al., 2008). Tlx3 is a genetic switch for selection of a glutamatergic over a GABAergic transmitter phenotype during nervous system development (Cheng et al., 2004; Cheng et al., 2005; Xu et al., 2008). We have found that forced expression of Tlx3 in ESCs combined with directed neural induction leads to sequential up-regulation of genes (and proteins) that are expressed in transiently-amplifying neural progenitors (Mash1), immature (Ngn1, NeuroD, Brn3a) and mature (GluR2, GluR4, Vglut2) SGNs (Kondo et al., 2008). Furthermore, these Tlx3-expressing ESC-derived neurons exhibited robust action potentials and excitatory post-synaptic currents, indicative of functional excitatory neurons.

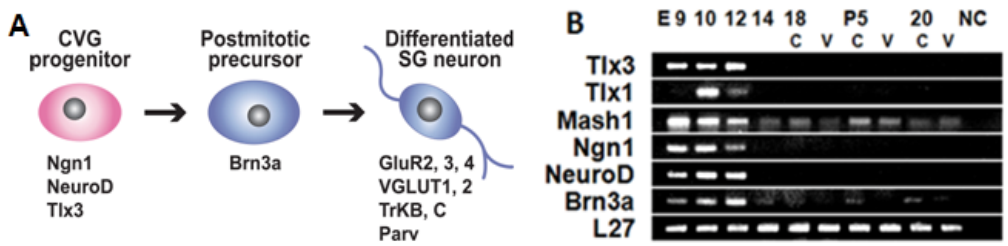

Fig. 3. (A) Sequential expression of Ngn1, NeuroD and Brn3a during SGN development. (B) Temporal changes in gene expression during inner ear development. C; cochlea, V; vestibule

Since Tlx3 is a transcription factor, we sought to identify a signaling molecule that controls Tlx3 expression with the ultimate goal of developing a means to extrinsically induce glutamatergic neuronal specification from ESCs (Kondo et al., submitted). Wnt/β-catenin signaling promotes neural differentiation by activation of the neuron-specific transcription factors, Ngn1, NeuroD and Brn3a, during neural development. The canonical Wnt pathway activation allows β-catenin to translocate to the nucleus, where it interacts with the T-cell factor (TCF) family of DNA-binding proteins and β-catenin/TCF complexes regulate transcription (Fig. 4). Based on this consensus signaling pathway, the β-catenin/TCF complexes are the prime nuclear effectors for canonical Wnt signaling. Since neurons in cranial sensory ganglia and dorsal root ganglia transiently express *Ngn1, NeuroD* and *Brn3a* during embryonic development, we hypothesized that Wnt(s) could instructively promote a sensory neuronal fate from stem cells undergoing neural induction. Consistent with our

hypothesis, Wnt1 induced expression of sensory neuron marker genes, including *Ngn1, NeuroD*, *Brn3a* and *P2X3*, as well as glutamatergic marker genes, such as *GluR2* and *GluR4*, in neurally-induced somatic stem cells in a dose-dependent manner (Fig. 4; Kondo et al., submitted). Additionally, Wnt1-induced up-regulation of these genes was suppressed by specific canonical Wnt antagonists, Dickkopf-related protein 1 (Dkk1) and Secreted frizzled-related protein 2 (sFRP2). The inhibitory effects of Dkk1 and sFRP2 were specific, as they had no effects on expression levels of *GATA3*, *Tau* and *TUJ1*, none of which is regulated by Wnt1. Furthermore, expression levels of Brn3a, GluR2, GluR4 and Vglut2 proteins in neurally induced stem cells grown in the presence of Wnt1 were significantly higher than those grown in the absence of Wnt1, indicating that Wnt1 up-regulates glutamatergic sensory neuron-specific proteins in neurally-competent stem cells.

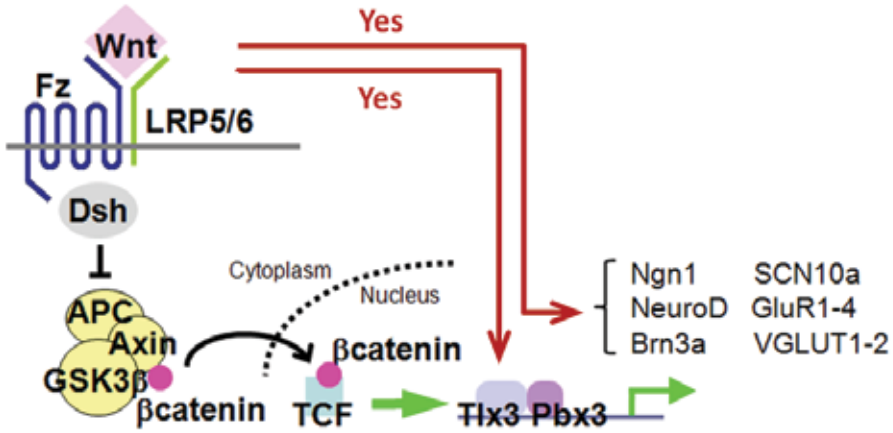

Fig. 4. Schematic figure showing canonical Wnt signaling pathway and our results

Given the consensus function of Tlx3 as a glutamatergic selector gene, we postulated that the effects of canonical Wnt signaling on sensory neuron and glutamatergic marker expression in stem cells may be mediated by Tlx3. We first confirmed that Wnt1 can indeed up-regulate *Tlx3* expression in somatic stem cells and the Wnt1-induced *Tlx3* up-regulation was entirely suppressed by Dkk1 or sFRP2 (Fig. 4; Kondo et al., submitted). Next, we demonstrated that forced expression of Tlx3 induced sensory and glutamatergic neuron markers after neural induction. Moreover, our chromatin immunoprecipitation assays revealed that TCF3/4 directly bind a regulatory region of *Tlx3* after neural induction (Kondo et al., submitted). To further characterize this binding site, we have cloned 2600 bp 5′- and 1700 bp 3′-non-coding regions flanking the Tlx3 coding region into a luciferase reporter vector and generated another reporter construct with a mutation in the TCF binding motif (Fig. 5A). Wild-type ESCs were transfected with a reporter construct containing the luciferase gene under the control of the wild-type or mutant *Tlx3* promoter and a constitutively expressing the *Renilla* luciferase construct for normalization of transfection efficiency. Transfected cells were incubated in neural induction medium in the presence or absence of 100 ng/mL Wnt1 for 2 days and luciferase activities were analyzed using a Dual Luciferase Reporter Assay System. Consistent with our hypothesis, the Wnt1-induced Tlx3 promoter activity was significantly reduced with a mutation in the region 3 TCF binding site, when compared to a wild-type (Fig. 5B). These results demonstrate that the TCF binding site in the Tlx3 promoter is required for Wnt-dependent transactivation of *Tlx3*.

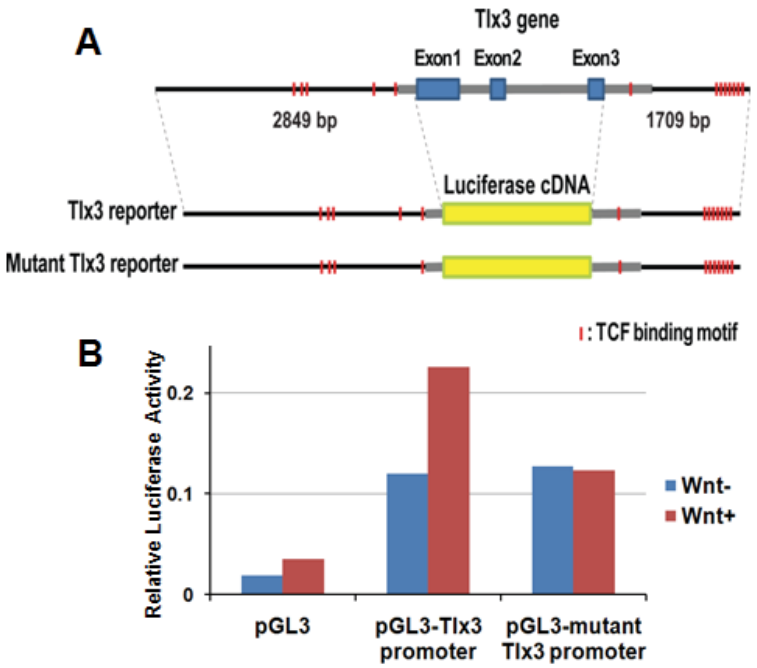

Fig. 5. Luciferase reporter activity of ESCs transfected with the empty vector (pGL3), wild-type or mutant Tlx3 reporter construct

4. ESC-derived neurons expressing Tlx3 form growth cones enriched with GAP43

We have characterized molecular and biochemical properties of Tlx3-expressing ESCs undergoing neural differentiation and found that Tlx3 instructively promotes these stem cells to acquire a glutamatergic phenotype, while suppressing a GABAergic phenotype (Kondo et al., 2008). To determine whether these Tlx3-expressing cells exhibit functional characteristics of excitatory glutamatergic neurons and make synaptic contacts with their targets after neural induction, we have performed subcellular fractionation and obtained evidence that ESC-derived neurons expressing Tlx3 form growth cones that are enriched with GAP43. Similar to primary neurons *in vitro*, GAP43 was co-localized with neurofilament 160 as well as F-actin in the distal tip of ESC-derived neurons at neural induction day 7 (Fig. 6A). Western blot analysis of whole cell lysates revealed that GAP43, Tau and the pan-sodium channel protein SP19 are abundant in ESC-derived neurons, but that none of these proteins was detectable in undifferentiated ESCs or EBs (Fig. 6B). To elucidate sub-cellular localization of GAP43, we isolated an enriched population of growth cone particles using a discontinuous density gradient sucrose (Fig. 6C). Our immunoprecipitation and western blot analyses detected a substantially higher GAP43 expression in the growth cone particle fraction (0.32/0.83M) when compared to whole lysate (before isolation) or the non-growth cone fraction (1.20/2.66M) (Fig. 6D). These results indicate that GAP43 is enriched in the growth cone of ESC-derived neurons expressing Tlx3 and suggest that the growth cone assembly, which is essential for axon guidance and target innervation, is already in place in these ESC-derived cells by neural induction day 7.

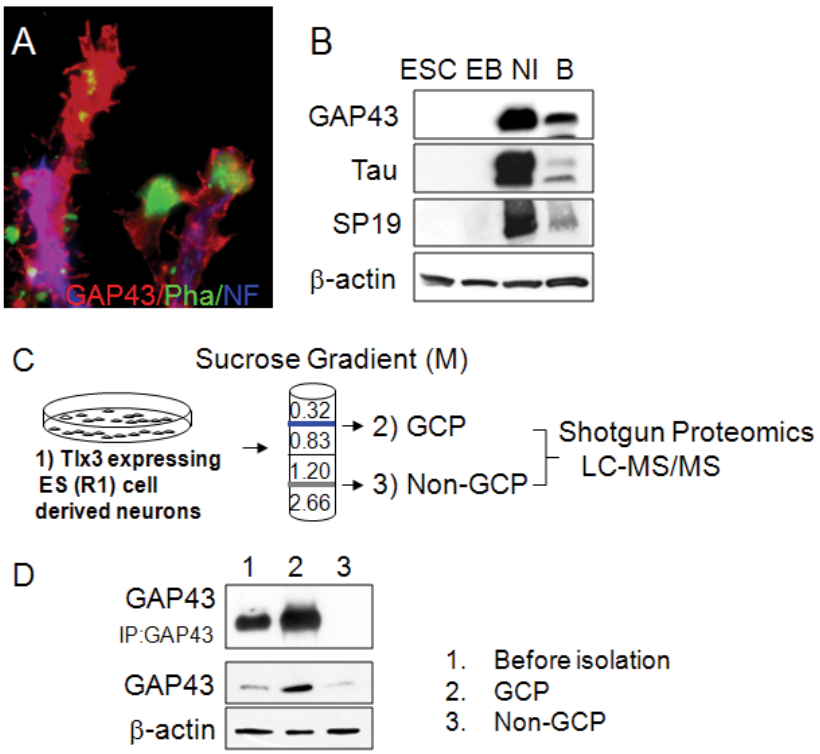

Fig. 6. GAP43 is enriched in growth cones of ESC-derived neurons expressing Tlx3. (A) Expression of GAP43 (red), Neurofilament 160 (blue) and F-actin (green) at neural induction day 7. (B) Western blot analysis for GAP43, Tau and SP19. ESC: undifferentiated ESCs; EB: embryoid bodies; NI: ESC-derived cells at neural induction day 7; B: brain (positive control). (C) Isolation of growth cone particle (GCP) fractions. (D) Immunoprecipitation and western blot analysis for GAP43 in GCP vs. non-GCP fractions

5. Tlx3-expressing ESC-derived neurons are attracted to hair cells in the cochlea

To study interactions between Tlx3-expressing ESC-derived neurons and embryonic inner ear tissues, GFP-positive ESCs were co-cultured with an E18 mouse organ of Corti, the peripheral target tissue for SGNs (Fig. 7). Organ of Corti tissues were removed from E18 mice and embedded into rat type I collagen in 8-chamber slide wells. Cochlear tissues were removed from wild-type E18 mice and embedded into rat type I collagen. Four to five hours after the start of incubation, Tlx3-expressing ESCs forming spheres were placed into collagen approximately 100 μm in the distance from a cochlear explant. On the following day, the medium was replaced with neural induction medium and incubation continued for an additional 5 days. In the presence of an organ of Corti explant, Tlx3-expressing ESCs survived and propagated vigorously, but upon exposure to the neural induction medium, withdrew from the cell cycle and differentiated into neurons. Additionally, these ESC-derived cells began expressing sensory neuron-specific antigens, such as GluR4 and calretinin, along with several pan-neuronal markers. Furthermore, ESC-derived neurons extended their processes towards the explant, formed ectopic synaptic contacts with cells in the organ of Corti.

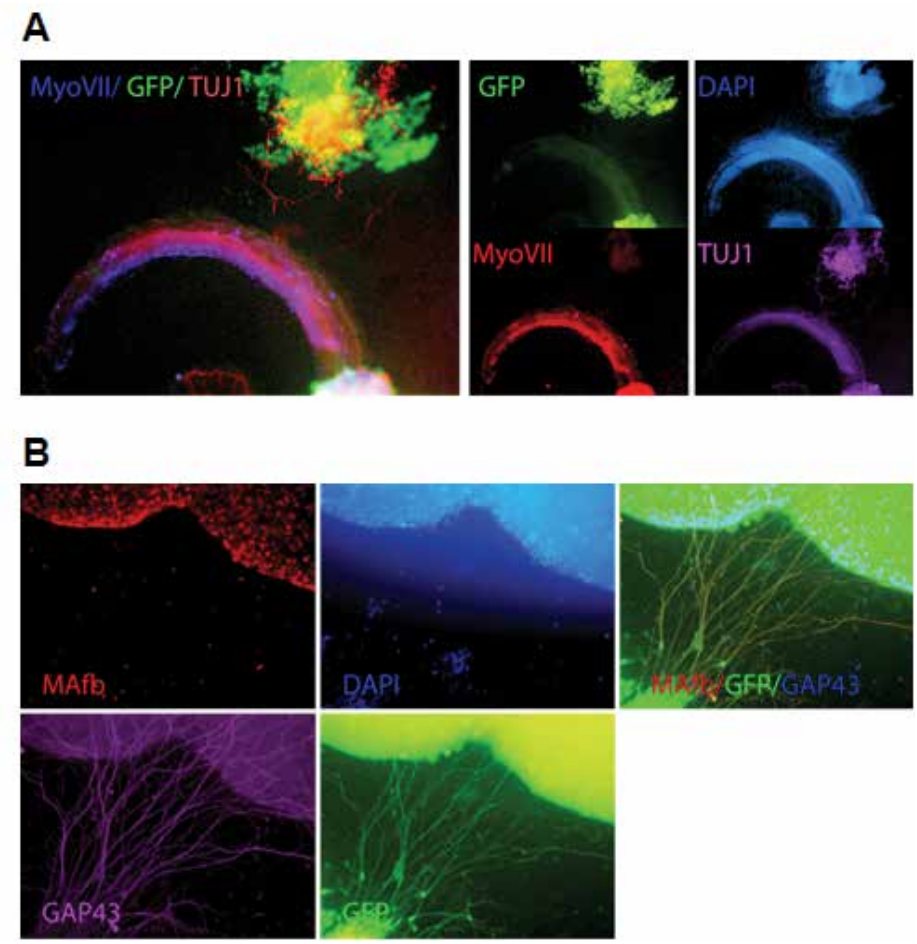

Fig. 7. (A, B) Tlx3-expressing ESC-derived neurons are attracted to both embryonic cochlea and auditory brain stem. Low-magnification fluorescence photo micrographs of 3-D collagen cultures with GFP-positive ESC-derived neurons and an E18 cochlear explant (A) or a brainstem slice (B). (A) MyosinVII (Red) and TUJ1 (Purple) immunoflorescence. Note that GFP-positive processes from ESC-derived neurons are extended towards the cochlear explants. CO, cochlear explant. (B) Mafb (Red) and GAP43 (Purple) immunofluorescence

6. Tlx3-expressing ESCs can survive and migrate towards endogenous SGNs in animal model of auditory neuropathy

We transplanted partially differentiated ESCs into the modiolus of the deafened gerbil cochlea that subsequently received neural induction medium for 3 days via osmotic pumps. Four-to-six month-old Mongolian gerbils were used as transplantation recipients. To deafen the animals, focal application of ouabain was performed based on a previously described procedure (Matsuoka et al., 2007) with minor modifications. Briefly, the animals, free of any signs of ear infection, were given atropine (0.2 mg/kg) to reduce secretion prior to the surgery. Following anesthesia with isoflurane, a small incision (less than 1.5 cm) was made to expose the right posterior aspect of the skull. A total volume of 5 μL ouabain solution (1

mM in normal saline) was slowly infused into the round window niche using a siliconized glass micropipette attached to a 10 µL Hamilton syringe. A small piece of gelform was placed in the round window niche to prevent leaking of excessive ouabain. The same amount of normal saline was infused into the left cochlea of the animal, which served as a control. Ouabain was washed off after 1 hour, the bulla will be closed with dental cement (Durelon, 3M ESPE), and a two-layer closure was made in the skin. The animals were allowed to recover for 4 weeks, during which progressive degeneration of spiral ganglion neurons took place. At the end of the recovery period, animals received a modiolar transplantation of ESCs stably expressing pBud-eGFP-cTlx3. Under anesthesia, a small fenestra was made in the wall of the basal turn scala tympani and a suspension of Tlx3-expressing stem cells ($1x10^6$ cells/µL) in 10 µL PBS (left ear) through a 30-gauge needle that was inserted into the bony wall of the basal turn of the gerbil cochlea.

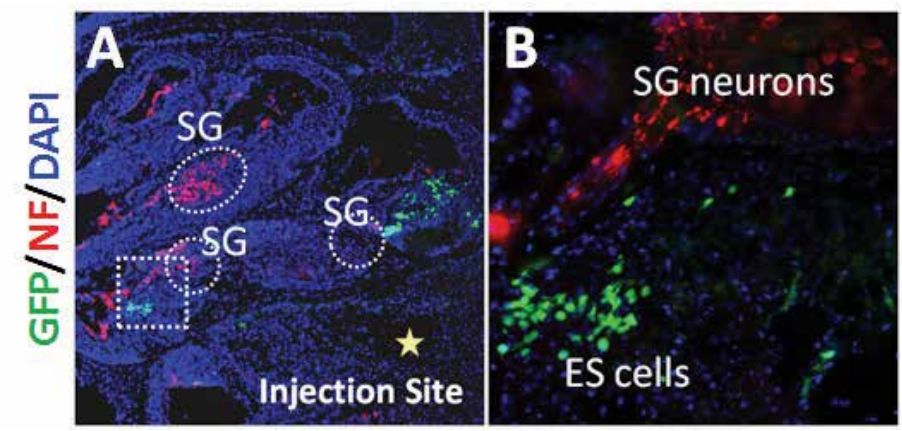

Fig. 8. Low (A) and high (B)-magnification micrographs of GFP-positive ESCs transplanted into the modiolus of the gerbil cochlea that has received neural induction medium. SG, spiral ganglion

We observed clusters of ESCs in the vicinity of SG of recipient animals 10 days after transplantation (Fig. 8). We were pleased with the extent of ESC migration as well as the number of engrafted ESCs. However, the percentage of engrafted ESCs expressing pan neural markers was low (less than 10%). We attributed this to (A) the short period in which neural induction medium was infused, and (B) the concentration of neural induction medium was not high enough to promote efficient neural induction, although we used a medium that contained 5-times higher concentrations of reagents used for our *in vitro* experiments. In order to increase the number of donor cells giving rise to neurons, we employ 3 approaches: first, we will increase the length of neural induction period from 3 days to 28 days; second, we will use a higher concentration of neural induction medium infused into the cochlea via osmotic pump; third, we will transplant stem cells that will have been incubated in neural induction medium for 2 days *in vitro*.

7. Magnetic resonance imaging of ESC in the cochlea

Monitoring transplanted cell delivery, homing, and trafficking is of the utmost importance for developing translational strategies. Magnetic resonance imaging (MRI) has recently emerged as one of the most predominant imaging modalities for tracking stem cells in live

animals in a noninvasive and repeated manner. To increase the resolution of images, specific contrast agents, such as iron-oxide nanoparticles, have been routinely used. In the present study, an alternative nontoxic agent, manganese chloride, was used to label only biologically active stem cells in host animals. Paramagnetic manganese ions (Mn^{2+}) are calcium (Ca^{2+}) analogs that are taken up by live cells through voltage-gated Ca^{2+} channels (Yamada and Yang, 2008). Thus, only cells with active Ca^{2+} channels are labeled with $MnCl_2$. This property makes $MnCl_2$ a unique contrast agent for functional live cell imaging.

ESCs were labeled with 0.1 mM $MnCl_2$ as previously described (Yamada and Yang, 2008). Following injection of $MnCl_2$ labeled cells into the basal turn of the cochlea as described above, the animals were anesthetized with 2% isoflorane in an anesthetic chamber and maintained during imaging with 1-2% isoflorane delivered via face mask. The animals were placed with the head centered within a custom-made birdcage head coil. Axial T1-weighted images were acquired using a 3D asymmetric spin-echo pulse sequence with the following parameters: TR = 100msec, TE = 6.5msec, 16 mm FOV, 256 x 256 matrix (Lane et al. 2005) to yield a maximum resolution of approximately 62 µm and minimum resolution of approximately 100 µm. Images were imported into Analyze (v9.0, Biomedical Imaging Resource, Mayo Clinic, Rochester, MN) and volume registered with the long axis of the basal turn.

Using a 9.4T MR system, one of the highest resolution MR systems available in the USA, we have obtained serial coronal images of the gerbil cochlea that received transplantation of $MnCl_2$-labeled ESCs. $MnCl_2$ is a strong T1 relaxation agent which, when imaged with T1 sensitive sequences, produces significant hyperintensity of the labeled cells. Images were taken 2 days after the animal received an intra-modiolar injection of 1 million $MnCl_2$-labeled ESCs. As expected, the injected cochlea exhibited significantly hyperintense contrasts when compared to the uninjected cochlea in the same animal (Fig. 9). Furthermore, the surrounding non-labeled structure of the cochlea appeared hypointense relative to labeled cells. No noticeable differences in the contrast between the left and right cochleae were observed in the uninjected control animal. Further optimization of imaging parameters and cell labeling protocols is under development.

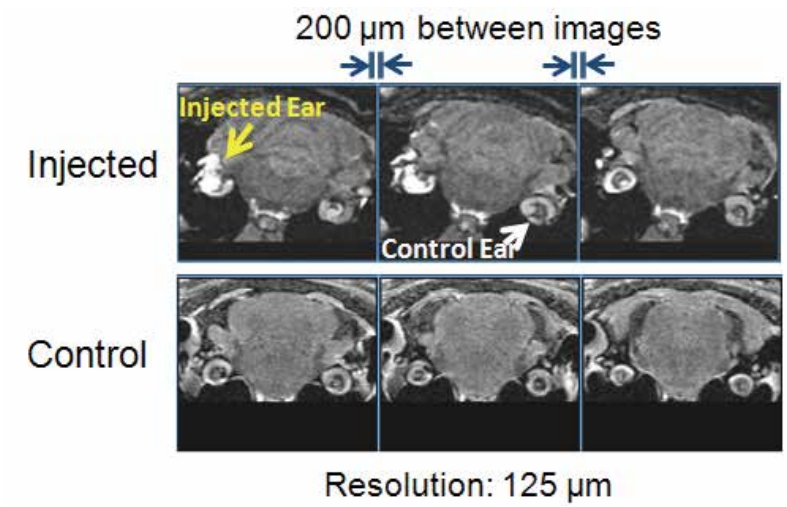

Fig. 9. Contiguous trans-axial Proton density weighted images of gerbil cochleae obtained with the 9.4T MR system. Regions in post-transplantation cochlea (Injected Ear) exhibit hyperintense contrasts when compared to the control ear

8. Translational approaches

Establishing a minimally-traumatic surgical technique to precisely place stem cells in the cochlea is a key for success in cell-based inner ear therapy. It addresses the unmet surgical challenge posed by the delicate cochlear structures being located deep in the hard temporal bone. Our previous studies using animal models demonstrated that transplanting stem cells directly into the modiolus of the gerbil basal cochlear turn yields a satisfactory degree of migration and engraftment (Matsuoka et al., 2006, 2007). However, our recent study using human cadaver temporal bones revealed that this approach would not be suitable for clinical applications due primarily to differences in anatomical structures between the rodent and human cochleae (Fritsch, 2009). For example, the human cochlea has a much larger scala tympani, scala vestibuli, and modiolus. Because of these anatomical differences, we recently started using cadaver human temporal bones to simulate surgeries for transplanting stem cells into the human cochlea. The large size of the human cochlear basal turn modiolus has actually made translation of gerbil studies into human temporal bone simulation surgeries less comparable. The reason is that the modiolus is less densely packed with cranial nerve VIII fibers in the human compared to the gerbil. Cerebro-spinal fluid (CSF) is present within the internal auditory canal in that area and is pulsatile in the living human due to brain pulsations. The net effect is that stem cells transplanted into the human basal turn modiolus are less apt to stay localized in the modiolus and more likely to either "squirt" into the larger internal auditory canal CSF during injection or be washed away by the pulsatile CSF. These two findings give rise to an incomplete or failed stem cell placement. For this reason, the investigators presently believe that the human middle turn modiolus will be the better target when translating gerbil studies to human subjects. It is much more likely to retain the stem cell dosage injected into the modiolus. This has been borne-out in early experiments on the gerbil and human cochleas with histological confirmation.

The human middle-turn cochlea and modiolus are comparable to the human basal turn anatomic structures except that they are smaller and the modiolus is solidly packed with nerve fibers. With injections into the middle-turn modiolus, the stem-cells are held in place by the gelatinous-consistency of the surrounding nerve fibers. From that placement position the stem cells are free to migrate under their own guidance rather than being mechanically washed-away by the CSF, as in the basal turn area. The exact mechanisms for how the stem-cells find their final location as replacement cells for originally damaged inner ear cells is still unknown. However, it is felt that surgical placement directly into the modiolus, which is adjacent to the SG and other inner ear structures, gives them a "head start" to their final location; this is compared to simply injecting them into the bloodstream or CSF space. For this reason, the middle turn of the cochlea is especially targeted in this study relative to the other surgical sites.

Unlike the basal turn, the middle turn structures cannot be simply accessed surgically by drilling a hole into the large promontory of the middle ear space. That basal turn surgical technique is commonly used every day by cochlear implant surgeons. Rather, the middle turn is buried under several structures and has no "promontory" to declare it's presence. To this end, Fritsch has performed experimental surgery on the human cadaver temporal bone to delineate a new surgical approach to the middle turn structures: the middle turn scala tympani and scala vestibuli are uncovered by surgical dissection followed by injection into the modiolus. Ongoing studies are underway to refine the techniques; it is obvious to the surgeon that some manipulations of instruments are beyond the delicate touch of the

surgeon's own human hands and would be better served by micro-manipulators. For multiple simultaneous entry sites, a small-diameter endoscope with a micromanipulator is currently under development (Fritsch, 2009).

For humans undergoing implantation with a cochlear implant device, the aforementioned drilling into the basal turn promontory (ie: cochleostomy) is sealed using tissue autograft plugs to seal the cochleostomy (after device electrode placement into the cochlea). For the gerbil experiments so far, we have used a plastic compound to seal the needle puncture site in the cochlea after injection of stem cells. In humans, plastic compounds often result in foreign-body reaction responses resulting in giant cell granulomas. Though tissue plugs are used to good success in cochlear implants, they are too large for needle punctures within the cochlea modiolus after stem cell injections. Purified collagen-matrix graft materials are being tested in animals, for tissue healing, and in human cadaver temporal bones for mechanical effectiveness of delivery and function. These materials are placed into the cadaver human temporal bone to check for extravasation into the inner ear (with histological confirmation) and into the gerbil with follow-up histological to check for healing and sealing of the cochleostomy opening.

9. Future directions

We plan on extending our research into 3 new directions. First, we will continue to fill in gaps of our understanding of molecular mechanisms underlying Tlx3-mediated glutamatergic neuronal cell-fate specification. Specifically, we aim to identify direct target genes for Tlx3 using a promoter array and elucidate their interactions with Tlx3 using chromatin immunoprecipitation and mutagenesis. We hypothesize that Tlx3, while activating Ngn1 transcription, triggers a counter-balance mechanism that suppresses GABAergic differentiation in ESC-derived neurons. Additionally, we aim to elucidate the epigenetic mechanisms underlying context-dependent transcriptional activation of target genes by Tlx3. Identifying and characterizing Tlx3-binding proteins that can alter chromatin structures via acetylation and/or methylation will greatly enhance our understanding of extrinsic modulation of neuronal cell-type specification. Second, we will extend our characterization of Tlx3-expressing ESC-derived neurons. To determine whether these Tlx3-expressing ESC-derived cells exhibit functional properties similar to those of endogenous SGNs and make synaptic contacts with their targets after neural induction, we will evaluate (A) glutamate release, (B) synaptic transmission, (C) target innervation and (D) electrophysiological properties of Tlx3-expressing vs. non-expressing control ESC-derived neurons. These studies will reveal the extent of functional and synaptic maturation of these transgenic neurons and provide insights into further improvements of directed differentiation of ESC-derived neurons. Third, we plan to further refine our surgical/MRI techniques and record auditory brain stem responses (ABRs) when a considerable number of engrafted cells expressing neural antigens are detected in the modiolas of the gerbil cochlea. These *in vivo* experiments will be instrumental in establishing translational strategies for successful cell-based therapy in the inner ear.

10. Conclusion

The present results reveal that Tlx3 confers ESCs undergoing neural differentiation with a glutamatergic neurotransmitter phenotype, which is accompanied by establishment of

proper synaptic assembly and axon outgrowth. Furthermore, Tlx3-expressing ESCs can migrate towards degenerating SGNs in the inner ear of host animals and these engrafted stem cells can be readily visualized by MRI. These results suggest that Tlx3-expressing ESCs can be used to replace damaged SGNs, which cause irreversible hearing loss in humans. Technical issues related to surgical approaches for safe and efficient transplantation of stem cells in the human cochlea, as well as, non-invasive monitoring of stem cell engraftment in the cochlea are discussed.

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11

Potential of Pluripotent Stem Cells for the Replacement of Inner Ears

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1. Introduction

The inner ear, which manages our senses of hearing and balance, has mechanosensitive hair cells, which convert vibration into electronic signal to depolarize auditory or vestibular neurons. Inner ear functions depend largely on hair cells, their associated neurons and cochlear lateral wall, and defects in these cells result in hearing loss and deafness. Although some investigations indicated hair cell regeneration in mammalian vestibular sensory epithelia, loss of mammalian auditory hair cells is currently irreversible, which is the reason why hundreds of millions of people worldwide with hearing impairment have no way of restoring their auditory function. To date, the cochlear implant, which is designed to electrically stimulate the auditory neurons, is the only available prosthesis for severe to profoundly deaf individuals. However, it depends on remaining auditory neurons, named as spiral ganglion neurons, and their loss severely compromises its efficacy. In this context, several research strategies are directed toward replacing the degenerating spiral ganglion neurons following hearing loss. Here we review recent advances in the field of inner ear regeneration using pluripotent stem cells.

2. Inner ear anatomy

The inner ear consists of the vestibule, three semicircular canals, and cochlea. The vestibular sensory epithelia are located on the maculae of the saccule and utricle, and the cristae of the three semicircular canals. The vestibular sense organs contain two types of hair cells: The type I hair cells with round bottoms and thin necks, and type II hair cells shaped like cylinders with a flat upper surface covered by a cuticle. A tuft of cilia, or the stereocilia protrudes from the apical surface of each hair cell. Most afferent fibers terminate on type I hair cells, whereas the small efferent fibers terminate on type II hair cells. The cochlea is divided into three chambers: the scala tympani and vestibuli, which are filled with perilymph, and the scala media, which is filled with endolymph, containing potassium ions at higher concentrations than perilymph. The organ of Corti, the excitatory structure of the cochlea, contains hair cells and supporting cells, including pillar cells, Deiters' cells, Hensen's cells, inner phalangeal cells, and inner and outer sulcus cells. The afferent innervation of the organ of Corti consists of the dendritic terminals of neurons whose cell bodies comprise the spiral ganglion in Rosenthal's canal in the modiolus. The major projection of the afferent input is to the ventral cochlear nucleus. When the organ of Corti vibrates in response to incoming sound waves, the stereocilia of each hair cells bend,

opening the mechanoelectrical trasduction channels that are in the wall of the stereocilia. The entry of potassium and calcium ions into the hair cells through these channels causes the hair cells to depolarize, releasing neurotransmitters to stimulate the afferent terminal of spiral ganglion neurons.

3. Stem cells in the inner ear

In mammals, some hair cell generation has been observed in vestibular sensory epithelia [8,49], however, lost hair cells were replaced by transdifferentiation of supporting cells, not by cell proliferation of hair cells or supporting cells [55]. The belief that no tissue stem cells might exist in the inner ear was overturned by the finding that stem cells were still present in the vestibular organs of adult mice [19]. Several laboratories adopted a sphere-forming assay to isolate stem/progenitor cells from complex cell mixtures [6,19,20,21,30,33, 39,37,48,51,53,54] derived from inner ear tissues. Sphere-forming cells from the utricle of adult mice are pluripotent and can give rise to a variety of cell types, including cells representative of ectodermal, mesodermal and endodermal lineages [19]. Unfortunately, the lack of regenerative capacity in the adult mammalian cochlea is explained by the findings that the adult cochlea loses the ability for sphere formation by the third week of age [30]. Although attempts to establish stem cells from embryonic rat otocysts [18, 53] have been made, it is not clarified that these established stem cells correspond to which developmental stage. Identification of stem cells in the human fetal cochlea [3] contributes to study stem cell biology of the auditory organ in humans, while advances in identification of stem cells heve been made in rodents.

4. Hair cell regeneration

The inner ear sensory epithelia contains less than 20,000 sensory cells, or hair cells, although there are about a million photoreceptors in the eye. Hair cells are damaged by various causes, including acoustic trauma, ototoxic drugs, and aging. Most non-mammalian vertebrates are able to regenerate sensory hair cells after injury. However, mammalian cochlear hair cells do not regenerate spontaneously, although vestibular hair cells in adult mammals regenerate at levels so low as to rule out any significant functional recovery [8, 49].

Recently, Oshima et al., reported on stepwise protocols to induce hair cell-like cells from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [31]. The early development of the inner ear occurs in three phases. The first phase is the formation of the otic placode, followed by the second phase, the transformation of the otic placode into the otocyst, and finally, regional patterning of the otocyst occurs. Taking advantage of the knowledge of early inner ear development, they reproduced the developmental events in vitro starting with undifferentiated ESCs and iPSCs of mice, which are directed toward the ectodermal lineage. ESC/iPSC-derived ectodermal cells respond to otic inducing growth factors, for example, basic fibroblast growth factor (bFGF). Finally, induced otic progenitors are subjected to chicken utricular stromal feeders, which promoted the differentiation of otic progenitors into epithelial clusters displaying hair cell-like cells with stereociliary bundles. These hair-cell like cells respond to mechanical stimulation with currents that are reminiscent of nascent hair cell transduction currents. Further studies are needed to elucidate the signals to specify hair cell subtypes such as auditory or vestibular, inner or outer hair cell, or type I or type II hair cell, so one possible use of hair cell-like cells from ES/iPS cells is to study the steps

leading to hair cell maturation. Another use of hair cell-like cells from ESCs/iPSCs is to evaluate effectiveness and toxicity of various drugs to hair cells in vitro. Finally, the generation of human iPSCs with mutations for genes required for hair cell development and function could elucidate the pathogenesis that causes hearing impairment.

Another approach for inner ear regeneration is stem cell transplantation. Ito et al., performed the first animal experiments in the auditory systems to examine the potential for repairing the central auditory pathway and reported that embryonic brain tissue transplanted into a lesion in the ventral cochlear tract resulted in tissue regeneration and associated functional recovery [14]. Tateya et al., examined the potential of neural stem cell (NSC) transplantation to restore inner ear hair cells in mice [47]. Although the majority of grafted cells differentiated into glial or neural cells in the inner ear, a few transplanted NSCs integrate in vestibular sensory epithelia and expressed specific markers for hair cells in vivo. However, a small number of hair cell-marker positive grafted cells and no evidence of synaptic connections between transplants and host spiral ganglion neurons hampered well-established methods for functional recovery.

5. Regeneration of spiral ganglion neurons

Spiral ganglion neurons (SGNs) are the neurons which relay auditory signals from hair cells to the central systems. Cochlear implants, which bypass the damaged hair cells, directly stimulate the SGNs in profoundly deaf patients. Some animal studies suggest that degeneration of SGNs may compromise cochlear implant function [9, 41], although some conflicting reports demonstrated no correlation between clinical performance and the number of surviving auditory neurons [2,7,25]. Many attempts have been made to regenerate SGNs by transplanting pluripotent stem cells into the inner ear. We review previous reports and discuss obstacles to overcome for successful functional recovery. Several kinds of pluripotent stem cells have been delivered into the cochlea for the regeneration of SGNs, including NSCs [10,13,46], ESCs [4,5,11,12,29,34,36,38], bone marrow stem cells (BMSCs) [26,28,40], and iPSCs [27].

Tamura et al., evaluated the ability of NSCs to achieve neural differentiation in the modiolus of the cochlea and demonstrated that some grafted NSCs expressed β-III tubulin, a neuronal marker, although the majority of them differentiated into glial cells [46]. However, NSC transplantation can be utilized for protection of SGNs, because transplantation of neurospheres can reportedly be utilized for local application of neurotrophins into the brain [32,42], and several neuroprophins are known to have protective effects for SGNs [24,43,50].

ESCs are promising candidates for restoration of SGNs, because they have the potential to replace the lost auditory nerve due to their pluripotency. Sakamoto et al., first examined the fate of ESCs transplanted into the inner ears of adult mice and demonstrated that damaged inner ear has some activity inducing ESCs to develop into ectodermal cells, but the effect was insufficient to induce inner ear specific cells, including SGNs and hair cells [36]. The methods for generation of neurons from ESCs, including retinoic acid treatment of embryoid bodies [1], and co-culture of ESCs with PA6 cells, stromal cells derived from skull bone marrow [17] have been utilized for neural induction of ESC to regenerate SGNs. In this context, the regenerative potential of ESC-derived neural progenitors transplanted into the modiolus of the gerbil cochlea was examined and extensive migration of transplants along the auditory nerve was demonstrated [5]. Furthermore, transplantation of neural progenitors recovered the function of auditory neurons [29]. The evidence that ESC-derived

neurons have the potential to make synapse formation with auditory hair cells justifies the strategies of stem cell transplantation for the regeneration of auditory neurons [22,23]. Toward successful replacement of damaged SGNs by ESCs, establishment of SGN-specific cell types from ESCs is important. Transient expression of Neurog1, which is expressed in developing otocysts and is required for SGN differentiation, migration and survival, and treatment with glial cell line-derived neurotrophic factor (GDNF) turned undifferentiated ESCs into auditory nerve-like glutamatergic neurons [35].

Although previous studies identified ESCs as the promising candidates as transplants, ESC-based therapy is complicated by immune rejection and ethical problems. In this context, iPSC-based regenerative medicine has been developed recently [44,45,52]. iPSC-derived neural progenitors survived and expressed glutamatergic neuronal marker, VGLUT1, one week after transplantation into the cochlea, which indicated iPSCs can be used as transplants for the regeneration of SGNs as well as ESCs [27].

BMSCs, which can be readily obtained from an individual's own bone marrow, are also good candidates as transplants, because recent studies have shown that BMSCs can produce not only osteoblasts, chondrocytes, adipocytes, or myoblasts, but also neurons [15,16]. The survival of autologous BMSCs grafted in the cochlea was proven [26,28,40]. The enhanced survival of BMSCs was confirmed in deafened cochleae [26]. Autologous BMSC-derived neurospheres transplanted into the cochlear modiolus of the deafened guinea pigs settled predominantly in the internal acoustic meatus [28]. Combined with those findings, BMSCs can be a source for replacement of SGNs.

6. Tables and figures

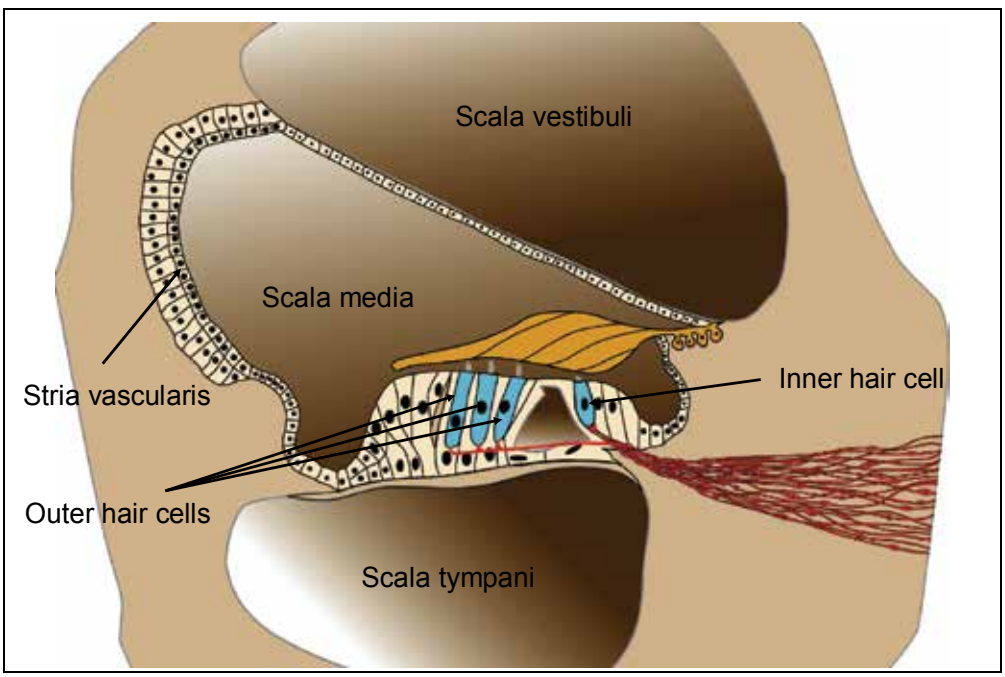

Fig. 1. The cochlea

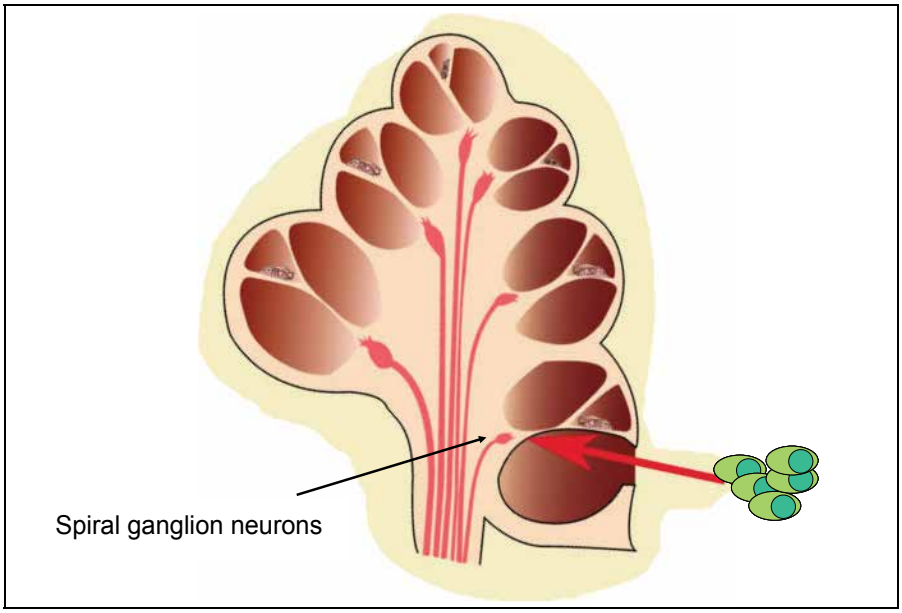

Fig. 2. Cell transplantation into the cochlear modiolus

7. Conclusions

Here we report the present status of development of stem cell-based therapy aiming for inner ear regeneration. Several experimental studies have demonstrated that pluripotent stem cells including ESCs and iPSCs are useful tools to examine detailed mechanisms of inner ear development, leading to reveal strategies for inner ear regeneration, and have the potential as a source of transplants for cell-based therapy for inner ear regeneration. However, many problems to be resolved still remain before realization of cell-based therapy for treatment of inner ears. More detailed analyses should be done to reveal key molecules that play critical roles in inducing differentiation of pluripotent stem cells into inner ear cells.

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12

Stem Cells and the Retina – Challenges for Regenerative Medicine

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1. Introduction

The retina, as one of the most accessible parts of the nervous system, has been extensively studied in different animal models. These studies have highlighted a remarkable conservation of the molecular mechanisms and gene regulatory networks involved in eye development and retinal differentiation. The initial phases of retinal specification see the activation and cooperation of a network of transcription factors (the Eye Field Transcription Factors, EFTFs) that allow the generation of a morphogenetic eye field in the anterior neural plate. The development of the Vertebrate eye then continues with a series of morphogenetic events, concomitant with the closure of the neural tube, that include evagination of the optic vesicles from the ventral forebrain and the subsequent formation of lens and retina. The retina comprises two juxtaposed parts: the neural retina represents the structure lining the inner surface of the eye and is involved in the reception and first elaboration of light stimuli. It presents a precise and stratified architecture based on seven different cell types: six types of neurons (cones, rods, amacrine cells, horizontal cells, bipolar cells and ganglion cells) and one type of glial cells (Müller glia). The Retinal Pigmented Epithelium is generated by the outer part of the neural retina and is fundamental for the correct functioning of retinal photoreceptors. Different molecular pathways that will be described are involved in retinal cell type specification.

As most adult organs, the adult eye has an endogenous population of stem cells – the retinal stem cells – localized in different anatomical structures (Ciliary Marginal Zone in amphibians and fish; Adult Ciliary Epithelial cells in Mammals). These cells remain multipotent throughout the lifetime of the organism, but in Mammals, especially in humans, they have lost the capability of reactivating after injury, thus being unable to repair the damaged retina. Identifying the molecular cues necessary to reactivate *in vivo* mammalian retinal stem cells is one of the strategies under study to repair damaged retinae, the other being the transplantation of differentiated cells. In the last years, many efforts have been devoted to the possibility to drive embryonic stem cells to become retinal cells. The aim is to initially generate retinal progenitors and subsequently mature retinal neurons of the types lost in degenerative diseases, such as photoreceptors and ganglion cells. Currently, only few protocols show promising results: we will describe and discuss them, analyzing their efficiency and capability of generating different mature retinal neuronal types. We will also discuss to which extent *in vitro* differentiation protocols recapitulate the developmental steps of embryonic development, and whether a more reliable representation of *in vivo*

processes could ameliorate the *in vitro* protocols. This type of analysis aims to elucidate the differentiation of retinal precursors into mature retinal cells and to give important suggestions to reproduce these processes *in vitro*. One important clue derives from our group, as we showed the capability of *Xenopus laevis* stem cells (Animal Cap Embryonic Stem cells, or ACES) to originate a functional retina when overexpressing Noggin, a secreted factor normally involved in the specification of the neural tissue during embryogenesis (Lan et al., 2009).
The last part of the chapter will describe the current applications and future perspectives of *in vitro* differentiated retinal cells. Retinal degenerative diseases affect many individuals and are the principal cause of irreversible and invalidating blindness in the western world. Cell replacement therapies represent one of the possible strategies to cure retinal dystrophies with regenerative medicine. We will describe what has been accomplished up to now in terms of stem cells transplantation in the retina, in animal models. Moreover, an emerging field of application of stem cell differentiation, especially since the generation of induced pluripotent stem cells, is the possibility to create patient-specific cellular models of retinal diseases. These will have great value for understanding the molecular mechanisms of the disease, screening for drug candidates that can prevent or slow down degeneration, understanding the variation in response of different patients.

2. Molecular regulation of Vertebrate eye development

The retina is an excellent model to study complex features of the nervous system, such as cell lineage, cell migration and connectivity. For these reasons, the eye represents one of the most important systems analysed during central nervous system development.
Genetic and molecular information acquired in eye and retinal development show that genes controlling retinal cell-fate are remarkably conserved among vertebrate species. These genes encode for different types of proteins, such as signalling molecules and transcription factors, and contribute both to retinal precursor specification as to their final differentiation. In the last years, the increasing know-how in transplantation techniques and more in general in regenerative medicine have cast a new interest on the genes and mechanisms involved in retina and eye formation, with the perspective of setting up cell replacement strategies to cure to retinal degenerative diseases (Lamba et al., 2009a).

2.1 Initial specification of retinal fate: the eye field

Most of the experimental approaches targeted to understand the initial phases of specification of retinal precursors were conducted in *Xenopus* embryos, which represents an accessible vertebrate animal model to study the role of cell-fate determinants, lineage effects and cell-cell interactions during stages prior to gastrulation (Sive et al., 2000). In *Xenopus*, lineage analyses showed that each retina originates from nine specific blastomeres at the 32-cell stage even if their contribution is not retina specific, as they participate in the formation of all three germ layers (Zaghloul et al., 2005). In this context, the first positional information is given by the expression of maternal factors such as VegT and Veg1, which are known to promote endo-mesodermal fates. The expression of these genes at the vegetative pole contributes to the localization of blastomeres competent to become retina in the animal pole of the *Xenopus* embryos (Yan & Moody, 2007).
A retina-specific presumptive territory becomes well defined only at neurula stages. This region, the “eye field”, is localized in the most anterior part of the neural plate, and will

subsequently split into two lateral domains that evaginate (optic vesicles). The eye field is composed by a non-homogeneous population of cells having different potentialities, constituted by early Retinal Progenitors Cells (RPCs) (Agathocleous & Harris, 2009). The RPCs comprise two groups of cells: multipotent progenitors, that are able to produce all retinal subtypes, and progenitors that produce only the retinal cells belonging to a single layer (Andreazzoli, 2009) (Fig.1).

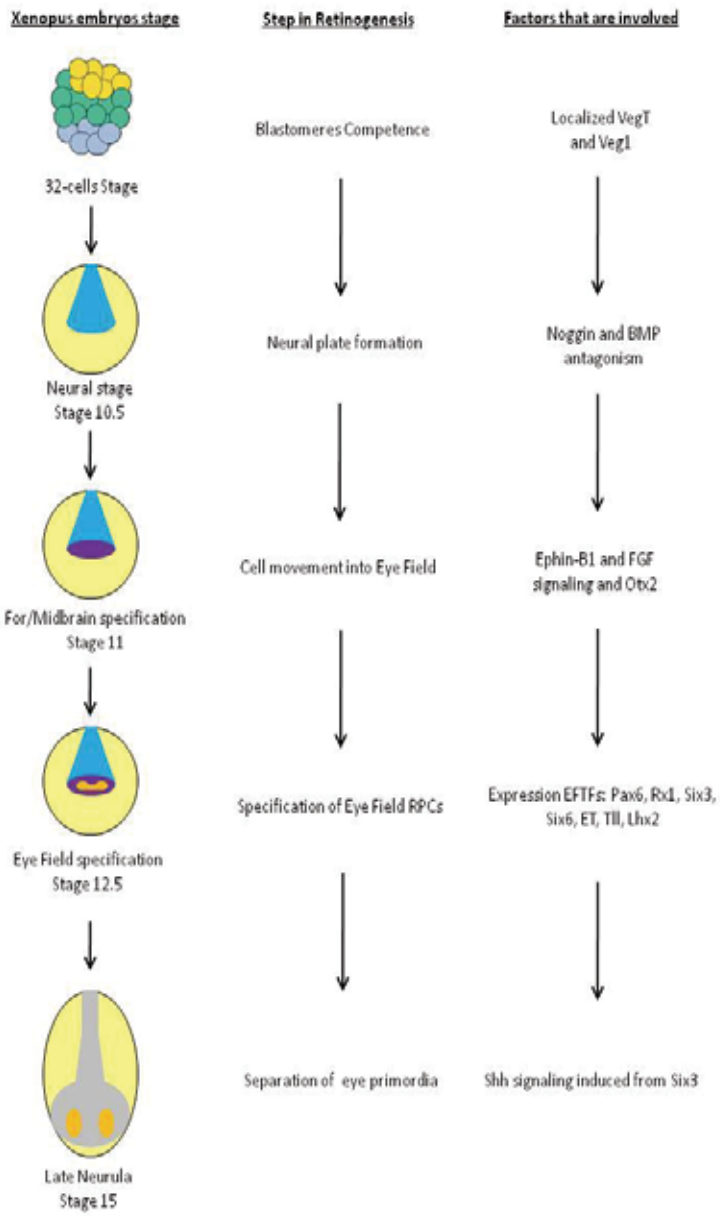

Fig. 1. Eye field and eye primordia development in *Xenopus* embryos, retinal specification steps and main known factors involved in each.

The first processes that are involved in the generation of the eye field are due to cell movements and rearrangements that start during gastrulation, continue with the identification of the two lateral retina primordia and end with the formation of the optic cup. In *Xenopus*, cells fated to form the anterior neural plate and the eye field show early cellular dispersal movement. This behaviour is promoted by Ephrin-B1 (Eph-B1) and inhibited by Fibroblast Growth Factors (FGFs), which modulate Eph-B1 intracellular ligands and down-regulate its signalling cascade (Moore et al., 2004). Other important factors involved are the inhibition of Wnt posteriorizing signaling and the inhibition of the Bone Morphogenetic Proteins (BMPs) cascade (Pera et al., 2001; Moore et al., 2004; Delaune et al., 2005). Moreover, our recent results suggest an important role of the secreted factor Noggin, a BMP antagonist, that is able to induce retinal fate in ectodermal explants (animal caps) of *Xenopus* embryos in a dose-dependent manner (Lan et al., 2009).
In all vertebrate species, the eye field and RPCs can be identified by the expression of several transcription factors. This set of homeobox transcription factors are named Eye Field Transcription Factors (EFTFs) and they are Pax6, Rx1, Six3, Optx2, ET, tll, Lhx2. They act as a self-regulating feedback network that specifies the eye field territory, and their over-expression is sufficient to induce ectopic eyes in *Xenopus* embryos (Zuber et al., 2003). The evidences about their relevance are shown by the fact that mutations in each of the EFTFs produce alterations of vertebrate eye development. For example, over-expression of Pax6 generates ectopic eyes in frogs while its loss-of-function leads to the "small-eye" or aniridia phenotypes in both mouse and humans (Chow et al., 1999, Hanson & Van Heyningen, 1995). Rx1/Rax is involved in eye field and pineal gland specification (Casarosa et al., 1997; Mathers et al., 1997). Overexpression of Rx1 in *Xenopus* produces an enlargement of the eye and ectopic retinal pigmented epithelium (RPE), while Rax knock-out mice present anophtalmia (Mathers et al., 1997; Voronina et al., 2004). Overexpression of Six3 in mice generates ectopic retinal tissue and a lack of it causes the absence of optical structures (Carl et al., 2002). ET/Tbx3, a T-box protein involved in the block of Rx1 transcriptional activity, is expressed in eye and cement gland primordia (Zuber et al., 2003). Moreover, both Six3 and ET are involved in Sonic hedgehog (Shh) signaling activity during the formation of the two bilateral eye fields from the initially single one (Takabatake et al., 2002; Geng et al., 2008). Lhx2, a LIM-homeodomain protein, is recruited in EFTFs transcriptional complexes, but it is involved in later events such as optic cup and lens formation (Yun et al., 2009). Moreover, Lhx2 promotes retinal proliferation *in vitro* (Tetreault et al., 2009). In the the eye field, the activation of the EFTFs and in particular of Rx1, that is fundamental for eye progenitors specification and proliferation, represses the anterior brain marker Otx2. This transcription factor is required for the initial specification of the eye field, but not for later stages of retinal development (Andreazzoli et al., 2003; Zuber et al., 2003).

2.2 Morphogenesis of the optic vesicle

As the neural tube closes, the two bilateral eye fields give rise to the optic vesicles, that evaginate from the lateral walls of the diencephalon. The optic vesicles come into contact with the epidermis and induce it to thicken and form the lens placode. As the lens differentiates, it invaginates until it pinches off from the epidermis. The lens acts as an inducer back to the optic vesicle to transform it into the optic cup and back to the epidermis to transform it into the cornea. The connection between the optic cup and the brain will form the optic nerve, while the optic cup delaminates into two layers: the outer layer of optic cup evolves in retinal pigmented epithelium (RPE) and the inner layer differentiates in neural retina (NR).

Optic cup development requires different signals deriving from surrounding tissues, such as: Shh, TGFß/BMP and FGF.
Shh is a secreted molecule, that as we said is involved in the formation of the two bilateral eye domains. Shh also activates the transcription of several factors that mediate the transition of the optic vesicle to optic stalk, RPE and NR, and enhances the differential identity of the ocular tissues. Shh binds its intramembrane receptor Patched. The binding produces a conformational change in Patched that fails to block Smoothened. The activation of Smoothened activates a transcriptional cascade that drives Gli family protein to transcribe specific transcription factors. At the end of this cascade: the proximal portion of each optic primordium, including optic stalk and ventral retina, starts to express Pax2 and Vax family homeoproteins (Liu et al., 2008; Vitorino et al., 2009); the distal portion, including RPE and dorsal retina, express Pax6 and Rx1 (Mathers et al., 1997; Futukawa et al., 1997). Shh persistence in pre-RPE tissue results in the down-regulation of Pax6 and in the transcription of Mitf (a RPE specific marker) and, transiently, of Otx2. The synergistic activity of Mitf and Otx2 converts pre-RPE in mature RPE (Martinez-Morales et al., 2001). In retina primordium, Pax6 and Rx1 expression persist. In conclusion, the cross-repression between Pax2 and Pax6 seems to form a boundary between optic stalk/ventral retina and RPE/dorsal retina (Yang, 2004).
TGFß/BMP factors are involved in dorso-ventral patterning of the neural tube and in neuronal differentiation. They also play a role in the morphogenesis of the optic vesicle. TGFß/BMP proteins in fact represent an important signal in later stages of eye development, when the optic cup evolves in the three ocular tissues (optic stalk, RPE and NR). In chick and mouse, BMP4 is expressed in distal optic vesicle structures and in dorsal retina and ventral RPE and, subsequently, in the peripheral margin of the differentiating retina (Sakuta et al., 2001). BMP4 expression, in chick, is able to inhibit Vax and Pax2 and to promote Tbx5, a gene expressed only in dorsal retina (Koshiba-Takeuchi et al., 2000), permitting to speculate on a competition among Vax and Pax2 (Shh effectors) in the ventral region, and Tbx5 (BMP4 effector) in the dorsal side to establish a correct dorso-ventral axis in eye structures. The important involvement of BMPs in eye development is shown by BMP7 null mice that frequently show an eyeless phenotype. In mouse, BMP7 is expressed in the surface ectoderm (pre-lens tissue) contacted by the optic vesicle. Many evidences suggest that the formation of the double-layered optic cup requires signals deriving from the pre-lens ectoderm (Dudley & Robertson., 1997; Hyer et al., 2003). Indeed, Activin, a TGFß factor secreted from the extraocular mesenchyme surrounding the optic vesicle, seems involved in RPE specification, repressing the neural retina specific markers Pax6, Six6 and Chx10 and promoting Mitf expression (Fuhrmann et al., 2000).
FGF signalling presents a regionalization function in optic cup morphogenesis. This family of Growth Factors is prevalently involved in neural retina development. In chick, mouse and *Xenopus* embryos, FGF8b is able to convert RPE in NR, while FGF2 treatments block differentiation of retinal layers and do not affect RPE. These data support the idea of a role of different FGFs in the organization of the double-layered optic cup (Pittack et al., 1997; Martinez-Morales et al., 2005).
The different factors that are involved in optic vesicle morphogenesis also contribute to the maturation of the three optic tissues that are formed during lamination processes (optic stalk, RPE and NR). The optic stalk evolves in the optic nerve when the choroidal fissure closes and retinal nerve fibers fill it. The RPE becomes pigmented and starts to produce trophic factors that are involved in NR successive lamination and maintenance and forms a barrier with blood vessels. NR precursors will give rise to a stratified neural retina.

2.3 Determination of retinal cells fates

Each retinal layer is characterized by the presence of specific classes of retinal neurons. A variety of basic helix-loop-helix (bHLH) transcription factors are expressed by a subset of retinal progenitors cells or by their post-mitotic progeny (Ohsawa & Kageyama, 2008). One of the first genes to be transcribed is Math5/Ath5 involved in ganglion cell precursors specification (Yang et al., 2003). Subsequently, in ganglion cell precursors are activated Brn3b and Isl-1, that confer a final differentiating stimulus, and Math5 is down-regulated (Pan et al., 2008; Qiu et al., 2008).

The specification of amacrine and horizontal cells requires the transcriptional activation of Foxn4 that mediates the activation of Pt1fa, transcriptional regulator of Prox1 (Li et al., 2004). Precursors in which Prox1 expression persists, become horizontal cells (Dyers et al., 2003). On the contrary, precursors in which are also expressed NeuroD and Math3 become amacrine cells. Different types of amacrine cells are generated under the activation of downstream factors, such as Barhl2 (glycinergic amacrine cells), BhlhB5 (GABA-ergic subtypes) and Isl-1 (cholinergic amacrine cells) (Elshatory et al., 2007) (Fig.2).

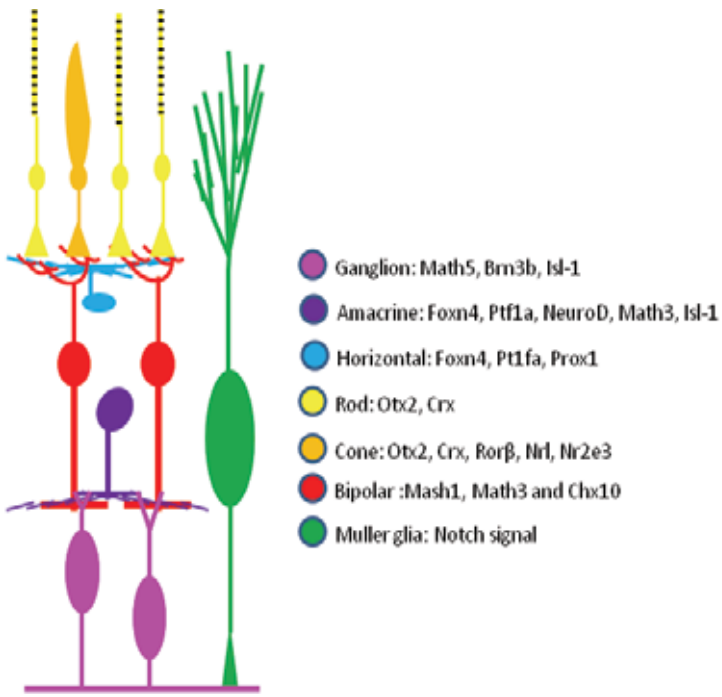

Fig. 2. Principal bHLH transcription factors involved in neural retina cells specification.

Otx2 is fundamental for photoreceptor fate. Otx2 acts through Crx, a cone-rod specific factor, involved in differentiation of photoreceptors (Reese, 2010). Crx activates the transcription of Rorβ, which in turn activates Nrl. Finally, Nrl activates Nr2e3. This transcriptional cascade switches the photoreceptor phenotype to rod fate (Corbo & Cepko, 2005; Oh et al., 2007).

Bipolar cells production requires the activation of Mash1 and Math3 that cooperate with the homeobox transcription factor Chx10. Down-regulation of only one of these genes produce Müller glia cells instead of bipolar cells (Tomita et al., 2000).

Müller glia cells are the last retinal cells produced during eye development. During the last division, the two daughter cells differentiate one into a bipolar cell or a rod photoreceptor and the other in a Müller glia cell. Notch signalling seems to repress proneural genes activation and contribute to the production of Müller cells (Jadhav et al., 2009).

In retina, an evolutionarily conserved aspect in the generation of cell diversity is represented by symmetric vs. asymmetric cell divisions. Symmetric divisions occur when a mother cell generates two equivalent daughter cells and asymmetric when it divides unequally (Huttner & Kosodo, 2005). Data acquired in transgenic zebrafish, in which GFP expression is under control of the Ath5 promoter (an essential proneural inducer of ganglion cells), suggest that retinal cells divide either symmetrically or asymmetrically. In fact, early retinal progenitors have a central-peripheral division and late progenitors a circumferential division (Poggi et al., 2005). Symmetric and asymmetric division occurs in relation to different cell fate determinants, such as Numb, an asymmetrical signal in mammalian retina. During division, Numb segregates to the apical part of daughter cells in asymmetric apico-basal divisions and it is equally distributed in both daughter cell following symmetric planar divisions. (Cayauette & Raff, 2003). Other information on retinal fates specification derives from positional information. Del Bene and collaborators (Del Bene et al., 2008) showed that signaling molecules, such as Notch, play a crucial role in determining retinal fate during interkinetic nuclear migration. Interkinetic nuclear migration is a process in which the nuclei of retinal progenitors, that generally contact the apical and basal surfaces of the retina, migrate along this apico-basal axis in different phases of cell cycle. This process is different in each type of early retinal progenitor and seems to be involved in the specification of a heterogeneous final retinal population. Cells with a reduced basal migration remain apical and proliferative, while cells that migrate to the basal side generate daughter cells that will become postmitotic (Andreazzoli, 2009).

The specification of retinal cell fates is also closely related to the retinal clock. The first evidences for a retinal clock are shown in the fruit fly *Drosophila Melanogaster* in which a complex of four factors Hunchback-Kruppel-Pdm-Castor seems to be involved in neuroblast competence. In fact, these neuroblasts divide asymmetrically and produce ganglion mother cells (GMCs) that express each of the genes of the complex transiently during different phases of GMCs divisions. Hunchback and Kruppel are necessary and sufficient to control early-born neuroblasts, while Pdm and Castor are expressed with further progression of the cell cycle. These observations allow to conclude that a cell cycle clock is involved in the timing of the generation of specific cell types (Isshiki et al., 2001). Recently, a similar mechanism has been shown in Mammals with the identification of Ikaros, a Hunchbach homologue, and Castor, both expressed in the mouse retina. Ikaros is expressed in early progenitors and its expression is switched off during cell cycle progression and retinal fates acquisition, suggesting a role for Ikaros in early retinal progenitors manteinance (Blackshaw et al., 2004; Elliot et al., 2008).

The length of cell cycle clock represents a crucial point to establish the transition between early and late progenitors. In fact, the first ones have a short cell cycle characterized by a great number of divisions, while the second ones have a long cell cycle, probably, due to the requirement of the transcription of specific factors that identify specific cell types (Decembrini et al., 2006). Many evidences suggest a role of Shh as regulator of cell cycle kinetics in retinal precursors (Decembrini et al., 2009) (Fig.3).

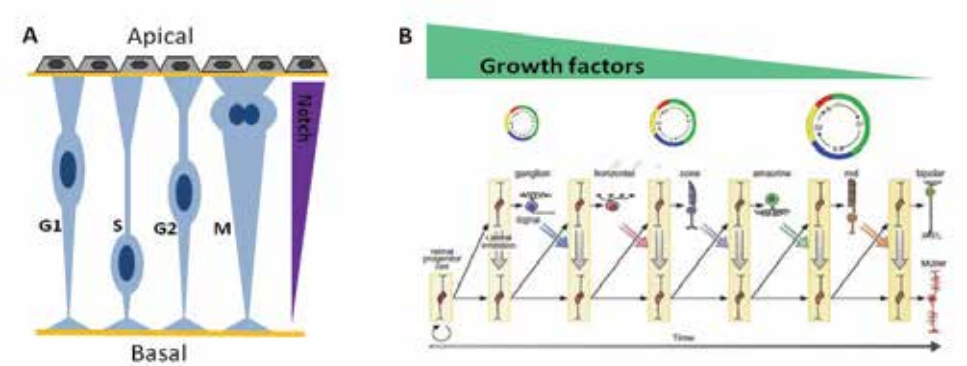

Fig. 3. Different processes involved in retinal cells fate specification. A) Interkinetic nuclear migration; B) Retinal clock in which Growth Factors, such as Shh, influence cell cycle elongation and retinal cell fates specification (*Andreazzoli, 2009*).

3. Retinal stem cells

Many evidences suggest that a population of stem cells, the retinal stem cells, persists in specific regions of the vertebrate retina such as the Ciliary Marginal Zone (CMZ) in amphibians and in Mammals, also called Circumferential Germinal Zone (CGZ) in fishes (Locker et al., 2009). In the last years, many other studies in fact suggest that retinal stem cells represent only a little group of cells with stemness properties in mature and adult retina. In fact, data support the idea that different mature cell types, such as Müller glia and retinal pigmented epithelium cells, if suitably stimulated, are able to transdifferentiate in other retinal specific subtypes.

3.1 Fish and amphibians

A first evidence of the existence of retinal stem cells in vertebrates derived from autoradiography experiments in fishes and amphibians. During the period between 1971 and 1977, Straznicky and Gaze and successively Johns showed that the retina of *Xenopus laevis* and *Carassius auratus* (goldfish) is constantly growing to fit to the growth of the animal body. They were able to identify the region in which proliferation was taking place and named it Ciliary Marginal Zone (CMZ) (Straznicky & Gaze, 1971; Johns, 1977). The CMZ is a circumferential region located at the periphery of the retina. Stem cells are found in the most peripheral part of the CMZ are able to give rise to both neural retina and pigmented epithelium cells, being thus equivalent to cells that form the earlier eye field (Harris & Perron, 1998; Locker et al, 2009). As they divide, their daughter cells move toward the central portion of the CMZ. These cells are mitotic but not stem cells and they are called retinoblasts. Retinoblasts differentiate in all the cell types that form the retina (Harris & Perron, 1998). The most internal part of the CMZ contains cells that have generally left the cell cycle and are committed to became specific retinal subtypes. The final fate of these cells is determined by extracellular interactions with the neighboring cell types and final localization of undifferentiated cell. The mentioned mechanism recapitulates the processes that occur during retinal development, in which, after differentiation in specific retinal neuronal subtypes, the newly formed cells inhibit the other to choose the same fate (Waid & Mcloon, 1998; Harris & Perron, 1998).

Specification of CMZ cells, in the three different regions, is under the control of the same factors that specify the eye primordia during embryonic development. Six3, Pax6, Rx1 and the other EFTFs are also expressed in the most peripheral part of CMZ in which are localized retinal stem cells in fish and *Xenopus* (Raymond et al., 2006; Locker et al., 2009). Moreover, components of the Shh pathway (Gli2, Gli3, X-Smoothened), and of Notch-Delta signaling, (XHairy1 and XHairy2, the respective orthologs of human Hes1 and Hes4), are expressed in the peripheral CMZ cells of *Xenopus laevis* supporting the idea of their stemness properties (Raymond et al., 2006; Locker et al, 2009). The central zone of *Xenopus* CMZ (in which are mitotic cells but not stem cells) expresses Xash1, a gene that drives stem cells to become neural and susceptible to differentiate in a specific neural subtype. In this region, the expression of athonal homologs (Xath5 and NeuroD) stimulates the generation of retinal precursors (Harris & Perron, 1998). During the progression toward the most central region, CMZ cells switch off the previously described genes and turn on specific retinal fate genes, such as Brn3, that promotes differentiation and survival of ganglion cells (Gan et al., 1996).
In the last years, analysis in fishes and other vertebrates showed that, in response to injury, Müller glia cells are able to start proliferating again and subsequently transdifferentiate in other retinal neuronal subtypes. This evidence suggests that Müller glia cells can represent another source of pluripotent stem cells in the adult eye (Locker et al., 2009). Retinal pigmented epithelium represents another source of mature retinal cells that can transdifferentiate in neural retina cells. In fact, the treatment of RPE cells with specific growth factors, such as FGFs, is able to convert pigmented epithelium in proliferative cells that can acquire a neural phenotype (Martinez-Morales et al., 2005).

3.2 Mammals

Ten years ago, evidence of a potential population of retinal stem cells is obtained in Mammals, even if it is not possible to identify a proliferating CMZ as in fishes and amphibians (Amato et al., 2004). In Mammals, two different types of cells are present in the adult eye that represent potential retinal stem cells: cells of the ciliary body (ciliary margin) and of the iris epithelium. The cells of ciliary body derive from epithelial cells and in particular conditions, such as damage, are induced to display retinal stem cell properties. Moreover, if cultured *in vitro*, they are able to generate retinal neurons. Similarly, the iris epithelium cells, which derive from the inner layer of iris tissue, express nestin (stem cells marker) and possess the ability to differentiate in multiple neuronal types including retinal neurons (Bi et al; 2009). Analyses in Mammals reveal that ciliary body and iris stem cells express Pax6, Rax, Chx10 and Six3, thus supporting the idea of retinal precursors properties and a conserved molecular mechanism in retinal stem cells generation among vertebrates (Locker et al., 2009).
The first evidences of the capacity of some mature retinal cell types to transdifferentiate in other types derive from studies in Mammals. In 2004, Ooto and colleagues proved the capacity of Muller glia cells to generate retinal neurons. Successively, an accurate analysis of gene expression in retinal precursors and Muller glia cells confirms an overlap between the profile of these two cell types and the potentiality of Muller glia cells as neural stem cells (Ooto et al., 2004; Bi et al., 2009). Transdifferentition of RPE cells into neural retina has been demonstrated in embryonic rat after the treatment with growth factors such as basic FGFs (Ballios & Van der Kooy, 2010).

4. Differentiation of stem cells into retinal cells: do *in vitro* protocols recapitulate embryonic development?

The retina is affected by a broad range of pathologies (collectively called retinopathies) often sharing a heterogenic genetic background and leading to cell depletion through a degenerative process. Commonly, photoreceptors represent the retinal cell population primarily affected by the degeneration: depending upon which type of photoreceptor dies first, we distinguish rod-cone degenerations from cone-rod degenerations. After the loss of most photoreceptors, the other retinal cell types show dendrite atrophy or are involved in a considerable remodeling of the entire circuitry (Strettoi et al., 2003; also reviewed in Marc et al., 2003). As retinal pigmented epithelium is fundamental for photoreceptors activity, also the impairment of its function may lead to retinal degeneration.

Among retinopathies, inherited retinal degenerations (such as Retinitis Pigmentosa) are a major cause of visual impairment in the juvenile-to-young adult population. As reviewed in Shintani et al., 2009, various approaches have been assessed for this type of retinopathies, but the results are often disaccording. For Retinitis Pigmentosa, it has been postulated that the assumption of nutritional supplements such as Vitamin A palmitate, Lutein and Docosohexanoic acid (DHA) can reduce the rate of the degeneration and preserve photoreceptors viability. Unfortunately, the effects seem to last for short periods and in some situations toxic side effects have been postulated.

Gene therapy is another potential approach and it has been carried out in three ways:

- through replacement of the mutated gene with a normal one, using vectors such as adenoviruses;
- using a ribozyme therapy aimed at disrupting the production of the aberrant protein;
- through RNA interference, for the same aim as the ribozyme.

However, the long-term safety of the procedure and the concrete effectiveness must both be assessed.

The common pharmaceutical strategies exert their positive effect in the presence of fully functional tissues, but they often fail where the target is mostly compromised. Like the previous approaches, the pharmaceutical one is actually unable to enhance cell viability, but it tries to slow down the course of the degeneration. The treatment with basic Fibroblast Growth Factor (bFGF) and Ciliary Neurotrophic Factors (CNTFs) seems to ameliorate the condition of rat models of Retinitis pigmentosa, by reducing the loss of photoreceptors. Retina pigmented epithelial (RPE) cells genetically modified to produce CNTFs can be encapsulated and implanted in the affected eye. This therapy is in phase I of clinical trial and it has been shown increasing the visual acuity of the patients. Antiapoptotic drugs used for Parkinson's Disease are currently under investigation: it is the case of Zelapar™ (Valeant Pharmaceuticals, Swindon, Wiltshire, United Kingdom).

The overall situation suggests that an efficient solution is still lacking, although it seems that embryonic stem cells and the newly described induced pluripotent stem cells (iPS), once correctly differentiated, may become an useful (yet challenging) tool to approach diseases having a degenerative substrate - a strategy generally referred to as cell-based substitutive therapy (see section 4 for the application in the retina). Embryonic stem cells may be differentiated either towards neural retina cells or towards retinal pigmented epithelium cells. A certain number of protocols have been described for the differentiation of mouse (Zhao et al., 2002; Hirano et al., 2003; Ikeda et al., 2005; Sugie et al., 2005; Osakada et al., 2008; Osakada et al., 2009a), human (Banin et al., 2006; Lamba et al., 2006; Osakada et al.,

2008; Aoki et al., 2009; Meyer et al., 2009; Osakada et al., 2009a; Osakada et al., 2009b; Nistor et al., 2010) and primate (Osakada et al., 2008) embryonic stem cells into retinal cells, and some of these have been demonstrated to work also on mouse (Hirami et al., 2009; Parameswaran et al., 2010) and human (Hirami et al., 2009; Meyer et al., 2009; Lamba et al., 2010) induced pluripotent stem cells (iPS cells). In a similar way, protocols of differentiation into retinal pigmented epithelium cells have been described for mouse (Kawasaki et al., 2002), human (Klimanskaya et al., 2004; Osakada et al., 2008; Idelson et al., 2009; Meyer et al., 2009; Osakada et al., 2009a) and primate (Yue et al., 2006; Osakada et al., 2008) embryonic stem cells, as well as for human (Buchholz et al., 2009) induced pluripotent stem cells.

Generally, it is believed that *in vitro* differentiating cells requires the simulation of the molecular environment to which the cell type of interest is exposed during embryogenesis. This simulation should lead the cultured cells to recapitulate the morphogenetic modifications also observed *in vivo* during the embryonic development. Regarding the retina, this means that the cells must be instructed to express in the right succession the combination of genes responsible for the specification of the structures we have seen previously (see Molecular regulation of Vertebrate eye development):

- eye field
- optic vesicles
- retinal progenitor cells
- immature photoreceptors
- photoreceptors

while activating neural genes and repressing the expression of genes related to a pluripotent condition. All the protocols aim to reproduce these conditions, as this represents the most reliable thread to follow.

4.1 Current *in vitro* protocols

Mouse embryonic stem cells are maintained and propagated in culture with different types of media depending on the cell line, but two key elements are always present: Leukemia Inhibitory Factor (LIF) and serum. Leukemia inhibitory factor can be either provided by a layer of mitotically-inactivated mouse embryonic fibroblasts (MEF), on which stem cells are cultured, or added in the medium if fibroblasts are not provided. Bone morphogenetic factor 4 (BMP-4) contained in Fetal Bovine Serum acts synergistically with LIF to inhibit differentiation and promote proliferation (Ying et al., 2003). Regarding human embryonic stem cells, the pathway activated by LIF seems to have no effect on the maintainance of pluripotency (Daheron et al., 2004; Humphrey et al., 2004). Instead, a role for basic Fibroblast Growth Factor and Activin-A has been described (Amit et al., 2000; Beattie et al., 2005; Dvorak et al., 2005; Vallier et al., 2005; Levenstein et al., 2006). The first published protocols about retinal differentiation *in vitro* describe a procedure to obtain retinal progenitors and photoreceptors from mouse embryonic stem cells by means of co-culture systems (Zhao et al., 2002; Hirano et al., 2003; Ikeda et al., 2005; Sugie et al., 2005).

Zhao and colleagues promote the formation of embryoid bodies from mouse stem cells by removal of LIF (Zhao et al., 2002). These embryoid bodies are three-dimensional structures consisting of few hundreds cells and they are often used to begin the differentiation simulating an embryonic-like environment (Doetschman et al., 1985). The formation of neural progenitors cells is obtained exposing embryoid bodies to Retinoic Acid (RA) or

plating them in the presence of a medium containing Insulin, Transferrin, Selenium, Fibronectin (ITSFn) and basic Fibroblast Growth Factor. In either ways, cells are then dissociated and expanded with basic Fibroblast Growth Factor. At this point, they are in active proliferation and they have acquired neural features, as demonstrated by the incorporation of Bromodeoxiuridine (BrdU) and the expression of Nestin, which is a marker of neuroectodermal stem cells (Lendahl et al., 1990). Moreover, the neural progenitors obtained in either way are mostly positive for Pax6 and Notch1, which are respectively involved in eye development and spatial-temporal organization of retinal neurons. When cultured with postnatal day 1 rat retinal cells, these neural progenitors start expressing genes involved in the specification of photoreceptors, such as Rx, Crx, Nrl and NeuroD. Prolonged time of co-culture leads to the expression of Rhodopsin in 6% of the overall population of cells but this evidence does not correlate with the acquisition of typical photoreceptor morphology.

A similar experiment has been conducted using chick retina harvested on embryonic day 6 for co-culture (Sugie et al., 2005). Mouse embryonic stem cells are first induced to acquire a neural phenotype using two possible protocols (Bain et al., 1995; Okabe et al., 1996), then they are cultured for 10 days on chick retinal tissue in the presence of the medium described previously (Zhao et al., 2002). In these conditions, 21.5% of total cells results positive for Rhodopsin when one protocol of neuralization has been used (Okabe et al., 1996) and 10% is positive following the other neuralization protocol (Bain et al., 1995). Crx, Interphotoreceptor Binding Protein (IRBP) and Recoverin (markers of both cones and rods) expression is detected by PCR during co-culture starting from day 4, 7 and 10 respectively.

Structures resembling eyes and containing photoreceptors positive for Rhodopsin and Recoverin are obtained also co-culturing mouse embryonic stem cells with the stromal cell line PA6 in the presence of basic Fibroblast Growth Factor, Dexamethasone and Cholera Toxin (Hirano et al., 2003). However, it seems that only a small fraction of cells produces these eye-like structures, thus a much smaller percentage of cells is thought to become photoreceptors. Almost the same result is observed when applying this protocol to human embryonic stem cells (Aoki et al., 2009).

Co-culture with retinal pigmented epithelium is also used to induce the formation of neural retina cells (Chiou et al., 2005). Human bone marrow stem cells can be induced towards a neural phenotype by treating them with Insulin, Progesterone, Putrescein, Selenium Chloride, Transferrin, Epidermal Growth Factor and basic Fibroblast Growth Factor. After 2 weeks they form spheroid aggregates and express Nestin (75.8% of total cells). When these spheroid structures are co-cultured with mitotically inactivated human pigmented cells, they give rise to neural cells expressing Opsin (29.6% of bone marrow stem – derived cells).

Sasai's group starting from 2005 has pursued a different strategy (Watanabe et al., 2005; Ikeda et al., 2005). Mouse embryonic stem cells are kept in suspension for 9 days in a medium containing KnockOut™ Serum Replacement (KSR, Euroclone) in order to form serum-free embryoid body-like aggregates (SFEB). Once treated with the anti-Wnt reagent Dickkopf-1 (Dkk-1) and the anti-Nodal reagent Lefty-A, these floating aggregates are plated and produce a good percentage (35%) of telencephalic precursors expressing the transcription factor Bf1 (Watanabe et al., 2005). Moreover, most of the floating bodies express Six3, which is the first eye field transcription factor expressed during the embryogenesis (Ikeda et al., 2005). These observations led the authors to think that the serum-free, floating culture of embryoid body-like aggregates could be used to obtain retinal neurons. Thus, in order to enhance the transcription of both Rx and Pax6, whose co-

expression identifies retinal progenitor cells, Fetal Calf Serum and Activin-A are added to the suspension culture. Once plated, 15% of total cells express Rx and 6.4% results positive for both Rx and Pax6 (Ikeda et al., 2005). Eventually, the co-culture with dissociated E17.5 mouse retinal cells leads 36% of differentiated cells to express Recoverin and Rhodopsin, but only when embryonic stem-derived cells and true photoreceptors are in close proximity.

When the procedure to obtain retinal progenitors is applied on mouse Rx-GFP knock-in cell line, cells expressing Rx can be isolated from the aggregates and subsequently sorted to produce an almost pure culture (Osakada et al., 2008; Osakada et al., 2009a). The treatment with the γ-secretase inhibitor DAPT from day 10 of differentiation induces the expression of Crx in 22.4% of total cells, although the percentage decreases significantly when a different cell line is used. The consecutive addition of acidic Fibroblast Growth Factor, basic Fibroblast Growth Factor, Taurine, Sonic Hedgehog (SHH) and Retinoic Acid then leads to the co-expression of Recoverin and Rhodopsin in 17.2% of total cells, but it has no effect on the production of Red/Green opsin+ and Blue opsin+ cones. Also in this case, the yield differs consistently depending on the cell line.

Monkey embryonic stem cells can be directed to retinal differentiation following almost the same procedure. Similarly, human embryonic stem cells can be used to obtain floating aggregates to be treated for 20 days with Dickkopf-1, Lefty-A. Y-27632, a Rho kinase inhibitor introduced to improve viability, is added to the medium. 15.8% of colonies originating from the aggregates results positive for both Rx and Pax6. As they are further treated with Retinoic Acid, Taurine and N2 supplement starting from day 90, 19.6% of total cells become Crx+. These eventually produce Rhodopsin+/Recoverin+ rods (8.5% of total cells), Red/Green opsin+ cones (8.9%) and Blue opsin+ cones (9.4%). To a different extent, the procedure has effect also on other human embryonic stem cell lines.

More recently, this protocol has been applied also on mouse and human induced pluripotent stem cells with some adjustments (Hirami et al., 2009). On day 120 of differentiation, nearly 3.5% of total cells (human iPS cells) expresses Recoverin and 50% of them is also positive for Rhodopsin.

In a therapeutic perspective, the differentiation of human stem cells obtained with compounds produced by animal cells or *E. coli* could lead to infection or immune rejection. For this reason, Osakada and colleagues have also described a second protocol having the same pipeline as before, but substituting factors such as Dickkopf-1 and Lefty-A with chemical compounds sharing equivalent effects: respectively, Casein Kinase I Inhibitor (CKI-7) and SB-431542 (Osakada et al., 2009b). Again, Y-27632 is used to improve cell viability. After 10 days from the replating of aggregates, 25.4% of colonies are Rx+, 79.2% are Pax6+. Moreover, 22.8% of total cells is Mitf+: 10 days later, 18.1% of total cells are pigmented and display a squamous, hexagonal morphology. These cells eventually form tight junctions (ZO-1+) after a whole 100 days of culture. As for photoreceptor induction, the treatment with Taurine and Retinoic Acid by day 140 produces Rhodopsin+ cells (nearly 6.5% of total cells) that don't acquire the morphology of mature rods. This population contains also cells positive for Recoverin, Phosphodiesterase 6b and 6c (respectively specific for rods and cones) and other markers correlated with phototransduction, indicating that these cells may respond to light stimuli.

A recent modification of Osakada's protocol (Osakada et al., 2008; Osakada et al., 2009a) has been applied on human embryonic stem cells to produce a three - dimensional tissue structure (Nistor et al., 2010). Differentiation is promoted in adhesion with B27 supplement, Insulin–Selenite–Transferrin (IST), triiodothyronine (T3), Taurine, Hyaluronic Acid (HA), Dickkopf-1,

LeftyA and Fibroblast Growth Factor. At day 7 cells are trypsinized and replated to promote aggregates formation. For the following 7 days, cells are cultured in a medium with B27 supplement, IST, T3, Taurine, HA, FGF and Retinoic Acid, which is added from day 10 to 13. At day 42, patches of retinal pigmented epithelium are isolated and replated to promote their expansion in a medium with B27 supplement, IST, T3, Taurine, HA, and Fetal Bovine Serum. Then, at day 50-60, neural cultures are replated onto the seeded RPE cells. With this procedure the production of a three – dimensional tissue is promoted, where it is possible to find cells expressing retinal markers such as Rx, Pax6, NeuroD, Mitf, Crx and Nrl. However, it is not clear what percentage of cells acquire each retinal phenotype.

Banin and colleagues obtain photoreceptors starting from human embryonic stem cells (Banin et al., 2006). The differentiation into neural precursors is enhanced by culture on feeders for 8 days in a medium containing Fetal Calf Serum and Noggin, which is a renowned neuralizing factor (Smith & Harland, 1992), and in the same medium without Noggin for an additional 5 days. Then, small patches of cells are explanted and replated in a serum – free medium containing B27 supplement, Epidermal Growth Factor and basic Fibroblast Growth Factor. Under these conditions, there is the development of floating aggregates that can be subcultured for 4 weeks (Reubinoff et al., 2001). At this point, nearly 98% of cells within the aggregates is positive for Nestin. Moreover, they express the common eye field and photoreceptors markers. Once plated, these aggregates give rise to a population of neurons that keep on expressing the above-mentioned markers but fail to produce the related proteins. This problem is partially overcome when the neural precursors are engrafted into rat retinas. Although the mature morphology is never acquired, nearly 1.5% of engrafted cells are positive for Rhodopsin protein.

An alternative approach is the one described by Lamba and colleagues (Lamba et al., 2006). Starting with human embryonic stem cells, the formation of embryoid bodies is promoted in a medium containing KnockOut™ Serum Replacement, B27 supplement, Dickkopf-1, Insulin-like Growth Factor 1 (IGF-1), whose overexpression in *Xenopus* embryos leads to the formation of ectopic eyes (Pera et al., 2001), and Noggin. Embryoid bodies are then collected and plated in a medium containing B27 supplement, N2 supplement, Dickkopf-1, Insulin-like Growth Factor 1, Noggin and basic Fibroblast Growth Factor. At the end of this procedure, nearly 12% of total cells expresses Crx, 5.75% expresses Nrl but only small percentages (less than 0.01%) of total cells express mature photoreceptors-specific markers, such as S-opsin and Rhodopsin. Similarly to Ikeda and colleagues, Lamba's group manages to co-culture their retinal progenitors with adult mice retinal explants. Interestingly, photoreceptor differentiation of these progenitors is seen only when retinal explants derive from mice models of photoreceptor degeneration.

The same protocol with some modifications has been recently applied on human induced pluripotent stem cells (Lamba et al., 2010). In this case, instead of promoting the formation of embryoid bodies, iPS cells are directly cultured with Noggin, Dickkopf-1 and Insulin-like Growth Factor 1 at lower concentrations. After 3 days, their concentration is the same as in Lamba et al., 2006 and the cells are kept in this medium for 3 weeks. Terminal differentiation is enhanced in a N2 - B27 containing medium for some months. After 2 months of induction, compatibly with the results of the previous protocol, nearly 12% of total cells expresses Crx. Late markers such as Recoverin, Rhodopsin and S-opsin are expressed in less than 1% of cells. However, better effects may be obtained when an IRBP – GFP cell line is used and Fluorescence Activated Cell Sorting (FACS) is performed to isolate a pure population of IRBP+/Crx+ cells.

Meyer and colleagues have recently described one more method, which is applied on both human stem cells and human induced pluripotent stem cells (Meyer et al., 2009). Stem cells are grown in suspension to allow for the formation of aggregates that are eventually transferred in a medium containing N2 supplement and Heparin. In the following days, the aggregates are harvested and plated onto Laminin – coated dishes, where they soon form neural rosettes. On day 16 of differentiation, the medium is converted in a B27 supplement – containing one. Rosettes are then explanted and put in suspension in order to produce floating neurospheres. At this point, there is a consistent expression of markers belonging to a general neural lineage (Sox1, Sox2) but also of markers of the eye field (Rx, Pax6, Lhx2, Six3, Six6), whose co-expression has been assessed in some cases. Western blot and qPCR analysis confirm the endogenous production of both Noggin and Dkk-1 in this culture and the use of antagonists suppresses the expression of Pax6 and Rx. Since this procedure allows for the production of eye field cells, the authors proceed to describe how to reach the acquisition of optic vesicle and optic cup cell phenotypes. By day 40 of neurosphere suspension, Mitf and Chx10 are co-expressed. Importantly, nearly 26% of total cells are positive for Chx10 and more than 99% of them express also Pax6. Taken together, this information indicate that the culture contains early retinal progenitors cells.
By day 80 of differentiation in suspension, nearly 12% of total cells expresses Crx and among them 46.4% of cells is positive for more mature photoreceptor markers (Recoverin, cone Opsin). These results are shared with the ones obtained by differentiating human iPS cell line IMR90-4, even if changing cell line drastically affects Pax6 expression levels during the procedure.
Recently, a new protocol has been described specifically for mouse induced pluripotent stem cells (Parameswaran et al., 2010) (Fig.4).

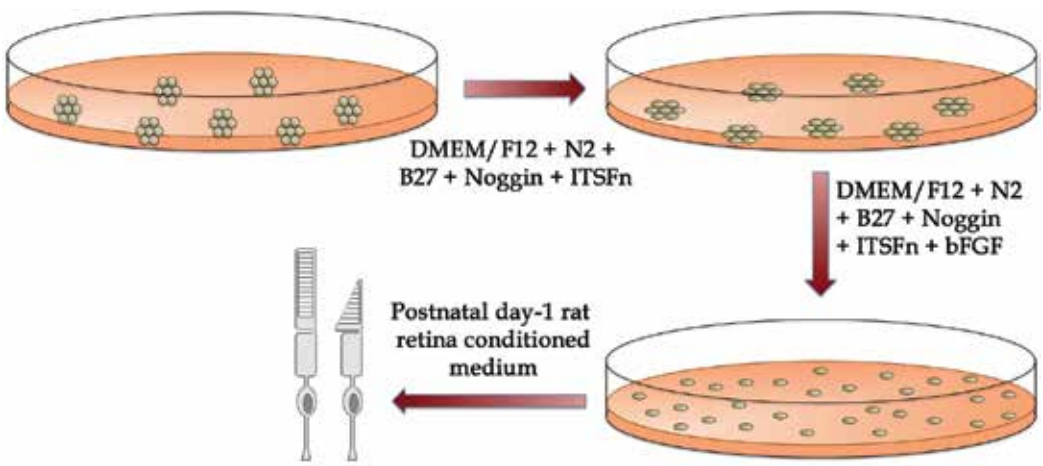

Fig. 4. Representation of the protocol of differentiation of mouse induced pluripotent stem cells into photoreceptors, described by Parameswaran et al., 2010.

A neural phenotype is obtained promoting embryoid bodies formation and culturing them in the presence of N2 supplement, B27 supplement, ITSFn and Noggin, following a previously described protocol (Okabe et al., 1996). Subsequently, the resulting colonies are trypsinized and expanded in the same medium added with bFGF for 25 days (day 40 of

differentiation). At this point, nearly 22.2% of total cells co-expresses Rx and Pax6. The differentiation is induced with N2 supplement and a medium conditioned by postnatal day-1 rat retinal cells. In 10 days, nearly 23% of total cells starts expressing Crx, while 15% expresses Nrl, 9% expresses Rhodopsin and 11.3% expresses S-Opsin.

Regarding the differentiation of stem cells into retinal pigmented epithelium cells, some of the protocols are shared with the ones described for neural retina cells induction.

Kawasaki and colleagues describe the production of neural cells from mouse embryonic stem cells using stromal cell – derived inducing activity (Kawasaki et al., 2000). In the same way, the co-culture of primate embryonic stem cells with the mouse stromal cell line PA6 allows for the induction of large patches of pigmented cells in nearly 8% of the colonies (Kawasaki et al., 2002). Further analyses confirm the presence of pigment granules and apical microvilli, both typical of RPE cells, and their capability to digest latex-beads as proof of the phagocytic function (Haruta et al., 2004). Pigmented cells may be obtained also by letting human embryonic stem cells spontaneously propagate and differentiate for 6-8 weeks as monolayer, in the absence of exogenous factors (Klimanskaya et al., 2004). Once they have reached confluence, patches of retinal pigmented epithelium appear and express typical RPE markers, such as Cellular Retinaldehyde-Binding Protein (CRALBP), Bestrophin and Pigmented Epithelium – Derived Factor (PEDF).

Culturing mouse embryonic stem cells on the stromal cell line PA6 in the presence of basic Fibroblast Growth Factor, Dexamethasone and Cholera Toxin, as previously mentioned (Hirano et al., 2003), leads to formation of eye – like structures. After 10 days, nearly 70% of colonies in culture comprises masses of cells displaying the first pigment granules (Aoki et al., 2006): depending on when these cells are collected and replated onto PA6 layer, it is possible to obtain other eye – like structures, RPE patches or single pigmented cells.

Retinal pigmented epithelium cells derived from embryonic stem cells can be used to induce the formation of neural retina cells. Primate embryonic stem cells can be differentiated into Pax6+ RPE cells by means of co-culture with Sertoli cells for 2 weeks (Yue et al., 2006). When primate embryonic stem cells are cultured for 3 days on these patches and Retinoic Acid is added to the medium, 25% of primate ES – derived cells are found expressing Rhodopsin in rosette – like structures (Yue et al., 2010). However Recoverin+ cells are only a few and they do not seem to be consistently affected by RA treatment (0.6% of total cells).

Following the protocol described by Osakada and colleagues, retinal pigmented epithelium cells can be obtained from monkey (Osakada et al., 2008) and human (Osakada et al., 2008; Osakada et al., 2009a) embryonic stem cells, positive for ZO-1 (40.1% and 34.7% of total cells respectively).

Meyer and colleagues describe using their protocol to obtain also RPE cells. If neural rosettes are kept in adhesion, by day 40 of differentiation patches of polygonal, pigmented cells positive for Mitf (25% of total cells) and ZO-1 are observed (Meyer et al., 2009).

Interesting experiments of retinal differentiation have been recently performed on *Xenopus laevis* using the animal cap essay (Yamada & Takata, 1961; reviewed in Ariizumi et al., 2009). Our group has demonstrated that Noggin overexpression is sufficient to induce the formation of a complete eye in this model, but only at high doses (Lan et al., 2009). Embryos at 2 – cells stage are microinjected with Noggin and GFP synthetic mRNAs: the animal caps are subsequently explanted at blastula stage and cultivated until stage 39 – when, during embryogenesis, the eye is almost completely formed. At this point, the cells within the aggregates express typical retinal markers (such as Opsin for photoreceptors, Vsx1 for

bipolar cells and Hermes for ganglion cells) and display a structure reminiscent of a wild type retina (Fig.5).

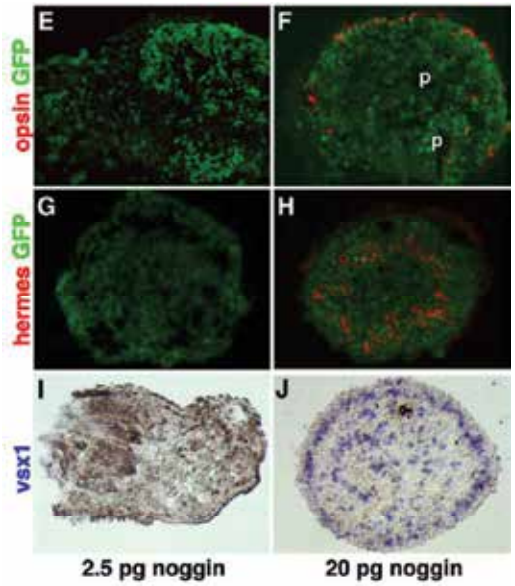

Fig. 5. Immunohistochemistry (E-H) and *in situ* hybridization (I-J) of animal caps treated with Noggin at low doses (2.5 pg) and high doses (20 pg). GFP fluorescence prove the correct procedure of microinjection. p: pigment (*from Lan et al., 2009*).

Moreover, an embryo precociously deprived of a single eye field can develop a complete eye when a treated animal cap is transplanted in the same position. Electrophysiological recordings demonstrate that the new eye elicits a correct response to light stimuli. Even more posterior transplants lead to the formation of an almost complete eye, but its cytoarchitecture seems to be more compromised (Fig.6).

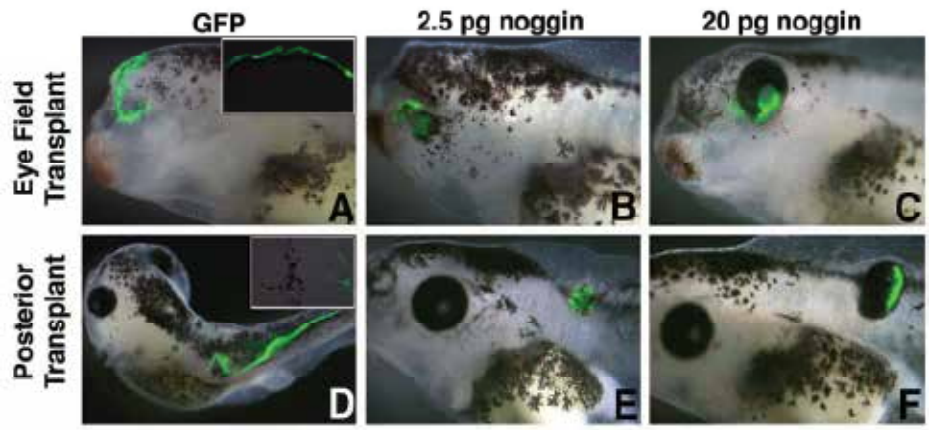

Fig. 6. Transplants of animal caps treated with GFP (A, D), GFP + Noggin at low doses (B, E) and GFP + Noggin at high doses (C, F). Anterior (A-C) and posterior (D-F) transplants are shown (*from Lan et al., 2009*).

Similar experiments have been carried out culturing *Xenopus* animal caps in the presence of mouse recombinant Noggin protein (Viczian et al., 2009). The results are equivalent to the ones we have described: Noggin enhances the formation of a complete eye, similarly to what the overexpression of the eye field transcription factors exert in the same model (Zuber et al., 2003).

4.2 Future strategies

The future of cell - based substitutive therapy seems to rely on direct reprogramming: instead of reprogramming somatic cells to a pluripotent state – and then using previously established protocols to induce differentiation toward specific fates – somatic cells can be directly reprogrammed into a neural phenotype (Vierbuchen et al., 2010). Mouse embryonic fibroblasts and tail – tip fibroblasts can be converted into mature neurons (19.5% of total cells) by a lentiviral transfection of Ascl1, Brn2 and Myt1l. Few experiments had been previously described in this direction, all focused on chick RPE cells reprogramming into retinal neurons (Ma et al., 2009; Li et al., 2010). However, at the moment there is no evidence of direct reprogramming of mouse or human fibroblasts into retinal progenitors or mature photoreceptors. Thus, a feasible strategy could aim to induce this type of differentiation, in order to avoid using embryonic stem cells and long manipulations. In a second moment, it will be necessary to perform the same procedure without using lentiviral delivery. This would bring research one step closer to actual therapy.

5. In vitro differentiated retinal cells as a tool for regenerative medicine and drug discovery

Retinal diseases are characterized by a permanent loss of retinal neurons that is accompanied by a gradual irreversible loss of visual capacity.

With the progression of the technologies and the adventure of stem cells era, many efforts are focused on the possibility to differentiate specific cell types *in vitro* and to transplant them in the injured organs including retina. During the initial phases of retinal degeneration, photoreceptors or RPE cells die, but the inner microarchitetture of the retina is not yet altered, suggesting the possibility to introduce *in vitro* differentiated retinal cells to replace the lost cells.

In parallel with regenerative medicine, another important application of *in vitro* differentiated retinal cells is represented by drug discovery. In fact, a large portion of current therapies for retinal diseases takes in consideration treatments with bioactive molecules (endobiotics or xenobiotics), that prevent or retard the pathology insurgence. The possibility to have an inexhaustible source of retinal cells *in vitro* could be a way to develop a strategy to screen a large numbers of new molecules and possible drugs in short periods of time.

5.1 Cell replacement strategies for retinal repair

Cell replacement therapies are innovative therapeutic strategies that allow the restoration of visual responses to the degenerate adult neural retina and represent an exciting area of regenerative medicine. So far, it has been shown that transplanted postmitotic photoreceptor precursors are able to functionally integrate into the adult mouse neural retina. Recent reviews discuss the differentiation of photoreceptor cells from both adult and embryonic-derived stem cells, their potential for retinal cell transplantation and possible strategies used

to overcome barriers present in the degenerate neural retina and improve retinal cell integration (Lamba et al., 2009b; West et al., 2009).

5.2 Future possibilities for drug discovery

The availability of libraries of chemical compounds is today a critical tool in drug discovery. There is now the possibility to perform High – Throughput - Screenings (HTS) using small molecule libraries, to search for new molecules able to reduce cell death and/or to enhance survival of degenerating photoreceptors. Photoreceptor cell death is the major hallmark of most inherited retinal dystrophies. Although the genetic causes are not always identified, and the mechanisms leading to photoreceptor degeneration poorly defined, some common features of the cell death have been identified (Marigo, 2007; Wright et al., 2010). It is thus possible to use as a readout of the HTS a TUNEL assay, that allows to efficiently isolate those molecules able to reduce cell death. The selected (and subsequently validated) molecules could be new interesting pharmaceutical leads, that could then be further analyzed and exploited. In this way, it is possible to identify new uses of drugs that are already pharmacologically and toxicologically well characterized. This can accelerate and speed up animal and clinical trials, permitting to obtain a new pharmacologically active drugs in a relatively short time (Osakada & Takahashi, 2009).

6. Conclusions

In the past years the molecular mechanisms underlying retinal development and differentiation have been at least partially elucidated, probably enabling the retina to become the best characterized region of the CNS and at the same time to be an attractive system for regenerative medicine. Moreover, the functional contribution of transplanted and integrated cells can be assessed by the analysis of visually guided behavior, thus making it possible to study in this system neuronal regeneration and replacement in the CNS. Over the next few years some of the hurdles to overcome to make cell replacement a clinical reality concern the efficiency of both in vitro retinal differentiation protocols, and functional integration of the transplanted cells. Non mammalian vertebrates will remain to be interesting systems to study retinal differentiation and regeneration mechanisms, that will allow to draw important conclusions, to be used also in mammalian retinal biology.

7. References

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Part 4

Tissue-specific Regeneration of Hematopoietic Systems

13

Generation of Blood Cells from Human Embryonic Stem Cells and Their Possible Clinical Utilization

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1. Introduction

All cellular blood components in adults are derived from hematopoietic stem cells resided in bone marrow (BM). However, along with the events in ontogeny, the process of hematopoiesis is a long and complex progression over time and space. It is classically assumed that the first blood cells generated in blood islands of the extraembryonic yolk sac are large nucleated erythroblasts, representing a primitive wave of the initial hematopoiesis. A second wave of the blood cell generation, named definitive hematopoiesis, has its origin in the aorta/gonad/mesonephros (AGM) region. These definitive hematopoietic cells (HCs) are endowed with property of hematopoietic stem cells that can rescue lethally radiated animals and hold the potential to generate all blood cell progenies. Consequently, hematopoiesis is shifted to fetal liver in the midgestation and later to BM, where hematopoietic stem cells inhabit life long. Recent findings support a model that yolk sac also provides committed and mature blood cells with multipotential property, allowing survival until AGM-derived hematopoietic stem cells can emerge, and then seed the liver and differentiate into mature blood cells.

Transplantation of human hematopoietic stem cells (HSCs) in clinical therapies has been well applied to the patients suffering with malignant dysfunction of the hematopoietic system or after deadly radiation therapy for cure of leukemia. Although knowledge about the mechanisms underpinning the early development of hematopoiesis during embryonic and fetal stages have been largely expounded by various gene-targeting technologies, because of the restriction to use living human embryos, the early genesis of the human hematopoietic system, especially during embryonic / fetal stages, is largely unknown.

Recently, the establishment of human embryonic stem cells (hESCs) greatly expanded our view to elucidate the events in early human ontogeny. The ESCs derived from the inner cell mass of the human balstocyst are capable of growing indefinitely while maintaining pluripotency, namely to differentiate into all tissues of the body, including blood cells.

The characteristics of both stemness and multipotency provide two main expectations on hESCs in basic research and clinical applications. First, they provide models for studies of basic disease mechanisms, screens for drug discovery, and tissue engineering for new treatments for diseases such as diabetes, spinal cord injury, Parkinson's disease, myocardial infarction and cancers. On the other hand, the pluripotency and embryonic property of these stem cells provide a unique tool in exploring the basic mechanisms of early development and differentiation of human beings, which never can be mimicked in lower level species.

During the past decade, hESCs have been utilized to characterize molecular and cellular mechanisms controlling the differentiation of hematopoietic progenitors and mature, functional blood cells. Almost all types of blood cells derived from hESCs have recently been reported, including functionally mature erythrocytes and neutrophils, platelets, megakaryocytes, eosinophils, monocytes, dendritic cells (DCs), nature killer (NK) cells, mast cells (MCs), and B-, T-lineage lymphoid cells. The advances in research are leading to a clinical translation of hESC-derived HCs as novel therapies in near future. Based on recent success, the initial clinical application of blood cells derived from hESCs will possibly be in the field of transfusion therapies (erythrocytes and platelets) and immune therapies (NK cells and DCs). However, hESC-derived hematopoietic stem cells capable of long-term, multilineage engraftment are still under searching. On the other hand, ethical recognition must be appropriately addressed before clinical utilization of hESC-derived cellular therapies.

This review outlines the current progress, including data collected in our laboratory, in the research on hESC-derived hematopoiesis and the aspects of what needs to be tackled in future in this research fields. The possibility of hESC-derived cellular therapies in clinical application will also be discussed.

2. Methodology

ESCs are cells capable of being indefinitely growing with multipotency if provided appropriate culture conditions. At the same time, they undergo spontaneous and synchronous differentiation into all cell lineages when deprived from the optimal conditions. Because ESCs under the undifferentiated state form teratomas when transplanted into the living body, the efficient in vitro induction of ESCs to differentiate into a specific cell lineage are of importance.

The earliest report for mouse ESCs to generate hematopoietic and mature blood cells was published two and half decades ago (TC. Doetschman, et al. 1985). By a coculture system, Nakano et al. successfully induced undifferentiated mouse ESCs into almost all lineages of mature blood cells (T. Nakano, et al. 1994), providing evidence that ESCs may play a role as sources of blood cells, experimentally as well as clinically. After then, the mouse stromal cell line they used, OP9, became a widely and standard matrix to be used to induce hematopoiesis both in mouse and human ESCs.

Since the first study on hESC-derived hematopoiesis reported (D. Kaufman, et al. 2001), derivations of mature blood cells from hESCs have been confirmed by many groups including ours. The efficiency and stability of the in vitro blood cell inducing system have also been improved. However, by different culture systems, hESC-derived blood cells are more or less diverse in their maturities. The methods commonly used to develop blood cells

from hESCs are categorized as: (1) the formation of embryoid bodies (EBs, three-dimensional colonies of differentiating ESCs) and (2) the coculturing of ESCs with stromal layers.

When formed EBs in suspension cultures, hESCs develop into a sac-like structure mimicking the early development of the zygote. Within the EBs, hESC-derived cells interact with each other among a microenvironment semi-separated from the culture medium, providing a suitable condition for differentiation, mainly spontaneous. Because the EBs mimic the early yolk sac structure, when with the stimulation of hematopoiesis-directing factors, the early blood cells including primitive erythrocytes can be gained. However, because accessibility of the external factors into this complex structure is limited, EBs may be disadvantageous in regulating differentiation of hESCs toward definitive hematopoiesis to generate fully matured blood cells. (H. Sakamoto, et al. 2010).

While EBs serve a microenvironment for initiation of primitive hematopoiesis, the coculture of ESCs with stromal cells, most of them derived from fetal /newborn hematopoietic niches, provide a more subtle and efficient way to generate mature blood cells. There are a variety of cell lines employed in coculture systems with mouse and human ESCs, among them the OP9 being most widely used (T. Nakano,et al. 1994; Y. Mukouyama, et al. 1998). OP9 was established from an op/op mouse deficient in macrophage colony-stimulating factor (M-CSF), and has some deleterious effects on the early development of HCs. Results accumulated from murine experiments showed that Flk1-expressing cells, representing the development of mesoderm, in EBs are detected up to day 4 and their number declines thereafter (WJ. Zhang, et al. 2005). While cocultures of mouse ESCs and OP9 cells give rise to high-level expression of Flk1 up to day 6 in differentiation, suggesting a prolonged mesodermal development may provide a proper environment for the ESCs to differentiate to HCs when cocultured with OP9.

By coculture with OP9 cells, differentiation of the HCs from mouse and human ESCs into various blood cell lineages can be observed. In mouse, coculture of ESCs with OP9 to generate erythrocytes (T. Nakano, et al. 1994; N. Motoyama, et al. 1999), B-lymphocytes (T. Nakano, et al. 1994; SK Cho, et al. 1999), megakaryocytes, NK cells and DCs (T. Era, et al. 2000; N. Nakayama, et al. 1998; S. Senju, et al. 2003) have been reported. OP9 cells expressing Delta-like ligand 1 (OP9-DL1), a ligand of Notch, also induce the differentiation of hematopoietic progenitors into T lymphocytes (TM. Schmitt, et al. 2004). OP9 and OP9-DL1 cells now have been widely used to induce the differentiation of HCs from both nonhuman primate and human ESCs (II Slukvin, et al. 2006; K. Umeda, et al. 2004; M Gaur, et al. 2006; N Takayama, et al. 2008; F. Timmermans, et al. 2009).

We also have reported efficient methods to induce human and non-human primate ESCs to differentiate into HCs by coculture with mouse AGM region-derived and fetal liver-derived stromal cells (MJ. Xu, et al. 1998; F. Ma, et al. 2001; F. Ma, et al. 2007; F. Ma, et al. 2008a; F. Ma, et al. 2008b). With these mouse fetal hematopoiesis-centered tissue stromas, human and non-human primate ESCs generate functionally mature blood cells through a first primitive hematopoiesis wave, mimicking the early hematopoiesis during the yolk sac stage, and then definitive hematopoiesis pathway.

3. Hematopoietic progenitor cells derived from hESCs

Since the first establishment of hESC lines had been done 12 years ago (JA. Thomson, et al. 1998), knowledge about the early hematopoiesis during human embryonic stage has been

extensively refreshed by applying hESC-differentiating methods. Through a large variety of experiments, it has been identified that the first progenitor cells holding hematopoietic activity mostly share an endothelial cell (EC) potential, thus they are named as hemangioblasts (ET. Zambidis, et al. 2005; M. Kennedy, et al. 2007). The onset of the EC and HC bipotential progenitors derived from hESCs express FLK-1, CD31, VE-Cadherin, CD34, but lacking CD45 on their surface (CD45- PFV cells) (L. Wang, et al. 2004), giving rise to both ECs and HCs when properly induced. Furthermore, this fraction of CD45- PFV cells can reconstitute the hematopoietic system in immunocompromised mice when injected into the bone marrow (L. Wang, et al. 2005a, L. Wang, et al. 2005b), suggesting that the CD45- PFV population containing hematopoietic stem cells.

When continuously maintained on an environment favoring hematopoiesis development, such as coculture on OP9 or on fetal liver stromal cells, these hemangioblastic progenitors further differentiate into mature blood cells (ET. Zambidis, et al. 2005; F. Ma, et al, 2008a). The hESC-derived hematopoietic progenitors coexpress CD34, CD43 and CD45, and give rise to myeloid and lymphoid cells (MA. Vodyanik, et al. 2006). Through a coculture with OP9 cells, a hESC-derived common myeloid progenitor cell fraction that share a phenotype of lineage specific marker- CD34+CD45+ CD43+ are capable of generating functionally mature myelomonocytic cells with high efficiency, including neutrophils, eosinophils, macrophages, osteoclasts, dentritic cells and Langerhans cells (KD. Choi, et al. 2009). These techniques for generating hESC-derived hematopoietic progenitor cells, especially multipotential myeloid progenitors, may play roles in searching and expanding new clinical approaches by generating large number of patient-specific cells for in vitro study and drug screening.

4. Functionally mature blood cells derived from hESCs.

By applying lineage-specific stimulation methods such as addition of cytokines or culturing on normal or genetically manipulated stromal cells, hESCs can be further induced to functionally mature cells along to a specific lineage with high purity. This ensures a distinct scientific base to trace the early development of human hematopoiesis along with a specific blood cell lineage, especially when hESCs are used as models. In addition, hESCs may provide a novel source for regenerative medicine. To fulfill this aim, induction of hESC-derived blood cells with full maturation is critical. Recently, various mature blood cells with functional maturation have been produced in vitro, challeging to translate use of these cells to clinical application.

Notably, the hESC-derived erytrocytes and pletelets should be the most feasible products in near future clinical applications, because both RBCs and platelets do not have a nucleus and are with minimal genetic material thus role out the possibility for malignant transformation of these particular cell types.

4.1 Erythrocytes

The limitation of blood sources hampers the sufficient utilization of red blood cells (RBCs) in transfusion medicine. Sufficient blood supply is always in great demand from a therapeutic standpoint. Since the mature RBCs lack nuclei and are free of concerns for tumagenicity, they represent an attractive, maybe the first generation of, product from the stem cell derivations. Large-scale production of erythroid cells from hESCs may provide

us a novel and safer source of RBCs for transfusions. hESC-derived mature erythrocytes with a universal blood type such as blood group O and suppressed expression of HLA molecules will be an ideal source of erythrocytes in transfusions. Large-scale productions of mature erythroid cells from hESCs have recently been reported by several groups, including ours (F. Ma, et al. 2008a; SJ Lu, et al. 2008; EN. Olivier, et al. 2006). By co-culturing hESCs with murine fetal liver-derived stromal cells, we first produced multipotential hematopoietic progenitors that could give rise to huge pure erythroid colonies. After harvesting these pure erythroid colonies, we successfully obtained large quantity of mature erythroid cells. When we traced these hESC-derived erythrocytes at clone level, we found that hESC-derived progenitors were fated mostly to become definitive erythrocytes that finally undergo enucleation, switching to adult-type β-globin at almost 100% along times in culture. Furthermore, these hESC-derived mature erythrocytes functioned as oxygen carriers. As much as 1 $x10^4$ undifferentiated hESCs roughly generated 1 x 10^6 mature erythrocytes (F. Ma, et al. 2008a). Our study not only provide evidence that hESC-derived erythrocytes can be induced to a definitive stage with functional maturity, but also offer a method to scale up the production of erythrocytes that may be employed in future clinical use. Actually, by a multistage protocol involving EB formation, defined cytokines plus a recombinant tPTD-HOXB4 protein to produce hematopoietic differentiation, Lu SJ et al also achieved up to 10^{10} to 10^{11} RBCs from one 6-well plate of undifferentiated hESCs (SJ. Lu, et al. 2008). However, although a promising direction has been provided, substantial effort should still be paid to bring hESC-derived RBCs to a scale needed for future clinical applications. Since the transfusion therapy is routinely applied in daily surgeries and the insufficiency of fresh blood sources always remains an headeache worldwidely, research on hESC-derived erythrocytes should be predominantly pushed up and hESC-derived RBCs may serve the first product from the benifit of stem cells.

4.2 Megakaryocytes and platelets

For the same reason as hESC-derived RBCs, platelets derived from hESCs will also meet the potential need for future transfusion medicin.When cocultured with murine bone marrow stromal (S17) and yolk sac endothelial cell (C166) lines, Kaufman et al produced hematopoietic progenitors that could generate mature megakaryocyte-containing clononies in semisolid culture (D. Kaufman, et al, 2001). Gaur et al applied a coculture with OP9 stromal cells to generate megakaryocytes from hESCs with characteristic DNA polyploid nucleus, specific cytoskeletal and surface proteins, and ability to signal through integrin αIIbβ_3 (M. Gaur, et al, 2006). However, they did not confirm the production of pletelets from these hESC-derived magakaryocytes. Subsequently, using coculture with either OP9 or C3H10T1/2 cells in the presence of thrombopoietin for longer periods of time (over 3 weeks), Takayama et al made a comparitively large production of mature hESC-derived megakaryocytes (2–5 x 10^5 platelet-producing megakaryocytes per 10^5 undifferentiated hESCs) (N. Takayama, et al. 2008). These hESC-generated mature megakaryocytes produced platelets with morphology and function similar to those human pletelets isolated from fresh plasma. However, the lower yeilding of hESC-derived pletelets when compared to in vivo process indicate that further improvement should be paid to reach a possible clinical trial.

4.3 Neutrophils

Human neutrophils are the most primary constituent of the peripheral blood leukocytes and play a central role in host defense against the invasion of microorganisms. In some cases, congenital leukocyte function deficiencies and myelosuppressions caused by chemo- or radiotherapies need granulocyte transfusion therapy to protect the patient from lethal infections.

Saeki et al reported a two-step method to generate mature neutrophils from hESCs (K. Saeki, et al, 2009). They first made formation of hESC-derived spheres by adding cytokines favoring the development of hematopoietic progenitor cells. After replated to adherent culture for 2 to 3 weeks, these hESC-derived spheres form sac-like structures holding round mature myeloid cell, with an approximately 40-50% ratio of mature neutrophils. Although these hESC-derived neutrophils phenotypically and functionally mimicked human mature neutrophils, their production is comparatively low (1×10^6 undifferentiated hESCs generate 1×10^6 mature neutrophils). A more efficient method by first making EB formation and then coculturing with OP9 cells had been applied to generate hESC-derived mature neutrophils (Y. Yokoyama, et al, 2009). In this system, high purity of mature neutrophils could be induced within 2 week in culture. These hESC-derived mature neutrophils showed various functions such as superoxide production, phagocytosis, bactericidal activity and chemotaxis that were similar to those with peripheral blood counterparts. Although these studies provided good culture system to research on the development and functional maturation of hESC-derived neutrophils, they are still difficult to be used clinically as a transfusion therapy model.

4.4 Nature killer cells

NK cells stand at the center in immune defenses against pathogens and malignant tumors. Human NK cells provide critical cell-mediated antitumor activities. Furthermore, clinical trials have already confirmed the transplantable NK cells in recipient patients, suggesting the possible new therapy may be conducted by the NK cell transfusion to cure cancers (JS. Miller, et al. 2005; L. Ruggeri, et al, 2002). Thus, if properly induced to be mature NK cells, the unlimited potential of hESC may provide an ideal source of human NK cells that can be used in extensive antitumor therapies.

Actually, the first confirmation of hESC-derived functional lymphocytes was NK cells (PS. Woll, et al, 2005). By a 2-step culture method, $CD56^+CD45^+$ lymphocytes with a function like mature NK cells could be induced from hESCs. The hESC-derived NK cells express killer cell-specific markers such as Ig-like receptors, natural cytotoxicity receptors, and CD16. These hESC-derived NK cells were able to lyse human tumor cells by both direct cell-mediated cytotoxicity and antibody-dependent cellular cytotoxicity, showing their full function of antitumor activities. More recently, interesting result has been reported by the same research group, showing that hESC-derived mature NK cells are more efficiently to clear human tumor cells in vivo than human cord blood derived NK cells, suggesting a potential clinical use for hESC-derived NK cells in cancer therapy (PS. Woll, et al. 2009).

4.5 T- and B-lymphocytes

Some earlier reports suggested development of lymphocytes from hESCs based on surface staining of markers such as CD3 (T cells) or CD19 (B cells) and RT–PCR analysis (X. Zhan, et al, 2004; MA. Vodyanik, et al. 2005), but without functional assays. By first using coculture with OP9 stromal cells to differentiate GFP-expressing hESCs into $CD34^+$ and $CD133^+$ cells and then injected them into human thymic tissues engrafted immunodeficient mice (SCID-

hu mouse model), Galic et al successfully made engrafts of hESC-derived mature T-lymphcytes in vivo (Z. Galic, et al, 2006). These hESC-derived T cells expressed T-specific surface markers such as CD4, CD8, CD1a, and CD7. Sequentially, the same research group has applied EB-mediated differentiation to generate T-cell progenitor cells in the SCID-hu model (Z. Galic, et al. 2009). In addition, function of the hESC-derived T cells has been tested based on increased expression of CD25 after CD3/CD28-mediated activation. However, the engraftment of the hESC-derived T cells in the SCID-hu model is low (1% or less). Comparing to myeloid cells, it has proven difficult to induce hESC-derived hematopoietic progenitors to further develop into mature T- and B-lymphoid lineage cells.

To allow more access to developing cells and improving conditions that support or inhibit development of T cells, a Notch ligand–expressing OP9-DL1 stromal cells have been used to derive T cells from multiple progenitor cell populations expressing CD34 and CD45 such as human BM, umbilical cord blood, and mouse ESCs (RF. de Pooter , et al. 2003; CH. Martin, et al, 2008; TM. Schmitt, et al, 2004; RN. La Motte-Mohs, et al. 2005). However, the same hESC-derived $CD34^{+}CD45^{+}$ cells that effectively produce NK cells from hESCs were unable to produce T cells in this in vitro system (D. Kaufman, 2009), suggesting a different condition may be needed for hESC-derived T cell development.

Recently, Timmermans et al reported that a specific population of hESC-derived $CD34^{+}CD43^{low}$ cells that were present in hematopoietic zones morphologically similar to blood islands (F. Timmermans, et al. 2009). By first coculture with OP9 and then with OP9-DL1 cells, they demonstrated in vitro development of mature T cells from hESCs. In their system, hESC-derived T cells typically developed through a sequential pathway, initially committed to a CD34+CD7+ T/NK common potential stage, then to CD7+CD4+CD8- single positive and CD4+CD8+ double positive satges, and finally to CD3+CD1-CD27+ mature T cell stage. This promising study provided a new approach to use hESCs to generate T cells for novel immunotherapy.

4.6 Other mature blood cells

Derivation of dendritic cells (DCs) from hESCs have also been reported (X Zhan, et al, 2004; II Slukvin, et al, 2006; S Senju, et al, 2007; Z Su, et al, 2008). These hESC-derived DCs expressed high levels of HLA class II molecules and showed an ability to stimulate leukocyte reactions as an in vitro measure of immune activity. Function of antigen uptaking and processing, and stimulating allogeneic and antigen-specific T-cell responses have been demonstrated on these hESC-derived DCs.

By culturing clonal hematopoietic cells derived from hESC in semisolid culture, we demonstrated the derivation of mature mast cells (MC) that held tryptase, but few chymase (F. Ma. et al, 2007). Recently, functionally matured mast cells (MC) have been induced from hESCs (M. Kovarova, et al. 2010). These hESC-derived MCs respond to antigen by releasing MC specific mediators, providing a useful model to analyze human MC development and may be possibly useful in drug screening for allergic diseases.

5. Future prospect

The establishment of hESCs brought forth a totally new generation of regenerative medicine. The unlimited potential of hESCs ensures their ability to derive almost all the tissue types in our living bodies, thus constructing a base for the future clinical use. However, before the clinical application of using hESC-derived hematopoietic cells, there

are still several gaps should be overcome. Firstly, an efficient and animal source-deprived culture system is needed to ensure the safety from infectious diseases and species-crossing genetic transfections. Secondly, for applying transfusion therapy by using hESC-derived RBCs, more efficient in vitro culture system should be promoted to ensure a large-scale production of enucleated hESC-derived RBCs. Third, since the real hESC-derived hematopoietic stem cells that can fulfill reconstitution has still yet been defined, efforts should be paid to search for a way by employing novel method to characterize the properties of the possible hESC-derived stem cells. Finally, to guarantee efficient and safe clinical use, attention should also be paid to develop standardization and stability of the cell culture system. The clinical need for new and better therapies by using hESC-derived cellular products should remain greater than any barriers and unanswered questions.

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14

Hematopoietic Differentiation from Embryonic Stem Cells

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1. Introduction

Since the development of human embryonic stem cells (hESCs) in 1998, the potential of stem cell-based manufacturing of tissues or organs as a form of regenerative medicine has drawn broad interest because hESCs are pluripotent and can proliferate infinitely without losing their pluripotency (Thomson et al., 1998). More recently, induced pluripotent stem cells (iPSCs) were generated from human fibroblasts (Takahashi et al., 2007) as well as other cell sources (Stadtfeld and Hochedlinger, 2010), thus accelerating the goals of research to realize regenerative medicine.

Theoretically, any organ can be generated from ESCs, but the obstacles to manufacturing solid organs *in vitro* remain great. Solid organs such as kidney and liver require well-functioning three-dimensional structures consisting of different kinds of cells as well as formation of and communication with blood vessels. Considering this, hematopoietic progenitors or mature blood cells derived from ESCs may be among the most attractive applications because blood cells can operate as single cells without forming a multicellular structure. Here, we describe methods of hematopoietic differentiation from ESCs, particularly focusing on hESCs, and some problems that need to be resolved before hESC-derived blood cells can be applied in the clinical setting.

2. Summary of hematopoietic differentiation from murine ESCs

2.1 Hematopoietic progenitor differentiation from murine ESCs

Since the days before and even after the development of hESCs, murine ESCs (mESCs) (Evans and Kaufman, 1981; Martin, 1981) have been a major tool in the study of differentiation of pluripotent stem cells into specialized cells. Although it is now recognized that there are a number of differences between mESCs and hESCs (Bhatia, 2007) and that it is indeed sometimes impossible to apply the differentiation protocols used in mESC experiments directly to those for hESCs, experiments using mESCs have provided us with important fundamentals for hESC research.

Methods developed to induce hematopoietic differentiation from mESCs can be classified into two categories: methods that use formation of embryoid bodies (EBs) (Doetschman et

al., 1985) and those that use coculture with feeder cells (Gutierrez-Ramos and Palacios, 1992). In addition, genetic manipulation was also adopted for derivation of hematopoietic cells from mESCs (Kyba et al., 2002; Perlingeiro et al., 2001).

Embryoid bodies are cystic structures obtained by culturing ESC colonies in floating conditions in liquid or semisolid media. Hematopoietic differentiation in EBs is induced effectively by appropriate cytokine stimulation (Johansson and Wiles, 1995; Nakayama et al., 2000). In the early reports, only erythroid cells were detected in EBs (Doetschman et al., 1985; Lindenbaum and Grosveld, 1990). In 1991, it was reported that macrophages, neutrophils, and mast cells were also differentiated by semisolid culture of EBs in the presence of interleukin (IL)-3 (Wiles and Keller, 1991). The same group reported that bone morphogenic protein-4 (BMP-4) mediated formation of ventral mesoderm and hematopoietic precursors from EBs (Johansson and Wiles, 1995). A later study revealed that BMP-4 promoted generation of both myeloid and lymphoid precursors from EBs, and that this effect of BMP-4 was enhanced by addition of vascular endothelial growth factor (VEGF), although VEGF alone did not have a positive effect on hematopoietic differentiation from EBs (Nakayama et al., 2000).

Although EB formation is a useful method for generating hematopoietic cells with myelopoietic and lymphopoietic potentials, early methods using EB formation did not succeed in generating lymphoid progenitors. The first report of simultaneous generation of both myeloid and lymphoid lineages from mESCs adopted a coculture system. In this report, mESCs were cultured on OP9 feeder cells derived from calvaria of newborn (C57BL/6×C3H)F2-*op*/*op* mice without addition of exogenous cytokines (Nakano et al., 1994). This simple culture system enabled mESCs to differentiate into hematopoietic progenitors that could differentiate into cells of various myeloid lineages as well as of B lymphocyte lineage. Thereafter, this cell line has been the standard feeder for hematopoietic differentiation not only from mESCs but also from hESCs. Other than OP9 cells, some cell lines, such as aorta-gonad-mesonephros (AGM) region-derived stromal cells (Weisel et al., 2006) or bone marrow-derived ST2 cells (Yamane et al., 2009), were also used but the OP9 cell-based method seems to be the most commonly used.

To advance the understanding of regulation of hematopoietic differentiation from mESCs, genetic manipulation of mESCs was frequently used. To demonstrate strictly that a single mESC-derived hematopoietic stem cell (HSC) could produce all lineages of mature blood cells *in vivo*, clonal analysis was performed using a gene transfer protocol (Perlingeiro et al., 2001). The chronic myeloid leukemia-associated oncogene bcr/abl was transferred to EB-derived hematopoietic progenitors. The cells were further cultured on OP9 cells and thereafter cloned and transplanted into irradiated recipient mice. These cloned bcr/abl-expressing cells differentiated into multiple myeloid lineages as well as into T and B lymphocytes *in vivo*, indicating that definitive HSCs could be generated from EB-derived progenitors by transformation using bcr/abl.

A homeotic selector gene, HoxB4, proved to be a key factor in transforming EB-derived hematopoietic progenitors into definitive HSCs (Kyba et al., 2002). Like transformation by bcr/abl, ectopic expression of HoxB4 switched EB-derived primitive progenitors into definitive HSCs capable of long-term multilineage hematopoiesis. These approaches may provide further understanding of the mechanisms of HSC emergence from ESCs as well as from primordial cells during embryogenesis, although ESCs generated by means of genetic manipulation may confront further difficulties when clinical application is directly considered.

2.2 Lineage-specific differentiation of mature blood cells from murine ESCs

In addition to HSC generation, lineage-specific differentiation of mature blood cells from mESCs has also been an important issue. Homogeneous populations of mESC-derived mature cells can be used in functional analyses and could form the basis of future hESC-based transfusion medicine. Studying the process of differentiation from mESCs to mature cells can lead to understanding of normal hematopoiesis as well. Similar to HSC induction, mature blood cells are usually generated by EB formation, coculture with feeder cells, or a combination of both. Lieber et al. described an effective three-step protocol for differentiating mESCs into mature neutrophils (Lieber et al., 2004). First, EBs were formed and cultured in Iscove modified Dulbecco medium (IMDM)-based fetal calf serum (FCS)-containing medium for 8 days. Second, the EBs were disaggregated and replated onto OP9 cells in IMDM containing fetal bovine serum (FBS) and horse serum supplemented with oncostatin M, basic fibroblast growth factor (bFGF), IL-6, IL-11, and leukemia inhibitory factor (LIF) for 3 days. Finally, the cells were terminally differentiated on OP9 cells in IMDM containing platelet-depleted serum, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6. During 7 to 14 days of terminal differentiation culture, 6×10^6 neutrophils were obtained from 8×10^4 mESCs.The purity of the mature neutrophils during this period reached 75% to 96%. These mESC-derived mature neutrophils expressed neutrophil-specific markers (Gr-1 and others) and contained gelatinase- and lactoferrin-positive granules. In the functional assays, mESC-derived neutrophils showed superoxide production and chemotaxis comparable to those of normal neutrophils harvested from murine bone marrow. Interestingly, neutrophils differentiated from MEKK1-deficient mESCs displayed impaired migratory ability. This result indicated that mESC-derived neutrophils could be used to study the genetic control of neutrophil differentiation and functions.

As regards the application of hESC-derived mature blood cells to transfusion medicine, the successful treatment of anemic mice by transfusion of mESC-derived erythroid progenitors was of great impact (Hiroyama et al., 2008). For the differentiation, mESCs were cultured on OP9 cells in IMDM-based medium with VEGF and insulin-like growth factor-II. On day 4, dexamethasone was added, and stem cell factor (SCF), erythropoietin (EPO), and IL-3 were substituted for the cytokines, although IL-3 could be omitted. After long-term culture in these conditions, immortalized erythroid cell lines were obtained. These mESC-derived erythroid progenitor lines expressed adult type α- and β-globins but did not express γ-, ε-, and ζ-globins, indicating that they were adult-type erythroid cells. The erythroid progenitors could be differentiated *in vitro* into mature and enucleated cells. The erythroid progenitors could proliferate and differentiate *in vivo*, and when transplanted into anemic mice in which acute anemia was induced by hemolysis, the anemia ameliorated and the mice showed greater survival rates. Notably, no tumors were observed in the erythroid progenitor-transplanted mice for at least 6 months. These results are encouraging for future transfusion medicine using hESC-derived cells, although thorough investigation into the possibilities of tumorigenesis is needed.

Megakaryocytes and platelets were effectively produced by combination of EB formation and coculture with OP9 cells (Nishikii et al., 2008). After EB culture for 6 days, megakaryocyte progenitors expressing both c-kit and integrin αIIb were sorted and further cultured on OP9 cells with TPO. For terminal differentiation, a mixture of TPO, IL-6, and IL-11 was substituted for the cytokines after 3 days. Using this method, 2×10^5 mESCs produced 1×10^6 megakaryocyte progenitors on day 6 and 2.5×10^6 megakaryocytes on day 12. After 2

to 8 days of coculture with OP9 cells, the culture supernatants contained proplatelets and platelets. Electron microscopy analysis revealed that they contained alpha and dense granules, same as platelets from adult mice. However, these mESC-derived platelets showed low levels of glycoprotein (GP) Ibα expression, and in the *in vitro* thrombus formation model, mESC-derived platelets had impaired ability to participate in thrombogenesis, which is triggered by binding of von Willebrand factor to GPIbα. Interestingly, shedding of GPIbα was prevented by addition of metalloproteinase inhibitors to the culture medium during differentiation, and this inhibition improved the thrombogenetic ability of the mESC-derived platelets. The effect of inhibition of metalloproteinase activity was further examined using an *in vivo* model. Murine ESC-derived platelets with or without metalloproteinase inhibition were transfused into irradiated mice with severe thrombocytopenia. Addition of metalloproteinase inhibitors increased the percentage of mESC-derived platelets in the peripheral blood of the transfused mice.

A simple, well-established method is used for T cell differentiation from mESCs: coculture with OP9 cells ectopically expressing the Notch ligand Delta-like 1 (OP9-DL1) (Schmitt et al., 2004). On day 14 of the coculture, mESC-derived cells contained CD4 and CD8 double-negative (DN) T lymphocyte progenitors, and on day 20, these cells contained double-positive (DP) cells. When mESC-derived $CD25^+$ DN progenitors were differentiated using deoxyguanosine-treated fetal thymic organ culture, they generated DP T cells and CD4 or CD8 single-positive (SP) T cells. Furthermore, when these thymic lobes containing mESC-derived T cells were implanted under the skin of sublethally irradiated *Rag2*-null mice, which are devoid of T and B lymphocytes, reconstitution with mESC-derived CD4 or CD8 SP cells was observed. Infection of these mice with lymphocytic choriomeningitis virus (LCMV) induced LCMV-specific cytotoxic T lymphocyte activity, indicating that mESC-derived mature T cells are capable of producing an effective antigen-specific immune response. As for B cells, coculture with OP9 cells induced B lineage development (Nakano et al., 1994), and this was enhanced by addition of Flt-3 ligand (FL) from day 5 of the coculture (Cho et al., 1999). After 4 weeks, more than 90% of the cells were $CD45R^+CD19^+$ B cells. In another report, knock down of PU.1 by small interfering RNA in $CD34^+$ cells produced by EB formation induced $CD19^+CD43^+CD45^+$ progenitor B (pro-B) cells (Zou et al., 2005). These mESC-derived pro-B cells produced precursor B (pre-B) cell colonies after a week of culture in a semisolid medium with IL-7 and IL-10, and a further 3-weeks culture enabled the pre-B cells to differentiate into mature B cells coexpressing immunoglobulin (Ig) M and CD19. These B cells produced by coculture with OP9 cells or by PU.1 knock down in EB cells showed up-regulation of CD80 and secretion of IgM by stimulation with lipopolysaccharide. Further detailed functional analyses, however, such as globulin class-switching, using mESC-derived B cells have so far not been performed.

3. Hematopoietic stem cells derived from human ESCs

Derivation of HSCs from hESCs, once successful, would have a great impact in both the clinical and basic research fields, given the wide range of potential applications. An unlimited amount of HSCs with various HLA and ABO blood types could be an ideal graft source in HSC transplantation, a starting material for manufacture of mature blood cells for blood transfusion, a gene transfer target for both clinical and experimental purposes, and so forth.

Besides mESCs, EB formation and coculture with feeder cells are the two major strategies used to produce hematopoietic cells from hESCs, although most of the protocols developed for mESCs cannot be applied for hESCs without significant modifications (Bhatia, 2007). For example, LIF is a critical factor for mESCs to be maintained in an undifferentiated state, but hESC maintenance is not dependent on LIF. For the maintenance of hESCs, bFGF is used, whereas bFGF induces neural differentiation from mESCs (Ying et al., 2003). As for the markers of the undifferentiated state, specific embryonic antigen (SSEA)-3 and SSEA-4 are used for hESCs, while SSEA-1 is used for mESCs. For hematopoietic differentiation, longer cultures are needed for hESCs than for mESCs. Given that species-adjusted hESC-derived cell transplantation experiments are impossible, evaluation of hESC-derived HSCs largely depends on phenotypic assays, colony-forming assays, and *in vivo* transplantation models using animals such as immunodeficient mice. Non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice have been widely used as the hosts for transplantation. For evaluation of hematopoietic ability, cells are transplanted into NOD-SCID mice and assayed by detection of SCID-repopulating cells (SRCs) (Ueda et al., 2000).

Kaufman et al. cultured hESCs on the murine bone marrow cell line S17 or the yolk sac endothelial cell line C166 in a medium containing FBS without any cytokines (Kaufman et al., 2001). This culture enabled hESCs to differentiate into progenitors capable of producing colonies with multiple hematopoietic lineages. As with somatic HSCs, these colony-forming cells were enriched in $CD34^+$ cells. Vodyanik et al. demonstrated that, as well as mESCs, hESCs could also be differentiated into $CD34^+$ hematopoietic progenitors by coculture on OP9 cells (Vodyanik et al., 2005). When hESC-derived $CD34^+$ cells were cultured on the murine bone marrow-derived cell line MS-5 in the presence of SCF, FL, IL-7, and IL-3, they could generate both myeloid and lymphoid cells.

Chadwick et al. formed EBs from hESCs and cultured them in the presence of SCF, FL, IL-3, IL-6, and G-CSF, with or without BMP-4 (Chadwick et al., 2003) and found that BMP-4 increased the number of hematopoietic progenitors from hESCs. The same group found that in these culture conditions including BMP-4, the primitive cells with ability to differentiate into both hematopoietic and endothelial cells would appear between day 7 and day 10 of the EB culture (Wang et al., 2004). These primitive cells expressed PECAM-1, Flk-1, and VE-cadherin, but not CD45 ($CD45^-$PFV). In a later report, they cultured $CD45^-$PFV cells for 7 days in serum-containing medium supplemented with SCF, FL, G-CSF, IL-3, and IL-6 and differentiated them into $CD45^+$ cells with SRC activity (Wang et al., 2005). These hESC-derived $CD45^+$ cells were transplanted directly into the femurs of sublethally irradiated NOD-SCID mice. Even at 8 weeks after transplantation, hESC-derived SRCs were detected, indicating that HSCs with reconstituting ability were obtained from the hESCs. But these hESC-derived HSCs could not repopulate in NOD-SCID mice when they were transplanted intravenously. Furthermore, hESC-derived HSCs showed lower levels of chimerism in the transplanted bone than did the somatic HSCs from human umbilical cord blood, and the same pattern was seen in the contralateral femur and other long bones. These results indicate that hESC-derived HSCs obtained by using this method are distinct from somatic HSCs in terms of the ability of proliferation and migration. Notably, the authors also mentioned that unlike in mESCs, ectopic expression of HoxB4 in hESCs accelerated proliferation of hematopoietic progenitors but had no effect on the repopulating capacity of hESC-derived cells.

The methods using coculture with feeder cells are also capable of generating hESC-derived HSCs. Tian et al. showed that hESCs cultured on S17 cells for 7 to 24 days differentiated into

hematopoietic cells with SRC activity even when they were transplanted intravenously (Tian et al., 2006). They also performed a secondary transplantation from the bone marrow of the primary recipient of hESC-derived HSCs into secondary donor mice and confirmed the long-term repopulating ability of hESC-derived HSCs.

As mentioned above, hESCs can differentiate into hematopoietic cells with SRC activity, but this activity of hESC-derived cells remains low when compared with that of somatic HSCs such as cord blood $CD34^+$ cells. We can thus conclude that no bona fide methods have been established that reproducibly generate true HSCs from hESCs. Recently, derivation of HSCs with higher SRC activity using a cell line derived from the AGM region was reported (Ledran et al., 2008). In that report, cell lines from the AGM region or fetal liver or primary cells from those organs were used as feeder cells. All hESC-derived hematopoietic cells differentiated on these feeders were capable of repopulating in immunodeficient mice when transplanted into the femurs of the recipient mice, and among the feeders the AGM-derived cell line AM20.1B4 was the best in terms of SRC activity of the hESC-derived cells. When this cell line was used, the chimerism of the hESC-derived cells in the peripheral blood of the recipient mice reached 16%. Notably, this chimerism is higher than that in previous reports. Considering these results, it may be important to place hESCs in an environment that closely mimics a hematopoietic niche in order to obtain true HSCs from them.

4. Mature blood cells derived from human ESCs

4.1 Neutrophils

Neutrophil transfusion can be beneficial for severe neutropenic patients with congenital diseases or who have undergone chemotherapy if a sufficient number of neutrophils are transfused at appropriate intervals. The current blood donation system, however, is incapable of providing sufficient amounts of neutrophils on schedule, given that the half-life of neutrophils *ex vivo* is less than 10 hours and thus, that multiple transfusions per day are necessary to ensure effectiveness. Human ESC-derived neutrophils might provide a solution to these difficulties. They could also offer a new tool for drug discovery, drug toxicity monitoring, and so on. Recently, a method to obtain mature neutrophils with high purity from hESCs was developed (Yokoyama et al., 2009).

The culture system consisted of 2 phases: EB formation and OP9 coculture with different combinations of cytokines at each phase. For the formation of EBs, hESC colonies were detached from the mouse embryonic fibroblasts used as feeder cells to maintain the hESCs, using collagenase. The removed colonies were then cultured in the IMDM-based medium for HSC expansion (Suzuki et al., 2006) in a serum-free condition, which resulted in the initial formation of EBs within 24 hours. The resulting EBs were collected and cultured for 17 days in IMDM containing 15% FBS supplemented with BMP-4, SCF, FL, IL-6/IL-6 receptor fusion protein (FP6), and TPO. For the preparation of feeder cells, irradiated OP9 cells were next plated onto gelatin-coated 6-well tissue culture plates at a density of 1.5×10^5/well 24 hours before use. The EBs were dissociated into single cells and suspended in IMDM containing 10% FBS and 10% horse serum supplemented with a combination of SCF, FL, FP6, IL-3, and TPO. Then, up to 5×10^5 EB-derived cells were seeded in a well with the OP9 cell layer. After 7 days, floating cells were collected, suspended in IMDM containing 10% FBS and G-CSF, and transferred onto the newly irradiated OP9 cells. Terminally differentiated cells were harvested 6 or 7 days later.

As determined by morphology, most of the hESC-derived cells at day 7 of the final coculture with OP9 cells were myeloblasts and promyelocytes. On days 9 through 11, myeloblasts and metamyelocytes were dominant. On days 13 and 14, 70% to 80% of the total cell population were differentiated mature neutrophils and the remaining 10% to 20%, metamyelocytes. Transmission electron microscopic observation also revealed characteristic segmented nuclei and cytoplasmic granules. On days 13 and 14, Wright-Giemsa staining also revealed that up to 10% of the cells were monocyte- or macrophage-like cells, but no other cell lineages such as erythrocytes, megakaryocytes, or lymphocytes were observed throughout the culture. Thus, this differentiation protocol made it possible to obtain hESC-derived neutrophils at a high purity. These hESC-derived neutrophils were positively stained for myeloperoxidase, which is a major constituent of the primary granules of neutrophils. Biosynthesis of lactoferrin, which is a major constituent of the secondary granules, was analyzed by comparison of mRNA expressions of the hESC-derived cells with those of mature neutrophils from the peripheral blood and mononucleated cells from the bone marrow of healthy volunteers. Lactoferrin mRNA was expressed in hESC-derived cells as early as day 7 of the final induction culture on OP9 cells, peaked at day 10, and declined at days 13 and 14. This pattern was consistent with the documented pattern of lactoferrin biosynthesis (Rado et al., 1984). These patterns of morphological maturation and lactoferrin mRNA expression during the culture indicated that hESC-derived cells differentiated into mature neutrophils by a process similar to physiologic neutrophil production, and thus, this method could be used to investigate the differentiation process of neutrophils.

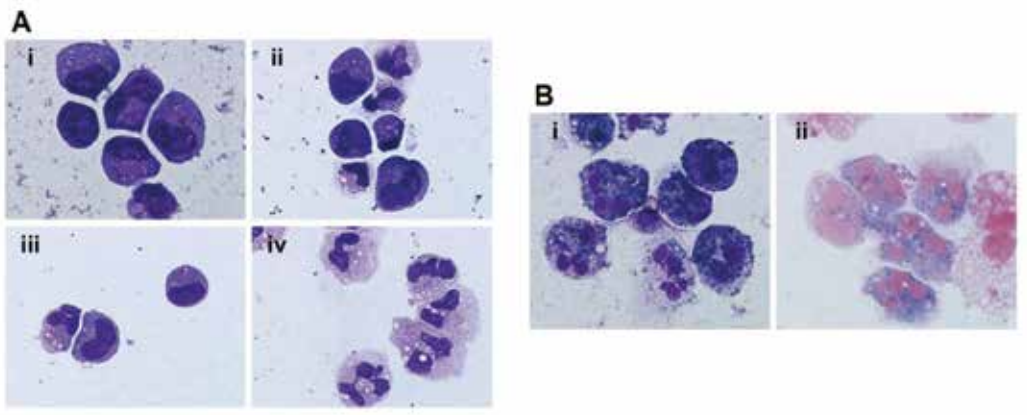

Fig. 1. Neutrophils derived from hESCs. (A) Wright-Giemsa staining of the hESC-derived cells at days 7 (i), 9 (ii), 11 (iii), and 13 (iv). (B) The hESC-derived neutrophils stained positive for myeloperoxidase and alkaline-phosphatase. This research was originally published in *Blood*. Yokoyama et al.. Derivation of functional mature neutrophils from human embryonic stem cells. *Blood*. 2009;113:6584-6592. © the American Society of Hematology.

Surface antigen expression of hESC-derived cells was analyzed at different time points by flow cytometry. The pattern of antigen expression was almost consistent with that of normal neutrophil differentiation, except for some G-CSF-related changes. Almost all the cells expressed the common blood cell antigen CD45 from days 7 through 13. A small population expressed the markers of immature hematopoietic cells such as CD34, CD117, and CD113 at

day 7 but lost the expression by day 10. The common myeloid antigens CD33 and CD15 were highly expressed from days 7 through 13, whereas CD11b expression increased as maturation proceeded. CD13 is also a common myeloid antigen, but only fewer than 20% of the cells expressed CD13 throughout the final culture. CD16 (Fcγ receptor [FcγR] III) is frequently used as the marker of mature neutrophils; it was already found on hESC-derived cells at day 7 and increased with maturation, which is consistent with the physiologic neutrophil maturation process. However, the proportion of $CD16^+$ cells was lower than that of the morphology-defined mature neutrophils on day 13. Other Fcγ receptors, CD32 (FcγRII) and CD64 (FcγRI), were also expressed on hESC-derived neutrophils. CD14 was expressed in 20% to 25% of the cells on days 10 and 13. In normal peripheral blood, mature neutrophils express CD16 but not CD64 and CD14 (van de Winkel and Anderson, 1991; van Lochem et al., 2004), but some of the hESC-derived mature neutrophils expressed CD14, but not CD16, and most of the cells expressed CD64. This aberrant expression pattern is similar to that of the neutrophils harvested from healthy donors who received G-CSF (Carulli, 1997; Kerst et al., 1993) and of the neutrophils derived from bone marrow $CD34^+$ cells *in vitro* by G-CSF stimulation (Kerst et al., 1993), and thus, hESC-derived neutrophils were thought to be also affected by G-CSF during the final culture.

The high purity and yield enabled subsequent functional analyses of the hESC-derived neutrophils. As seen in the expression of surface antigens, G-CSF used in the induction culture might affect the functions of hESC-derived neutrophils. Therefore, hESC-derived neutrophils were restimulated with G-CSF before the assay and compared with peripheral blood neutrophils with and without G-CSF stimulation.

Chemotaxis is the first step in innate immune system by neutrophils and important for neutrophils to be able to move to the inflammatory site effectively. Chemotaxis was analyzed using a modified Boyden chamber method (Harvath et al., 1980). In this method, reaction medium with or without chemotactic factor formyl-Met-Leu-Phe (fMLP) was placed into each well of a 24-well plate, and a semipermeable membrane with 3.0-μm pores was placed into each well to divide the well into upper and lower sections. Neutrophils were added to the upper section and allowed to migrate from the upper to the lower side of the membrane. After incubation, the number of neutrophils on the lower side of the membrane was counted. The neutrophils that migrated to the lower side without fMLP were considered to have migrated randomly. This random migration of peripheral blood neutrophils was accelerated by G-CSF, but, despite the stimulation by G-CSF, the hESC-derived neutrophils showed an extent of random migration that was only similar to that of the peripheral blood neutrophils without G-CSF stimulation. The number of cells that showed chemotaxis to fMLP was calculated by subtracting the number of migrated cells without fMLP from that of migrated cells with fMLP. This chemotaxis was not significantly different between hESC-derived neutrophils and peripheral blood neutrophils with or without G-CSF stimulation.

The next step in innate immune system by neutrophils is phagocytosis, and subsequently, killing of ingested microorganisms occurs mainly depending on superoxide production. We adopted a unique assay that simultaneously visualizes phagocytosis and superoxide production. Autoclaved baker's yeast was suspended in 0.5% nitroblue tetrazolium (NBT) solution (0.5% NBT and 0.85% sodium chloride in distilled water). When these NBT-coated yeasts are ingested by neutrophils, the yeasts change their color from brown to purple or black because of reduction of NBT and formation of formazan in response to superoxide produced by neutrophils. We incubated these NBT-coated yeasts with hESC-derived and

peripheral blood neutrophils. Ingested yeast cells that changed color in the cells were NBT-reaction positive. The difference in the number of positive yeasts yeilded by the hESC-derived neutrophils and peripheral blood neutrophils was not significant. G-CSF stimulation had no effect on the peripheral neutrophils in this assay.

Superoxide production by oxidative burst is the most important function for neutrophils to perform efficient bactericidal activity. In addition to the above-mentioned NBT reduction, we used dihydrorhodamine123 (DHR) to evaluate superoxide production. In the test, DHR was added to the neutrophil suspension with or without stimulation by phorbol myristate acetate (PMA), and rhodamine fluorescence from the oxidized DHR was detected by flow cytometry (Richardson et al., 1998). When DHR was added to the neutrophil suspensions, rhodamine-specific fluorescence was detected, indicating basal production of superoxide without PMA stimulation. Stimulation by PMA strongly increased rhodamine fluorescence in hESC-derived neutrophils and peripheral blood neutrophils, indicating that hESC-derived neutrophils had sufficient capability of superoxide production and adequate response to stimulation.

Finally, we evaluated actual bactericidal activity *in vitro* using viable *Escherichia coli* (Decleva et al., 2006). Opsonized *E coli* were added to the neutrophil suspension at a neutrophil/bacteria ratio of 2:1 or to the control medium. After 1 hour of incubation, the neutrophils were lysed, and the samples were added to molten tryptic soy broth with 1.5% agar and plated on dishes. The colonies derived from the surviving *E coli* were counted after overnight incubation. When the *E coli* were incubated with hESC-derived neutrophils and peripheral blood neutrophils with or without G-CSF stimulation, the numbers of the colonies were similarly reduced to approximately 40% those of the control, indicating that the hESC-derived neutrophils had bactericidal activity against *E coli* comparable to that of normal neutrophils.

Generation of functional neutrophils using a feeder-free culture system was also reported by another group (Saeki et al., 2009). In this method, EBs were cultured in IMDM supplemented with FBS, insulin-like growth factor II, VEGF, SCF, FL, TPO, and G-CSF. After 3 days, the EBs were transferred onto gelatin-coated dish and cultured in the same medium as that of the EB culture. After 2 weeks of adherent culture on the gelatin-coated dish, sac-like structures (SLSs) emerged, and within a few days, round cells appeared in the sacs. These round cells had the potential to produce granulocyte, macrophage, or erythroid colonies. After 4 to 6 weeks of adherent culture, immature and mature myeloid cells were obtained, including mature neutrophils, although the purity of the mature neutrophils was relatively low (30%-50%). These hESC-derived neutrophils showed chemotaxis to fMLP and IL-8, phagocytosis of zymosan, and NBT-reduction. Interestingly, the authors of this report evaluated the chemotactic activity *in vivo* using a zymosan-induced air pouch inflammation model (Doshi et al., 2006). In this model, neutropenia was induced in immunodeficient NOD-SCID/γc^{null} (NOG) mice by injection of 5-fluorouracil, and a subcutaneous air pouch was formed on the back of the NOG mice. After 3 days, 2×10^6 hESC-derived or human cord blood CD34-positive cells were transfused. Injection of both zymosan and IL-1β into the air pouch caused inflammation of the pouch, and accumulation of neutrophils in the pouch was observed. Among the massive murine neutrophils, hESC-derived neutrophils accounted for 0.54% of the total cells that were accumulated in the pouch. This percentage was the same as that for cord blood $CD34^+$ cells. For the establishment of fundamentals for clinical application, *in vivo* analysis of neutrophil functions, especially the bactericidal activity and prolongation of survival of infected mice by neutrophil transfusion, is needed.

4.2 Erythrocytes

Adult-type erythrocytes derived from hESCs could be a new and ideal transfusion source if large-scale production can be achieved, given that they could be free from infectious organisms. Furthermore, hESC-derived erythrocytes from rare blood-type donors might resolve the difficulty of availability of such types of RBCs.

In normal human erythroid development, the expression pattern of hemoglobin subunits in erythrocytes changes according to the developmental stage. In primitive yolk sac erythropoiesis, embryonic-type ζ– and ε–globin are expressed. In definitive erythropoiesis, ζ–globin and ε–globin switch to fetal-type α-globin and γ-globin, respectively, and γ-globin further switches to adult-type β-globin (Peschle et al., 1985). When evaluating erythrocytes derived from hESCs, in addition to the efficiency of the induction culture, it is important to examine the globin expression pattern to determine the erythrocyte type.

As described in section 3, culturing EBs in the presence of SCF, FL, IL-3, IL-6, G-CSF, and BMP-4 accelerates the generation of hematopoietic progenitors (Chadwick et al., 2003), and when VEGF was added to these basal cytokines, both the number and the frequency of erythroid colonies derived from the EBs were augmented (Cerdan et al., 2004). Evaluation of globin expression by detection of mRNA of each globin revealed that the cells from EBs treated with only basal cytokines expressed only ε-globin, but addition of VEGF to the basal cytokines promoted expression of both ε- and ζ-globins. β-globin expression was not proven in either culture condition. Thus, the erythropoiesis in the EBs cultured with this combination of cytokines was thought to recapitulate primitive erythropoiesis with embryonic globin expression. However, the erythrocytes picked up from the EB-derived erythroid colonies in a semisolid culture expressed β-globin in addition to ε-globin, but not ζ-globin, indicating the possibility of globin switch during the colony-formation culture. Expression of embryonic and fetal globins, but not adult β-globin in hESC-derived erythrocytes was also reported by different groups (Chang et al., 2006; Olivier et al., 2006).

Other groups showed successful expression of β-globin in hESC-derived erythrocytes. Ma et al. developed an efficient method of inducing erythrocytes using coculture with feeder cells (Ma et al., 2007; Ma et al., 2008). In this method, the hESC colonies were cultured on irradiated primary murine fetal liver stromal cells without any cytokines. At days 11 to 12, hESC-derived cells formed SLSs containing hematopoietic cobblestone-like cells. On day 14, 1×10^4 original hESCs had given rise to 1×10^6 total cells including 5×10^3 cobblestone-like cells. When the mixture of stromal cells and all hESC-derived cells were prepared as a single cell suspension and cultured in a semi-solid medium with EPO, SCF, IL-3, IL-6, TPO, and G-CSF, they generated mainly erythroid colonies including erythroid bursts, although approximately 25% were non-erythroid colonies. Erythroid bursts accounted for about 5% of the total colonies, and each large erythroid burst contained approximately 2×10^5 erythroid cells. Importantly, about 60% of the hemoglobin-containing erythroid cells in each erythroid burst derived from hESC after 12-day co-culture on murine fetal liver stromal cells expressed adult β-globin, and the proportion reached nearly 100% when the coculture was extended to 18 days. In contrast, the proportion of ε-globin-expressing erythroid cells in each erythroid burst decreased from 100% to 60%. Globin switch could also be observed when the day 12-erythroid bursts were transferred to a suspension culture for an additional 6 days; the expression of ε-globin decreased, whereas β-globin expression increased to about 100%, and, notably, β-globin-expressing enucleated RBCs were observed. The hESC-derived erythroid cells could function as oxygen carriers showing oxygen dissociation curves similar to those of human cord blood RBCs, although their curves were left-shifted when compared

to those of adult peripheral blood RBCs. The hESC-derived erythroid cells had higher glucose-6-phosphate dehydrogenase activity than did the adult peripheral blood RBCs.

Lu et al. showed two methods of producing erythrocytes using hemangioblasts derived from hESCs as starting materials: one, the massive production of nucleated erythrocytes without adult β-globin expression, and the other, induction of enucleation of hESC-derived erythrocytes with some β-globin expression (Lu et al., 2008). By the method for massive production, they generated 10^{10} to 10^{11} erythrocytes from one 6-well plate of hESCs. In the first step, EBs were formed and cultured in serum-free medium containing BMP-4, VEGF, and bFGF. After 48 hours, half the medium was exchanged for fresh medium with the same cytokines and additional SCF, TPO, and FL, and cultured for a further 36 hours. In the second step, EBs were then dissociated into single cells, which were cultured for 10 days in blast-colony growth medium (BGM) consisting of IMDM, 1.0% methylcellulose, bovine serum albumin, insulin, iron-saturated transferrin, GM-CSF, IL-3, IL-6, G-CSF, EPO, SCF, VEGF, and BMP-4. Dependent on the hESC lines, TPO and FL were added to the cytokine combination. This culture condition induced and expanded the hESC-derived hemangioblasts that had been described in a previous report (Lu et al., 2007). To optimize the method, they used a fusion protein consisting of HoxB4 and triple protein-transduction domains (tPTD-HoxB4). The PTD used here was a modified form of PTD embedded in the transactivator of transcription protein of the human immunodeficiency virus (Ho et al., 2001; Lu et al., 2007). Maximum efficiency was achieved when tPTD-HoxB4 and bFGF were added to the BGM. In the third step, equal volumes of BGM containing additional EPO were added to the existing BGM, and the cells were further cultured and differentiated into erythroid cells for 5 days. The erythroid cells were then transferred to serum-free medium containing SCF, EPO, and 0.5% methylcellulose, and expanded for 7 days. In the final step, for the purification of the erythroid cells, the resulting cells were plated in tissue culture flasks overnight to allow nonerythroid cells to attach to the flasks, and the nonadherent cells were collected. By this method, numerous erythroid cells (10^{10} to 10^{11} cells from one 6-well plate of hESCs) could be obtained; however, these hESC-derived erythroid cells were nucleated and contained embryonic ζ- and ε-globins, and fetal ^{G}γ-globin, but neither fetal ^{A}γ-globin nor adult β-globin. Nevertheless, the hESC-derived erythroid cells showed an oxygen equilibrium curve comparable to that of normal adult RBCs.

A modification of this method allowed enucleated hESC-derived erythrocytes with adult β-globin to be obtained. The protocols in the first step and up to day 7 in the second step were the same. After 7 days of culture in the second step, the cells were cultured in serum-free medium containing bovine serum albumin, inositol, folic acid, transferrin, insulin, ferrous nitrate, and ferrous sulfate, and supplemented with hydrocortisone, SCF, IL-3, and EPO. After 7 days, SCF and IL-3 were removed. In these conditions, 10% to 30% of the hESC-derived erythrocytes were enucleated. Importantly, considering that the hESCs were maintained without feeder cells, the enucleated erythrocytes were generated in completely feeder-free conditions. By this method, however, hESCs showed expansion of only 30- to 50-fold. Furthermore, even after enucleation, the hESC-derived erythrocytes expressed mainly embryonic ζ- and ε-globins, and fetal γ-globin, but not β-globin. However, survival and enucleation of the erythrocytes were enhanced when they were cocultured on OP9 cells, and long-term culture of the cells induced adult β-globin expression from 0% at day 17 to 16% at day 28, indicating the potential of globin switch of hESC-derived erythrocytes.

Dependent on the methods, the expression patterns of the hemoglobin subunits were different. Comparison of the methods would be useful to understanding the mechanisms of

erythroid development and globin switch. If hESC-derived fetal erythrocytes could be successfully changed to adult erythrocytes and high efficiency achieved, it would open up the way to clinical use.

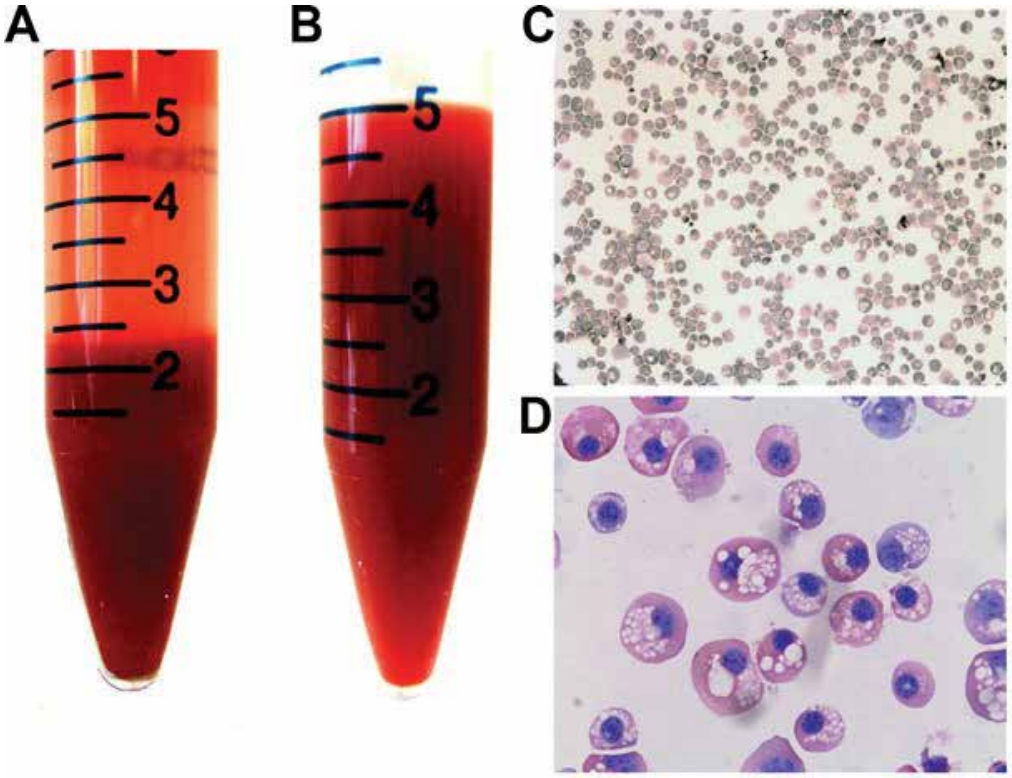

Fig. 2. Erythrocytes derived from hESCs. (A) Erythrocytes derived from 2×10^6 hESCs. (B) Suspension of erythrocytes from panel A in equivalent hematocrit of human whole blood. (C, D) Wright-Giemsa staining of hESC-derived erythrocytes. Original magnification, C: ×200, D: ×1000. This research was originally published in *Blood*. Lu et al.. Biologic properties and enucleation of red blood cells from human embryonic stem cells. *Blood*. 2008;112:4475-4484. © the American Society of Hematology.

4.3 Megakaryocytes and platelets

Platelet derivation from hESCs is also of concern for transfusion medicine. Platelets can be stored for only 3 to 4 days, and more donors are needed to secure sufficient amounts of platelet concentrates than are needed for RBCs. Two groups so far reported specific methods for megakaryocyte/platelet derivation from hESCs, and both used coculture with feeder cells (Gaur et al., 2006; Takayama et al., 2008). In the first report, small clumps of hESCs were cultured on OP9 cells in the presence of 100 ng/mL TPO. The cells were transferred onto fresh OP9 cells on days 7 and 11. After 15 to 17 days of culture, 20% to 60% of the hESC-derived cells were positive for both CD41a and CD42b, which are representative markers of the megakaryocyte lineage. In this culture, 1×10^5 starting hESCs yielded 1 to 4×10^4 $CD41a^+CD42b^+$ cells. These cells showed megakaryocytic morphology with

polyploidy. The hESC-derived megakaryocytes showed substantial increase of fibrinogen-binding capacity compared to baseline in response to thrombin receptor-activating agonists or adenosine di-phosphate. This result indicated the presence of appropriate inside-out signaling of integrin αIIbβ3 in hESC-derived megakaryocytes, which controls affinity and avidity of integrin αIIbβ3 for fibrinogen. Moreover, when hESC-derived megakaryocytes were plated on fibrinogen-coated glass cover slips, they showed extensive lamellipodia formation, F-actin formation, and vinculin localization, indicating proper outside-in signaling of integrin αIIbβ3. However, these apparently functional megakaryocytes rarely differentiated to proplatelets. These data imply that terminal differentiation to mature platelets might not be observed in this culture system. On the other hand, Takayama et al. confirmed the first report of the derivation of megakaryocytes from hESC using coculture with OP9 cells, and developed a new method of generating megakaryocytes capable of releasing platelets (Takayama et al., 2008). Coculture of hESCs on either C3H10T1/2 or OP9 cells without transfer to new feeders for 2 weeks led to emergence of SLSs. Addition of VEGF to the culture medium increased the number of the SLSs. These SLSs contained hematopoietic progenitors with multilineage colony-forming potential, and those progenitors could be further differentiated into mature proplatelet-forming megakaryocytes when transferred onto new feeder cells and cultured in the presence of TPO for an additional 7 to 9 days. $CD41a^{+}CD42b^{+}$ platelets were then detected in the culture supernatants. The maximum yield was achieved when the medium was supplemented with SCF and heparin in addition to TPO, resulting in approximately 5×10^{6} platelets produced from 10^{5} hESCs. The hESC-derived platelets had appropriate inside-out and outside-in signaling of integrin αIIbβ3. This method is expected to be useful for studies of the developmental mechanisms and functions of megakaryocytes and platelets, and for transfusion medicine, although extreme improvement in the efficiency of platelet generation from hESCs is needed.

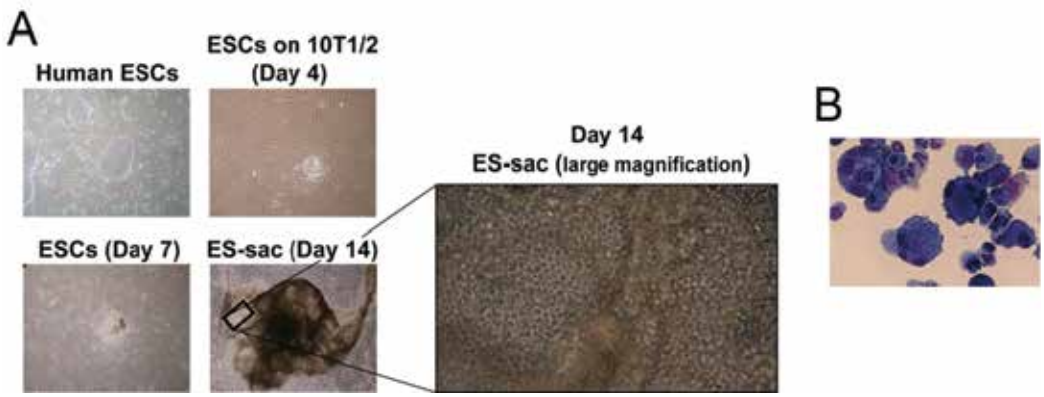

Fig. 3. Sac-like structures and megakaryocytes derived from hESCs. (A) Sac-like structures with distinct morphology. They contained hematopoietic progenitors. (B) Megakaryocytes derived from hESC. This research was originally published in *Blood*. Takayama et al. Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood*. 2008;111:5298-5306. © the American Society of Hematology.

4.4 Natural killer cells

Natural killer (NK) cells have cytotoxic enzymes and play a major role in innate immunity. They also have antitumor activity, and the possibility of safe and efficaciously adoptive immunotherapy using NK cells has been shown in the setting of allogeneic hematopoietic stem cell transplantation or studies of NK cell transfusion for malignancies (Ljunggren and Malmberg, 2007; Miller et al., 2005; Ruggeri et al., 2002). Derivation of NK cells with antitumor activity from hESCs could be a possible means of immunotherapy. The most functional hESC-derived NK cells so far were generated by sequential coculture on different feeder cells (Woll et al., 2005; Woll et al., 2009). Firstly, hESCs were cultured on S17 or a murine bone marrow stromal cell line M210-B4 for 17 to 20 days. After the first coculture, $CD34^+CD45^+$ cells were sorted and transferred to a murine fetal liver-derived stromal cell line, AFT024, and cocultured in medium containing human AB blood-type serum with a cytokine cocktail consisting of IL-3, SCF, IL-15, FL, and IL-7. At 3 to 5 weeks of culture, approximately 70% of the hESC-derived cells were CD45- and CD56-positive NK cells, with expression of receptors typically found on adult NK cells such as CD16, CD94, NKp46, and killer-cell Ig-like receptors (KIR or CD158). Interestingly, hESC-derived NK cells showed higher cytolytic activity against various tumor and leukemia cell lines than did NK cells derived from cord blood progenitors under the same conditions. Higher antileukemic activity *in vivo* with hESC-derived NK cells was also demonstrated in a mouse model for human leukemia using the human erythroleukemia cell line K562. These results indicate that hESC-derived NK cells are potentially a good source for immunotherapy.

4.5 T and B lymphocytes and other lineages

T and B cells have central roles in acquired immunity, but derivation of these cells from hESCs could be more difficult than that of cells of other lineages. As described in section 2. 2, mESCs can be easily differentiated into T cells using OP9-DL1 cells. However, Martin et al. reported that hESC-derived $CD34^+$ progenitors could not be differentiated into the T-cell lineage *in vitro* even by co-culture with OP9-DL1 cells or by fetal thymic organ culture (Martin et al., 2008). The first successful specific derivation of mature T cells from hESCs was achieved by an *in vivo* procedure using SCID-hu mice (Galic et al., 2006). The SCID-hu mice were constructed by insertion of human fetal thymus and liver under the renal capsule of SCID mice, and provide the environment for T lineage differentiation (Akkina et al., 1994; McCune et al., 1988). Human ESC-derived $CD34^+$ or $CD34^-CD133^+$ hematopoietic progenitors, obtained by coculture with OP9 cells for 7 to 14 days, were injected into thymus/liver implants in sublethally irradiated SCID-hu mice. After 3 to 5 weeks, biopsy of the thymus/liver implants demonstrated repopulation of hESC-derived cells in the implants accounting for up to 6.2% of the total cells. Phenotypic analysis revealed differentiation of hESCs into immature $CD4^+CD8^+$ T cells and mature $CD4^+CD8^-$ and $CD8^+CD4^-$ T cells. Later, the same group modified the methods and adopted EB formation instead of coculture with OP9 cells (Galic et al., 2009), and they showed normal V(D)J recombination during differentiation of hESC-derived T cells and CD25 expression on the cells in response to stimulation. However, complicated and cumbersome *in vivo* procedures, particularly the use of human fetal thymus and liver, obviously hamper the further progress of the study of hESC-derived T-cell development.

Contrary to the previous report by Martin et al., Timmermans et al. reported an *in vitro* method of T cell differentiation using coculture with OP9-DL1 (Timmermans et al., 2009). In

this method, hESCs were cocultured on OP9 cells. After 10 to 12 days, endothelium-lined cell clumps emerged that resembled the hESC-derived SLSs described in Takayama's method of megakaryocyte differentiation. These structures were transferred onto OP9-DL1 cells and cultured in medium supplemented with FL, IL-7, and SCF. After 14 days of coculture on OP9-DL1 cells, CD4 SP cells and CD4 CD8αα DP cells were detected within the cytoplasmic $CD3\varepsilon^+CD5^+$ cell population. After 21 days, CD4 CD8αβ DP cells appeared, and on day 28, DP cells accounted for 25% of the cells. After 30 days of culture, 15% to 50% of hESC-derived cells were T lineage cells expressing surface CD3 and TCRαβ. In addition to the $CD3^+TCR\alpha\beta^+$ cells, $CD3^+TCR\gamma\delta^+$ cells also emerged. These results suggested that hESC-derived T cells differentiated phenotypically in a way similar to that in thymic development. In response to stimulation, hESC-derived T cells showed a 2,500-fold increase, and all surface $CD3^+$ T cells had the mature $CD27^+CD1a^-$ phenotype. Restimulation of the expanded T cells induced interferon-γ production. These results indicated that phenotypically and functionally mature T cells could be generated from hESCs, although detailed functional analyses have yet to be performed.

B cell differentiation from hESCs is also challenging compared with that of other lineages. No effective methods for achieving B cell differentiation from hESCs have so far been devised. Martin et al. reported that hESC-derived $CD34^+$ hematopoietic progenitors lacked B lineage differentiation capability when cocultured with MS-5 cells that support B cell differentiation from cord blood $CD34^+$ progenitors (Martin et al., 2008). Thus, an additional cue is required to establish an environment sufficient for B cell differentiation, in addition to the cytokines and feeders that have been used so far. Given the success in B cell differentiation from mESCs, differences between mESCs and hESCs or species specificities of the feeder cell-expressed proteins may explain this hurdle.

Other lineages of blood cells, such as macrophages (Anderson et al., 2006) and dendritic cells (Slukvin et al., 2006), can also be generated from hESCs. As described in this section, hESC-derived mature blood cells including neutrophils, erythrocytes, megakaryocytes, and NK cells are commonly very similar to their normal counterparts in morphology, phenotype, and function. Therefore, if sufficient amounts of mature blood cells derived from hESCs can be obtained, they can be expected to be used for a variety of purposes, for example, as substitutes for normal blood cells for *in vitro* drug screening and as blood transfusion sources.

5. Future directions

Coculture with feeder cells and EB formation are the two major strategies for hematopoietic differentiation from hESCs commonly used to generate both progenitors and mature blood cells. However, no methods for generating bona fide HSCs from hESCs have yet been established, despite the fact that feeder cells derived from bone marrow, fetal liver, and AGM should provide a hematopoietic microenvironment similar to the physiologic one. As regards the preparation of an ideal microenvironment for inducing HSCs from hESCs, the combined use of an *in vitro* culture system with an animal body may prove a powerful method. Recently, a sensational report of the generation of rat pancreas in mouse was published (Kobayashi et al., 2010). Injection of rat wild-type iPSCs into blastocysts of a Pdx1-null mouse, which is devoid of pancreas and dies soon after birth, resulted in the development of a compensatory pancreas entirely derived from rat iPSCs. This result indicated that when a developmental niche for a certain organ is empty, pluripotent stem

cell-derived cells can occupy the niche and compensate for the missing organ. Considering application of this finding for hematopoiesis, it may be possible to obtain pluripotent stem cell-derived HSCs using a mouse that is devoid of HSCs, for example, GATA2- (Tsai et al., 1994), SCL/Tal1- (Porcher et al., 1996), Runx1/AML1- (Okuda et al., 1996), or Notch1- (Kumano et al., 2003) null mice. This approach of interspecific blastocyst complementation might contribute to overcoming the issue of yield, which still represents a high barrier to reaching clinical applications. If large animals, such as pigs, without hematopoietic ability become available, injection of human ESCs or iPSCs into their blastocysts might make it possible to obtain massive amounts of human HSCs and mature blood cells, although contamination with xenogeneic constituents is still a problem, and ethical arguments must be addressed before proceeding to the generation of human-animal hybrid embryos, particularly given that human cells could be differentiated into mature cells other than blood cells in the animal.

To achieve magnitudes of increase in cell number yields, which is necessary for virtually all the protocols hitherto reported, one potential goal is generation of progenitor cell lines that can proliferate infinitely and produce mature blood cells. As described in section 2. 2, mESC-derived erythroid progenitor lines could differentiate into functional mature red blood cells both *in vitro* and *in vivo* and ameliorate anemia in mice (Hiroyama et al., 2008). Although these erythroid progenitor lines were generated by coculturing with feeder cells under cytokine stimulation, genetic manipulation of hESCs or their progenies can also be considered. Gene manipulation has a risk of causing tumorigenesis; however, this concern is much smaller in the case of RBCs and platelets, given that these are unnucleated cells.

With the accumulated findings of hematopoietic differentiation from hESCs, hESC-derived HSCs and mature blood cells are now or will soon be good resources for functional analyses, drug-screening tests, research into the differentiation process, and so forth. Remarkable progresses in this field are continuously being made, which is encouraging for the achievement of clinical application of hESC-derived blood cells in the not-too-distant future.

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15

ES Cell-derived Erythroid Cell Lines Able to Produce Mature Red Blood Cells

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1. Introduction

Transfusion therapies involving red blood cells (RBCs), platelets, and neutrophils depend on the donation of these cells from healthy volunteers. However, unpredictable adverse results can ensue from transfusion therapies because of the donation of cells from a very large number of anonymous volunteers. For example, transfusion of blood products that include hazardous viruses or prions is difficult to prevent completely because, occasionally, tests to detect them yield pseudo-negative results. This comment is, of course, not intended as a criticism of the current system that is dependent on volunteers who act as blood donors from their own good will. However, there is little doubt that RBCs, platelets, and neutrophils produced in vitro might be a preferable means of producing such cells, thereby reducing, or even eliminating, the need for a large pool of anonymous donors. To date, however, the use of hematopoietic cells produced in vitro has not proved practical for routine therapeutic applications.

RBC transfusion was the first transplantation procedure to be established and is now routine and indispensable for many clinical purposes. However, in many countries the supply of transfusable materials is not always sufficient. In Japan, for example, the supply of RBCs with an AB/RhD(-) phenotype is always lacking because individuals with this RBC phenotype are rare. This problem of inequalities in the supply and demand for RBCs has stimulated interest in the development of in vitro procedures for the generation of transfusable and functional RBCs from hematopoietic stem cells or progenitor cells present in bone marrow or umbilical cord blood (Figure 1) (Neildez-Nguyen et al., 2002; Giarratana et al., 2005; Miharada et al., 2006; Douay and Andreu, 2007).

In addition, it is important to realize that clinical risk factors associated with RBC transfusions have not been entirely excluded. One notable and very severe complication of the procedure can be transfusion-related acute lung injury (TRALI), which has only recently been recognized and has not yet been eliminated (Silliman et al., 2009; Looney et al., 2010). One of the possible causes of TRALI may be a factor in the transfused materials, such as antibodies in the transfused materials against antigens on the leukocytes. This type of adverse outcome also results from the dependence of blood transfusion on the supply of blood from a large number of anonymous individuals. Problems may arise if donated blood is utilized without sufficient preliminary trials being carried out on each sample. The use of RBCs derived from selected human resources may help to alleviate these problems, since they can be intensively tested for pathogens before clinical use. Trial transfusions of a minimal amount of material into each

recipient could also be performed to determine if there are unexpected complications. Therefore, the establishment of resources for in vitro production of RBCs (Figure 1) will provide a means to alleviate many problems associated with RBC transfusion.

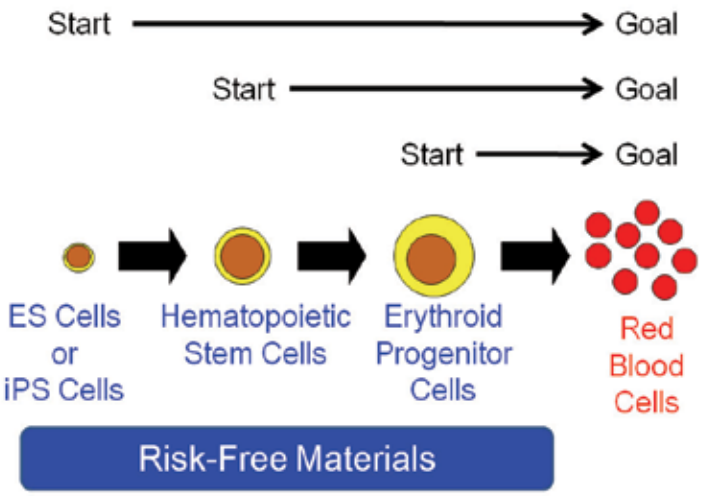

Fig. 1. A summary of some of the ways in which RBCs could be produced in vitro.

2. RBC production from hematopoietic stem cells

The hematopoietic stem cells present in bone marrow and umbilical cord blood are promising materials for in vitro production of RBCs and this has stimulated interest in the development of in vitro procedures for the generation of functional RBCs from these tissues (Neildez-Nguyen et al., 2002; Giarratana et al., 2005; Miharada et al., 2006). Umbilical cord blood cells are of particular interest as they are readily available but are usually discarded. Provided the mother of a neonate consents to use of the umbilical cord blood, this material can provide a useful resource without any further complicating critical or ethical concerns.

It was reported that human erythroid cells (nucleated cells) produced on a large scale ex vivo could differentiate in vivo into enucleated RBCs (Neildez-Nguyen et al., 2002). This study demonstrated that erythroid progenitor cells produced in vitro from hematopoietic stem and progenitor cells could have a clinical application as an alternative method for transfusing terminally differentiated RBCs. More recently, the same group described an ex vivo methodology for producing fully mature human RBCs from hematopoietic stem cells (Giarratana et al., 2005). The enucleated RBCs produced by this approach are potentially even more valuable as they should be functional immediately after transfusion without requiring the long latency period for enucleation normally necessary for erythroid cells.

The mechanism of erythroblast enucleation, a critical step in RBC production, has not yet been fully elucidated (Lee et al., 2004; Kingsley et al., 2004). The role of interactions between erythroblasts and other cells, such as macrophages, in this process is a controversial topic (Ohneda and Bautch, 1997; Yanai et al., 1997; Hanspal et al., 1998; Iavarone et al., 2004; Spike et al., 2004). Macrophages in retinoblastoma gene (Rb)-deficient embryos are unable to physically interact with erythroblasts and RBC production is impaired in these embryos (Iavarone et al., 2004). In addition, in vitro production of enucleated RBCs from immature

hematopoietic stem/progenitor cells proceeds efficiently in the presence (Giarratana et al., 2005) but not in the absence (Neildez-Nguyen et al., 2002) of feeder cells.
Of note, however, enucleation can apparently be initiated in vitro in erythroblasts that have been induced to differentiate in vivo to a developmental stage that is competent for nuclear self-extrusion (Spike et al., 2004; Yoshida et al., 2005). Moreover, we have developed a method to produce enucleated RBCs efficiently in vitro without use of feeder cells (Figure 2) (Miharada et al., 2006). The culture system has allowed erythroid cells to differentiate to a developmental stage competent for nuclear self-extrusion (Miharada et al., 2006). Taken together, although it has generally been thought that efficient enucleation of erythroblasts is largely dependent on signals mediated by cells in their local environment (Ohneda and Bautch, 1997; Yanai et al., 1997; Hanspal et al., 1998; Iavarone et al., 2004), the interaction of erythroblasts with other cells is not necessary for efficient erythroblast enucleation (Miharada et al., 2006). Signals mediated by humoral factors appear to be sufficient for the efficient autonomous completion of erythroblast enucleation.

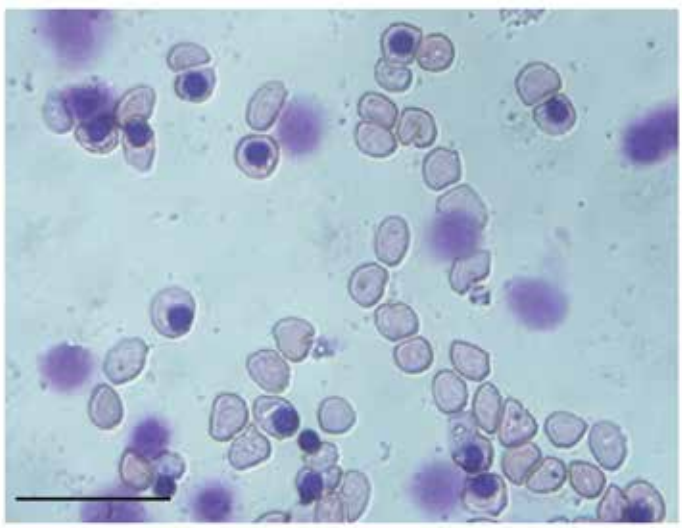

Fig. 2. Enucleated RBCs produced in vitro from hematopoietic stem cells. Scale bar indicates 50 μm.

Since culture without the use of feeder cells is technically easier and less expensive, the method we established (Miharada et al., 2006) has the potential to be a cost-effective means of producing transfusable RBCs on a large scale from immature hematopoietic stem/progenitor cells. Currently, however, cost factors means that it is not yet realistic to produce RBCs on a large scale, approximately 200 ml or more, using our in vitro culture system. In particular, patents on the growth factors used in the culture system are a major obstacle, because these growth factors are very expensive, at least at the moment. After the relevant patents expire, our in vitro culture system will become a more realistic scenario.

3. RBC production from ES cells

ES cells possess the potential to produce various differentiated cells able to function in vivo and thus represent another promising resource for RBC production. Furthermore, since ES

cell lines are immortalized, they can be used repeatedly and have potential to produce abundant differentiated cells in the quantities required for clinical use. However, it will be important to carry out routing screening of the ES cell lines for de novo chromosomal aberrations and/or genetic mutations that may arise in vitro, before these long term cell cultures are applied in the clinic. Unsurprisingly, there is now a widespread and enthusiastic debate on standardization of the characteristics of ES cells for regenerative medicine protocols that exploit these cell lines. In my opinion, since chromosomal aberrations and genetic mutations are inevitable in long term cell cultures, only ES cell lines that have been cultured for a limited period, e.g., less than 30 passages, should be selected for clinical use.

Hematopoietic cells, including those in the erythroid lineage, have been generated from mouse ES cells (Keller et al., 1993: Nakano et al., 1994; Nakano et al., 1996; Carotta et al., 2004), non-human primate ES cells (Li et al., 2001; Umeda et al., 2004; Kurita et al., 2006), and human ES cells (Kaufman et al., 2001; Chadwick et al., 2003; Cerdan et al., 2004; Vodyanik et al., 2005; Wang et al., 2005; Olivier et al., 2006). We have also established a long-term in vitro method for culturing hematopoietic cells derived from ES cells of the non-human primate, the common marmoset (Hiroyama et al., 2006). Recently, abundant production of enucleated RBCs from human ES cells was reported (Lu et al., 2008).

Taken together, we can now produce mature RBCs by in vitro culture of ES cells or the hematopoietic stem/progenitor cells present in umbilical cord blood. In practice, however, the efficiency of RBC generation varies with the quality of the ES cell line or the umbilical cord blood sample. Since ES cell lines can be utilized repeatedly, derivation of RBCs from ES cells appears to be more practical. However, even with optimal experimental procedures and the most appropriate ES cell line the generation of abundant RBCs directly from primate ES cells is a costly and time-consuming process (Hiroyama et al., 2006; Lu et al., 2008). If human erythroid progenitor cell lines can be established that have efficient production of mature RBCs, they would provide a much more useful resource than ES cell lines.

4. Establishment of mouse RBC progenitor cell lines

Several mouse and human erythroid cell lines have been established. However, to the best of our knowledge, there is no cell line that can efficiently differentiate into enucleated RBCs. For example, the human erythroid cell line K562, derived from chronic myelogenous leukemia cells, can differentiate to mature erythroid cells and produce haemoglobin but cannot produce enucleated RBCs.

It is generally difficult to establish hematopoietic cell lines from adult hematopoietic stem or progenitor cells, since these somatic cells are quite sensitive to DNA damage and are unable to maintain the lengths of their telomere repeats on serial passage (Lansdorp, 2005). In contrast, ES cells are relatively resistant to DNA damage and maintain telomere length on serial passage (Lansdorp, 2005). Therefore, we speculated that these characteristics of ES cells might be advantageous for the establishment of cell lines, since differentiated cells derived from ES cells should retain these beneficial characteristics. In addition, mouse cells tend to immortalize more readily than human cells. Hence, we attempted to evaluate the feasibility of establishing hematopoietic cell lines, erythroid cell lines in particular, from mouse ES cells.

4.1 Establishment of RBC progenitor cell lines from mouse ES cells

To induce differentiation of hematopoietic cells from mouse ES cells, we cultured ES cells using OP9 feeder cells (Nakano et al., 1994; Nakano et al., 1996; Kodama et al., 1994) in the presence of specific factors (Hiroyama et al., 2008). OP9 cells were used not only for induction of hematopoietic differentiation but also for establishment of cell lines in the early phase of long term culture of the induced hematopoietic cells (Hiroyama et al., 2008). In most cases, the induced cells stopped proliferating within two months of the initial induction of differentiation from ES cells (Hiroyama et al., 2008). This phenomenon is similar to that observed in primary culture of human cells such as fibroblasts, the so-called "crisis" of primary cells. In general, normal human cells cannot bypass this crisis stage and thus it is impossible to obtain immortalized cells from normal cells by continuous culture alone.

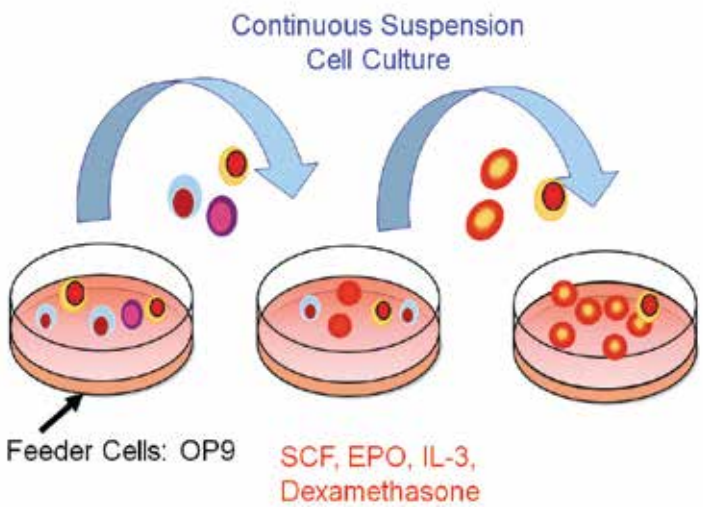

Fig. 3. A diagrammatic outline of the method to establish erythroid cell lines from ES cells.

Induced cells that could proliferate continuously for approximately two months (60 days) were cultured in the absence of OP9 cells and in the presence of hematopoietic humoral factors (Figure 3). Cells that continued to proliferate in the absence of OP9 cells were selected to establish cell lines. These cell lines acquired independency from OP9 cells within three months of the initial induction of differentiation from ES cells (Hiroyama et al., 2008). At approximately four months after initial induction, we sought to determine the factor(s) that were essential for the proliferation of each cell line. After this evaluation, each cell line was cultured in the presence of these essential factor(s) alone with changes of the medium every two or three days.

In addition to the method described in Figure 3 (Method A), we developed a second protocol (Method B) identical to Method A but omitting IL-3 at all stages. We attempted long term cultures of 63 lines, 51 with Method A and 12 with Method B. Five independent immortalized cell lines were successfully established, 4 with Method A and 1 with Method B (Hiroyama et al., 2008). These five cell lines continued to proliferate for more than a year. Morphological and flow cytometric analyses suggested that three of the lines were erythroid in nature, while the other two were mast cell-like (Hiroyama et al., 2008). We designated the erythroid cell lines MEDEP (mouse ES cell-derived erythroid progenitor line) and the mast

cell-like cell lines MEDMC (mouse ES cell-derived mast cell line). MEDEP-E14, MEDEP-BRC4, and MEDEP-BRC5 were derived from E14TG2a (129 strain), BRC4 (C57BL/6N strain), and BRC5 (C57BL/6N strain) mouse ES cell lines, respectively. The presence of IL-3 in the culture medium (Method A) may not be necessary for the establishment of erythroid cell lines, as we were able to establish one erythroid line, MEDEP-BRC4, following culture of the cells in the absence of IL-3 (Method B) (Hiroyama et al., 2008). MEDEP cells could proliferate from single cells following sorting by flow cytometry, i.e., cloning was possible.
All three MEDEP cell lines retained the morphological characteristics of erythroid cells and also showed cytokine dependency after cloning (Hiroyama et al., 2008). MEDEP-E14 and MEDEP-BRC5 were dependent on erythropoietin (EPO) and stem cell factor (SCF), respectively (Hiroyama et al., 2008). Although MEDEP-BRC5 appeared to respond to EPO, it could not proliferate long term in the presence of EPO alone. MEDEP-BRC4 proliferated most efficiently in the presence of SCF, EPO and dexamethasone (Hiroyama et al., 2008). The cytokine dependency of these MEDEP cell lines has not changed since they were induced to differentiate from ES cells more than a year ago.
RT-PCR analyses demonstrated that all MEDEP lines expressed genes specific for erythroid cells: GATA-1, EKLF (Erythroid Krüppel-like factor) and EPOR (erythropoietin receptor) (Hiroyama et al., 2008). In addition, all MEDEP lines expressed α- and β-globin, but not γ-, ε-, or ζ-globin (Hiroyama et al., 2008), indicating that they were adult and not primitive erythroid progenitor cells. Since it has been reported that definitive erythropoiesis can be induced in mouse ES cells, i.e., the induction of adult type erythroid cells (Nakano et al., 1996), all MEDEP lines appear to be derived from adult type erythroid progenitor cells.

4.2 In vitro differentiation of MEDEP

Next, we evaluated the potential of MEDEP cells to differentiate into more mature erythroid cells and found that the various lines could be induced to differentiate by the following treatments: MEDEP-E14 by deprivation of EPO; MEDEP-BRC5 by deprivation of SCF and addition of EPO; and, MEDEP-BRC4 by deprivation of SCF and dexamethasone and addition of EPO (Hiroyama et al., 2008). EPO appeared to be necessary for MEDEP-BRC5 and MEDEP-BRC4 cells to maintain cell viability during the differentiation process.
The three MEDEP lines exhibited differential expression of TER119 (a cell surface antigen specific for mature erythroid cells) and CD71 (transferrin receptor). For example, expression of CD71 was slightly higher in MEDEP-E14 cells than in MEDEP-BRC5 cells (Hiroyama et al., 2008). $TER119^{-}CD71^{-}$ cells differentiate first to $TER119^{-}CD71^{+}$ cells, subsequently to $TER119^{++}CD71^{+}$ cells, and then finally to $TER119^{+}CD71^{-}$ cells (Miharada et al., 2005). Consistent with the differences in their cytokine dependency, the three MEDEP cell lines appeared to represent different stages of erythroid differentiation. Nevertheless, after induction of differentiation in vitro by the methods described above, expression of TER119 and CD71 in each of the MEDEP lines exhibited a pattern consistent with a more mature lineage (Hiroyama et al., 2008). This expression pattern suggests that each of the three lines was able to differentiate into a more mature lineage. At present, the cause of the variability between MEDEP cell lines remains uncertain. However, these results clearly demonstrated that erythroid progenitor cells could be immortalized at different stages of their differentiation.
Of note, the vast majority of cells in each MEDEP line could differentiate into more mature cells, although each MEDEP line included cells possessing abnormal karyotypes (Hiroyama et al., 2008). This result strongly suggested that cells with abnormal karyotypes still retained

the potential to differentiate into more mature erythroid cells. In general, most immortalized cell lines are not necessarily homogenous in many aspects such as karyotype, genotype, phenotype etc., even after cloning. The emergence of cells possessing different characteristics is often observed following long term utilization of immortalized cell lines. Hence, periodic recloning and selection of cell lines is recommended to maintain cell cultures with desirable characteristics for clinical application.

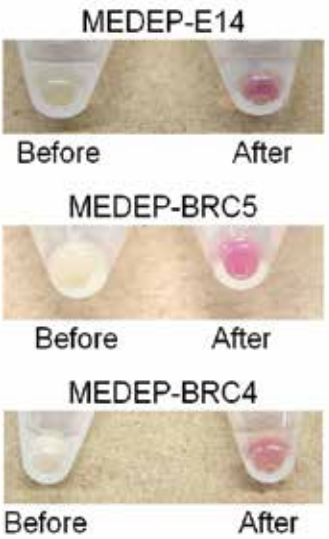

Fig. 4. MEDEP cell pellets show different coloration before and after differentiation.

Following induction of differentiation of MEDEP cells in vitro, the cell pellets collected after centrifugation appeared red while those before differentiation were white (Figure 4). In addition, following differentiation, enucleated cells could be identified by flow cytometric analysis using SYTO85 staining (Hiroyama et al., 2008). Morphological analysis confirmed that enucleated RBCs were present in addition to very mature erythroblasts (Figure 5).

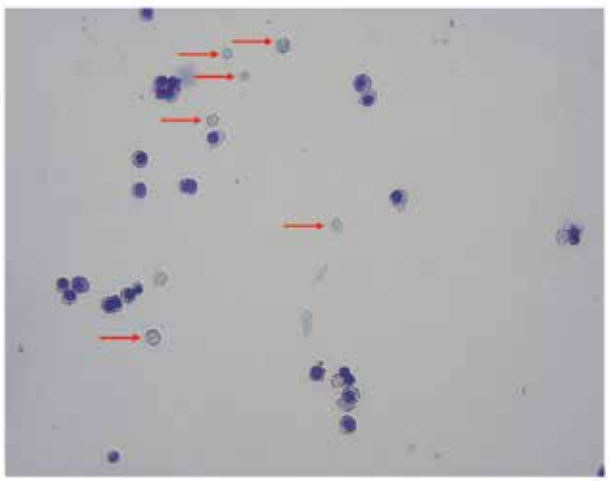

Fig. 5. Enucleated RBCs produced from MEDEP cells. Arrows indicate enucleated cells.

4.3 In vivo proliferation and differentiation of MEDEP

To evaluate the functional potential of MEDEP cells in vivo, we established a subline of MEDEP-E14 expressing the GFP marker Venus (Nagai et al., 2002). Although the expression of TER119 was slightly higher in MEDEP-E14-Venus cells than in parent MEDEP-E14 cells, the MEDEP-E14-Venus cells retained the ability to proliferate and differentiate into more mature erythroid cells in vitro (Hiroyama et al., 2008).

In general, the ablation of endogenous hematopoietic cells in mice is required to allow efficient detection of transplanted hematopoietic cells. Acute anemia induced by phlebotomy or hemolysis is commonly used in the study of urgent erythropoiesis (Alter et al., 1982; Miharada et al., 2005). We induced acute anemia in mice by intraperitoneal injection of phenylhydrazine, an inducer of hemolysis, and transplanted MEDEP-E14-Venus cells ($2x10^7$ cells/mouse) 24 hours later. Three days after transplantation, Venus-positive cells were present in the bone marrow and spleen (Hiroyama et al., 2008). Since the spleen is the major organ supporting urgent erythropoiesis in mice (Miharada et al., 2005), the transplanted cells were more abundant in the spleen than the bone marrow (Hiroyama et al., 2008). Venus-positive cells (the transplanted cells) demonstrated a phenotype consistent with differentiation into more mature erythroid cells compared to their phenotype just before transplantation (Hiroyama et al., 2008). Of note, MEDEP-E14-Venus cells differentiated into much more mature lineages in vivo than they did in vitro (Hiroyama et al., 2008).

To investigate whether transplanted cells could proliferate in vivo, we determined the absolute number and proportion (%) of Venus-positive cells in the spleen in a cell transplantation experiment. The absolute number of Venus-positive cells was elevated approximately two fold at three days compared to one day after cell transplantation (Hiroyama et al., 2008). This result indicates that transplanted cells can proliferate in vivo.

The expression of Venus in the transplanted cells decreased following their differentiation, i.e., the expression of Venus was lower in $TER119^{++}$ cells than in $TER119^{+}$ cells (Hiroyama et al., 2008). Thus, although we could not detect Venus-positive cells in peripheral blood, this was likely the result of disappearance of Venus following terminal differentiation. We therefore sought to confirm that MEDEP cells could differentiate into terminally-differentiated RBCs in vivo.

4.4 Increase of RBC number in mice suffering from acute anemia following transplantation of MEDEP

MEDEP cells ($2x10^7$ cells/mouse) were transplanted 24 hours after induction of acute anemia. As a control experiment, MEDMC cells ($2x10^7$ cells/mouse) were transplanted into control mice. Since $2x10^7$ transplanted RBCs correspond to a mere 2 μl of transfused cells, the number of RBCs in the transplanted mice will only increase if these transplanted MEDEP cells proliferate to some degree and differentiate into terminally-differentiated RBCs in vivo. Five days after transplantation, blood cell counts were performed in peripheral blood. The transplantation of MEDEP-E14 significantly ameliorated anemia compared to the control (Figure 6). The data obtained from the mice transplanted with control cells did not differ significantly from the data obtained from anemic mice that were not transplanted with any cells.

Since the RBC count in peripheral blood reflects the number of enucleated cells, whereas erythroblasts (nucleated cells) are included in the count of white blood cells (WBC), the

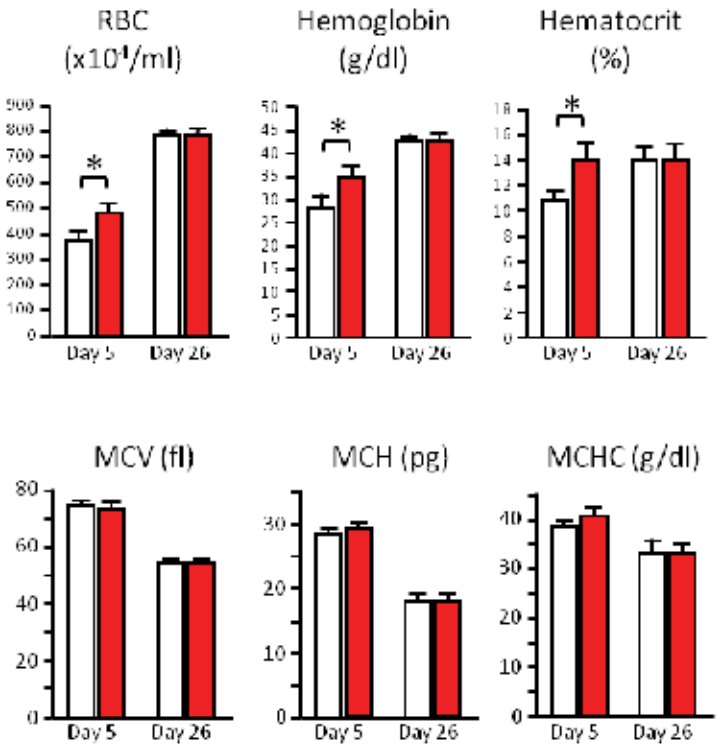

Fig. 6. Blood counts in control and MEDEP transplanted mice. White bars and red bars indicate mice transplanted with control cells and MEDEP cells, respectively. Asterisks indicate statistically significant differences ($P<0.05$).

increased number of RBC observed in mice transplanted with MEDEP cells indicated that the transplanted MEDEP cells could efficiently differentiate into enucleated cells (Hiroyama et al., 2008). The life span of RBCs is approximately 50 days in the mouse; therefore, it is highly likely that the RBCs produced from the transplanted MEDEP cells accumulated in the transplanted mouse.

Increases in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC) (Figure 6) are commonly observed in the recovery phase of acute anemia (Miharada et al., 2005). In addition, an increase in the number of WBC is observed in the recovery phase of acute anemia (Hiroyama et al., 2008) and is due to the presence of erythroblasts in the peripheral blood, since erythroblasts are counted as WBC by the automatic counter (Miharada et al., 2005). Given that there was no difference in MCV, MCH and MCHC levels between the transplanted and control mice in the recovery phase of acute anemia (Figure 6), RBCs derived from MEDEP cells in vivo appeared to possess characteristics similar to those derived from erythroid progenitor cells in the host mice. Twenty-six days after transplantation (27 days after the induction of acute anemia), all mice had recovered from the anemia and there were no differences in the blood counts of the two groups (Figure 6).

The transplantation of MEDEP-E14-Venus and MEDEP-BRC5 cells also ameliorated anemia compared to the control (Hiroyama et al., 2008). However, the transplantation of MEDEP-

BRC5 cells appeared to be less effective for amelioration of anemia than MEDEP-E14 cells (Hiroyama et al., 2008). Given that the in vitro proliferation activity of MEDEP-BRC5 cells was lower than that of MEDEP-E14 cells, the in vivo proliferation activity of MEDEP-BRC5 cells might have also been lower than that of MEDEP-E14 cells (Hiroyama et al., 2008). In addition, hemoglobin synthesis in MEDEP-BRC5 might have been less efficient than in MEDEP-E14 (Hiroyama et al., 2008).

Immunogenicity of human ES cell derivatives is one of the potential obstacles to their clinical use (Drukker and Benvenisty, 2004; Boyd et al., 2005). Indeed, transplanted MEDEP cells do not ameliorate acute anemia in mouse strains other than those from which the individual lines were derived or in immuno-deficient mice, suggesting immunological rejection by the heterologous strains. Hence, if human erythroid cell lines are to be established, the clinical application of these cells might involve application of a number of lines that express different major histo-compatibility (MHC) antigens.

4.5 Lack of tumorigenicity of MEDEP

Approximately three months after transplantation, Venus-positive cells were absent from the bone marrow and spleen of mice transplanted with MEDEP-E14-Venus cells (Hiroyama et al., 2008). In addition, although we examined all other transplanted mice up to 6 months after transplantation, no tumors were observed in MEDEP-transplanted mice or MEDMC-transplanted control mice (Hiroyama et al., 2008). Furthermore, subcutaneous transplantation of MEDEP cells ($2x10^7$ cells/injection site) did not give rise to any tumors, whereas subcutaneous transplantation of the same number of parent ES cells led to the formation of a teratoma (Hiroyama et al., 2008).

What mechanism underlies the lack of tumorigenicity of MEDEP? MEDEP cells can only be successfully cultured in the presence of excess growth factor(s) and cannot proliferate or survive without such growth factor(s). Therefore, MEDEP cells cannot proliferate or survive in vivo in the presence of the normal range of growth factors. Indeed, when MEDEP-E14-Venus cells were transplanted into mice that were not in an anemic condition, an increase in RBC numbers in the peripheral blood was not observed and Venus-positive cells were not detected in the bone marrow or spleen by flow cytometry a few days after transplantation. By contrast, when MEDEP cells were transplanted into mice suffering from acute anemia, the concentration of growth factors was upregulated in the mice due to anemia and thus MEDEP cells proliferated. Following recovery from anemia, there was a reduction in growth factors in the serum and MEDEP cells no longer proliferated or survived. We suggest that the concentration of growth factors determines whether or not MEDEP cells can proliferate with the potential for tumorigenicity.

Therefore, establishment of growth factor-dependent erythroid cell lines may be of particular value for clinical applications. Nevertheless, when human erythroid cell lines are established, the tumorigenic potential of these lines will still need to be exhaustively analyzed prior to their use in the clinic (Vogel, 2005; Hentze et al., 2007). It may also be advisable to engineer such cells so that they can be eliminated should a malignant phenotype arise for any reason (Schuldiner et al., 2003).

4.6 RBCs derived from MEDEP are functional in vivo

To confirm that the RBCs derived from the transplanted MEDEP cells are functional in vivo, we monitored the response of transplanted mice to a second induction of hemolysis.

Hemolysis was induced and followed by cell transplantation; a second induction of hemolysis was performed five days after cell transplantation (Figure 7) (Hiroyama et al., 2008). Analysis of blood counts was not performed at any time point in this experiment, because collection of peripheral blood would affect the results. We observed that one of the eight mice in the group transplanted with MEDEP-E14 cells died, while seven of the eight mice in the group transplanted with control cells (MEDMC-NT2) died (Figure 7). Mice that did not receive any transplanted cells showed a mortality rate similar to that of mice transplanted with control cells. This result is consistent with observed increase in RBC numbers five days after cell transplantation (Figure 6). In other words, this result indicated that RBCs derived from MEDEP cells were functional in vivo and that mice transplanted with MEDEP cells could survive a severe acute anemia caused by a second induction of hemolysis.

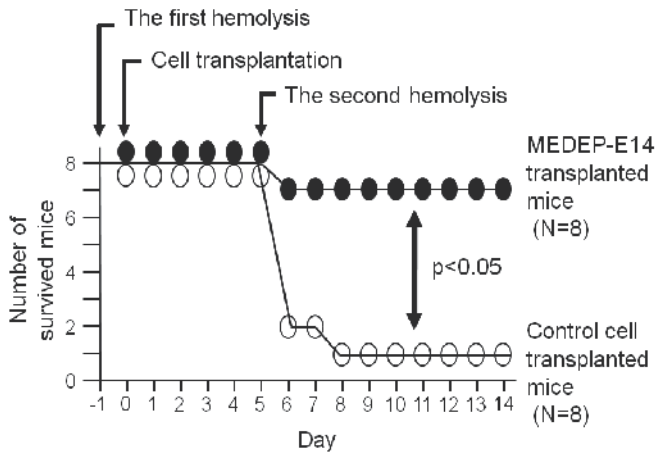

Fig. 7. Survival curves of mice transplanted with MEDEP or control cells following severe acute anemia.

5. Strategy for clinical use of hematopoietic cells produced in vitro

The most critical obstacle to use of ES cell-derived cells is the potential for tumorigenicity. First, there is a risk that the transplanted cells include ES cells and that such contaminant cells could be tumorigenic. Second, even if ES cells can be completely excluded from the transplanted sample by some method, the transplanted cells may revert to an ES-like state and could be tumorigenic. Therefore, when we consider the possibility of clinical application of ES cell-derived cells, thorough preclinical studies need to be carried out.

Establishment of erythroid progenitor cell lines from human ES cells would provide a valuable source of material for further utilization. However, the risk of tumorigenicity of such cells is similar to that of ES cells since they are immortalized. As mentioned above, growth factor dependent cell lines that can proliferate only in the presence of excess growth factor(s) in vitro might offer candidate cell lines for use in the clinic. Those cells could be transplanted with a simultaneous injection of the requisite growth factors and, after achievement of their clinical purpose, the cells would be eliminated due to eventual deprivation of growth factor(s). In any case, the risk of tumorigenicity must be taken into account in the transplantation of nucleated cells derived from immortalized cells, since we cannot predict at present how these cells will behave after transplantation.

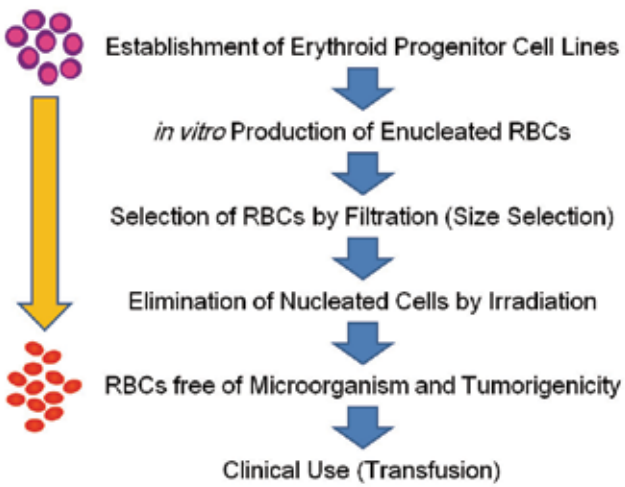

Fig. 8. Strategy for production of transfusable RBCs from immortalized erythroid cell lines.

On the other hand, RBCs and platelets are very specific cells in the body that lack nuclei following terminal differentiation. These anuclear cells cannot form tumors in vivo. Therefore, RBCs and platelets, even if they are derived from immortalized cells, could be transfused without concerns about possible tumorigenicity. Following production of RBCs or platelets in vitro, pure populations can be selected by size using filtration, since they are much smaller than normal nucleated cells. In addition, any nucleated cells, which are still present in the sample after size selection, could be eliminated by irradiation. Irradiation of RBC samples is already routinely performed to eliminate lymphocytes in some clinical protocols. Therefore, if we can establish an in vitro culture system that enables abundant production of RBCs or platelets, then the cells could be applied in the clinic using the procedures described above (Figure 8). To establish such a culture system, progenitor cell lines able to produce enucleated cells in vitro will be essential.

6. Establishment of RBC progenitor cell lines from human ES cells or human iPS cells

The reproducible establishment of MEDEP cell lines described above strongly suggests that similar erythroid cell lines could also be established from human ES cells. We, therefore, sought to establish human erythroid progenitor cell lines. The methods used to induce hematopoietic cells from ES cells and to culture the induced hematopoietic cells are similar to those established for MEDEP cell lines (Figure 3), with the exception that the corresponding human factors were applied and IL-3 was not used at all. Exclusion of IL-3 was based on our finding that the compound was not necessary for establishment of MEDEP cell lines (see above).

Initially, we used three human ES cell lines, KhES-1, KhES-2 and KhES-3, that had been established in Japan. However, we were unable to induce hematopoietic cells from all three lines and, compared to mouse ES cells, the efficiency of production of hematopoietic cells was extremely low. As a result, we have yet been successful in establishing immortalized cell lines from the three ES cell lines.

During the course of the experiments using these human ES cell lines, a breakthrough discovery in the field of regenerative medicine was reported, namely, the establishment of human iPS cells (Takahashi et al., 2007) following that of mouse iPS cells (Takahashi and Yamanaka, 2006). This discovery prompted us to establish human iPS cells, since the characteristics of pluripotent stem cells, such as ES cells, differ among cell lines. In other words, we speculated that we could obtain iPS cell lines that could have the ability to differentiate into hematopoietic cells. We were able to establish a number of human iPS cell lines using fibroblast-like cells derived from neonatal tissues (Fujioka et al., in press). Fortunately, we were able to induce abundant numbers of hematopoietic cells from some of these iPS cell lines and also to establish immortalized hematopoietic cell lines from the induced hematopoietic cells. Currently, we are investigating the characteristics of these immortalized hematopoietic cell lines. Some seem to be erythroid cell lines.

7. Concluding remarks

We propose that by utilizing ES cells or iPS cells it will be possible to establish human erythroid progenitor cell lines able to produce enucleated RBCs. RBCs produced by in vitro culture of such erythroid cell lines could be applied in the clinic following size selection and elimination of nucleated cells by irradiation.

Once an erythroid cell line able to produce O/RhD(-) RBCs is established, it could be used as necessary to produce RBCs for transfusion into patients around the world. Obviously, there would be a few patients for whom this approach would not be practicable, such as those possessing Rh-null RBCs.

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Part 5

Tissue-specific Regeneration of Other Tissues

16

Differentiation of Hepatocytes from Mice Embryonic Stem Cells in Three-Dimensional Culture System Imitating in vivo Environment

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1. Introduction

Liver transplantation is accepted as the standard treatment for saving patients affected with serious liver disease. However the demand for donated livers for transplantation far exceeds the supply. One potential way to compensate for the chronic shortage is the development of bioartificial (Aoki et al., 2008, Mizumoto et al., 2004, Qian et al., 2003) and secondary livers (Kosone et al., 2008, Nguyen et al., 2009, Strom & Fisher, 2003). A necessity for development of these liver systems is the availability of sufficient high quality hepatocytes (Dan & Yeoh, 2008, Haridass et al., 2009, Mizumoto et al., 2008, Umehara et al., 2008). In addition, the high quality hepatocytes have great potentials to be source for pharmaceutical models to assess toxicity of new drugs, a critical step in drug discovery and development. In this review, we describe recent progress towards using embryonic stem (ES) cells differentiated in three-dimensional (3D) culture systems that imitate *in vivo* environment of hepatic histogenesis and liver regeneration. We suggest that the combination of the ES cells and 3D culture systems have the potential to provide high quality hepatocytes.

Pluripotent ES cells, which are derived from the inner cell mass of blastocysts, are able to replicate and differentiate into various cell types composed of whole body. Thus, ES cells have great potentials to serve as sources of hepatocytes to construct liver systems, bioartificial or secondary livers. Hepatocyte-like cells differentiated from ES cells by various methods have already been reported (Hamazaki et al., 2001, Matsumoto et al., 2008, Rambhatla et al., 2003). This review introduces our culture systems for differentiation into the hepatocyte-like cells and describes the characteristics of the ES cell-derived hepatocyte-like cells induced by the culture systems.

2. Produce of three-dimensional (3D) culture systems

We maintain undifferentiated mice ES cells of the cell line 129/sv strain. The cells are cultured in Dulbecco's modified eagle medium supplemented with 20% Knockout Serum Replacement, 100 mM non-essential amino acids, 100 μM sodium pyruvate, 100 mM 2-mercaptoethanol, 50 units/ml penicillin and 50 μg/ml streptomycin, 1000 units/ml leukemia inhibitory factor on 0.1% gelatin-coated dishes with STO fibroblasts treated with mitomycin C. The cultures are maintained at 37°C in humidified air with 5% CO_2.

Following, we form embryoid bodies (EBs) including germ layers with our original method, conical tube (CT) method (Kurosawa et al., 2003). The ES cells are dissociated using 0.1% trypsin–EDTA, and resuspended at $2x10^4$ cells/ml with EB-formation medium composed of Iscove's modified Dulbecco's medium containing 20% fetal bovine serum, 100 mM non-essential amino acids, 100 μM sodium pyruvate, and 100 mM 2-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin. One milliliter of the cell suspension is placed into polypropylene 1.5-ml conical tubes with round bottoms and the screw caps loosely closed for gas exchange. The cells are cultured for 5 days in 5% CO_2 at 37°C.

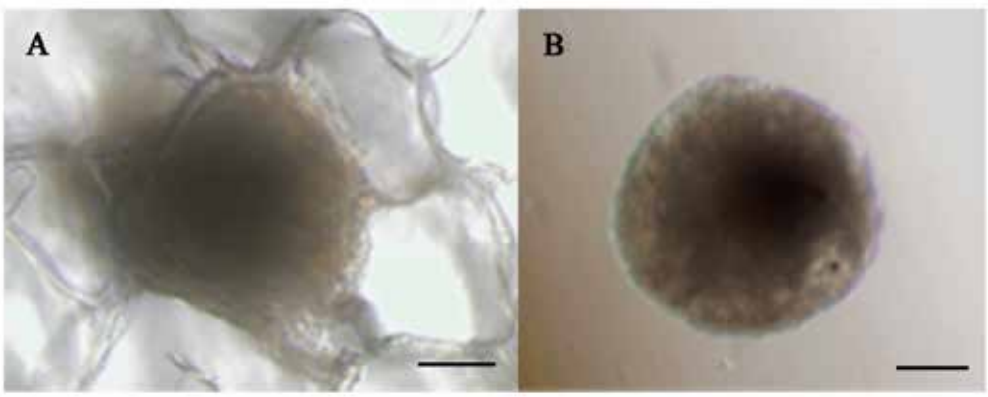

Fig. 1. (A) Day 0 EB derived from $2.0x10^4$ ES cells formed by the CT method inserted into collagen scaffold, 3D culture system. Bar=100 μm. (B) EB is put on the culture dish, 2D culture sysytem. Bar=100 μm

We construct 3D culture system by inserting a single EB prepared by the CT method into the three-dimensional space of collagen scaffolds (Fig. 1A). The scaffold is composed of atelocollagen type I derived from cow skin, and the diameters of the scaffold pores are approximately 200-400 μm. To estimate the 3D culture system, we put a single EB on the collagen-coated dishes (two-dimensional (2D) culture system, Fig. 1B). The EBs inserted into each scaffold, or put on each dish are cultured for 24 days with originally cocktailed EB-differentiation medium. The medium are composed of EB-formation medium supplemented with 100 ng/ml acidic fibroblast growth factor, 20 ng/ml hepatocyte growth factor, 10 ng/ml mouse oncostatin M, 10^{-7} M dexamethasone, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite. Every 3 days, one half of the old medium is removed and an equal volume of fresh medium is added to replace it.

3. Hepatocyte-like cells induced from ES cells in the 3D culture system

To induce hepatocyte from the EBs, we imitate environments of hepatic histogenesis by using acidic fibroblast growth factor, hepatocyte growth factor, oncostatin M, dexamethasone, insulin, transferrin and sodium selenite. These exogenous growth factors and hormones are known to relate with liver development (Hamazaki et al, 2001). In the 3D culture system, the cells derived from the EB fill many of the scaffold pores, and proliferating cell nuclear antigen (PCNA)-positive cells are present inside the collagen

scaffolds. The cells in the 2D culture system also proliferate and spread. Both culture systems induce formation of various cell types including spontaneously beating cardiac muscle cells.

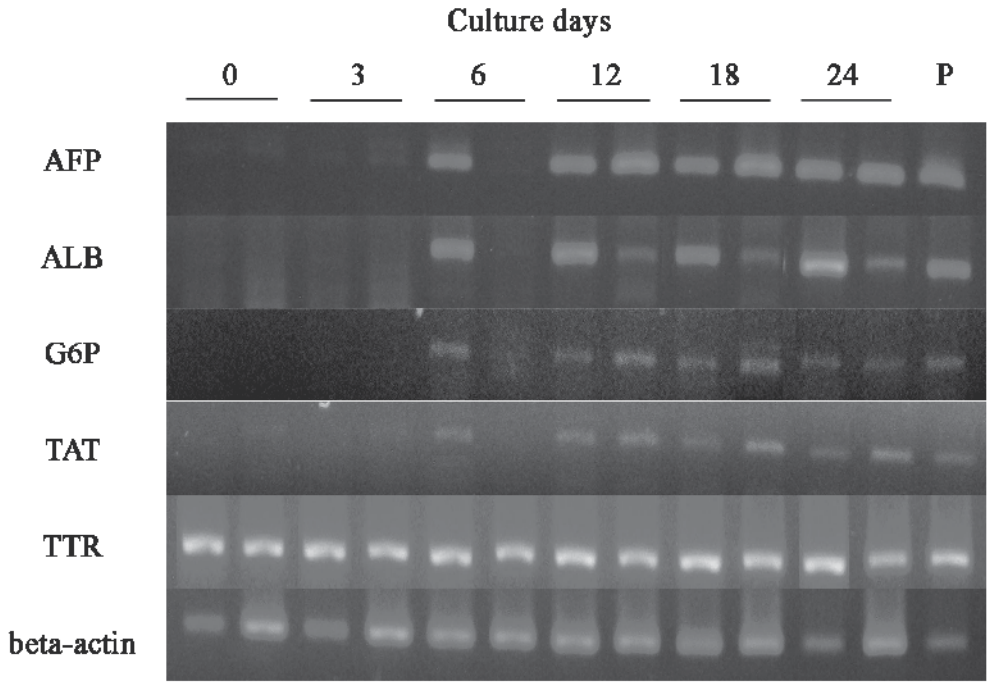

Fig. 2. In the 3D culture system, the cells cultured from 6 to 24 days express AFP, ALB, G6P and TAT genes. In contrast, these genes in 2D culture systems are expressed only after Day 12. P, positive control from fetal liver of pregnant ICR mouse at Day 12

The cells derived from EB are analyzed to determine if hepatocyte-like cells are induced in both culture systems. Reverse transcription polymerase chain reaction (RT-PCR) data show that the cultured cells express hepatocyte-specific mRNAs alpha-fetoprotein (AFP), albumin (ALB), glucose-6-phsphatase (G6P), tyrosine aminotransferase (TAT) and the endodermal transthyretin (TTR) mRNA (Fig. 2). Because EBs include endoderm, TTR expression is apparent at Day 0 and is maintained for the period of culture in both 3D and 2D culture systems. The 3D culture system shows that cells express all of the hepatocyte marker mRNAs on Day 6, and these are maintained throughout the culture to Day 24. In the 2D culture system, these mRNAs appear at Day 12 and each is maintained to Day 24.

Some of the cells derived from EBs in both systems were positively stained with anti-albumin antibody. These albumin-positive cells have large nuclei and polyhedral contours. In 3D culture systems, the albumin-positive hepatocyte-like cells formed cord-like structures (Fig. 3A). In 2D culture systems, the albumin-positive cells are usually distributed singly, but also occasionally form small clusters (Fig. 3B). However, the cells do not show cord-like structures.

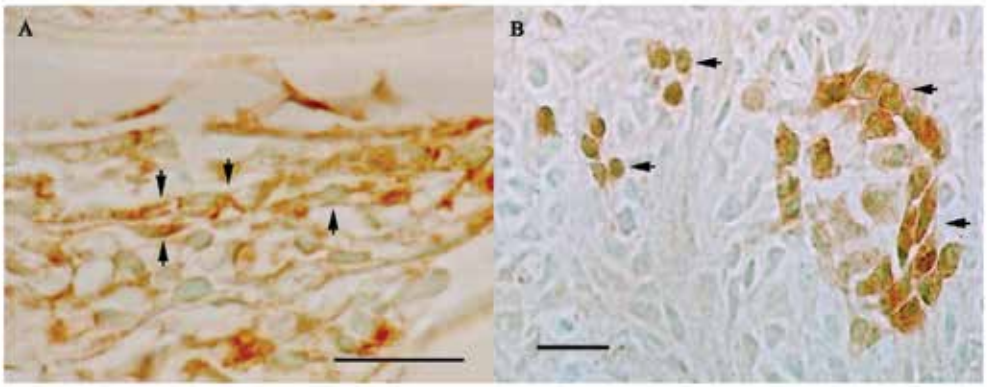

Fig. 3. (A) At 24 days, cells cultured in a 3D culture system contain albumin-positive cells. The albumin-positive cells form cord-like structures (arrows). Bar=20 μm. (B) Outgrowths derived from EBs in monolayers of 2D cultures systems also include albumin-positive cells. The albumin-positive cells distribute singly or in small clusters (arrows). Bar=50 μm

These results show that the both culture systems induce hepatocyte-like cells from EBs. However, there are two differences between 3D and 2D culture systems. One is that on the sixth day of culture, all hepatocyte-specific genes are detected in cells of the 3D culture system, but not in the 2D culture system. Another is that the albumin-positive cells induced in the 3D culture system form cord-like structures that are not present in the 2D culture system. These differences suggest that the 3D culture system promotes differentiation of ES cells and formation of tissue-like structures better than the 2D culture system. This may be due to the presence of cell-to-cell and/or cell-to-matrix interactions in the 3D culture system, which is one of the advantages, similar to those likely to occur *in vivo*.

4. Hepatocyte-like cells induced from ES cells in the 3D culture system

We have investigated the embryologic characteristics of the hepatocyte-like cells that are differentiated from ES cells within the 3D culture system *in vitro*. Firstly, RT-PCR shows that the cells derived from EBs express the following genes: AFP, an endodermal marker of early fetal hepatocyte differentiation; ALB, an early fetal and mature hepatocyte differentiation marker; G6P, predominantly expressed in the hepatocytes in late gestational or perinatal stages; and TAT, a marker for perinatal or postnatal hepatocyte-specific differentiation (Hamazaki et al., 2001). Although AFP and ALB are expressed in other cells, G6P and TAT are selectively expressed in developmental hepatocytes (Harada et al., 2003).
Secondly, immunohistochemistry shows that the ALB-positive EB-derived hepatocyte-like cells are positive for AFP, a marker of hepatoblasts, and cytokeratin 19, a marker of hepatic-progenitor cells, but not for cytokeratin 18, a marker of mature hepatocytes. On embryonic day 9.5 in mice, hepatoblasts, immature liver epithelial cells expressing both albumin and alpha-fetoprotein, appear in the hepatic bud. The hepatoblasts then develop into hepatic-progenitor cells, bipotential cells that are capable of differentiating into hepatocytes or bile duct epithelial cells, both of which express albumin and cytokeratin 19 (Dabeva et al, 2000). The cells develop into mature hepatocytes expressing both albumin and cytokeratin 18 (Rambhatla et al., 2003).

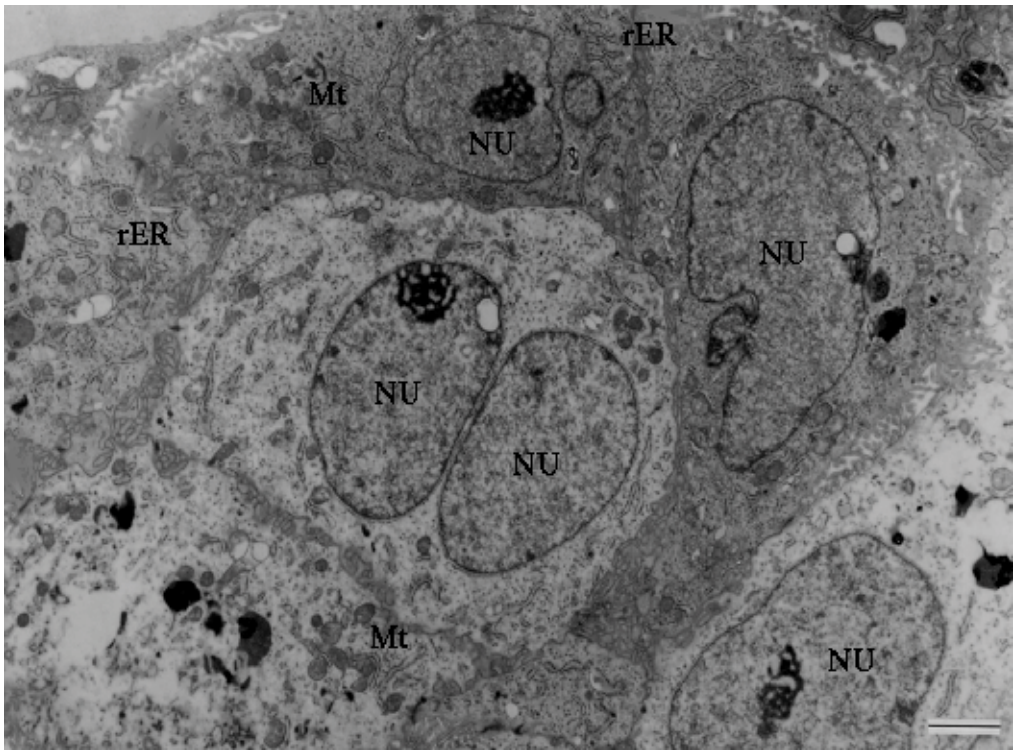

Fig. 4. Electron microscopy shows that hepatocyte-like cells in the collagen scaffold have numerous free ribosomes, moderate numbers of mitochondria and rough surfaced endoplasmic reticulum. The nuclear-cytoplasm ratio is high, and some nuclei are slightly pleiomorphic. Bar=2 μm. NU: nuclear; Mt: mitochondria; rER: rough surfaced endoplasmic reticulum

Finally, electron microscopy shows that the hepatocyte-like cells have typically immature intracellular organelles including numerous free ribosomes, mitochondria and rough-surfaced endoplasmic reticulum (rER) (Fig. 4). However they do not have glycogen particles, smooth-surfaced endoplasmic reticulum (sER), lipid droplets, peroxisomes, or iron deposits that are typically found in mature hepatocytes. In addition, the nuclei are occasionally irregular in shape, with a high nuclear-cytoplasmic ratio. Developmentally, cytoplasmic structures associated with hepatocyte synthetic and secretory function, i.e., rER and Golgi apparatus, are the first to differentiate (Bielanska-Osuchowska, 1996, Kanamura et al., 1990, Luzzatto, 1981), and glycogen particles, sER and peroxisomes appear later. Therefore, the hepatocyte-like cells induced from EBs in the 3D culture system *in vitro* resemble an immature type, which are developmentally intermediate between embryonal and fetal types (Nonoyama et al., 1988).

5. Hepatocyte-like cells derived from ES cells in the liver regeneration environment

We have attempted to use the *in vivo* environment of regenerating liver to induce the formation of mature hepatocyte-like cells. To provide an *in vivo* environment of liver

regeneration, we use partially hepatectomized livers of BALB/c nu/nu male nude mice at postnatal week 5. The nude mice are anesthetized with an intraperitoneal injection of pentobarbital sodium solution (0.05 mg/g-body weight), and then approximately 60% of the liver is removed. At that time, we transplant a single EB-collagen scaffold cultured for 24 days into the median lobe. We have analyzed the cells within the scaffolds implanted for 7 and 14 days. The transplanted cells are clearly demarcated with a capsule composed of collagen fibers. The scaffolds have many translucent structures that contained red blood cells, and PCNA-positive cells are present inside the transplanted scaffolds. In the recipient liver, the cells derived from EB do not form teratomas, and then these cells or host tissues do not expand and invade each other.

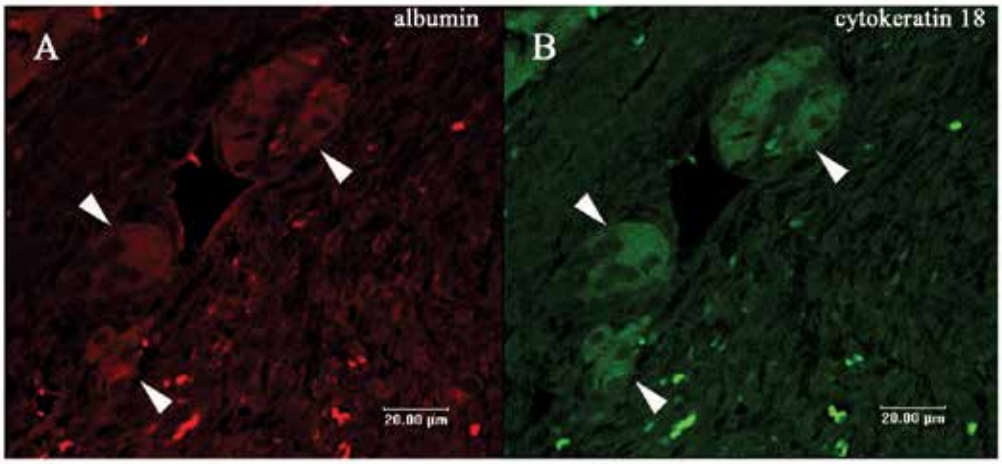

Fig. 5. At 14 days after implantation, the EB-derived hepatocyte-like cells expressing both albumin (A, red) and cytokeratin 18 (B, green) form hepatic lobule-like aggregates (Bar=20 μm)

We are investigating whether the hepatocyte-like cells derived during *in vitro* culture developed into mature cells or, alternatively, if other transplanted cells differentiated into them. At 7 days after transplantation, albumin positive cells are routinely present but alpha-fetoprotein positive hepatocyte-like cells disappear, even though cytokeratin 19 positive cells remain in the scaffolds. Importantly, some hepatocyte-like cells are clearly stained with both anti-albumin and anti- cytokeratin 18 antibodies. These are not recognizable in the cells after 24 days of *in vitro* culture. At 14 days, the hepatocyte-like cells positive for both albumin and cytokeratin 18 progressively develop from the cord-like structures into aggregates similar to hepatic lobules (Fig. 5). These results clearly indicate that immunohistochemically mature hepatocyte-like cells are induced from ES cells, and these cells construct hepatic lobule-like structures in the *in vivo* environment of liver regeneration.
The differentiation of hepatocyte-like cells from ES cells is complex. It requires not only an appropriate supporting matrix, but also an abundance of chemical mediators. During liver regeneration, nonparenchymal hepatocytes and parenchymal hepatocytes secrete various factors including tumor necrosis factor-alpha, interleukin-6, heparin bridge-epidermal growth factor, tumor growth factor, hepatocyte growth factor, and vascular endothelial growth factor (Duncan et al., 2009, Ishii et al., 1995). These factors might be responsible the

induction of immunohistochemically mature hepatocyte-like cells in the ES cell-implanted livers. Therefore, culture systems that imitate the *in vivo* environment and include co-cultured freshly isolated hepatocytes supplemented with growth factors and sufficiently high oxygen tension (Kimura et al., 1998), have the potential to promote development of new hepatocytes from the ES cells. Culture systems using scaffolds make this possible because they can provide an optimal structure that imitates *in vivo* histoarchitecture (Kamiya et al., 2002, Takimoto et al., 2003).

6. Advantage of the 3D culture systems and imitation of in vivo environment

This review shows two advantages of 3D culture systems. One is that 3D culture systems using scaffolds can provide cell-to-cell and/or cell-to-matrix interactions. These interactions promote differentiation of ES cells and construction of tissue-like structures (Carlberg et al., 2009, Elisseeff et al., 2006, Gao et al., 2010, Tian et al., 2008, Yim et al., 2006). Another benefit of the 3D culture system is that it can be manipulated to retrieve the cells or tissues derived from EBs without injuring or destroying them. Although hepatocyte-like cells can be induced from EBs in monolayer culture systems, these cells must be harshly treated to dislodge them from the culture dish before use. This operation can injure cells and destroy tissue structures. In the case of the scaffold culture system, we show that hepatocytes and hepatic structures derived from EBs can be used without any prior treatment.
Furthermore, this review shows that two points are worth highlighting regarding the development of bioartificial and secondary livers. The first is that ES cells have the potential to differentiate into hepatocytes and hepatic structures in suitable environments. The second point is that the best of these environments are ones that closely imitate the *in vivo* environment.

7. Conclusion

This review has shown that EBs formed from ES cells in collagen scaffolds when cultured *in vitro* for 24 days with exogenous growth factors and hormones associated with liver development. The 3D culture system induces hepatocyte-like cells from ES cells, and these cells form cord-like structures that are not present in monolayer cultures. However, the hepatocyte-like cells produced under these conditions are immature. Seven days after the cultured cells in scaffolds are transplanted into mice livers after partial hepatectomy, immunohistochemically mature cells, expressing both albumin and cytokeratin 18 are induced. At 14 days after transplantation, the cells are configured into hepatic lobule-like aggregates. Threfore, the combination of ES cells and culture systems imitating the *in vivo* environment warrant further development as a potential source of hepatocytes and liver structures for *in vivo* use.

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Abbreviations: ES cell: embryonic stem cell; EBs: embryoid bodies; 3D: three-dimensional; 2D: two-dimensional; CT method: conical tubes method; ALB: albumin; AFP: alpha fetoprotein; TTR: transthyretin; G6P: glucose-6-phsphatase; TAT: tyrosine aminotransferase; CK19: cytokeratin19; CK18: cytokeratin18; PCNA: Proliferating Cell Nuclear Antigen; rER: rough surfaced endoplasmic reticulum; sER: smooth surfaced endoplasmic reticulum

17

Rejuvenation of the Thymic Microenvironment by ESC-derived Thymic Epithelial Progenitors

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1. Introduction

T-cells play a critical role in the adaptive immune system, providing protection against neoplasia and bacterial, viral, fungal and parasitic infections. Unfortunately, T-cell deficiencies can occur in a number of physiological and pathological situations. The thymus is the primary organ for T cell generation, and while it continues to export T-cells throughout life, the thymus undergoes age-dependent involution, resulting in decreased numbers and functional capacity of T-cells in the elderly (Dorshkind et al., 2009; Zediak and Bhandoola., 2005; Lynch., 2009; Taub and Longo., 2005). Various genetic and infectious diseases, such as AIDS, are associated with T-cell deficiencies. In addition, intensive chemotherapy or radiotherapy for cancer patients and preparative regimens for foreign tissue or organ transplants often result in severe and protracted T cell deficiencies. Furthermore, the recovery of T cells after hematopoietic stem cell transplantation is often slow and incomplete compared with that of myeloid, NK, or B-cells (Williams et al., 2007; Wils et al., 2005). T-cell deficiencies contribute to increased morbidity and mortality from opportunistic infections, the occurrence and relapse of cancers, and the failure of vaccinations and other immunotherapies (van den Brink et al., 2004).

There are two major pathways for T-cell reconstitution: thymus-dependent regeneration and thymus-independent homeostatic expansion (Williams et al., 2007; Mackall et al., 1996; Mackall and Gress., 1997). The former recapitulates thymic ontogeny and generates T-cells from bone marrow (BM)-derived T-cell progenitors that undergo positive and negative selection in the thymus. Therefore, T cells generated from this pathway usually have a diverse TCR repertoire, are capable of responding to a variety of foreign antigens, and tolerate self-antigens. In contrast, the thymus-independent pathway usually occurs by expansion of residual mature T cells in the periphery, thus producing T-cells with a limited TCR repertoire and the possible loss of self tolerance. Therefore, the thymus-dependent pathway is generally a preferred pathway for T-cell regeneration.

T-cell development in the thymus is dependent on the thymic microenvironment, in which epithelial cells are the major components (Anderson et al., 2006; Chidgey etal., 2007). Thymic epithelial cells (TECs) can be divided into cortical (cTEC) and medullary (mTEC) subpopulations. The former are thought to mediate positive selection and the latter are thought to control the negative selection process in which potentially autoreactive T-cells

are deleted. The importance of TECs has been underscored by the fact that defects in these cells results in T-cell deficiencies. For example, gene mutation or deletion of the forkhead transcription factor Foxn1 in human or mouse affects thymic epithelial differentiation, resulting in loss of intrathymic T-cell development and severe immunodeficiency (Anderson et al.,2006; Chidgey etal., 2007). Furthermore, reductions in the number of TECs results in a reduced number of thymocytes (Jenkinson et al., 2008; Jenkinson et al., 2007; Revest et al., 2001).

Many studies have demonstrated that during aging, TECs undergo both a qualitative and quantitative loss believed to be one of the major factors responsible for age-dependent thymic involution (Dorshkind et al., 2009; Zediak and Bhandoola., 2005; Lynch., 2009; Taub and Longo., 2005). Transfer of young BM into aged recipients is not capable of restoring the thymic architecture to that of a young thymus, as these aged recipients still exhibit diminished thymocyte numbers as well as significantly reduced numbers of naïve T cells in the periphery. This suggests that there are some irreversible alterations in the aged thymic microenvironment (Zediak and Bhandoola., 2005). In addition, TECs are vulnerable to injury from radiation, chemotherapy, infection, and graft-versus-host disease following bone marrow transplantation (Adkins et al., 1988; Rossi et al., 2002; Ye et al., 2004; Mackall et al., 1995). Therefore, there is a need for the development of strategies to enhance or restore TECs. Administration of keratinocyte growth factor or sex steroid ablation has been shown to increase the number of TECs, resulting in enhanced thymopoiesis (Kelly et al., 2008; Min et al., 2002; Rossi et al., 2007; Min et al., 2007; Alpdogan et al., 2006). Additionally, transplantation of cultured thymus fragments has been used to provide the thymic microenvironment for T-cell regeneration in patients and experimental animals with T-cell deficiencies or as a method for the induction of tolerance in organ transplantation (Markert et al., 2003; Hong et al., 1979; Yamada et al., 2000; Kamano et al 2004). However, the applications of cultured thymus fragments are limited by tissue availability.

During development in mice, the thymus initially arises from the third pharyngeal pouch (Anderson et al., 2006). Previous studies have suggested a dual origin for thymic epithelium, with cortical epithelium deriving from ectoderm and medullary epithelium deriving from endoderm (Manley, 2000). However, recent functional data strongly supports the theory that thymic epithelium is derived from a single germ layer, the endoderm (Manley, 2000; Rodewald, 2008; Blackburn & Manley 2004; Zhang et al., 2007). This single endoderm-origin model is consistent with data suggesting a common progenitor gives rise to both types of TECs (Gordon et al., 2004; Bleul et al., 2006). The thymic epithelial progenitors (TEPs) in the embryonic thymus have been reported to be MTS24+ cells due to the finding that purified MTS24+ cells can reconstitute a functional thymic epithelial microenvironment capable of supporting T-cell development *in vivo* (Markert et al., 2003; Hong et al., 1979). However, another recent study showed that thymic architecture could be formed *in vivo* by a large number of MTS24- cells (Yamada et al., 2000). While the expression of a common surface marker on these progenitor cells remains controversial, ontogenetic analysis of epithelial cells during thymic development has shown that TEPs co-express cytokeratins (k) 5 and K8,, then proliferate and differentiate into mature K5-K8+ cTECs and K5+k8- mTECs (Anderson et al., 2006; Klug et al., 2002). However, because cytokeratins are intracellular antigens, which requires that cells are permeabilized before antibody staining and analysis, the patterns of the cytokeratin expression allow only phenotypic analysis, not the isolation of live cells for functional studies. Recently, Rossi et al reported that a fraction

of MTS24+ cells in the embryonic thymus expressed the cell surface marker, EpCAM1 (Rossi et al., 2006). Importantly, a single EpCAM1+ cell isolated from the embryonic thymus could develop into both types of TECs *in vivo* (Rossi et al., 2006). The identification and characterization of TEPs has important clinical implications for restoring thymic function. However, use of TEPs for this purpose has been restricted thus far by the limited availability of such cells.

2. Generation of thymic epithelial progenitors from mouse embryonic stem cells

It is well known that embryonic stem cells (ESCs) have the dual ability to propagate indefinitely *in vitro* in an undifferentiated state and to differentiate into many types of cells that derive from all three germ layers (Murry & Keller 2008). We have investigated whether mouse ESCs can be selectively induced to generate TEPs *in vitro*. By using both three-dimension embryoid body formation and two-dimension monolayer culture systems in the presence of four growth factors [fibroblast growth factor (FGF)-7, FGF-10, bone morphogenetic protein 4 and epithelial growth factor], we have shown for the first time, that murine ESCs (mESCs) can be selectively induced to differentiate into TEPs (Lai & Jin, 2009). Under these culture conditions, EpCAM1+ cells can be generated from mESCs (Lai & Jin, 2009). Importantly, most of the mESC-derived EpCAM1$^+$ cells co-expressed K5 and K8, a phenotype of TEPs (Gordon et al., 2004; Bleul., 2006; Rossi et al., 2006). The EpCAM1+ cells also expressed the Pax1, Pax9, FoxN1, and Plet1 genes that are known to be highly expressed in TEPs from the embryonic thymus (Manley, 2000). Together, these findings indicate that the mESC-derived EpCAM1$^+$ cells contained TEPs.

To determine whether the mESC-derived EpCAM1$^+$ cells could differentiate into cTECs and mTECs, and form normal thymic architecture, we purified cultured mESC-derived EpCAM1$^+$ cells and mixed these cells with CD4$^-$CD8$^-$CD45$^+$ immature thymocytes from adult mice. The mixtures were subjected to cell reaggregation *in vitro*, and then were transplanted under the kidney capsule of syngeneic mice. Six weeks later, the grafts were examined by immunohistochemical staining. We found that discrete K8$^+$K5$^-$ cortical and K8$^-$K5$^+$ medullary epithelial areas were present in the grafts. Some of the cells co-expressed K8 and K5, suggesting they were residual, or self-replicating, TEPs (Lai & Jin, 2009). In controls, transplantation of CD4$^-$CD8$^-$CD45$^+$ immature thymocytes and/or mESC-derived EpCAM1$^-$ cells could not form normal thymic architecture in vivo. Further analysis showed that all stages of T cells were generated in the EpCAM1$^+$, but not in the EpCAM1$^-$ cell grafts (Lai & Jin, 2009). These data suggest that the mESC-derived EpCAM1$^+$ cells can form normal thymic architecture that supports T-cell development *in vivo*.

To further confirm that the mESC-derived TEPs can give rise to both cTECs and mTECs *in vivo*, we injected a single EGFP$^+$ EpCAM1$^+$ cell into irradiated thymic fragments that were then implanted under the kidney capsule of syngeneic mice. We examined the grafts six weeks later and found that EGFP$^+$ EpCAM1$^+$-derived cells were comprised of K5$^+$K8$^+$ TEPs, K5$^-$K8$^+$ cTECs, and K5$^+$K8$^-$ mTECs (Lai & Jin, 2009). These results further suggest the mESC-derived TEPs are able to differentiate into both types of TECs and to self-renew *in vivo*.

We then determined whether transplantation of ESC-derived EpCAM1$^+$ cells could enhance thymopoiesis following bone marrow transplant (BMT). Because the mESC-derived

EpCAM1$^+$ cells cannot efficiently migrate from the blood into the thymus (Lai & Jin, 2009), we injected the EpCAM1$^+$ cells intrathymically (i.t.) into irradiated syngeneic mice followed by intravenous (i.v.) injection of T cell deleted (TCD)-BM. Either mESC-derived EpCAM1$^-$ cells or PBS were transplanted as controls. One month after transfer, thymic cellularity was analyzed. We found that the number of thymocytes in the EpCAM1$^-$ cell- or PBS-treated BMT mice was significantly reduced, as compared to that in the normal non-BMT control mice. In contrast, the number of thymocytes in the EpCAM1$^+$ cell-treated mice approximated that of normal, non-BMT control mice. Further analysis showed that the relative distribution of thymocyte subsets in the EpCAM1$^+$ cell-treated mice was comparable to that of the normal non-BMT controls (Lai & Jin, 2009). Importantly, the number of TECs in the EpCAM1$^+$ cell-treated mice was significantly increased (as compared to EpCAM1$^-$ cell- or PBS-treated mice), and about two thirds of TECs were derived from mESCs. Further analysis revealed that the mESC donor-origin TECs expressed CCL25, delta-like Notch ligands, and MHC I and MHC II molecules (Lai & Jin, 2009). All of these proteins are involved in attracting T-cell progenitors to the thymus, and/or in supporting T-cell development within the thymus.

We then analyzed peripheral T cells in these recipients and found that the numbers of total and naïve CD4$^+$ and CD8$^+$ T-cells in the spleens of the EpCAM1$^+$ cell-treated BMT mice were significantly higher than those in the EpCAM1$^-$ cell- or PBS-treated BMT mice (Lai & Jin, 2009; and Figure 1). We also evaluated the functions of these peripheral T cells and found that splenic T cells from EpCAM1$^+$ cell-treated BMT mice had a higher rate of proliferation in response to T cell mitogen or alloantigen stimulation than those from

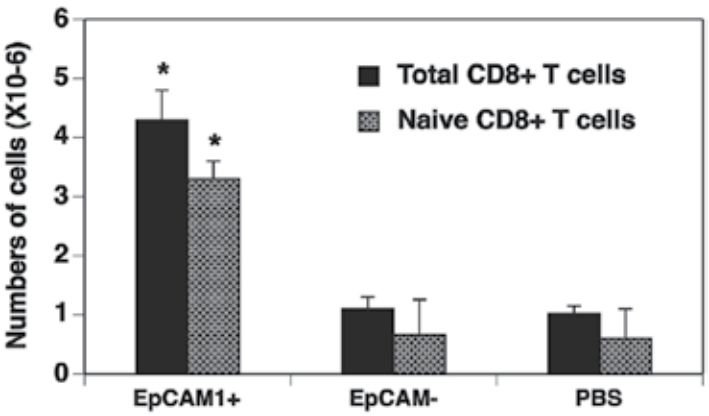

Fig. 1. Mouse ESC-derived EpCAM1$^+$ cell treatment increases the numbers of total and naïve CD8$^+$ T cells after syngeneic BMT. Lethally irradiated syngeneic mice were injected intrathymically with 5X10^4 mESC-derived EpCAM1$^+$, EpCAM1$^-$ cells, or PBS, and injected i.v. with TCD-BM (2X10^6). One month later, the numbers of total CD8$^+$ T cells and naïve CD8$^+$ T cells (CD62^{hi}CD44^{lo}CD8$^+$) were analyzed by flow cytometry. Data are presented as means $\pm$ standard deviation (S.D.) from 4-6 mice per group. * $P<0.05$ compared to PBS-treated BMT mice.

EpCAM1- cell-, or PBS-treated BMT mice (Lai & Jin, 2009). Furthermore, a significantly higher percentage of splenic CD4+ and CD8+ T cells from the EpCAM1+ cell-treated BMT were able to produce interferon (IFN)-γ, tumor necrosis factor-α (TNFα), and interleukin-2 (IL-2) than those from EpCAM1- cell-, or PBS-treated BMT mice (Lai & Jin, 2009; and Figure 2). To determine whether the induction of antigen-specific T cell-mediated immunity was intact in the EpCAM1+ cell-treated BMT mice, groups of BMT recipients were vaccinated with ovalbumin (OVA) in complete Freund's adjuvant 2 weeks after BMT. OVA-specific T cell responses in these mice were evaluated 2 weeks after vaccination. As shown in Figure 3, T cells purified from OVA-vaccinated, EpCAM1+ cell-treated mice exhibited higher rates of proliferation in response to in vitro stimulation with OVA, as compared to T cells purified from vaccinated PBS or EpCAM1- cell-treated mice.

These results suggest that mESCs can be selectively induced to differentiate into EpCAM1+ cells with the phenotypes and genotypes of TEPs. When placed *in vivo*, these mESC-derived EpCAM1+ cells develop into both cTECs and mTECs, reconstitute the normal thymic architecture, and enhance thymopoiesis, resulting in increased numbers and functions of T cells in the periphery.

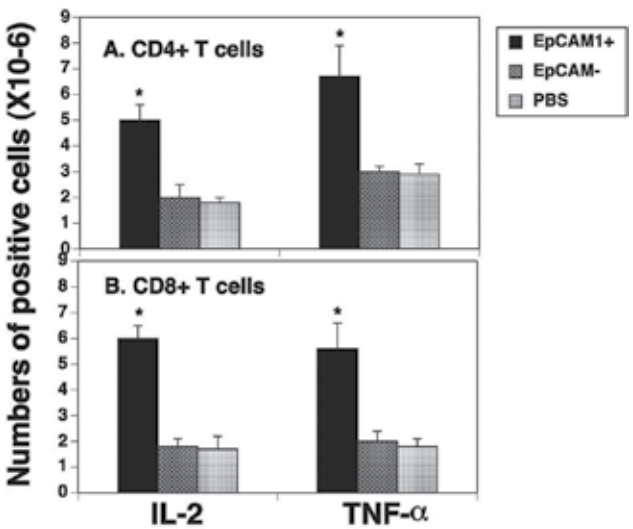

Fig. 2. Significantly higher fractions of splenic T cells from the mESC-derived EpCAM1+ cell-treated BMT recipients are able to produce TNFα and IL-2 after stimulation. Lethally irradiated, syngeneic mice were injected intrathymically with mESC-derived EpCAM1+, EpCAM1- cells, or PBS, and injected i.v. with TCD-BM as in Figure 1. One month later, splenocytes were stimulated with PMA and ionomycin and stained for cell surface markers and intracellular cytokines using antibodies against CD4, CD8, IL-2, and TNFα. The percentages of IL-2 and TNFα positive cells in (A) CD4+ and (B) CD8+ T cells were determined by flow cytometry. Data are presented as means + S.D. from 4-6 mice per group. * P<0.05 compared to PBS-treated BMT mice.

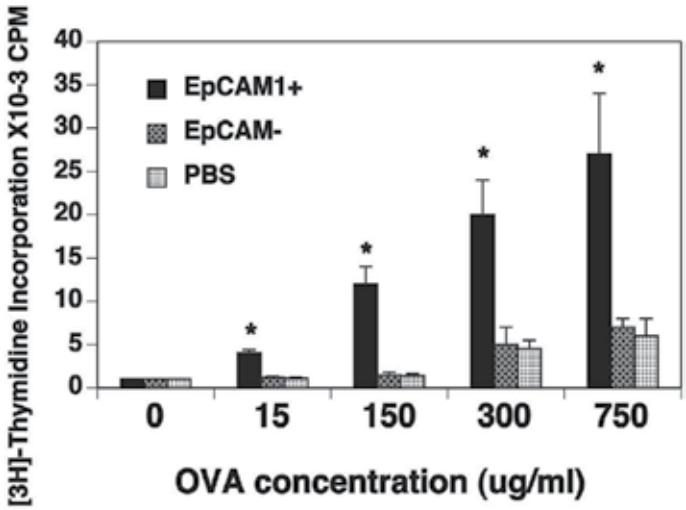

Fig. 3. Antigen-specific T cell-mediated immunity was in EpCAM1$^+$ cell-treated BMT mice. Lethally irradiated syngeneic mice were injected intrathymically with mESC-derived EpCAM1$^+$, EpCAM1$^-$ cells, or PBS, and injected i.v. with TCD-BM as for Figure 1. The recipients were vaccinated with 50 µg of OVA emulsified in Complete Freund's Adjuvant two weeks after BMT. Two weeks later, freshly isolated CD3$^+$ cells were cultured in the presence of irradiated normal splenocytes + OVA for 4 days. T cell proliferation was determined by [3H] thymidine incorporation. Data are presented as means + S.D. from 4-6 mice per group. * $P<0.05$ compared to PBS-treated BMT mice.

3. Future directions

The generation of ESC-derived TEPs has important applications at both basic and translational levels. Although some molecules have been implicated in early thymic organogenesis, molecules involved in the development of TEPs and TECs remain poorly defined mainly due to the lack of a suitable system for these studies. Our demonstration that mESCs can be selectively induced to generate TEPs *in vitro* provides a powerful model with which to study the molecules associated with the development and differentiation of TEPs and TECs. These studies are important not only for gaining a better understanding of the mechanisms underlying thymic development, but also for strategies aimed at maximizing the generation of ESC-derived TEPs and regenerating adult TEPs and TECs.

With modern advances in medicine, our average lifespan is increasing. Currently, individuals aged 60 years and older constitute about 10% of the total world population, and this segment of the population is projected to increase to approximately 22% - 25% by 2050 (Dorshkind et al., 2009). However, advanced age is often accompanied by chronic diseases that have a significant impact on both an individual's quality of life and on the health-care systems that treat those patients (Dorshkind et al., 2009). Aging affects several organ systems and the immune system is one of the systems most significantly affected. Thymic involution is a hallmark of immune system aging, and this results in decreased numbers and functional capacities of T-cells, which in turn leads to increased rates of infections,

autoimmunity, and cancers (Dorshkind et al., 2009; Zediak and Bhandoola., 2005; Lynch., 2009; Taub and Longo., 2005). It is important to determine whether transplantation of ESC-derived TEPs can be used to prevent or reverse age-dependent thymic involution. It will also be important to determine whether transplantation of ESC-derived TEPs can enhance T cell regeneration after chemotherapy and radiotherapy for cancer patients, as well as after preparative regimens for foreign tissue and organ transplants. If successful, human ESC-derived TEPs might one day be used in the treatment of these patients.

Other ESC-derived cells have the potential to be used in the treatment of various degenerative diseases. However, one of the major challenges in ESC-based therapies is the potential for ESC-derived tissues to be rejected by the host immune response (Li et al., 2004; Hyslop et al., 2005; Chidgey et al., 2008). Several studies have shown that the inherent immune privileged status of ESCs was insufficient to prevent rejection across a multiple minor histocompatibility mis-match, even when the major histocompatibility complexes were the same (Li et al., 2004; Hyslop et al., 2005; Chidgey et al., 2008). Therefore, strategies to induce tolerance to ESC-based transplants will be required. Although current strategies to establish tolerance to foreign grafts by inhibiting pre-existing host T cells has achieved some success, these strategies often lead to immune deficiency (Chidgey et al., 2008). Therefore, strategies to induce donor-specific tolerance in the host, whilst maintaining generalized immunocompetence, are required. The best way to induce ESC-specific tolerance would be to use the same thymus-induced tolerance mechanisms that cause potentially autoreactive T cells to be deleted in the thymus. However, to achieve this the thymus must be functional and ESC antigens have to be incorporated into the thymus. As mentioned above, the thymus is subjected to age-dependent involution that affects its functions. ESC-derived TEPs should provide a powerful tool to prevent rejection of the ESC-derived tissues by supporting both restoration of thymic function and incorporation of the ESC antigens into the thymus.

4. References

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18

Actual Achievements on Germ Cells and Gametes Derived from Pluripotent Stem Cells

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1. Introduction

Germ cells (GC) appeared very early in embryonic development and are maintained over life period in order to give rise to the gametes of organisms that reproduce sexually (Fig. 1). These cells provide continuous tie between the generations (Donovan & de Miguel 2003, McLaren 2003).

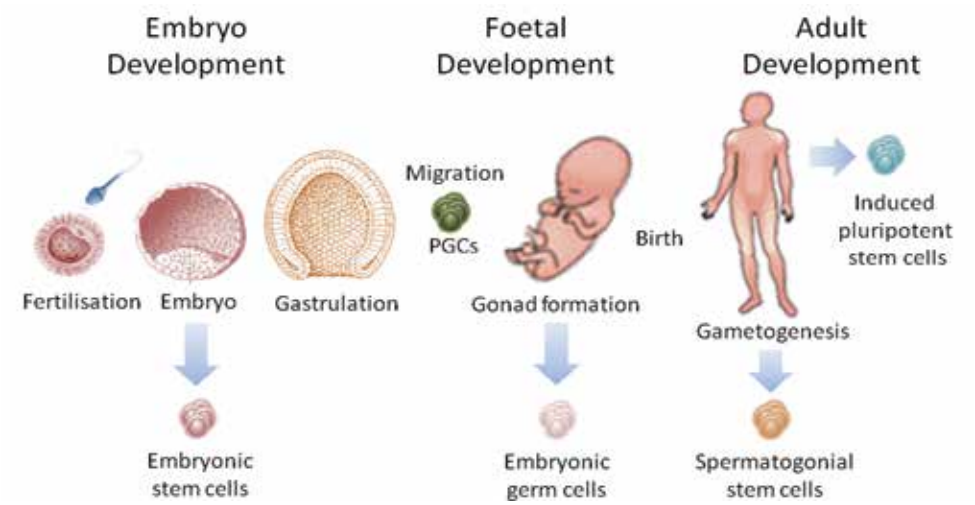

Fig. 1. Germ cells lifespan.

In mammals, the specification of GC begins during cleavage; GC first appears near the gut and further migrates to the developing gonads. The lineage of GC is called germ line. They are unique cells, which undergo cell division of two types, mitosis and meiosis, in contrast to somatic cells of mammal's body, which only divide by mitosis. Accordingly to Fig. 2 following further differentiation GC can be transformed into mature gamete, either eggs or sperm (Adams & McLaren, 2002). There is growing evidence for effects of environmental

chemicals on the various early stages of GC and gametes development (Baillie et al., 2003). Therefore, elucidating of the mechanisms controlling GC and gametes development is crucial for understanding an etiology of various aspects of infertility. Due to the complexity of natural process of mature gametes formation from GC and in order to provide *in vitro* model of GC development and fate, embryonic stem (ES) cells were recently used (Nagy et al., 2008).

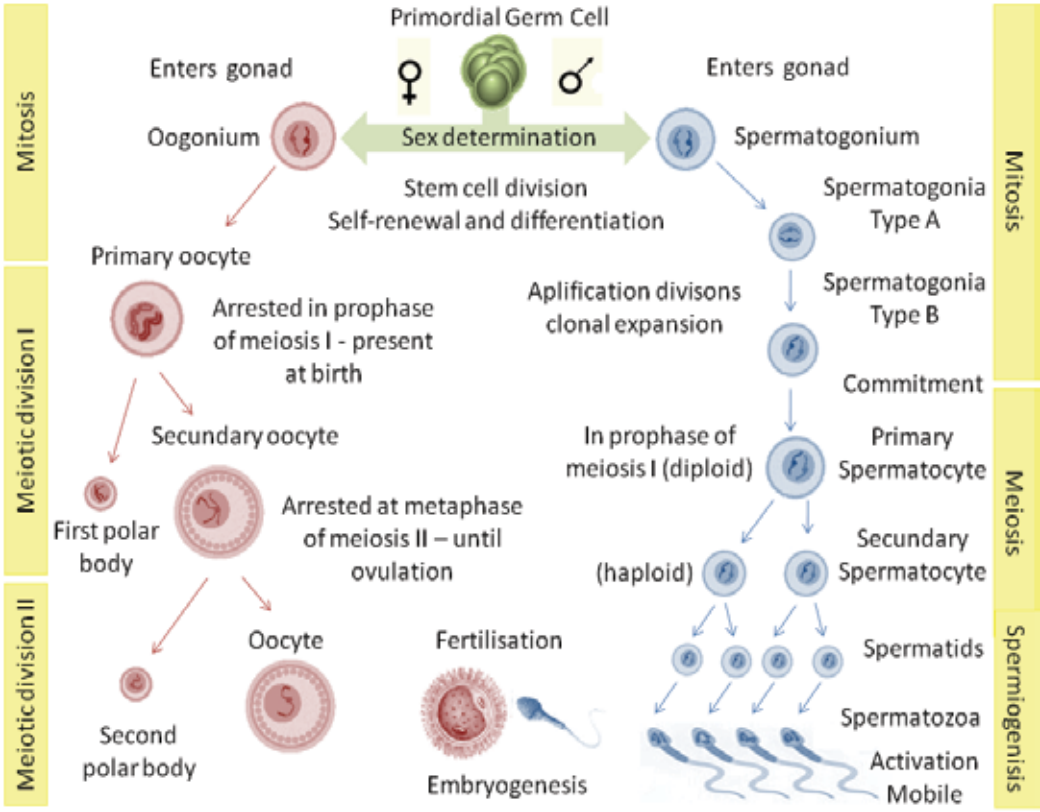

Fig. 2. Gametogenesis.

In vitro derivation of GC and male- and female-like gametes from pluripotent mouse embryonic stem (mES) cells was achieved using adherent cells (monolayer cell culture) and three-dimensional structures called embryoid bodies (EB), which present *in vitro* model of pre-implantation embryos, however chaotically organized. A variety of culture media have been also tested. These protocols are contradictory in respect of timing needs for GC generation from mES cells, which varies from 11 to 43 days in different studies; summarized in Fig. 3 and 4. The other difficulty is a lack of appropriate molecular markers for the characterization of the undifferentiated GC. The markers of early primordial GC (PGC) such as Stella, C-kit and Fragillis have key roles in GC competency and development, while Dazl and Vasa express in premeiotic GC. However, all these genes are also expressed in ES cells (Aflatoonian and Moore, 2006; Ko & Schöler, 2006; Nagano, 2007; Daley, 2007; Hua and Sidhu, 2008; Marques-Mari, 2009).

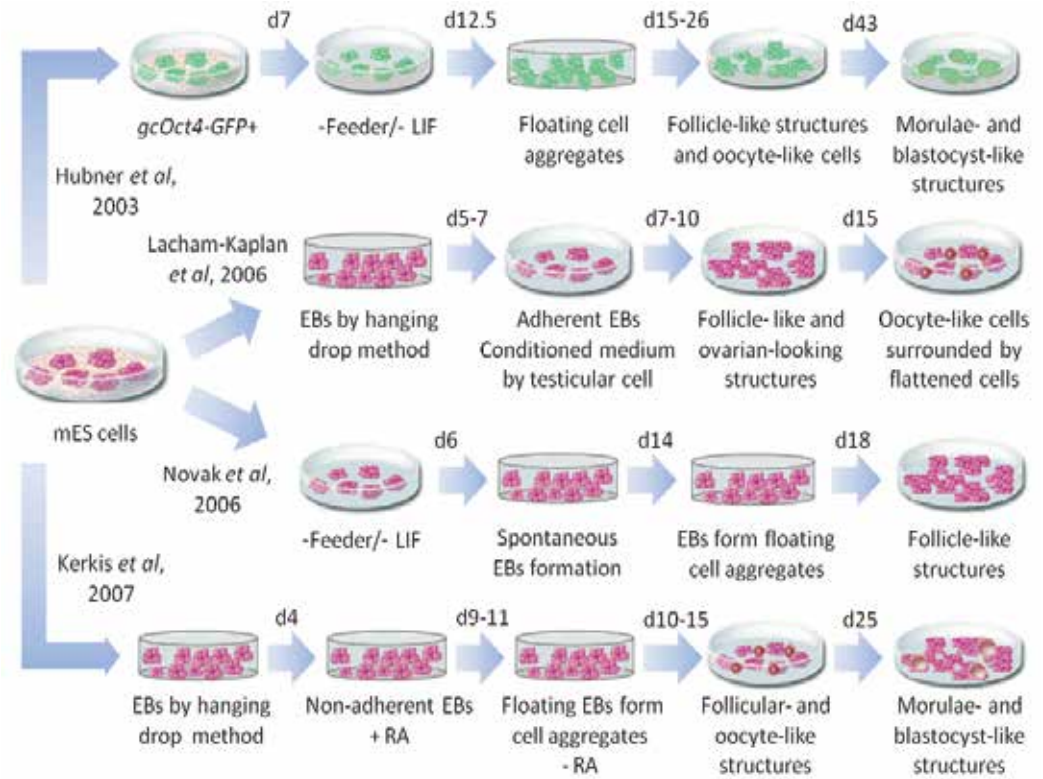

Fig. 3. Presumptive female GC and gametes derived from ES cells.

Genetically modified ES cells using fluorescent proteins linked to specific gene promoters (genes implicated in pluripotency or GC line fate) have been used in order to pre-select committed PGC and to provide more efficient harvesting of mature GC (Hubner et al., 2003, Toyooka et al., 2003, Nayernia et al., 2006). Although genetic modification facilitates the process of GC isolation, in the future such genetically modified GC (GM-GC) would have limited practical utilization especially in clinical procedures. Therefore, other research groups have a propensity to establish efficient protocols of GC isolation from native ES cells (Geijsen et al., 2004; Lacham-Kaplan et al., 2006; Kerkis et al., 2007).

The data about morphological and ultrastructural features, GC and gametes stage-specific proteins expression and GC epigenetic modification pattern are incomplete and need to be deepened (Aflatoonian and Moore, 2006; Nagy et al., 2008; Hua and Sidhu, 2008; Marques-Mari, 2009). Meiosis is pre-requisite for functionally normal gametes formation and its investigation is indeed insufficient (Novak et al., 2006; Kerkis et al., 2007). A single attempt was made to demonstrate the functional state of gametes obtained from mES cells. It showed that the integration of artificial gametes with genetic material of normal eggs theoretically can occur. However, live offspring, which were obtained in this study died soon after birth (Nayernia et al., 2006). Therefore, experiments of other research groups are needed in order to confirm this study.

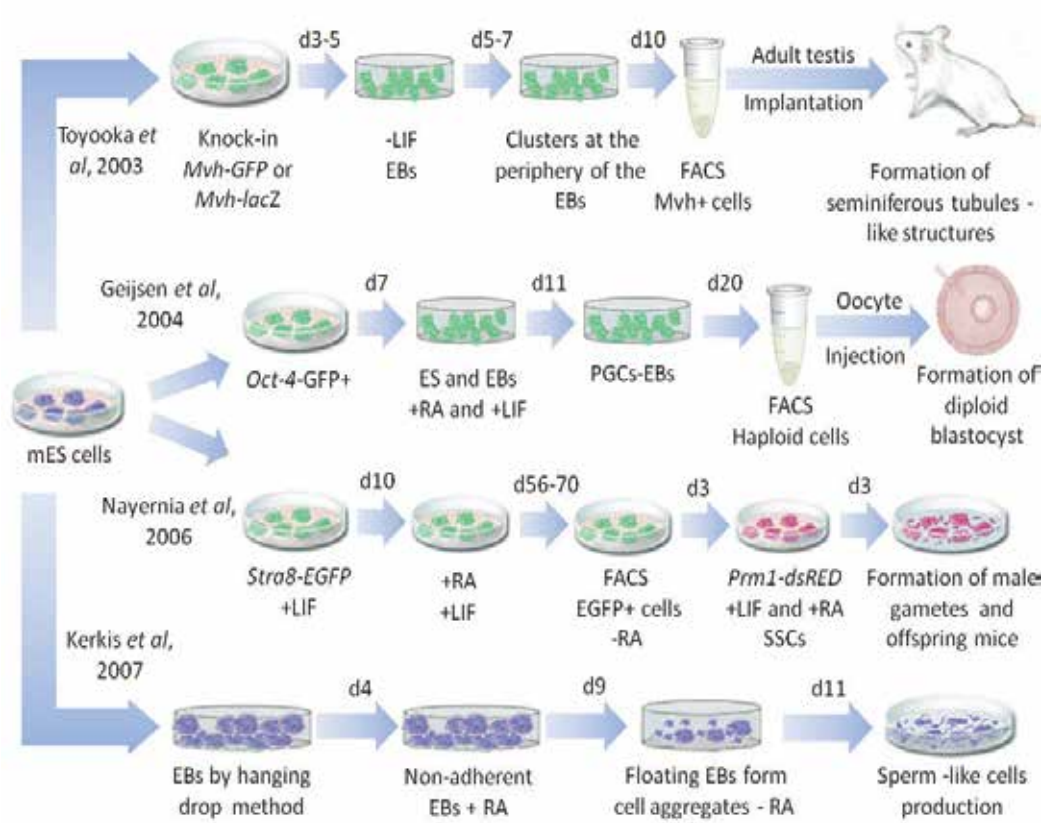

Fig. 4. Presumptive male GC and gametes derived from ES cells.

This chapter is focused on critical discussion of the actual achievements on GC and gametes-like *in vitro* generation from pluripotent stem cells, such as, mES and human embryonic stem (hES) cells (Evans & Kaufman 1981, Martin 1981, Thomson et al., 1998) and reprogrammed cells: induced pluripotent (iPS) cells (Takahashi & Yamanaka 2006) and somatic cell hybrids (Mittmann et al., 2002; Lavagnolli et al., 2009).

2. Embryonic stem cells

The ES cells are derived from early embryo, more precisely from morulae or inner cell mass (ICM) of blastocysts. ES cells can be maintained and expanded indefinitely in culture, while retaining their ability to produce all cell types in the body. This ability, which is also denominated as a pluripotency, is an extremely important property of ES cells. These cells are growing in monolayer and express in undifferentiated state a set of pluripotent markers among which Oct3/4, Nanog and Sox2. The mES cells additionally require leukemia inhibitory factor (LIF) in order to maintain undifferentiated state. Differentiation potential of ES cells provides a unique tool to generate various cell types *in vitro* and represent unlimited experimental model to study cell lineage commitment, which can be modified by cell culture conditions, external factors and spatial orientation (Evans & Kaufman 1981, Martin 1981, Thomson et al., 1998).

2.1 Differentiation assay

Generally two basic methods are used for GC differentiation from ES cells. One of them consists in adherent ES cell culture and differentiation after removing factors that promote pluripotency as feeders and basic fibroblast growth factor (bFGF) or LIF (Hübner et al., 2003; Nayernia et al., 2006b; Novak et al., 2006; Chen et al., 2007), whereas the second one implicates with the formation of three-dimensional structures known as EB, which further allowed to adhere on plastic dishes and differentiate (Clark et al., 2004; Geijsen et al., 2004; Lacham-Kaplan et al., 2006; Kerkis et al., 2007). Wei et al., (2008) compared GC, which were derived via attachment culture technique and via EB method. They demonstrated that the process of PGC derivation was more faithfully recapitulated using the EB method. Next, the ES cells can undergo spontaneous or induced differentiation. Spontaneous differentiation occurs in ES cells culture medium without MEF or growth factors besides those present in heat-inactivated serum, while induced differentiation required addition of growth factor or other chemicals: e.g. bone morphogenic protein 4 (BMP-4) or retinoic acid (RA) are usually used to induce GC differentiation *in vitro*. RA is a potent growth activator of mouse PGC (Koshimizu et al., 1995). During gametogenesis, the exposure to RA controls the progress of GC through meiosis and the differentiation of GC into male or female phenotypes (McLaren, 2003; Bowles et al., 2006; Bowles and Koopman, 2007; Doyle et al., 2008).

2.1.1 Derivation of female GC and artificial gametes

For the first time female GC were obtained *in vitro* by Hübner and colleagues (2003), which used female mES cells carrying gcOct4-GFP gene reporter. Prior differentiation experiments these cells were tested in transgenic animals and expression of gcOct-4-GFP gene in GC but in blastocyst or epiblast-stage embryo were not demonstrated. These cells were maintained adherent in ES cells culture medium without any feeder cells or growth factors besides those present in heat-inactivated serum. Large cell colonies, which formed by day 12 (d12) were composed by cells expressing GFP+ or GFP+/ Vasa+ or Vasa+ suggesting a presence of premigratory, migratory and early postmigratory GC. Vasa+ cells, which were physically separated from each other, detached simultaneously from large colonies and form small floating aggregates. Further these aggregates were collected and transferred in new plates where they were cultured for 2 weeks forming adherent or floating three dimensional follicle-like structures. The expression of growth differentiation factor 9 (Gdf-9), which is required for ovarian folliculogenesis, was observed between d16 and d22. Floating follicle-like structures collected at d26 did not express Gdf-9 indicating that follicular growth was completed. Two of three zona pellucida (ZP) proteins ZP2 and ZP3 were detected between d16 and d30. The lack of ZP1 expression could be because of thin and fragile zona of the ES-derived oocytes. Blastocyst-like structures were found at about d43, suggesting spontaneous parthenogenetic activation. These structures expressed a set of stage-specific molecular trophectodermal markers, such as Hand1, Pl-1, Mash-2 and TpBp. Morulae-like structures showed signal of Oct-4 protein with appropriate nuclear localization, which differs from GC where Oct-4 localizes in the cytoplasm (Hübner et al., 2003; Salvador et al., 2008). Native mES cells (XY) without any genetic modification also were able to produce putative oocytes. The EB were obtained and cultivated adherent on the plastic dish, in a testicular cells conditioned medium, once testis of newborn males contain most growth factors required for the transformation of germ stem cells into differentiated GC. At about 2 weeks oocyte-like cells surrounded by one to two layers of flattened cells were obtained, which expressed

Vasa protein as well as Oct4, Dazl and Stella. Although they did not have visible pellucid zone, a structural hallmark of maturing oocytes, the expression of oocyte-specific markers, such as Fig-α, Stra-8 and ZP3 were detected (Lacham-Kaplan et al., 2006; Quing et al., 2007). Novak and colleagues (2006) induced differentiation of native ES cells toward female GC by their cultivation in ES cells culture medium without LIF and MEF. Under such conditions EB were formed around d7. These EB produced cells of variable morphology, which after detaching from EB form floating aggregates. Follicle-like structures derived from the aggregates were observed from day 18 and afterward, which expressed several oocyte-specific markers.

Our group contributed to these studies demonstrating that male ES cells can generate both female and male (described below) presumptive GC and gametes (Kerkis et al., 2007). In contrast to previous studies, which used the same ES cells basal culture medium, we used serum-free B27/neurobasal medium for differentiation. This medium supported the growth and long-term viability of nearly pure populations of neural cells without the need of any feeder layer. It contains hormones in its composition, which can enhance differentiation process. Thus, we suggested that serum-free B27/neurobasal medium can provide additional advantages. First, the EB were obtained by hanging drop technique in ES cells basal culture medium. Further, they were transferred into serum-free B27/neurobasal medium supplemented with RA. Under such conditions non adherent EB were cultivated for additional 4 days. RA was removed and the cells were further maintained in serum-free B27/neurobasal medium. After 9 days floating EB started to aggregate. The structure composed by several EB underwent further differentiation and within 10–15 days inside EB aggregates the formation of follicular-like structures and of presumptive germinal vesicle has occurred. Floating oocyte-like structures were also found and they expressed Dazl protein, which is overexpressed in normal oocyte during GV-MI–MII (Assou et al., 2006). Although we did not expect to observe spontaneous *in vitro* fertilization of the oocyte-like structure by the presumptive sperm cells, both formed in the same Petri dish during the process of differentiation, we could identify structures resembling fertilized oocyte with two pronuclei. These "fertilized" oocytes underwent further development into morulae, blastocyst-like structures with well defined but fragile zona pellucida, and hatching blastocyst-like structures. The expression of the genes Gdf9, Zp2, and Zp3, which are indicative for female GC differentiation, was detected. In accordance to Hübner et al., (2003), Zp1 did not express in those structures. For about 25 days, the blastocyst-like structures, attached on the monolayer of differentiating ES cells and formed the cell layer resembling trophoblasts with inner cell mass. This supposed pre-embryo expressed Oct-4 in the inner cell mass-like cells (inside), while trophoblast-like cells (outside) expressed Mash-2 (mammalian achaete-scute homologous protein-2) protein, which is specific for extraembryonic trophoblast lineage (Fig. 5). It is worth mention, that estradiol (~189.0 pg/ml) was found in culture medium used for ES cells differentiation. It is not included in serum-free B27/neurobasal medium composition, suggesting that it was produced by differentiated GC. Hübner et al., (2003), also reported estradiol production (50–100 pg/ml) after 12 days in mES cell cultures that generated follicle-like structures.

2.1.2 Derivation of male GC and artificial gametes

In order to produce male GC from mES cells Toyooka et al., (2003) generated knock-in mES cells, in which GFP or lacZ was expressed from the endogenous mouse vasa homolog (Mvh), which is specifically expressed in differentiating GC. The EB formation occurred

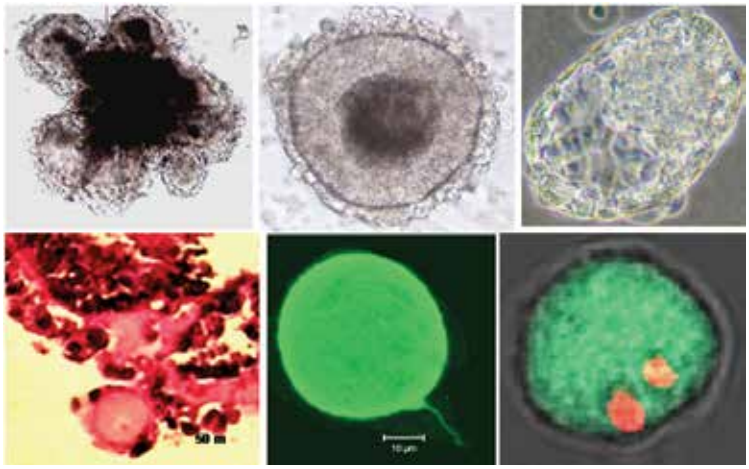

Fig. 5. Female GC development (follicle-, oocyte-, blastocyst- and zygote-like).

when these cells were cultivated in suspension in a LIF-free medium. The knock-in cells, which expressed the reporter gene products clustered together at the periphery of the EB by days 5–7. The Mvh$^+$ cells purified (flow cytometry) from EB were equivalent to *in vivo* fetal gonad GC. Then, Mvh+ cells were aggregated within male genital ridge cells of wild-type embryos. Following implantation into adult mouse testis, the cell aggregates composed by Mvh$^+$ positive cells undergo further maturation, formed seminiferous tubules-like structures that have a capacity to support complete spermatogenesis and to produce mature putative sperm cells. Additionally, the authors also found that the exposure of EB to Bmp4 led to the emergence of Mvh$^+$ cells within 24 hours. Although the ability of spermatozoa to activate eggs was not examined, this study suggested that the germ line specification and the emergence of postmigratory PGC can occur spontaneously or be induced in EB, which can be completed in testis environment. Geijsen et al., (2004) isolated GC from male ES cells carrying EGFP reporter gene. The EB were produced in basal medium and after 7 days of cultivating, RA was added for additional 5 days. After RA removal the EB were collected by day 20-22. The FEJ1 antibody (Fenderson et al., 1984) was used in order to identify haploid round-spermatid-like cells. The positive cells for this marker and EGFP$^+$ were further selected by flow cytometry and intracytoplasmatic injection into recipient oocytes was performed. These presumptive GC were capable of generating diploid blastocysts, which did not undergo further development. Although the isolated cells did not produce cells resembling spermatozoa and analyses of further embryonic development was not complete, this study suggested that male PGC aroused from ES cells can become postmeiotic cells capable of eggs activating. Nayernia et al., (2006) reported not only the induction of male gametes from ES cells but also the production of offspring; however this work should be carefully evaluated (Daley, 2007). They modified genetically ES cells in order to obtain spermatogonial stem cells (SSC). First, they introduced a promoter (Stra8) active in early male GC linked to a marker gene encoding EGFP. The selected cell population Stra8$^+$/EGFP$^+$ already had the characteristics of male PGC ready to enter the initial stages of meiosis. In order to enrich this cell population the cells were further cultivated for 10 days in the presence of RA and 8-10 weeks in RA-free culture medium. Next RA was added 12 hours following selection of EGFP cells. Then authors performed another round of selection by introducing the promoter of a gene expressed in more mature haploid male GC (Prm1)

linked to another fluorescence marker gene, dsRED. Two genetically modified SSC cell lines were established and designated as SSC7 and SSC12. For differentiation, the cells were cultured on gelatine-coated dishes, without LIF. The appearance of red fluorescent cells suggested that the emerging haploid cells had undergone the final stages of spermatogenesis. Nayernia et al., (2006) claimed that they established SSC cell lines that undergo meiosis and produce male gametes after only 72 hr. The shape of the resulting sperm was, however, abnormal. They injected these presumptive sperm cells into eggs. When the resulting 65 embryos were transferred to surrogate mothers, seven live pups carrying the Prm1-reporter gene were derived, which apparently had growth abnormalities and died short time after birth. Certainly, the production of progeny needs to be confirmed by other laboratories.

We demonstrated generation of male GC from XY ES cells without the use of any genetic modification or pre-selection (Kerkis et al., 2007). In our experiments the spermatogenesis – like process took place between 9 and 11 days: EB were obtained using "hanging drop" by day 3, then they were kept floating and RA was added for additional 4 days. During this period we already observed migration of presumptive PGC from EB surface and formation of aggregates composed by different cell types, such as spermatogonia, spermatocytes, spermatids and sperm-like cells between 9 and 11 days. We described that gamete-like cell formation occurred in the correct manner based on the expression of early and late GC specific genes, such as Oct-4, Mvh, Stella, Dazl, Piwil 2, Pdrd 1, Rex 14, Rnf 17, Bmp8b, Acrosin, Stra-8, Haprin, LH-R, Gdf9, Zp3, Zp2, Sycp1 and Sycp3. Our immunofluorescence analysis of morphologically well-formed GC and presumptive gametes showed positive labeling with SSEA-1, Oct-4, EMA-1, FE-J1, Dazl, Fragilis, Mvh, Acrosin, and acetylated α-tubulin (Fig. 6).

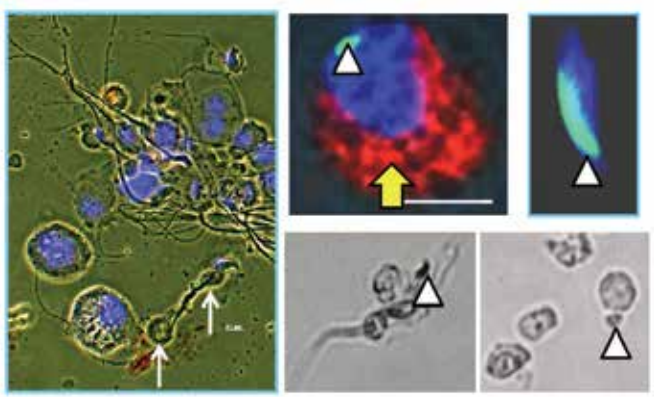

Fig. 6. Morphological aspect of male GC formation and immunocytochemistry analyses for acrosin (green) and alpha-tubulin (red).

Routine cytogenetic analysis demonstrated that GC were able to undergo chromosome reduction, since diploid and haploid chromosome plates were detected. Moreover, we presented undiscussable proofs of sperm-like cells formation *in vitro,* which are morphologically similar to normal ones. Electron microscopy images showed acrosomal phase of differentiation, which presented by elongated and flattened nucleus, acrosomal granule has become the acrosome and it follows the shape of the nucleus, tail filaments called axoneme further elongate and mitochondria aggregate about the axoneme cytoplasm

is moved by a cylindrical sheath of microtubules called the manchette in the area of the developing tail. At maturation phase, we observed final formation of sperm and residual body (Fig. 7).

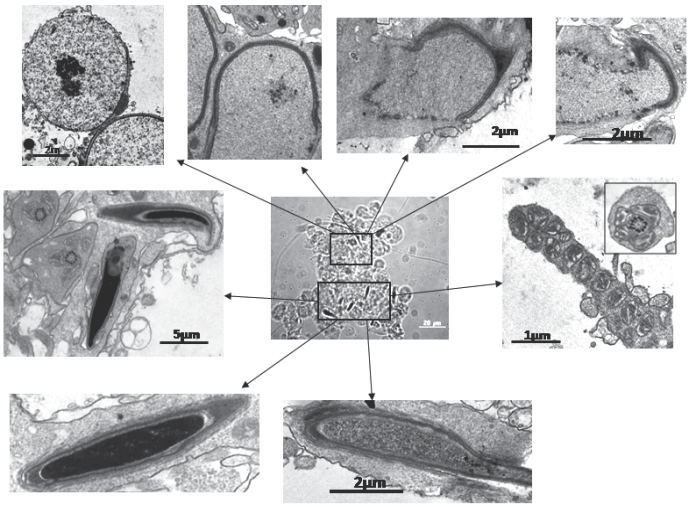

Fig. 7. Transmission electron microscopy of male GC formation.

2.1.3 Fate determination during XY ES cells differentiation into GC

During murine and human ES cells culture, markers of female GC are expressed in both XX and XY cell lines (Toyooka et al., 2003, Hübner et al., 2003; Geijsen et al., 2004, Clark et al., 2004; Nayernia et al., 2006: Kerkis et al., 2007; Lavagnolli et al., 2009). Previous studies involving XX↔XY mouse chimeras have shown that GC whether XX or XY were able to enter meiosis in developing ovary, but not in testis, suggesting that initial sex determination depends on environment rather than on chromosome composition (Palmer and Burgoyne, 1991; McLaren, 2003). For that reason, both male and female ES cell lines can display female germ cell markers, since culture conditions may be sub-optimal and lack meiosis inhibition. Recently it has been shown, that GC in order to enter meiosis responds to the external signal of RA and its metabolism, (Bowles et al. 2006, Koubova et al., 2006). Thus, in the embryonic mouse ovary, RA induces germ cells to express the pre-meiotic marker Stra-8 (stimulated by) retinoic acid and initiate meiosis. By contrast, in the embryonic mouse testis, RA is metabolised and inactivated by the P450 enzyme CYP26 (B1) thereby preventing early germ cell entry into meiosis with down-regulation of genes such as SCP3 (synaptonemal complex protein; associated with meiotic events). Therefore, the induction of presumptive PGC into meiosis in culture medium containing RA might be expected although local concentrations within cell aggregates may differ significantly and affect the timing. However, the sensitivity of cells to RA can vary considerably depending on composition of medium.

3. Putative male and female GC and artificial gametes derived from mouse reprogrammed cells

Cell fusion between embryonic stem (ES) and somatic cells usually yields ES-somatic cell hybrids (ES-SCH), which retain pluripotency in spite of the presence of ‘‘somatic’’ chromosomes in their genomes (Fig. 8). These reprogrammed near-diploid ES-SCH shows a

developmental potential similar to ES cells: they express molecular ES cell markers, present reactivation of the X chromosome, form EB *in vitro* and produce chimeras with contribution to different tissues (Mittmann et al., 2002; Vasilkova et al., 2007).
Our group was the first to show that reprogrammed cells obtained from the fusion of mES cells and mouse splenocyte were also able to undergo GC differentiation *in vitro* (Lavagnolli et al., 2009). Differentiation of ES-SCH was induced through EB formation and by the addition of retinoic acid following previously described protocol (Kerkis et al., 2007). Presumptive GC obtained reacted positively with anti-EMA, Vasa, Fragilis and Dazl antibodies and expressed GC-specific genes, such as Vasa, Stella, Dazl, Piwil2, Tex14, Bmp8b, Tdrd1 and Rnf17. Fluorescent in situ hybridization analysis (FISH) indicates chromosome reduction in the GC-like cells. Expression of meiotic and postmeiotic GC-specific genes such as Haprin, Acrosin, SYCP1, SYCP3 and Stra-8 was also detected. Transmission electron microscopy confirmed ES-SCH differentiation into presumptive GC. The presence of several autosomes and the X chromosome originated from the ''somatic'' partner did not prevent ES-SCH differentiation towards presumptive GC. Overall our study suggests an interesting *in vitro* model, which allows the study of GC differentiation in reprogrammed somatic cells. This study represents significant advance, showing future prospect to obtain partly patient-matched GC and gametes.

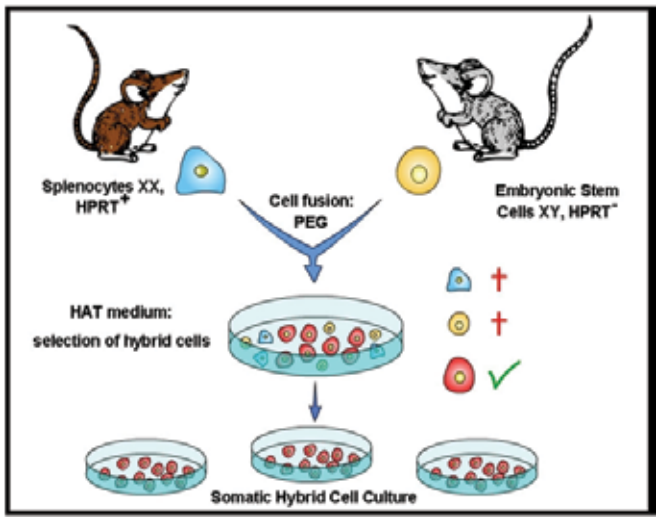

Fig. 8. Somatic cells reprogramming by fusion

4. Putative GC derived from hES and induced pluripotent stem cells

The ability of hES cells to enter the germ line was examined by Clark and colleagues (2004). This and further studies, based mainly on molecular analysis of stage specific genes expression, suggested that hES cells of both sexes may spontaneously through EB formation generate presumptive GC and supposedly undergo meiosis (Clark et al., 2004, Aflatoonian et al., 2005). More recently other groups improved previous protocol by the addition of growth factor in order to improve GC differentiation within the EB (Kee et al., 2006; Tilgner et al., 2008; West et al., 2008; Park et al., 2009). The molecular analysis revealed the expression of the germ-line stage-specific genes (e.g., VASA, DAZL) and of the meiotic

marker (SYCP3). However, expression of germ-line stage-specific proteins was only marginally studied (West et al., 2008, 2010). Until now, only two studies showed the image of follicular-like structures appearing within monolayer or EB of differentiated ES cells whose morphology resemble those of normal oocytes (Chen et al., 2007; Aflatoonian et al., 2009). The first study did not present any evidences if they do indeed oocytes-like structures (Chen et al., 2007), while other failed to detect expression of ZP proteins in these structures (Aflatoonian et al., 2009). Induced pluripotent stem (iPS) cells are differentiated somatic cells reverted to a pluripotent state. After reprogramming, they acquire properties of ES cells in morphology, proliferation, gene expression, epigenetic profile, and differentiation potential (Takahashi & Yamanaka, 2006, Takahashi et al., 2007). This approach would allow the creation of patient-specific cells, which is advantageous for cell therapy due to immune compatibility. The derivation of GC from human (h)iPS cells constitutes both a desirable model for reproductive geneticists, and a potential method for treating couples with infertility due to germ cell defects. It has been shown that derivation of GC from (h)iPS cells following 7 days of differentiation results in the generation of immature GC corresponding to a developmental stage *in vivo* between specification and less than 9 weeks of gestation (Park et al., 2009). However, the doubts about the usefulness of (h)iPS cells for GC generation raised by the study of massive epigenome reorganization. It was unclear whether reprogramming of female human cells reactivates the inactive X chromosome (Xi), as in mouse. In order to clarify this question, (h)iPS cells were derived from several female fibroblasts under standard culture conditions carrying a Xi. These cells showed the lack of Xi reactivation. This finding critically implicates with the use of (h)iPS cells for clinical applications and disease modelling, and could be exploited for a unique form of gene therapy for X-linked diseases (Tchieu et al., 2010). Furthermore, (h)iPS and hES are distinguished by gene expression signatures (Chin et al., 2009). Overall, the methodologies of hES cells derived presumptive GC and gametes characterization are often limited to molecular profile of genes expression involved in GC specification leaving aside the other important characteristics. Therefore, derivation of GC from hES and (h)iPS cells is still at initial stage.

5. Timing of *in vitro* ES-derived GC differentiation

The genes involved in the differentiation of the three germ layers, and genes specific for several cell lineages are expressed in EB over the same time period as in gastrulating embryos. In contrast, all studies showed that derivation of GC from ES cells takes place much faster *in vitro* than *in vivo*. Several studies showed that ES cells in culture have already acquired the capacity to form PGC expressing a set of PGC genes in undifferentiated state e.g. PGC founder-specific genes Stella and Fragillis are detectable in ES cells before the onset of differentiation, whereas those genes are not expressed in the inner cell mass and early epiblast cells *in vivo* (Aflatoonian and Moore, 2006; Ko & Schöler, 2006; Nagano., 2007; Daley, 2007; Hua and Sidhu, 2008; Marques-Mari, 2009). The environmental factor can also influence the timing of differentiation, therefore progression of PGC differentiation may be suppressed in the embryonic environment until the PGC reach an appropriate position near the developing gonads. Additionally, some of the factors that regulate the timing of PGC differentiation in the embryo are absent from *in vitro* culture systems. The discrepancies in timing observed in different studies may be related with properties of ES cell lines and culture conditions used in each work.

6. Molecular characterization of putative GC

Independently of the methodologies of ES cells differentiation toward GC, the molecular characterization is indispensable. Using simple or sophisticated methods, the studies need to show an expression of PGC markers (specification and specific gametogenesis) during development of both types of gametes. Understanding of PGC molecular mechanisms of differentiation and fate is important to elucidate infertility causes. The molecular analyses are an important tool to characterize this complex process, which is necessary not only to confirm a success of applied protocol, but also to evaluate their efficiency and effectiveness. In table 1 we summarized information about genes markers for different stages of gametogenesis used in different studies. A careful study of *in vitro* PGC specification is difficult by the fact that mammalian *in vivo* or *in vitro* cultured PGC derived from native tissues and ES cells shared a significant number of markers e.g. Oct4 and SSEA1. In addition, PGC markers, such as Blimp1, Mvh, Fragillis and Stella and even GC markers, such as Piwil2, Rnh2, Tdrd1 and Tex14, are also detected in undifferentiated ES cells. Among these markers Stella was appointed as an excellent marker to be used for cell sorting of GC *in vitro* (Wei et al., 2008). A systematic analysis of single cell showed a gene expression dynamics in GC line during PGC specification *in vivo* and revealed differential pattern of several genes expression between ES cells and PGC e.g. Eras, T and Fgf8 (Yabuta et al., 2006). A core of genes as *Oct4*, *Sox2,* and *Nanog* which regulates pluripotency in stem cells also performs an important role in GC development regulation. The repression of this core machinery regulating pluripotency is an early event involved in differentiation of GC. Furthermore, in mice fetal male-specific germ line methylation of functional elements as *Nanog* and *Sox2* promoters and genes was shown suppressed during early male GC differentiation. The Oct4 translation is suppressed post-transcriptionally following male GC differentiation allowing entrance in the mitotic arrest (Western et al., 2010). Therefore, the determination of single cell PGC and GC molecular markers in a challenge for the future studies will allow improving currently established protocols of both GC derivations from appropriate tissues and from ES cells *in vitro*.

Specification								
Blimp1								■
Bmp8b		■					■	
C-kit	■				■	■■		■
Ddx4					■			
eFgls		■	■		■		■	
Fgf8								■
Nanog								■
Oct4	■	■	■	■	■	■■	■■	■■
Piwil2			■		■		■	
Rbm					■■			
Rnf17			■		■		■	
Rnh2			■					
Sox2								■

Sox17								■
Stella		■	■		■	■	■	■
Tdrd1			■				■	
Tex14			■				■	
SSEA1			■	■			■	
Studies	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Gamete determination								
Dazl			■		■	■	■■	
Stra8					■■	■	■	
Vasa (Mvh)	■■	■■			■	■■	■■	■
Gametogenesis								
Acrosin					■		■■	
Acetylated α-tubulin							■	
Dmc1	■				■			
DsRed (condesen Nuclei)					■			
Ema-1							■	
FE-j1			■				■	
Figα						■		
Gcna1 (Meiosis)		■						
Gcnf			■					
Gdf9	■						■	
Haprin			■				■	
hnRNPGT (sperm meiosis)					■			
Hsp90α					■			
Oam (sperm pos meiotic)					■			
Prm1 (Pos Meiotic)					■			
Rec8 (Meiosis)				■				
Scp1 (Meiosis)				■			■	
Scp2 (Meiosis)				■				
Scp3 (Meiosis)	■■	■		■	■		■	
Smc1-β (Meiosis)				■				
Stag3 (Meiosis)				■				
Tp2 (Pos Meiotic)* **					■■			
Zp1			■			■	■	
Zp2	■		■			■	■	
Zp3			■			■	■	

■ **RT-PCR** ■ Immunoassay

1– Hübner et al. (2003); 2 – Toyooka et al. (2003); 3 - Geijsen et al. (2004); 4 – Novak et al. (2006), 5 - Nayernia et al. (2006); 6 – Lacham-Kaplan & Trounson (2006); 7 – Kerkis et al. (2007), 8 – Wei et al. (2008).

Table 1. Markers of GC differentiation.

7. Epigenetic modifications of ES derived GC

An important emerging theme from recent studies is that epigenetic modification can be implicated in GC development itself, contributing to the gene expression program that is required for GC development, regulation of meiosis and genomic integrity. Understanding of epigenetic regulation in GC has implications for reproductive engineering technologies and human health.

Epigenetics has been defined as a collection of mechanisms and phenomena that will generate the phenotype without affect the genotype. The mechanisms involved in this regulation are represented by a range of chromatin modifications, including DNA methylation, histone modifications, remodelling of nucleosomes and higher order chromatin reorganization. These epigenetic modifications define a cellular identity regulating gene expression and are unique in each cell. Epigenetic profiles are adjustable during cellular differentiation, but remains inherited: it ensures that daughter cells have the same phenotype as the parental cell (Goldberg et al., 2007).

The process of GC development is regulated by both genetic and epigenetic mechanisms. This type of cell can give rise to a new organism. Therefore, during fertilization, the products of GC development - the oocyte and sperm cell, fuse to form a zygote, which is considered totipotent. To acquire this totipotency, the GC and the zygote undergo to extensive epigenetic reprogramming. In mammalian GC, reprogramming also erasure the existing parental imprints and establishes new ones, which are different in male and female gametes. Genomic imprinting gives rise to differential expression of paternally and maternally inherited alleles of certain genes. Thus, unlike most genes in our genome, which are either expressed or silenced from both parentally inherited alleles (biallelic expression), monoallelic expression of imprinted genes occurs in a tissue and developmental stage-specific manner during development (Allegrucci et al., 2005; Reik, 2007). Imprinted genes comprise a small subset of the genome, perhaps 100 out of the total 30 000 genes whose epigenetic reprogramming in the germ line is imperative for subsequent normal development of the embryo (Miozzo & Simoni, 2002). Almost all these imprinted genes could be used as a strategy to confirm the potential status of GC differentiation.

The role of epigenetics in GC and in somatic cells occurs in different way. During somatic cell differentiation, cells initiate in a pluripotent state followed by decision of their fate, being able to originate a range of different cell types (Reik, 2007). Their gene-expression profile become more restricted and potentially protected from epigenetic modifications. On the other hand, GC are different in that, because their fate has been determined during early development. The GC have specific fate suffering a series of epigenetic events that are unique to this cell type. GC undergo meiosis and the particular importance of maintaining genomic integrity. It is important to study how imprints are re-established in the male and female GC and their contributions to GC-specific functions at each stage.

Recent studies have shown that changes in epigenetic modifications also have important roles in the regulation of post-migratory PGC-specific genes. Genes such as Ddx4 (also known as Mvh), Sycp3 (synaptonemal complex protein 3) and Dazl (deleted in azoospermia-like) are induced after migrating of PGC toward genital ridge. DNA-methylation analysis revealed that, despite the genome-wide decrease in DNA methylation after E8.0, the flanking regions of these genes remain methylated at E10.5, but become hypomethylated by E13.5 when they are expressed. The results suggest that DNA methylation regulates the timing of activation of these genes. In mice, when PGC arrive at

the genital ridge (E11.5), they undergo extensive epigenetic reprogramming, including the erasure of parental imprints. The erasure of imprints is reflected by demethylation of the imprinted loci, which occurs concomitantly with demethylation of other regions. Once the parental imprints have been erased, new imprints must be re-established according to the gender. This re-establishment occurs only after sex determination has been initiated, and male and female GC development diverges to give rise to sperm or oocytes, respectively (review in Sasaki & Matsui, 2008). It should be considered the GC formation from ES cells.

Another important event that occurs in mammalian female cells is the X-chromosome inactivation. In somatic cells of female mammals, one of the two X chromosomes is inactivated so that the dosage of the genes on this chromosome is equalized between males and females. The inactive X chromosome is reactivated during female GC development. Extensive studies have been made to understand when this mechanism occurs and how they can be maintained by the generation. It had been thought that this reactivation occurs around the time of imprint erasure (Monk & McLaren, 1981; Tam et al., 1994) However, more recent studies showed that it is initiated in the migratory stage (de Napoles et al., 2007) or at an even earlier stage (Sugimoto & Abe, 2007). Therefore, X-chromosome reactivation occurs in a progressive manner in prolonged period and is completed in post-migratory PGC.

Geijsen et al., (2004) showed in their experiments haploid round spermatids displayed somatic-like imprinting status of the Igf2r and H19 genes by day 4 of differentiation. The imprinting methylation profile was erased by day 7, demonstrating that the PGC derived from EB may be able to mimic the epigenetic reprogramming features of PGC developing *in vivo*. Nayernia et al., (2006) obtained sperm cells, which were able to fertilize oocytes after ICSI resulting in a number of pregnancies, although the majority died in uterus, the others development to term. The resultant pups, however, had abnormalities in DNA methylation at imprinted loci and survived only up to five months, indicating that reprogramming of the GC genome was not properly accomplished.

Recently, Kee et al., (2009) explored adherent differentiation of human ES cells carrying VASA-GFP reporter in the medium supplemented with or without BMP4, and observed that both XY- and XX-bearing human ES cells (approximately 0.8–5%) reproducibly gave rise to PGC. The epigenetic status was analyzed and showed erasure of methylation (hypomethylation) globally and at the differentially methylated regions (DMRs) of imprinted loci. The authors found that the H19 locus was hypomethylated in GFP1 cells relative to GFP2 cells. Results from other imprinted loci (PEG1/MEST, SNRPN, and KCNQ) confirmed that the GFP1 cells also showed significantly lower levels of methylation at these DMRs relative to other cell types. Furthermore, examination of global DNA methylation levels provided strong evidence that the VASA–GFP1 population was in the process of erasing methylation globally. When DAZL and BOULE were overexpressed in XX human ES cells, PGC formation was enhanced suggesting potential formation of haploid gametes.

Regarding to iPS cells, Park e co-workers (2009) showed that VASA expressed during germ cell formation in the first trimester of human development *in vivo*. They used these surface markers to isolate putative (h)iPGC from hES cells and (h)iPS cell lines after differentiation on primary human fetal gonadal stromal (hFGS) cells *in vitro*. They found that imprinting control center that were differentially methylated in undifferentiated hES cells (H19 and SNRPN in HSF-1 and H19, PEG1, and SNRPN in HSF-6) initiated the process of imprint erasure by day 7 in the iPGC. However, (h)iPGC derived from hIPS cells do not initiate imprint erasure as efficiently. Regarding Xi, these (h)iPGC showed the lack of Xi

reactivation. This work suggests that GC originated from iPS cells are not as efficiently as from ES cells in the epigenetic remodelling. If these results will be confirmed by other studies, it could be a barrier in case of therapy of infertility using iPS cells to produce gametes in infertile patients.
When we fully understand the complete mechanisms of germ-cell reprogramming, we might be able to derive appropriately reprogrammed and functional gametes from cultured cells. This will allow new approaches to reproductive engineering, although ethical and safety issues must be carefully considered.

8. Meiosis in ES derived GC

The meiotic process is an essential step in the gamete formation in organisms with the system of sexual reproduction and this process is indeed studied inadequately. A better understanding of the meiosis and its regulation, together with advances in genomics, will allow us to predict a perfect establishment of fertility disorders. Meiosis is a cellular division mechanism based on separation and diminishes of the chromosomal set followed by haploid cells formation (gametes). In eukaryotes is characterized by an extended prophase, followed by two divisions. A sexual meiosis dimorphism should be considered: first, in male, one single cell that enters in meiosis can produce four haploids gametes (sperm cell). On the other hand, in the female, one single cell will originate only one gamete (oocyte), which completes second division after the fertilization (Fig. 2).
Meiosis is preceded by DNA replication in a pre-meiotic S phase, usually longer than mitotic S phase. The pre-meiotic DNA replication generates primary gametocytes; each chromosome is comprised of two chromatids (4C DNA content). The S phase is proceeded by the long meiosis I prophase (this phase is divided in other four substages), during which homologous chromosomes starts to be paired and undergo recombination in a series of events that define the substages of meiosis I prophase. This crossing-over (CO) recombination in meiosis is required to guarantee an accurate segregation of homologous chromosomes at the first meiotic division. The absence of CO can result in random disjunction (separation of chromosomes or chromatids during anaphase of mitosis or meiosis) and might form an aneuploidy gamete, that may leads to embryonic death or developmental abnormalities (review in Handle & Schimenti, 2010). In fact, aneuploidy present in gametes is a principal cause of birth defects in humans. Most of these aneuploidies are formed during oogenesis, mainly during the first meiotic division, and the frequency of such errors increases with female age (Hassold et al., 2007).
The substages of meiosis I prophase are defined by chromosome configurations and structure: pairing, which occurs during the leptotene and zygotene stages. These events are accompanied by synapsis, a process that is mediated by a specialized meiotic structure, the synaptonemal complex (SC). The recombination structure is defined by the formation of a chiasma (the point that the chromatids of the homologous chromosomes undergo the recombination - exchange). The first meiotic division (metaphase I, followed by anaphase I and telophase I) is reductional and separation homologous chromosomes, producing secondary gametocytes. Each gametocyte has haploid chromosome content in which still comprised of two chromatids). In male gametogenesis, it results in two secondary spermatocytes and in females it results in one secondary oocyte and a polar body. The second meiotic division — an equational division, separates sister chromatids. This phase is also sexually dimorphic, but in this case in both of timing and in the products formed. In

males it occurs immediately after the first division and the chromatids are separated and produce four haploid immature spermatids that contain the haploid chromosome number and 1C DNA content. In female GC, the timing of the second meiotic division is coordinated with ovulation and occurs after fertilization, producing a haploid oocyte (fertilized egg) that contains two haploid pronuclei, one paternal and the other maternal as well as three polar bodies (review in Handle & Schimenti, 2010).

Based on the architecture of the meiosis, the chromosome pairing and the CO are crucial for the appropriate segregation of homologous chromosomes at the first meiotic division of most organisms. The fidelity of recombination and chromosome segregation that avoid aneuploidy are dependent of the dynamics of chromosome pairing and synapsis during meiotic prophase. The chiasma formed by CO events is essential and we can see the occurrence of at least one CO per chromosome in humans. This protein structure (lateral elements – LE, cohesin REC3 and axial elements – AE, synaptonemal complex (SC)-specific proteins, such as SYCP3 and SYCP2 formed in the chromosome pairing) can physically maintain chromosome homologues attached during the end of prophase. In this moment, the cohesins are removed and the structure progress to the metaphase I and chromosome homologue division (Fledel-Alon et al., 2009). Failure of a chromosome pairing to undergo at least one CO can result in both homologues segregating to the same daughter cell at the metaphase I, leading to aneuploidy.

A lot is known about of the proteins that contribute to formation of the chromosomal axes and the SC, but little is known about exactly how the separable events of pairing and synapsis come about, mainly when we consider this events in GC derived from ES cells. Some proteins related with this structure such as SYCP1, SYCP2 or SYCP3, cohesins and the telomere length of the chromosome have been studied to a better understanding of the meiosis process in the GC derived from ES cells. Several reports have demonstrated the formation of oocyte-like structures and postmeiotic male GC in embryonic stem (ES) cells culture (Clark et al., 2004; Geijsen et al., 2004; Hübner et al., 2003; Lachan-Kaplan and Trounson, 2006; Nayernia et al., 2006; Toyooka et al., 2003). Molecular analysis of the expression of these genes required in the initial and progression of the meiosis and the SC formation have been done suggesting that the cell formed in this type of differentiation could be GC in different phases of maturation. But in almost all of these studies the analyses is based on molecular gene profile only or in some cases in immunocytochemistry showing the presence of the proteins involved. According to Novak et al., (2006), the meiotic process in germ cell-like cells derived from ES cells probably fail to correctly initiate and progress due to the absence of complementary meiotic proteins, even though they express SYCP3, indicating that only the molecular expression analysis is not enough to confirm the CG formation and maturation. Their results demonstrated that the SC did not occur efficiently, and the FISH results revealed two separate signals of same chromosome homologue (when the expected are together), which is an indicative of the absence of bivalent formation. This study suggested that the chromosome organization of these cells was associated to a mitotic division instead of meiotic. Resumption of meiotic progress and entry into the meiotic division phase is controlled by somatic cells and *in vivo* is hormonally prompted (Hsieh et al., 2009). In this case described by Novak, we can suggest that the oocyte-like cells did not progress into meiosis due to the fact that the development observed *in vivo* are arrested in prophase I of meiosis I until there is a hormone stimulus that triggers their meiosis I accomplishment. We should consider that in their work, it was analyzed a low number of follicle-like structures, that could not represent the remaining cells. Another important thing is that, *in vivo* in each cycle a lot of primordial follicles were required

in hormone response, but just one completes the meiosis I, being ovulated. In this work, it might occur and the samples could represent the oocytes that not complete the meiosis phase. The correct segregating chromosomes in meiosis and formation of a haploid cell is a prerequisite for the derivation of CG from ES cells in both - animal model of study or cellular basement therapy in the treatment of infertility. Our group (Kerkis et al. 2007), which showed the occurrence of both types of cells female and male GC in the same culture conditions. The cytogenetic analyses demonstrated cells with the chromosomes organized in similar manner as during meiosis I. FISH data showed cells with two signals of both X and Y chromosomes, as well as, the cells with only one signal of X or Y chromosome in each, indicating that the meiotic process occurred (Fig.9). However, both studies (Novak et al., 2006; Kerkis et al., 2007) are not completely conclusive. Further analyses should be provided to explain the details of meiosis. We need to analyze all aspects of meiosis associating them with epigenetic modifications.

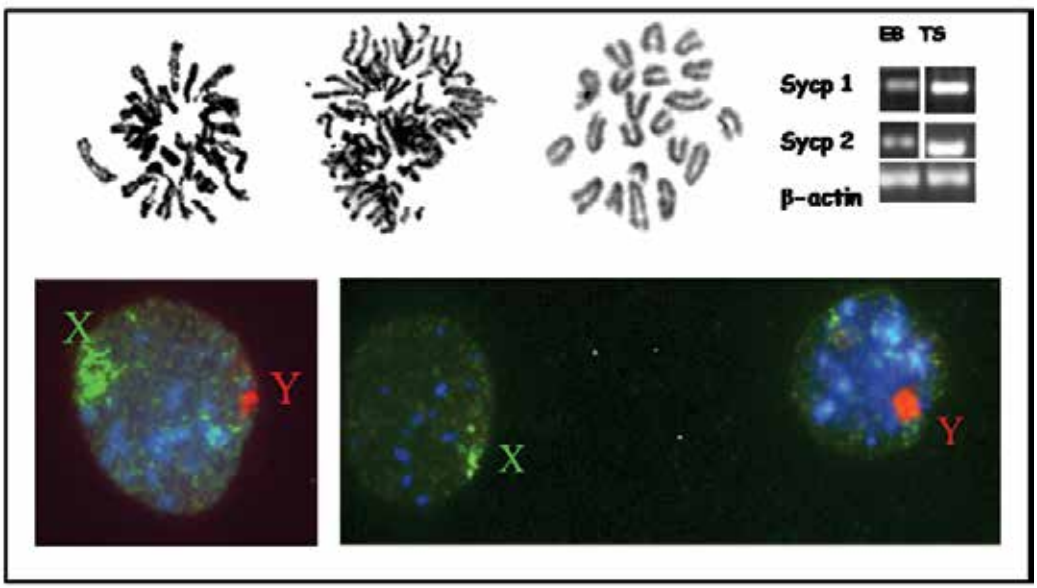

Fig. 9. Meiosis progress during male GC differentiation (conventional cytogenetic, molecular and FISH analyses).

Although, the substantial knowledge of PGC and gamete development in the mouse and many mechanisms are highly conserved in mammals, little is known about human CG formation and there remains necessary to investigate (Moore & Aflatoonian, 2007), mainly when we consider the differentiation from ES cells. Although PGC development can be formed from these systems, post-meiotic cell phenotypes have been difficult to identify and recover. Recently, Kee et al., (2009) used γH2AX (indicator of meiotic recombination) and SYCP3 (synaptonemal complex formation in meiotic prophase I) to examine meiotic progression and showed low levels of SYCP3 in rare cells and no signal of γH2AX. These results indicate that these cells were in a pre-meiotic stage, and rare cells entering in meiosis. DNA content analyses by flow sorter showed that less than 2% of the cells were 1N

(haploid) confirmed by FISH. This percentage of cells that complete meiosis process is consistent with the natural process *in vivo*.

Several studies have been characterized the transcriptome during male meiosis in mice (Schlecht et al., 2004; Shima et al., 2004), but the regulators of the mammalian meiotic program continuous unidentified. Up to now, the major advance was the discovery that the onset of meiosis in mice is regulated by RA and is mediated by Stra8 (Bowles et al., 2006; Koubova et al., 2006; Bowles & Koopman, 2007). The effect of RA on Stra8 induction and initiation of meiosis is sexually dimorphic in timing. In fetal ovaries the RA is secreted by the mesonephroi and can induce the Stra8 expression and the GC enter in meiosis. These events can be detected by waves of expression of meiotic markers, for example: Dmc1 (disrupted meiotic cDNA 1 homologue) and Sycp3. The fetal testis are also exposed to RA, but the Stra8 expression is not induced because the presence of a CyP26B1 and P450 family. This gene is expressed only in Sertoli cells with consequence of male GC do not enter meiosis during the fetal life; the entry of GC into meiosis in the adult testis appears to be controlled, at least in part, by stage specific expression of CyP26B1. The role of STRA8 and RA in the regulatory process of the meiotic initiation in both spermatogenesis and oogenesis was confirmed by genetic analysis (Anderson et al., 2008). The regulation of meiotic initiation by RA could involve germ cell intrinsic factors, such as the RNA binding protein DAZL (deleted in azoospermia-like), which may act upstream of Stra8 in the pathway of meiotic induction (Lin et al., 2008).

Based on these results, the presence of RA and expression of STRA8 and possible the DAZL are necessary for the entry of GC in the meiosis in different times of life. In almost all works published showing the derivation of germ cell from mES cells have been demonstrated a molecular profile of gene expression and in almost all of them, the presence of RA in the cell culture is totally necessary for the meiotic progress (review in Kerkis et al., 2007). Nayernia and co-workers (2006) developed SSC lines from ES cells able to undergo meiosis and capable to generate a functional haploid male gametes *in vitro*. The authors demonstrated the increasing of the expression of Stra8 after the addition of RA in culture. A FACS analysis showed that approximately 30% of these cells undergo meiosis and produce a haploid cell population after 72 hr of RA induction that was not observed in culture without RA induction. The authors showed the formation of SC after electron microscopy analyses. Lacham-Kaplan and Trounson (2006) reported the formation of putative oocytes by using testicular cells conditioned medium. We showed both types of differentiation, male and female CG (Kerkis et al., 2007) in the same culture condition by RA induction. Our results demonstrated an increase of gene expression after RA induction and the expression of meiosis markers.

Finally, much effort has been devoted recently to the generation of functional gametes from embryonic stem cells or iPS cells. We should consider that mammalian GC are surrounded by specialized somatic cells (Sertoli cells and granulosa cells) in which secrete substances that influence their homeostasis and meiotic status. For these reasons, it has not been completely possible to successfully sustain initiation and continuous execution of all steps of meiosis *in vitro*. In any attempt to generate mammalian gametes *in vitro*, it will be challenging to mimic the roles of the somatic cells that act in both instructive and permissive roles to support meiosis and gametogenesis. Any use of *in vitro* derived mammalian gametes must be predicated on rigorous proof of the execution of key steps in meiosis and the fidelity of chromosome segregation.

9. Functional status of artificial gametes

The gametes are a key for conservation and preservation of the species (Hua & Shidu, 2008). The haploid DNA, generated on gametogenesis, will be joined by fertilization to develop a new individual. Due to the importance of this event, the gametogenesis is a complex and regulated process, difficult to be mimicked *in vitro* (Daley, 2009). Spermatogenesis involves mitosis, meiosis, spermiogenesis and spermiation, a cascade of events that must occur precisely and with the involvement of the Sertoli cells (Wong & Cheng, 2009). After leaving the testis, the sperm pass through the epididymis, formed by a pseudo-stratified epithelium with several cell types (Cornwall, 2009) and a specific and specialized condition is created. The changes in the spermatozoa arising from active secretion and absorption of water, ions, organic solutes and proteins, beyond the function of maintaining blood barrier. Besides the changes, epididymal secretions maintains the vitality of the sperm, allowing the development of motility and protect against harmful agents (Robaire et al., 2006), essential for complete maturation of sperm, which involves the development of progressive motility the ability to recognize, bind and penetrate the oocyte (Moore, 1990). These modifications include changes in composition and biophysical properties of the sperm membrane, stabilization of chromatin and other organelles with disulfide bridges, as well as morphological changes such as the migration of cytoplasmic droplets (Bassols et al., 2005). One mature sperm is mobile, able to perform the fertilization process and support the development of a new individual. The maturity of gametes differentiation *in vitro* was hardly questioned (Daley, 2007). Several groups have succeeded in obtaining sperm-like cells in different conditions. None of them showed typical progressive motility and nobody known whether the culture conditions and differentiation *in vitro* causes any change such as occurs *in vivo* in the epididymis. In contrast with other studies, we obtained mature sperm cells (Fig. 6 and 7); however we also did not succeed to register any sperm motility (Kerkis et al., 2007). Embryos (Geijsen et al., 2004) and pups (Nayernia et al., 2006) performed by ICSI using sperm-like cells derived *in vitro*, died quickly, demonstrating that this process still have failures. Another important point to consider is the efficiency of gamete production *in vitro*. Rare sperm-like cells are generated, regardless of culture condition and genetic manipulation.

10. Conclusions

Infertile couples caused by genetic and epigenetic or any other problems which requires chemotherapy or radiotherapy, justify the challenge to enlarge our knowledge about the complex gametogenesis process and develop diagnostic approaches; gene therapies and any other techniques for fertility preservation and treatment. Only the first's steps were taken. The knowledge produced can be used to better understand this complex process - the gametogenesis and help these couples studying the failures. Now, we should focus on increasing the efficacy of the process and better characterize the cell types produced. In animals, is possible to test the functionality of these cells, and see how close the differentiation process is from normal. Then, it will be possible to test male contraceptive drugs evaluating the toxicity effects of new drugs on the germinal tissue. Another side of this promising technology is the application in transgenesis, since stem cells are easily genetically engineered and selected. After the process of differentiation, these gametes could be used to generate transgenic animals.

Differentiation toward gametes represents the extraordinary advances in the developmental biology, biology of reproduction, stem cell biology. Further investigation of this amazing type of differentiation implicates with great biotechnological progress in regards of genetic amelioration of livestock and preservation of genetic fund of the animals in extinction. In humans, this research can contribute significantly for understanding of genetic and cellular mechanisms of infertility. However, the use of artificial gametes in assisted reproductive technologies is implicated with huge ethical problems, which should be well thought and discussed before this technique will become a reality in Reproductive Medicine (Testa & Harris, 2004).

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Part 6

Side Effects of Pluripotent Stem Cell Therapies

19

Self-Renewal, Pluripotency and Tumorigenesis in Pluripotent Stem Cells Revisited

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1. Introduction

Embryonic stem cells (ESCs) are derived from preimplantation embryos and are capable of both long-term proliferation (self-renewal) and differentiation into cell types of all three germ layers (pluripotency). The self-renewal and pluripotency of ESCs are sustained by certain essential transcription factors. Intriguingly, the viral transduction of these transcription factors into differentiated adult somatic cells results in reprogramming of the developmental process that the somatic cells have undergone. Consequently, pluripotent cells similar to ESCs, termed induced pluripotent stem cells, can be artificially established from specialized cells. These two types of pluripotent stem cells (PSCs) have held the promise of providing customized tissue replacements as well as platforms for drug screening since they were derived from human tissues and embryos. However, the heterogeneous nature of PSC cultures, which may reflect the plasticity of early embryonic cells, hampers the establishment of a definitive and reproducible culture microenvironment. In addition, the induction of PSC differentiation is dependent on random events and generates heterogeneous populations of specialized cells. Furthermore, PSCs, by definition, are able to generate benign tumors called teratomas, which consist of cell types of three germ layers. To prevent the growth of teratomas in therapeutic transplanted tissue replacements, it is necessary to establish techniques for efficiently manipulating cell fate decisions in PSCs and to understand the mechanism responsible for tumorigenesis in the stem cells. To our surprise, the mechanism of teratoma formation from PSCs has received little attention to date. Thus, in order to better understand self-renewal, pluripotency and tumorigenesis in PSCs, this chapter will address the following three simple but overlooked questions:

1. Does every pluripotent stem cell possess identical self-renewal capability?
2. Are current standard culture conditions optimal for maintaining pluripotent stem cells?
3. Is tumorigenesis an inherent feature of cellular pluripotency?

Accumulating experimental evidence, including our recent studies using mouse ESCs as a model, indicates that the self-renewal of PSCs can be easily compromised by extrinsic factors in the culture microenvironment that can turn the stem cells tumorigenic. Thus, the safety of PSC-based therapy may be significantly improved by more careful manipulation and definition of the cellular microenvironment.

2. Pluripotent stem cells generate heterogeneous populations

2.1 Pluripotent stem cells

Pluripotent stem cells (PSCs) are an excellent model to study mechanisms of cellular pluripotency and differentiation *in vitro* because of their capacity for self-renewal and their capability to become most kinds of specialized cells, including germ cells. The identification and characterization of a mouse strain that naturally develops testicular teratoma (Stevens & Little, 1954; Stevens, 1973) contributed to demonstrating that teratomas originate from PSCs (Solter, 2006). A benign teratoma, normally found in 1 out of 40,000 live births (Barksdale & Obokhare, 2009), is a "monstrous" tumor consisting of specialized cells derived from all three germ layers (ectoderm, mesoderm and endoderm). The first PSCs, embryonic carcinoma cells (ECCs), were derived from malignant teratocarcinomas, which were experimentally generated by transplantation of peri-implantation embryos into the testes of host animals (Stevens, 1970). ECCs are transplantable, in that they will develop into teratocarcinomas when transplanted. Because ECCs are pluripotent, the original study established an *in vitro* system to study the cell fate decision mechanism. Furthermore, this study indicated that there could be another kind of PSCs in early embryos that could be directly established by *in vitro* culture, but not by transplantation, of early embryos. During mouse preimplantation development, the first cell differentiation event gives rise to the pluripotent inner cell mass (ICM) and the lineage-committed trophectoderm. When cultured on embryonic fibroblasts, the ICM gives rise to pluripotent stem cells. Mouse embryonic stem cells (ESCs) were successfully derived in 1981 (Martin, 1981; Evans & Kaufman, 1981) and have been the primary model used to investigate mechanisms of cell fate decision. Similar PSCs were later established from primordial germ cells, namely embryonic germ cells (Matsui *et al.*, 1992). These studies on mouse embryos paved the way for the derivation of embryonic stem and germ cells from human embryos (Thomson *et al.*, 1998; Shamblott *et al.*, 1998). The derivation of PSCs from human embryos shed light on regenerative medicine and helped to expand this field of research (Tanaka, 2010). ESCs have been derived from a variety of species (Tanaka, 2010). Studies on self-renewal and pluripotency using ESCs further enabled the establishment of other kinds of PSCs, including early primitive ectoderm-like stem cells (EPLCs; Rathjen *et al.*, 1999) and epiblast-derived stem cells (EpiSCs; Brons *et al.*, 2007; Tesar *et al.*, 2007). Because EpiSCs are derived from, and EPLCs are thought to be equivalent to, cells of post-implantation embryos, their capabilities to generate differentiated cells are more restricted than those of ESCs (Hiratani *et al.*, 2010). That is, embryonic development proceeds by restricting a cell's ability to generate specialized cells. Therefore, a method to erase such acquired restrictions in specialized cells was sought in order to restore differentiated cells to the pluripotent state. This was first achieved by transferring somatic cell nuclei into enucleated oocytes (Briggs & King, 1952; Campbell *et al.*, 1996; Wakayama *et al.*, 1998; Rideout *et al.*, 2002; Gurdon & Melton, 2008). Intriguingly, recent studies have shown that delivering extra copies of four transcription factors that orchestrate self-renewal and pluripotency into differentiated cells results in the reprogramming of the specialized cells into PSCs, called induced pluripotent stem cells (iPSCs; Takahashi & Yamanaka, 2006). Since the successful derivation of iPSCs from human cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007), iPSCs have been considered to hold great potential for developing customized replacement tissues and for providing platforms for drug screening. However, cells differentiated from PSCs *in vitro* that have been transplanted into animal disease models (for example, Kerr *et al.*, 2003; Brederlau *et al.*, 2006; Jomura *et al.*,

2007) tend to develop into teratomas due to residual populations of undifferentiated PSCs. Thus, a better understanding of extrinsic and intrinsic factors involved in cell fate decisions and tumorigenesis in PSCs is necessary to significantly improve iPSC-based stem cell therapy.

2.2 Extrinsic factors for maintenance of self-renewal

The derivation of ESC lines from human and mouse embryos could not have been accomplished without feeder layers of embryonic fibroblasts. Although cultured ECCs do not require a layer of feeder cells for growth, both embryonic germ cell and iPSC cultures do. Interestingly, conditioned medium (CM) from embryonic fibroblasts was sufficient to support the culture of undifferentiated mouse ESCs in the absence of feeder layers (Smith & Hooper, 1983). Analysis of components in CM led to the identification of the leukemia inhibitory factor (LIF) as a differentiation inhibitor (Smith *et al*., 1988; Williams *et al*., 1988). These studies laid the foundation for investigating the dependence of self-renewal and pluripotency of ESCs on other extrinsic factors. In addition to LIF, the maintenance of mouse ESC culture requires Bone morphogenetic protein 4 (Bmp4; Ying *et al*., 2003), vitamin A (retinol and retinoic acid; Chen & Khillan, 2008; Wang *et al*., 2008; Chen & Khillan, 2010), threonine (Wang *et al*., 2009) and a decreased oxidation state (Yanes *et al*., 2010). The existence of another extrinsic factor independent from the LIF/Stat3 signal, namely ES cell renewal factor, has also been postulated (Dani *et al*., 1998). The supplementation of basal culture media with animal sera, such as fetal bovine serum (FBS), provides all of these extrinsic factors except LIF. Although human ESCs are similar to mouse ESCs with respect to their self-renewal and pluripotency, the extrinsic factors necessary for mouse ESC culture failed to support the culture of human ESCs. For example, the combination of LIF and serum could not support long-term self-renewal of human ESC lines (Bongso *et al*., 1994). Furthermore, Bmp4 promoted differentiation of human ESCs into trophoblasts (Xu *et al*., 2002), whereas long-term proliferation of these cells was maintained in the presence of Noggin, an antagonist of Bmp4 (Wang *et al*., 2005; Xu *et al*., 2005b). Instead, the maintenance of human ESC self-renewal and pluripotency mainly relies on basic fibroblast growth factor (bFGF; Xu *et al*., 2005a). In addition, members of the transforming growth factor β (TGFβ) superfamily, especially TGFβ, activin and Nodal, are essential for maintaining the pluripotency of human ESCs in combination with bFGF (Beattie *et al*., 2005; James *et al*., 2005; Vallier *et al*., 2005). Mouse and human iPSCs exhibit dependency on extrinsic factors similar to mouse and human ESCs, respectively. Mouse and rat EpiSCs are dependent on activin and bFGF to sustain self-renewal and pluripotency, and thus human ESCs are more similar to these EpiSCs. These discrepancies are attributed to differences in development between mouse and human embryos, even though mouse and human ESCs have been derived from embryos at similar developmental stages. Very interestingly, it has been suggested that the reprogramming process makes human iPSCs more similar to mouse ESCs (Hanna *et al*., 2010). ECCs do not exhibit dependency on extrinsic factors, whereas the maintenance of embryonic germ cells requires LIF, bFGF and the c-Kit ligand, Steel factor (Matsui *et al*., 1991; Matsui *et al*., 1992). Thus, signals from these extrinsic factors may converge in maintaining the activity of a common set of intrinsic genetic factors that define cellular "stemness".

2.3 Intrinsic factors to maintain self-renewal

Maintenance of the self-renewal and pluripotency of mouse ESCs relies on the activity of the downstream target of the LIF signal, the *Stat3* transcription factor (Niwa *et al*., 1998;

Matsuda *et al.*, 1999). However, key players further downstream of Stat3 are essential for these processes because the LIF/Stat3 signaling pathway is not required for the maintenance of pluripotent cells in developing embryos or for the self-renewal and pluripotency of human ESCs (Dani *et al.*, 1998; Tanaka, 2009). This pathway may interact with the transcription factors *Oct3/4*/ *Pou5f1* (Nichols *et al.*, 1998; Niwa *et al.*, 2000), *Sox2* (Avilion *et al.*, 2003; Masui *et al.*, 2007), *Nanog* (Chambers *et al.*, 2003; Mitsui *et al.*, 2003), *Klf4* (Li *et al.*, 2005) and *c-Myc* (Cartwright *et al.*, 2005). In a steady state, a balance of the relative expression levels of these genes is essential for fate decisions of mouse ESCs (Fujikura *et al.*, 2002; Niwa *et al.*, 2005). The genetic network of these transcription factors and the expression of their downstream target genes have been elucidated by genomic approaches (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002; Tanaka *et al.*, 2002; Boyer *et al.*, 2005; Loh *et al.*, 2006; Matoba *et al.*, 2006; Walker *et al.*, 2007). These genomic approaches revealed that cellular pluripotency is characterized by the expression of a unique set of genes that suppress transcripts associated with cellular differentiation. Recently, the self-renewal of mouse ESCs was shown to be maintained by simple pharmacological inhibition of Erk, which is downstream of FGF receptors, and the inhibition of Gsk3β activity (Ying *et al.*, 2008). Because mouse ESCs express Fgf4 (Wilder *et al.*, 1997), these studies indicate that ESCs maintain self-renewal by competing against their own differentiation-inducing signals. Mouse and human ESCs express Wnt (Nordin *et al.*, 2008; Lako *et al.*, 2001; Okoye *et al.*, 2008), which is the biological inhibitor of Gsk3β, and the pharmacological inhibition of Gsk3β alone promotes self-renewal of both mouse and human ESCs (Sato *et al.*, 2004) as well as derivation of ESCs from the ICM (Umehara *et al.*, 2007). However, exogenous Wnt promotes the differentiation of mouse (Lindsley *et al.*, 2006) and human (Wang & Nakayama, 2009) ESCs. Thus, the role of Wnt in the self-renewal of ESCs requires further investigation. Finally, a comparison of global gene expression profiles of mouse ESCs of different genetic backgrounds, teratocarcinoma cells (ECCs) and embryonic germ cells showed that the expression of *Rex1* was higher in cells with greater pluripotency (Sharova *et al.*, 2007). The zinc-finger protein *Rex1*/*Zfp42* was originally identified as one of the genes whose expression was downregulated when the teratocarcinoma cell line F9 was induced to differentiate by retinoic acid (Hosler *et al.*, 1989). However, the targeted knockout of *Rex1* revealed that it is not required for the maintenance of self-renewal (Masui *et al.*, 2008). There are several genes expressed specifically in pluripotent embryonic cells at significant levels, which do not play any essential role in pluripotency (e.g., *Esg1*/*Dppa5*; Western *et al.*, 2005; Amano *et al.*, 2006; Tanaka *et al.*, 2006).

2.4 Transcriptional heterogeneity in pluripotent stem cells

One of the challenges in understanding the mechanism of self-renewal and pluripotency of PSCs is that cultured ESCs consist of cell populations that show fluctuating expression of genes. That is, a bulk preparation of ESCs may only show an averaged state of ESCs and thus obscure the presence of distinct ESC populations. Therefore, a better understanding of gene expression at the cellular level is critical. In fact, several groups have performed expression microarray analyses at the single-cell level and have revealed populations of cells that differ in their transcript profiles (Crino *et al.*, 1998; Chiang & Melton, 2003; Kurimoto *et al.*, 2006; Ramos *et al.*, 2006; Tang *et al.*, 2010). Several studies, including ours, have found that well-maintained mouse ESC cultures consist of a small percentage of cells that show fluctuating expression levels of genes such as *Dppa3* (*Stella*/*Pgc7*; Payer *et al.*, 2006; Hayashi

et al., 2008), *Nanog* (Chambers *et al*., 2007; Singh *et al*., 2007), *Pecam1* (Furusawa *et al*., 2004; Furusawa *et al*., 2006), *Rex1* (Toyooka *et al*., 2008) and *Zscan4* (Falco *et al*., 2007; Zalzman *et al*., 2010), or genes associated with cell differentiation, such as *Brachyury/T* (Suzuki *et al*., 2006a; Suzuki *et al*., 2006b), *Rhox6/9* (Carter *et al*., 2008), *Tcf15* and *Twist2* (Tanaka *et al*., 2008). These genes are either downregulated (*Nanog* and *Rex1*) or expressed (the rest) in about one-tenth of cells in culture as a steady state (Fig. 1; Tanaka, 2009). Mouse ESCs showing fluctuating expression of *Nanog*, *Rex1*, *T*, *Dppa3* and *Zscan4* have been extensively characterized. When mouse ESCs were sorted according to expression levels of one of these genes and cultured separately, the resulting ESC populations eventually showed similar fluctuating expression of the gene. For example, when sorted *Zscan4*-positive and -negative subpopulations were replated and cultured separately, both subpopulations regained *Zscan4*-negative and -positive cells, respectively (Zalzman *et al*., 2010). Each subpopulation possessed a unique differentiation potential. Thus, the heterogeneous nature of PSCs may reflect the plasticity of early embryonic cells (Hayashi *et al*., 2008; Zalzman *et al*., 2010). The underlying mechanism

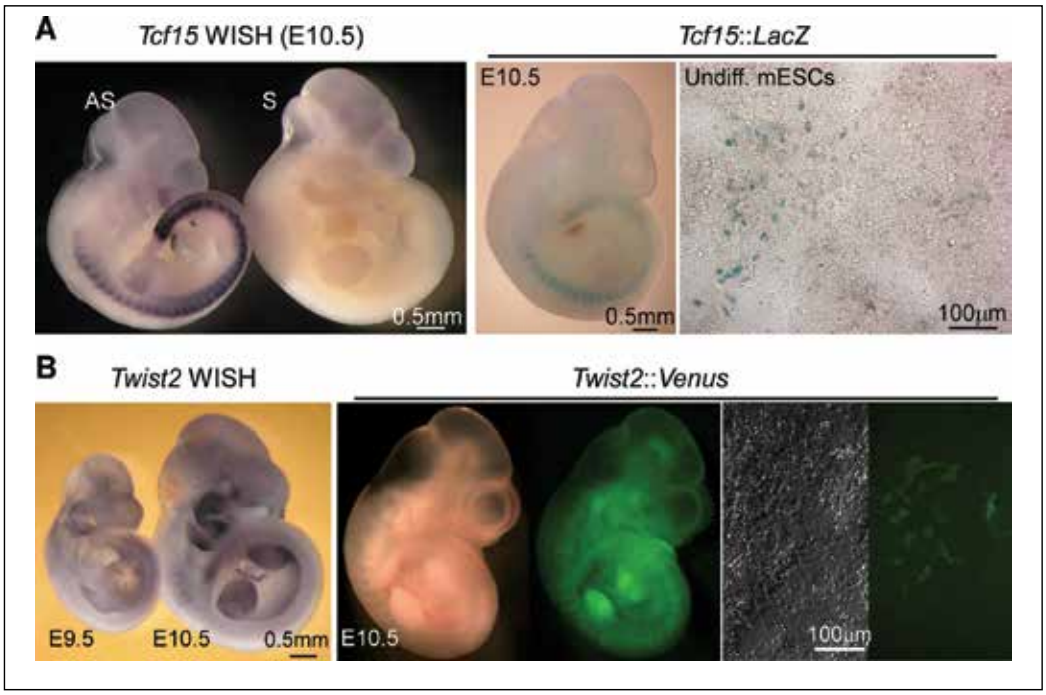

Fig. 1. **Standard culture of mouse embryonic stem cells (ESCs) exhibit fluctuating expression of genes** (modified from Tanaka *et al*., 2008). (**A**, **left**) The *Tcf15* expression pattern in a 10.5 days post-conception (d.p.c.) embryo shown by whole-mount *in situ* hybridization (WISH). S, sense (negative) control. (**A**, **right**) Expression of a reporter (*LacZ*) under the *Tcf15* promoter in a 10.5 d.p.c. embryo derived solely from the mouse ESCs by tetraploid aggregation and in undifferentiated mouse ESCs plated on gelatin-coated dishes (Undiff. mESCs). (**B**, **left**) *Twist2* expression patterns in 9.5 and 10.5 d.p.c. embryos examined as in A. (**B**, **right**) Expression of a fluorescent reporter (*Venus*) under the *Twist2* promoter in a 10.5 d.p.c. embryo derived solely from mouse ESCs and in undifferentiated mouse ESCs.

responsible for inducing the transcriptional heterogeneity in ESCs remains largely unknown. However, as will be discussed in the following sections, ESCs in culture may have received some signals from the microenvironment, such as the stiffness of culture dishes and serum components, which initiate the heterogeneous transcription of these genes.

3. Impacts of culture conditions on the self-renewal of pluripotent stem cells

3.1 Stiffness of a culture dish

When LIF is supplied in the culture medium, mouse ESCs can be maintained on gelatin-coated plates without a layer of embryonic fibroblasts as feeders (Robertson, 1987). Similarly, human ESCs can be maintained on plates coated with Matrigel (a basement membrane preparation extracted from a murine Englebreth-Holm-Swarm sarcoma) independent of a feeder layer in a chemically defined culture medium. Interestingly, other extracellular matrix proteins elicit different responses from ESCs. For example, collagen IA promotes the self-renewal of mouse ESCs (Furue *et al.*, 2005), and fibronectin and laminin

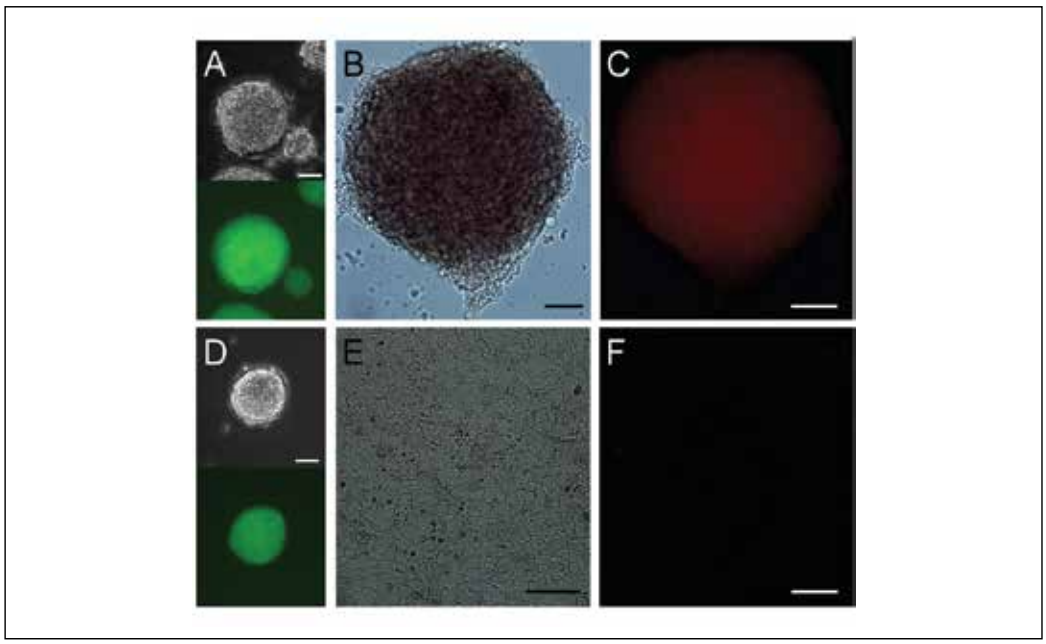

Fig. 2. **Soft substrates promote mouse embryonic stem cell self-renewal.** Mouse ESCs were plated on substrates that have the same stiffness as mouse ESCs (**A-C**) or on glass-bottomed dishes (**D-F**) and maintained under standard culture conditions with LIF (**A & D**) or without LIF for 5 days (**B**, **C**, **E & F**). Bars, 50 μm. (**A & D**) In the presence of LIF, mouse ESCs typically formed round colonies (**top**) on collagen type IA-coated surfaces and maintained *Oct3/4* expression, indicated by the enhanced green fluorescent protein (EGFP) driven by the *Oct3/4* promoter (*Oct3/4::EGFP*, **bottom**). (**B & C**) Mouse ESCs on soft substrates without LIF for 5 days formed round colonies that maintained active alkaline phosphatase (**B**) and the expression of Nanog (**C**). (**E & F**) Mouse ESCs on a glass-bottomed dish without LIF for 5 days exhibited appearance of differentiated cells with no detectable alkaline phosphatase activity (**E**) or Nanog expression (**F**).

help decrease their differentiation potential (Hayashi *et al*., 2007; Hayashi *et al*., 2010). Collagen IV is an inducer of mesoderm lineages for both mouse and human ESCs (Schenke-Layland *et al*., 2007). Intriguingly, the analysis of Matrigel components has led to the discovery of synthetic polymers that can support the long-term self-renewal of human ESCs (Melkoumian *et al*., 2010; Rodin *et al*., 2010; Villa-Diaz *et al*., 2010). Recently, it has become evident that cell fate decisions in stem cells are regulated by matrix elasticity or substrate stiffness (Discher *et al*., 2009). For example, synthetic soft substrates (Elasticity, E = ~1 kPa) that mimic the elasticity of the brain induced the differentiation of neurons from mesenchymal stem cells, whereas stiffer substrates (E = ~40 kPa) that mimic the elasticity of collagenous bone induced the differentiation of osteoblasts (Engler *et al*., 2006). In contrast, we found that mouse ESCs are intrinsically soft and respond optimally to physical forces when cultured on substrates that match their intrinsic softness, which is 0.6 kPa (about 7000-fold softer than plastic culture dishes; Chowdhury *et al*., 2010). In culture conditions, mouse ESCs are grown on much harder substrates than any tissue *in vivo*. To investigate the effect of soft substrates on the self-renewal of mouse ESCs, we plated a mouse ESC line expressing enhanced green fluorescent protein (EGFP) under the *Oct3/4* promoter (**Fig. 2A & D**; Walker *et al*., 2007) on either soft substrates or glass-bottomed dishes in the absence of LIF for 5 days. Remarkably, mouse ESCs on the soft substrate grew as uniformly round colonies without any noticeable differentiating colonies (see **Fig. 2E**) and were able to maintain the expression of markers for pluripotent cells: *Oct3/4* (data not shown), alkaline phosphatase (**Fig. 2B**) and Nanog (**Fig. 2C**). Mouse ESCs cultured on a glass-bottomed dish fully differentiated and downregulated these markers (**Fig. 2E & F**). Therefore, these results strongly indicate that substrate stiffness is a critical extrinsic factor to sustain the self-renewal of mouse ESCs (Chowdhury *et al*., 2010).

3.2 Culture conditions with animal serum

Animal serum provides nutrients, hormones, growth factors, steroids and matrix proteins to cultured cells. It also contains remnants of plasma components used for the activation and processing of blood clots as well as other substances that do not normally pass through the endothelial barrier (Hewlett, 1991; Holliday, 1999; Sato *et al*., 2010). Despite the fact that animal serum is similar but not identical to the interstitial fluid (i.e., lymph) that surrounds cells *in vivo*, animal serum is preferred for cell culture because it significantly improves the growth of cells. However, animal serum is also known to negatively impact cells in culture (Sato, 1975). For example, complement in serum may inhibit cell growth; these components may be inactivated by heat (Robertson, 1987). In addition, serum promotes aneuploidy in cultured cells (Loo *et al*., 1987) that may contribute to the incidence of chromosomal instability in mouse ESCs (Rebuzzini *et al*., 2008). In fact, no cell types *in vivo* are exposed to serum for extended periods, except the ones in the vicinity of a wound where clotting has taken place (Barnes & Sato, 1980). Because animal serum provides cell culture with many other uncharacterized components that may compromise the capability of PSCs to self-renew and differentiate, only qualified animal serum can be used for PSC culture (Robertson, 1987). Furthermore, animal products cannot be used to maintain human iPSCs for transplantation purposes (Ludwig *et al*., 2006b). Although attempts have been made to culture human ESCs in human serum, these cells exhibited extensive differentiation (Rajala *et al*., 2007). Chemically defined culture is a preferable alternative, as it not only allows us to obtain more consistent results for better manipulation of PSC differentiation, but can also be applied to practical therapeutic uses for iPSCs.

3.3 Serum-free culture conditions

To eliminate the effects of unknown components in animal serum, chemically defined serum-free culture methods have been established for PSCs (Ying *et al.*, 2003; Furue *et al.*, 2005; Ludwig *et al.*, 2006a; Ludwig *et al.*, 2006b; Furue *et al.*, 2008). Typically, these defined culture media are composed of critical growth factors (e.g., LIF and Bmp4) and other factors present in animal sera, such as hormones (e.g., insulin and transferrin), vitamins, fatty acids and minerals. In addition, a pre-mixed serum replacement that claims to include no animal serum components was introduced in 1998 (Goldsborough *et al.*, 1998; Cheng *et al.*, 2004). Although the exact components in the serum replacement cannot be disclosed by its patent (Price *et al.*, 1998), the patent indicates that it contains at least albumin, amino acids, vitamins, transferrin, antioxidants, insulin, collagen precursors and some trace elements. In spite of the fact that the serum replacement successfully supported the growth of primate ESCs (e.g., Suemori *et al.*, 2001), human ESCs cultured with this preparation indicated the presence of some BMP-like factors that induced the differentiation of trophoblasts (Xu *et al.*, 2005b). The maintenance of the undifferentiated state of both mouse and human ESCs using defined culture media has been well documented (Ludwig *et al.*, 2006a; Ludwig *et al.*, 2006b; Hayashi *et al.*, 2007; Ying *et al.*, 2008), and the pluripotency of these mouse ESCs has been validated by their differentiation *in vitro* (Furue *et al.*, 2005; Hayashi *et al.*, 2007) and by the development of chimeric mice (Ying *et al.*, 2003).

4. Tumorigenesis in pluripotent stem cells

4.1 Intrinsic factors involved in tumorigenesis

The ability of cells to grow as a teratoma after transplantation into a host animal is a hallmark of cellular pluripotency (see **"2.1 Pluripotent stem cells"**; Chambers & Smith, 2004; Solter, 2006; Jaenisch & Young, 2008; Damjanov & Andrews, 2007; Lensch & Ince, 2007). Testing this cellular ability requires no special techniques or equipment and reduces the use of experimental animals, and it is particularly useful and widely accepted for the validation of pluripotency in human PSCs (Yu & Thomson, 2008). However, this cellular ability is the major critical safety issue hampering the therapeutic application of human iPSCs (Yamanaka, 2009). According to Lawrenz *et al.* (2004), two mouse ESCs were sufficient able to grow into a teratoma only when mixed with 2×10^6 non-tumorigenic fibroblasts (MRC-5) prior to transplantation into immunocompromised mice. To date, little is known about the tumorigenic property of PSCs, except that the oncogene *Eras* is responsible for the tumor-like growth of mouse ESCs (Takahashi *et al.*, 2003). It is interesting to note that Eras activates Akt (Takahashi *et al.*, 2003) and that constitutive activation of Akt is sufficient to drive self-renewal of mouse and non-human primate ESCs (Watanabe *et al.*, 2006). In addition, Akt mediates the inactivation of Gsk3β by insulin via phosphorylation (Bechard & Dalton, 2009; Wu & Pan, 2010; Cross *et al.*, 1995). Gsk3β inhibits its downstream target c-Myc through β-catenin (He *et al.*, 1998; Bechard & Dalton, 2009), so Eras may indirectly activate c-Myc, which is responsible for the self-renewal of mouse ESCs (Cartwright *et al.*, 2005) and for tumorigenesis in mouse iPSCs (Okita *et al.*, 2007; Nakagawa *et al.*, 2010). However, this model may involve other uncharacterized gene products, as human ESCs do not express human *ERAS* (Kameda & Thomson, 2005; Tanaka *et al.*, 2009) but develop into teratomas.

4.2 Extrinsic factors responsible for tumorigenesis

Interestingly, mouse PSCs contribute to the development of normal chimeras, instead of forming teratomas, when mixed with mouse preimplantation embryos (Bradley *et al.*, 1984; Auerbach *et al.*, 2000; Polo *et al.*, 2010). Thus, mouse PSCs may require proper extrinsic signals or niches (Voog & Jones, 2010) to differentiate normally and to contribute to the development of chimeras. The fact that mouse PSCs behave differently when exposed to different microenvironments raises the question of whether PSCs are inherently tumorigenic or are provided with extrinsic signals *in vitro* that promote tumor-like growth. To address this question, we transferred mouse ESCs maintained under standard conditions (**Fig. 3A**) using fetal bovine serum (FBS) into chemically defined serum-free (CDSF) conditions (**Fig. 3B**).

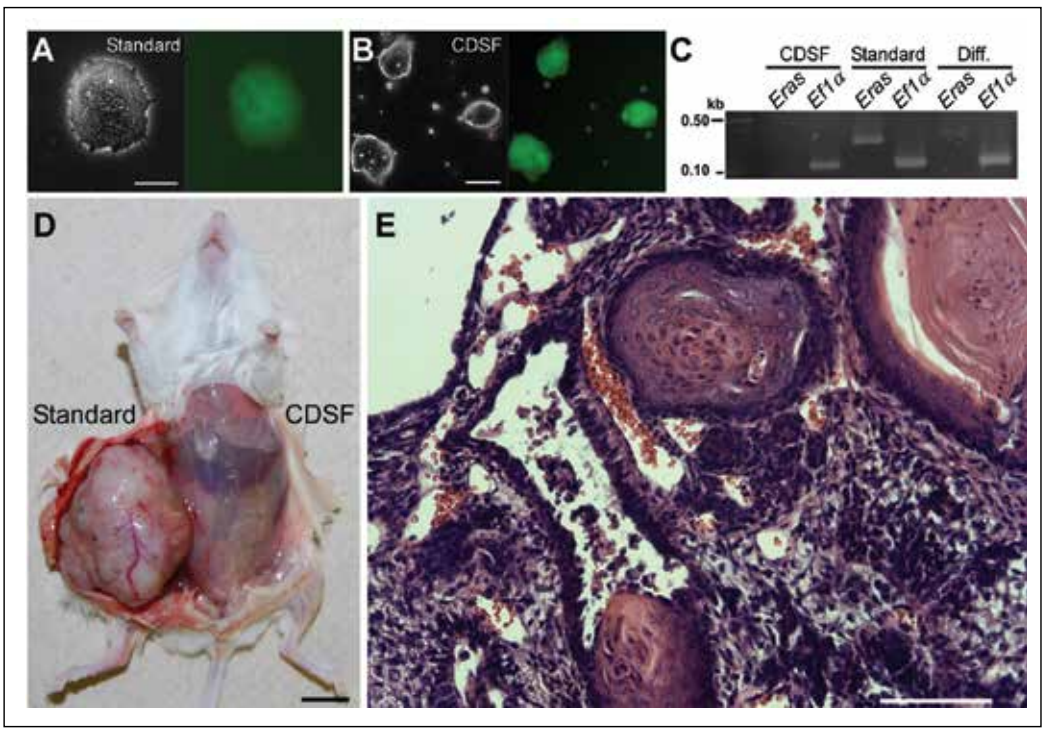

Fig. 3. **Mouse embryonic stem cells gain tumorigenicity from animal serum.** (**A**) A mouse ESC line that harbors an EGFP reporter driven by the *Oct3/4* promoter (right) was maintained under standard conditions using fetal bovine serum (FBS). Bar, 50 μm. (**B**) The same ESC line shown in (A) was plated on a collagen IA-coated plate and cultured under chemically defined serum-free (CDSF) conditions. The transcriptional activity of *Oct3/4* is evidenced by the green fluorescence (right). Bar, 50 μm. (**C**) Expression of *Eras* was examined in mouse ESCs cultured under the indicated conditions. Diff., ESC differentiation was induced by the withdrawal of LIF for 5 days. *Ef1a* is shown as a control. (**D**) 1 x 10^6 cells maintained under each indicated condition were transplanted subcutaneously into NOD-SCID mice, and their growth was monitored for 11 weeks. Bar, 1 cm. (**E**) Histological image of a teratoma consisting of a variety of specialized cells. Bar, 100 μm.

These ESCs were maintained under CDSF conditions for three passages before being subcutaneously transplanted into immunocompromised mice. Surprisingly, the ESCs failed to produce teratomas for up to six months, whereas mouse ESCs maintained under standard conditions generated well-developed teratomas within five weeks (**Fig. 3D & E**). When mouse ESCs were cultured under CDSF conditions supplemented with FBS, or when the cells were cultured under CDSF conditions followed by standard culture conditions, they consistently developed into teratomas. The tumorigenic plasticity of mouse ESCs appears to be unique; ECCs (F9; Bernstine *et al*., 1973) cultured in CDSF formed teratomas when transplanted (data not shown). Because serum is different from interstitial fluid (i.e., lymph), it is suggested with our present data that interstitial fluid will not provide tumorigenicity. Mouse ESCs cultured under CDSF conditions proliferated significantly more slowly than mouse ESCs cultured under standard conditions. Their slower proliferation was accompanied by the downregulation of *Eras* (**Fig. 3C**), which is responsible for the tumorigenicity of mouse ESCs. However, mouse ESCs cultured under CDSF conditions maintained the expression of transcripts associated with cellular pluripotency, *Oct3/4* (**Fig. 3B**), *Sox2* and *Esg1* (data not shown; see "**2.3 Intrinsic factors to maintain self-renewal**"). These results indicate that the tumorigenicity of mouse ESCs is reduced without compromising the pluripotency by short-term serum-free culture (Li & Tanaka, submitted). Perhaps these mouse ESCs exhibited cell death after transplantation due to the absence of a continuous supply of LIF (Furue *et al*., 2005), even though mouse ESCs express their own LIF transcripts (Shen & Leder, 1992). Because the effect of long-term serum-free culture on tumorigenesis in mouse ESCs has not yet been evaluated, we cannot rule out the possibility that undifferentiated mouse ESCs that have adapted to long-term serum-free culture may regain tumorigenic properties.

5. Conclusion

Here we present experimental evidence to suggest that soft substrates promote mouse ESC self-renewal and that short-term serum-free culture reduces the tumorigenicity of mouse ESCs. The underlying mechanisms involved in the cell-substrate interaction and tumorigenesis in mouse ESCs are currently unknown. However, these studies using mouse ESCs provide a basis for further study and help establish simple strategies to significantly enhance the control of differentiation and increase the safety of human iPSCs.

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Embryonic and Cancer Stem Cells - two views of the same landscape

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1. Introduction

According to the Cancer Stem Cell (CSC) theory, there is a small subset of neoplastic cells in the tumors, which retain unlimited proliferative capacity. These cells give also rise to a differentiated cancer progeny that does not have spreading potential but makes up the bulk of the tumor. Thus, CSCs would be the ultimate cause of tumor growth, maintenance and recurrence (Figure 1).

CSCs are experimentally defined by the ability to recapitulate the heterogeneity of the original tumor when transplanted into immunocompatible or nude mice. In 1867 Julius Cohnheim proposed that tumors are derived from embryonal cells that rest in the adult tissues. Later on, in the middle of the following century, Furth and Kanh (1937) and Pierce and Dixon (1959) proved the stem cell properties of a subset of cells in leukemia and testicular germ cell tumors (TGCTs) and Till and McCulloch (1961) transplanted colony forming units (CFU) from the bone marrow into lethally irradiated mice. Additionally, the group of Barry Pierce showed the *in vitro* modulation of cancer cell differentiation (Pierce & Verney, 1961) and the *in vivo* cloning of single embryonal carcinoma (EC) cells, proving their pluripotency (Kleinsmith & Pierce, 1964). The differentiation of cloned leukemic cells (Pluznik & Sachs, 1965) and the reprogramming of embryonal carcinoma (EC) cells when injected into early embryos (Brinster, 1974) were also outstanding results. All these discoveries paved the way for the isolation of embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981) and one of the above groups also studied the differentiation of CSCs from distinct tumors, like squamous cell carcinoma, chondrosarcoma and adenocarcinoma (Pierce & Wallace, 1971; Pierce, 1974; Pierce et al., 1977) seeding the concept of cancer differentiation therapy (Pierce & Speers, 1988). A new progress in this matter was done with the isolation of CSCs in human acute myeloid leukemia (Lapidot et al., 1994) and, particularly, with the discovery of new markers for progenitor cells in several solid tumors, such as breast (Al-Hajj et al., 2003), brain (Singh et al., 2003) and colon (O'Brien et al., 2007) cancer.

During the evolution of CSC research, there have been several technical improvements that have undoubtedly contributed to the success in the engraftment of the tumor cells in the host mice and the subsequent tumor formation. Firstly, it has been proved that the immune system of the host notably affects the survival of the transplanted cells, and thus, the use of

mutant mice with less effective immune systems increases the calculated CSC number within the studied tumor. Similarly, CSCs have been shown to be more prone to successfully form tumors when transplanted accompanied by either carcinoma-associated fibroblasts or *Matrigel®* (Hwang et al., 2008, Quintana et al., 2008).

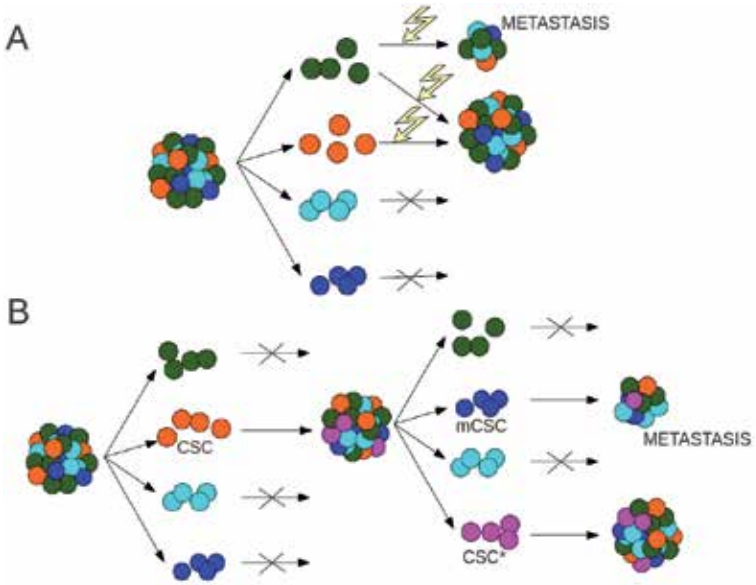

Fig. 1. Hierarchical and stochastic models of CSC in solid tumor growth. (A) According to the stochastic model of cancer progression, every cell present in the neoplasia is able to generate all the cell lineages of the tumor. Nonetheless, the malignant potential of the cells may depend on external factors. (B) The CSC hypothesis states that only a small population of cells, is responsible for tumor growth, due to their self-renewal capacity and unlimited proliferative potential. As tumor progresses, distinct CSCs (CSC*) may originate due to additional mutations or epigenetic modifications. Some of these new CSCs may undergo the epithelial-mesenchymal transition, retaining stem cell characteristics, giving rise to migrating CSCs (mCSCs), the ultimate cause of metastasis

It is important to consider that the CSC model has not yet been proven true for all the existing tumor types. Thus, it is also thinkable that some cancers follow the random or stochastic model that states that every cell within the tumor may have the ability to seed tumors under the required conditions. The importance of getting to a better distinction between tumors that follows one model or the other lies on the increase of the effectiveness of cancer treatments, which will be condemned to failure if right cells are not targeted.

2. Cancer stem cell origin

Most cancers are believed to arise from a single cell that undergoes malignant transformation triggered by genetic mutations or epigenetic modifications, followed by clonal selection of cells that gain the ability to adapt to the microenvironment. Although CSCs have been described and even isolated in several tumor types, the identity of the pre-cancerous cell that first acquires the tumorigenic potential is still a matter of debate. On one

hand, tissue-specific stem cells residing in normal tissues or organs, may acquire malignant characteristics and turn into CSCs. This hypothesis is supported by the fact that tumorigenic modifications require years to accumulate. Given that stem cells present the longest life span in the organism, they are the ideal targets of the neoplastic process. Furthermore, adult stem cells share many functional characteristics with CSCs, e.g. self-renewal and the potential to differentiate into several cell types.

It is likewise possible that the first mutagenic "hit" affects somatic cells responsible for the support of the specialized stem cell niche. This specific microenvironment is necessary for the maintenance of the stem cell identity. Hence, alterations in one or more of the supporting cells may cause that non-tumorigenic stem cells receive aberrant signals which trigger their transformation into malignant cells.

Nevertheless, adult stem cells may not be the source of every type of cancer. CSCs could arise from normal somatic cells, that as a consequence of genetic mutations are re-programmed into "defective" stem cells. This is likely the case for T-lymphoid leukemia that develops from T-cells in which the T-Cell Receptor (TCR) gene rearrangement has already occurred. This genomic modification happens during T-Cell maturation in the thymus and is unique and irreversible. Interestingly, T-Cell leukemias present clonal TCR rearrangements and thus, it seems clear that in this case tumors arise from somatic cells that undergo de-differentiation and recover stem cell properties (Schmidt & Przybylski, 2001).

Experiments that comprise the analysis of cell lines cultured in 3D systems, have shown the ability of certain cells to adopt a stem cell-like phenotype under particular culture conditions. Liu and coworkers reported that embryonic fibroblasts from retinoblastoma (Rb) knockout mice, are able to form spherical structures that express specific stem cell markers when cultured in suspension, and some cells in the spheres adopted CSCs characteristics (Liu et al., 2009). This process has also been reported in the 293T cell line, derived from human embryonic kidney (Debeb et al., 2010). Furthermore, Meyer and colleagues have suggested the possibility of reprogramming differentiated cancer cells into CSCs (Meyer et al., 2009). They have reported that non-invasive CD44+/CD24+ breast cancer cells are able to give rise to malignant CD44+/CD24- progeny *in vivo* and *in vitro*.

In 2006, Takahashi and Yamanaka reported that the overexpression of a cocktail of 4 transcription factors (Oct4, Sox2, c-Myc and Klf4) in adult fibroblasts turned them into pluripotent stem cells (Takahashi & Yamanaka, 2006). Recent studies have demonstrated that these so-called induced pluripotent stem (iPS) cells and ES cells exhibit similar gene expression signatures and potentiality. Because they are genetically modified cells, the idea of using these first generation iPS cells in therapeutic treatments has been seriously questioned. Furthermore, two of the four genes used in the cocktail have been shown to behave as oncogenes (Geoghegan & Byrnes, 2008). In order to solve these issues, new methods for iPS generation have been established, which instead of transfecting cells, are based on the use of recombinant proteins (Zhou et al., 2009).

Taken together, these experiments reveal the capacity of somatic cells to acquire pluripotency. Thus, it could also be possible that alterations in the expression of one or more of those genes triggers the transformation of a given normal somatic cell into a malignant cell capable of forming a tumor. Indeed, the overexpression of Oct4 can lead to epithelial dysplasias by blocking the differentiation of progenitor cells (Hochedlinger et al., 2005). Moreover, several tumor types, for example bladder carcinoma, lung adenocarcinoma, ovarian carcinoma and testis tumors present abnormally higher levels of Oct4 when compared to their normal counterpart tissues. Similarly, Klf4, Sox2 and c-myc appear

upregulated, either alone or together, in a variety of hematological malignancies and solid tumors, including brain, breast, bladder, lung, pancreas, colon and kidney cancer (Schoenhals et al., 2009).

Interestingly, non-CSCs present in the tumor may as well gain stem cell properties due to the acquisition of further genetic and epigenetic modifications and in response to changes in the tumor microenvironment. Therefore, the cell population responsible for tumor growth at a given tumor stage, may not be the same during tumor evolution or metastasis (Roesch et al., 2010). In this regard, the previously mentioned CSC model proposes that CSCs are the only cells within the tumor that can acquire the ability to spread and grow in distant sites.

3. Cancer types in which CSCs have been identified

Although the existence of CSCs has been reported in several tumor types, so far it has not been described any marker that exclusively labels CSCs. Normally, these cells are isolated using antibodies specific for normal stem cells of the same tissue from which the tumor is originated. Flow-cytometry-based cell-sorting, enables the isolation of a side population which is transplanted into host mice to test their tumorigenic potential and conclude whether indeed, it is enriched in CSCs. CD24, CD44, CD133, epithelial specific antigen (ESA) and ATP-binding cassette B5 (ABCB5) are some of the cell surface markers that have been proved to be differentially expressed by those CSC enriched populations (Table 1).

Tumor type	Surface markers used to purify the CSCs	References
Accute myeloid leukemia	CD34+/CD38-	Lapidot et al., 1994
Breast cancer	CD44+/CD24-/CD24low	Al-Hajj et al., 2003
Brain cancer	CD133+	Singh et al., 2003
Head and neck squamous cell carcinoma	CD44+	Prince et al., 2007
Colon cancer	CD133+	O'Brien et al., 2007
Pancreatic adenocarcinoma	CD44+/CD117+	Li et al. 2007
Melanoma	ABCB5+	Schatton et al., 2008
Ovarian cancer	CD44+/CD24+/ESA+	Zhang et al., 2008
Prostate cancer	CD133+/α2β1integrin/ CD44+	Maitland & Collins, 2008

Table 1. Distinct surface markers have been extensively used to isolate CSC-enriched subpopulations from different cancer types

Leukemia and TGCTs were the first tumor types in which CSCs were experimentally described (Furth & Kanh, 1937; Pierce & Dixon, 1959). Furth and Kahn proved that a single cell from a murine cancer cell line was able to transmit leukemia. Regarding TGCTs, Kleinsmith and Pierce (1964) dissociated teratocarcinoma-derived embryoid bodies and transplanted single cells into host mice. Around 2.5% of the grafted cells had the ability to form new tumors, albeit their differentiation potential was highly variable. Later on, Stevens

(1968) managed to generate testicular teratocarcinomas by grafting 3 and 6-day embryos into testicles of adult mice and to serially transplant the formed tumors. It was not until 1994 when surface markers were used to isolate an enriched fraction of tumor-initiating cells. In that year, Lapidot and coworkers (1994) separated a subpopulation of CD34+/CD38- cells from human accute myeloid leukaemia (AML) patients and verified their CSC properties after transplanting them into severe combined immune-deficient (SCID) mice observing that they were able to recapitulate the disease.

Regarding solid tumors, it is likely that additional technical issues, such as the difficulty in obtaining homogeneous single cell suspensions for their further separation, delayed the achievement of a well-characterized CSCs population from a human tumor. The first solid malignancy from which CSCs were isolated was breast cancer. Al-Hajj and colleagues described a CD44+/CD24-/low cell population that was significantly enriched in tumor-initiating cells (Al-Hajj et al., 2003). Shortly after these findings, CSCs of brain tumors were also isolated using CD133 as a marker and characterized by the expression of other markers for non-pathological neural stem cells (Singh et al., 2003) . Interestingly, different pathologic subtypes of brain tumors, like medulloblastomas, astrocytomas and gangliogliomas, share common CSCs. However, the self-renewal capacity of CSCs varies depending on the tumor subtype and its aggressiveness.

Prince and colleagues (2007) developed an immunodeficient mouse model to test the tumorigenic potential of different populations of cancer cells derived from primary human head and neck squamous cell carcinoma (HNSCC). They reported that a population of CD44+ cancer cells was capable of giving rise to new tumors that reproduced the original tumor heterogeneity and could be serially passaged. Moreover, these CSCs had a primitive cellular morphology and did express the basal cell markers Cytokeratin 5 and 14. On the contrary, CD44- cells did not show tumorigenic potential and were similar to differentiated squamous epithelium as assessed by the expression of differentiation markers such as Involucrin.

Later on, it was established that in colon cancer, CSCs are characterized by the expression of the CD133 marker (O'Brien et al., 2007). In humans, colon cancer-initiating cells represent only around 0.06% of all cells within the tumor, but 0.4% of them are CD133+. Thus, it is still necessary to find additional cell surface markers in combination with CD133 in order to further purify the CSC fraction from this type of cancer.

In 2007, Li and colleagues analyzed the tumor initiating ability of different subpopulations from primary human pancreatic adenocarcinoma. They reported that those pancreatic cancer cells with a CD44+/CD24+/ESA+ phenotype, that represent around 0.2 to 0.8% of pancreatic cancer cells, had a 100-fold increased tumorigenic potential compared to non-tumorigenic cancer cells. Furthermore, the genetic analysis of pancreatic CSCs revealed an increased expression of the signaling molecule Sonic Hedgehog (Li et al., 2007).

Schatton and colleagues (2008) identified an ACBB5+ subpopulation of melanoma cells that represents 0.0001% of the total tumor cells and shows high capacity to re-establish the malignancy after xenotransplantation into mice. In addition, they proved that the specific targeting of these CSCs using monoclonal antibodies against ABCB5, resulted in inhibition of tumor growth. However, Quintana and collaborators (2008) have recently shown that the amount of CSCs in human melanoma may change dramatically depending on the conditions of the xenotransplantation assay. This finding raises the question whether tumors in which CSCs are rarely detected, may in fact have a higher number of these cells. For example, tumor microenvironment as well as site of inoculation may have an influence on the tumorigenic potential of presumptive CSCs. The frequency of CSCs in human

melanoma reported by this group is indeed much higher than reported for any other cancer type that follows the CSC model. Although efforts were made to characterize those melanoma-seeding cells, they could not find phenotypic differences between tumorigenic and non-tumorigenic cell populations.
Zhang and colleagues (2008) have also identified the surface phenotype of CSCs from ovarian adenocarcinoma. These cells are characterized by a higher expression of CD44 and CD117 (c-kit), Bmi-1, stem cell factor, Notch-1, Nanog, nestin, ABCG2 and Oct4 when compared to non-malignant ovarian tumor cells (Zhang et al., 2008). Finally, Maitland and Collins (2008) have reported that tumor-initiating cells are enriched in a CD133+/α2β1integrin+/CD44+ subpopulation from human prostate cancer. Moreover, prostate CSCs have a unique genetic fingerprint that makes them useful to predict the tumor staging and clinical outcome. Remarkably, these putative CSCs do not express androgen receptor, which makes them refractory to the widely used androgen-based therapies (Maitland & Collins, 2008).

4. CSC and ES cell similarities

Three different types of non-malignant stem cells have so far been described *in vivo*: ES cells, chord blood/placental stem cells and adult stem cells. Among them, ES cells are the only ones that show pluripotency, being capable of giving rise to cell derivatives of the three germ layers: ecto-, endo- and mesoderm. ES cells are obtained from the inner cell mass (ICM) of the blastocyst and can be cultured *in vitro* using specific conditions to prevent their differentiation. In turn, adult and chord blood/placental stem cells are multipotent and can differentiate into a limited number of cell lineages (Rogers & Casper, 2003).
The ability of cancer cells to grow indefinitely led to the belief that CSCs were similar to adult stem cells. However, detailed gene expression analysis of both cell types reveals that actually, CSCs share more characteristics with ES cells. In fact, long before the CSC hypothesis was enunciated, it was observed that tumor and embryo development share multiple common features. For instance, many tumors have been histologically classified due to their differentiation state, being this characteristic also relevant to prognosis. This is the case of TGCTs that are characterized by the presence of embryonic and extra-embryonic tissues, together with embryonal carcinoma (EC) cells, a population of pluripotent stem cells. EC cells, the CSCs of TGCTs, are considered the pathological counterpart of ES cells, due to their ability to lose their malignant phenotype and participate in normal embryo development when transplanted into blastocysts. In the resulting chimeric mice EC cells appear in tissues derived from the three germ layers, including germ cells (Brinster, 1974; Mintz & Illmensee, 1975). Based on these similarities, EC-derived post-meiotic neurons have been implanted in damaged regions of the brain in a clinical trial (Hara et al., 2008). Nonetheless, due to the karyotypic instability of EC cells, these regeneration therapies have some safety concerns.

4.1 Gene signature in CSC and ES cells

A key goal in cancer research is to identify the molecular mechanisms by which CSCs arise and acquire their stem-like characteristics. Nowadays, extensive databases, mainly obtained by microarray analysis, have been generated, offering the possibility of comparing the expression profile of a huge number of both tumor and normal cells or tissues. Based on the fact that ES and cancer cells share properties such as self-renewal and differentiation

capacity, there has been a recent increased interest in finding out whether CSCs and ES cells present a similar gene signature. As previously mentioned, Oct4, Sox2, c-Myc and Klf4 comprise the gene cocktail that induces pluripotency in somatic cells by a process known as "somatic cell reprogramming" (Takahashi & Yamanaka, 2006). Thus, it is believable that alterations in the expression of one or more of these ES-defining genes may trigger the transformation of a normal somatic cell into a malignant CSC.

Oct4 is the commonly used synonym for POU5F1 (POU class 5 homeobox 1). This transcription factor is active from the fertilized oocyte throughout the whole preimplantation period of embryo development. Oct4-deficient mouse embryos fail to form the ICM, lose pluripotency and differentiate into trophectoderm. Therefore, the level of Oct4 expression in mice is crucial for regulating pluripotency and early cell differentiation since one of its main functions is to maintain the undifferentiated state of the embryo (Zaehres et al., 2005). Moreover, an Oct4 overexpression leads to epithelial dysplasias by blocking the differentiation of progenitor cells (Hochedlinger et al., 2005). It is likewise known that several tumor types, as for example bladder carcinoma, lung adenocarcinoma and testis tumors among others, present increased levels of Oct4 expression compared to their normal counterpart tissues (Schoenhals et al., 2009). It has been recently reported the existence of a subpopulation of cells from ovarian cancer that express Lin 28 and Oct4 genes, both highly expressed in human ES cells. In fact, the up-regulation of these genes in tumor samples is correlated with advanced tumor grade (Peng et al. 2010). Interestingly, the CSC population isolated from ovarian cancer has an up-regulated expression of Oct4 (Zhang et al., 2008). The expression of these two factors seems to be essential for cell growth, since their inhibition using iRNA results in a significant reduction in cell growth and survival (Peng et al., 2010).

Sox2, also known as Sry (sex determining region Y), is a key transcription activator during early embryonic development and its activity is also important in adult stem cells, since it has been reported to maintain the proliferative potential of neural stem cells (Episkopou, 2005). Oct4 forms an heterodimer with Sox2 that drives the expression of several pluripotent-specific genes, including Nanog, FGF-4, UTF1, Fbx15 and Lefty1, together with Oct4 and Sox2 themselves. The expression of at least three of these genes (Nanog, Sox2 and Oct4) is essential to maintain the pluripotent ES cell phenotype. With the possible exception of Lefty1, the expression level of each of the genes regulated by the Oct4/Sox2 complex is substantially reduced upon differentiation of both ES and EC cells, due to the down-regulation of Sox2 and Oct4 (Boer et al., 2007). It has also been reported that the CSCs of multiple myeloma express high levels of Sox2, together with Oct4 and c-Myc, being the latter a well-known proto-oncogene. c-Myc was first described in Burkitt's lymphoma patients, and its function has been proved to be crucial for early embryo development and adult stem cell maintenance. For example, antisense DNA inhibition of c-Myc expression in preimplantation mammalian embryos results in developmental arrest at the eight-cell morula stage (Paria et al., 1992). Over-expression of c-Myc has been described in several hematological malignancies, such as leukemia, lymphoma, smoldering myeloma and multiple myeloma. Wang and colleagues (2008) studied the importance of this transcription factor in CSCs using glioma CD133+ cells as a model. Inhibition of c-myc using lentivirally transduced short hairpin RNA (shRNA) resulted in cell cycle arrest in the G(0)/G(1) phase, reduced proliferation and increased apoptosis. Furthermore, glioma CSCs with decreased c-Myc expression levels failed to form neurospheres *in vitro* or tumors when xenotransplanted into brains of immunocompromised mice. Overall, c-Myc seems to play a essential role in the regulation of the stem cell characteristics of CSCs. Finally, the transcription

factor Klf-4 (Krüppel-like factor 4), necessary for somatic cell reprogramming, is also required for ES cell self-renewal and maintenance of pluripotency (Zhang et al., 2010). Klf4 appears up-regulated in most hematological malignancies, such as acute lymphoblastic leukemia, hairy cell leukemia and multiple myeloma, being yolk sac tumors, a germ cell tumor subtype, one of the few solid tumors that present elevated levels (Schoenhals et al., 2009).
A better understanding of the acquisition and regulation of the self-renewal and proliferation potential by CSCs will improve the design and generation of anti-neoplastic drugs and accelerate the discovery of novel molecular targets for clinical application. This knowledge may as well help to use the existing treatments in a more CSC-specific and effective manner.

4.2 Signaling pathways shared between CSCs and ES cells

Besides the gene signature that defines the ES cells, there are some signals that can extrinsically regulate several stem cell properties such as self-renewal. Many studies show that embryonic development and tumorigenesis share common regulatory mechanisms. Thus, it would not be surprising to find out that the cellular process, which leads to the generation of both, ES and CSCs, is regulated by the same factors. Hereby, we list the most important pathways that modulate ES function, mentioning their relationship with CSCs.
The Notch signaling pathway is highly conserved in mammals, playing an important role in embryonic development and adult tissue repair. It is known that Notch signaling is down-regulated during the cellular differentiation, being a promoter of stem cell survival, proliferation and undifferentiation of some adult stem cells, like for example hematopoietic and brain stem cells. It has been reported that the inhibition of this signaling pathway in some cancer types, such as medulloblastoma, reduces their proliferation *in vitro* and their capacity of forming tumors when transplanted into mice (Fan et al., 2006). These authors showed the blockage of Notch signaling by gamma-secretase inhibitors (GSI) reduced neurosphere growth and clonogenicity *in vitro*, whereas the activation of this pathway increased tumor growth. Furthermore, the expression of glioblastoma CSC markers such as CD133, Nestin, Bmi1, and Olig2 was reduced when Notch signaling was blocked. Xenograft transplantation experiments revealed that GSI-pretreated cells did not form tumors and even the implantation of drug-impregnated polymer beads in the tumor beds also effectively reduced tumor growth and significantly prolonged survival (Fan et al., 2010). Thus, it is likely that Notch pathway inhibition depletes CSCs through reducing proliferation and increasing apoptosis associated with decreased AKT and STAT3 phosphorylation.
Regarding the Wnt pathway, it is known to play an essential role in cell proliferation and stem cell maintenance. Sato and coworkers (2004) demonstrated that the overactivation of the Wnt/b-catenin pathway was responsible of the maintenance of pluripotency in ES and adult stem cells, as assessed by the expression of markers such as Oct4, Nanog and Rex1. Mutations within this signaling pathway occur frequently in human cancers. In fact, experiments in mice have shown that the overactivation of the Wnt/b-catenin pathway promotes oncogenic transformation of different cell types. For instance, mice that overexpress Wnt1 under de MMTV (mammary tumor virus) promoter develop salivary and mammary gland tumors and the accumulation of b-catenin due to mutations in APC gene may be linked to the appearance of colorectal tumors in humans (Markowitz et al., 2009).
Fibroblast growth factors (FGFs) are intercellular signaling molecules that, among other functions, participate in embryonic development, affecting several cell functions such as proliferation, differentiation, survival, adhesion and migration (Szebenyi & Fallon, 1999).

The FGF signaling pathway regulates the specification events that occur in the early embryo, when the cells composing the ICM of the blastocyst give rise to the three germ layers. Since ES cells are derived from the ICM it is believable that these cells conserve the same pathways. One of the most interesting members of the FGF pathway is FGF-2, also known as basic-FGF or bFGF. It has been reported that elevated expression levels of FGF-2 in the microenvironment of metastatic prostate tumors induce chemotherapy-resistance in the malignant cells. It has been speculated that the dysregulation of this factor in tumor cells is acquired as an adaptation to gain self-renewal and unlimited proliferative ability, two of the main features that define ES cells (Villegas et al., 2010).
During normal embryo development, the Hedgehog (Hh) signaling pathway is related to the epithelial-mesenchymal transition (EMT) by means of the up-regulation of the E-cadherin repressor SNAIL1. EMT is related to tumor progression in correlation with the loss of the epithelial characteristics and the acquisition of metastatic phenotype (Hay, 1995). According to the CSC theory, metastatic cells retain stem cell properties, thus cells that undergo EMT have to conserve stem cell properties. To describe these cells, Brabletz and colleagues (2005) proposed the term migrating CSCs (mCSCs).
A better knowledge of the pathways that regulate ES cell functions is crucial to understand how CSCs self-renewal, survival, proliferation and metastasis are regulated. This will help to target specifically those stem cells that may be the source of recurrent tumors and are able to escape from the majority of the currently used cancer therapies (Takebe & Ivy, 2010).

5. Testis germ cell tumors as a CSC model

Early pathologists noted that certain germinal tumors contained a mixed population of adult tissues, but looked like a "caricature" of them (Aréchaga, 1993). Therefore, these malignancies were named teratocarcinomas, being "terato" a prefix meaning "monster". Testis germinal cell tumors (TGCTs) form a heterogeneous group of neoplasias that are usually classified according to their histological aspect. Teratocarcinomas are characterized by the presence of embryonic and extra-embryonic tissues together with a population of pluripotent stem cells, named EC (Kleinsmith & Pierce, 1964). EC cells represent the most aggressive cell population in germ line tumors and are as well responsible for the transplantability of the tumors between immunocompatible animals. Interestingly these cells are frequently referred as the "malignant counterpart" of ES cells, due to the similar features that both cell types share (Aréchaga, 1993, Andrews et al., 2005). For example, as previously noted, when injected into a blastocyst, EC cells lose their malignancy and participate in the normal development of the embryo, just like ES cells (Brinster, 1974). Furthermore, it has been described that when cultured, ES cells acquire karyotypic changes that resemble those of EC cells, being their general *in vitro* behavior also similar.
TGCTs derive from a precursor lesion called carcinoma *in situ* (CIS) of the testis, which is found forming a single row at the basement membrane of seminiferous tubules. It was described for the first time when "atypical spermatogonia" were found in testis samples of patients that later developed TGCTs (Skakkebaek, 1972). It is known that CIS cells are already present in a latent state at the moment of birth, but it is not until puberty, when triggered by hormonal changes, the cells start to proliferate and to invade the testis stroma (Díez-Torre et al., 2004).
Two possible origins for the CIS of the testis have been proposed. Taking in consideration that germ cell tumors appear mostly in the gonads and that the transplantation of mouse embryo genital ridges into testis originates tumors (Stevens, 1967), it seems that CIS cells

result from germ cells that are unable to differentiate correctly. However, ES cell origin is supported by the fact that these tumors can be also generated by the transplantation of early embryos (Stevens, 1968) and that the gene expression pattern and the differentiation potentiality of the tumor cells is similar to that of the ES cells. Furthermore, the existence of, in terms of histology, identical tumors that appear in extra-gonadal locations, suggests its non-germinal origin. However, it is possible that these rare neoplasias develop from primordial germ cells (PGCs) that are retained during their migration towards the gonads. In fact, extra-gonadal germ cell tumors mostly arise in the body mid-line, which is the path that PGCs follow in their migration.

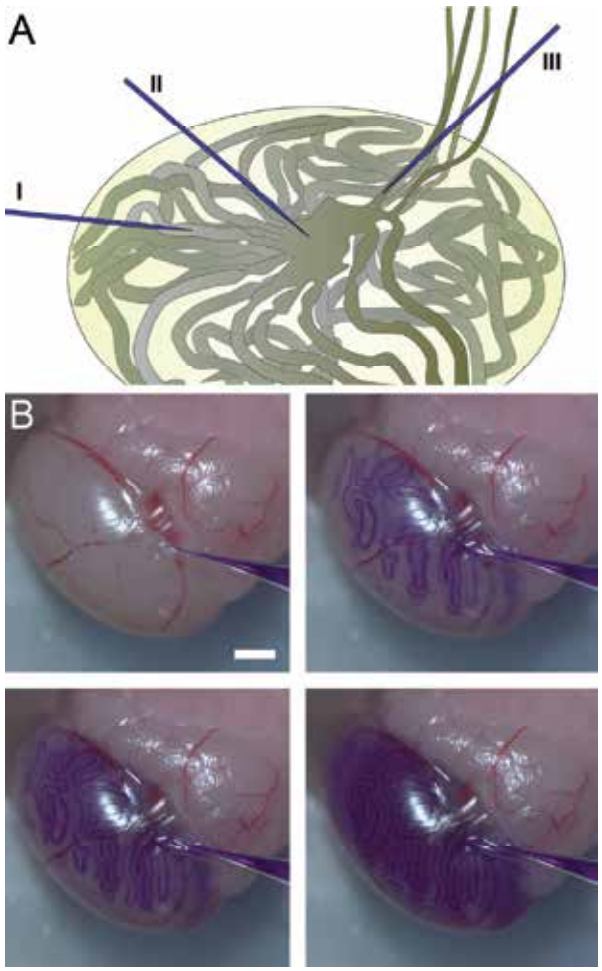

Fig. 2. Technique of cell transplantation into the seminiferous tubules. (A) Scheme showing the three different approaches for cell transplantation into the seminiferous tubules. The sharpened micropipette can be inserted directly in the seminiferous tubules (I), through the efferent ducts (II) or into the *rete* testis.(B) Time lapse photographic series of the microinjection of a blue-colored cell suspension. The microinjection pipette is inserted through the efferent ducts in this case. The process finishes when almost all tubules are filled. Scale bar represents 1mm

Since non-human mammals rarely develop TGCTs until now no suitable animal models are available to study this malignancy. Leroy Stevens (1973) made one of the first attempts to establish an animal model of TGCTs describing an inbred subline of mice termed 129-ter*Sv* which shows a high incidence of those tumors. However, most of the spontaneous tumors were teratomas, the differentiated and benign variant of teratocarcinoma, and thus not adequate for the study of human TGCTs, which generally are malignant (Stevens, 1973).
In the middle 90s of the last century Brinster and Zimmerman (1994) developed the technique of cell transplantation into the seminiferous tubules of azoospermic animals. Their initial approach consisted of microinjecting a spermatogonia-enriched cell suspension into single seminiferous tubules. Later on, two more effective approaches were described, namely the microinjection into the *rete testis* and through the efferent ducts (Figure 2 A and B; Ogawa et al., 1997). Using this technique, it was possible to determine that germinal cells transplanted from one animal into another are capable of nesting in the seminiferous epithelium and differentiate into fertilization-competent spermatozoa. At the same time, Brinster and Avarbock (1994) tested the ability of the seminiferous compartment of reprogramming ES cells and driving their differentiation towards the germ cell lineage. However, ES transplantation resulted in tumors. Later on, a TGCT model using this technique was developed. It consists of the transplantation of EC cell lines into the seminiferous tubules of germ-depleted mice (Li et al., 2008a). The experiments involved the transplantation of two human lines derived from TGCTs, the JKT-1 seminoma cell line and the 833K EC cell line. Transplantation of both resulted in tumors that expressed TGCT markers.

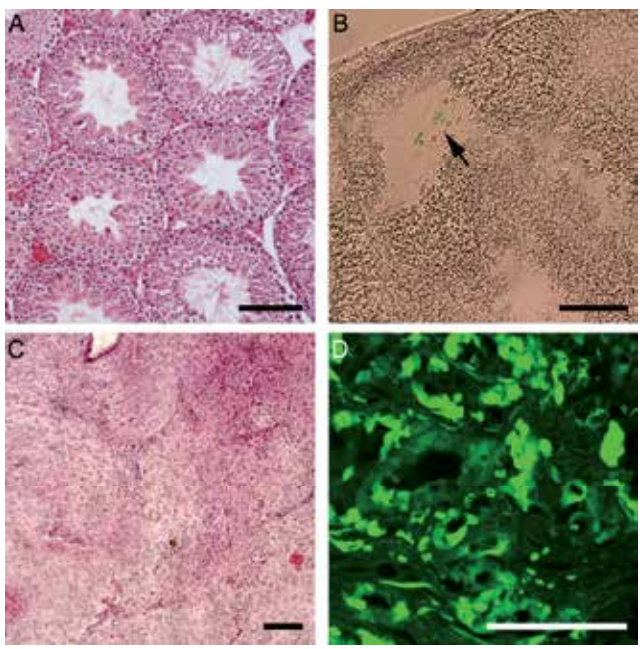

Fig. 3. Histology of an experimental testis teratocarcinoma. (A) Normal testicular tissue is composed of dense packed seminiferous tubules. (B) Cryostat section of a mouse testis few minutes after transplantation of autofluorecent ES cells (arrow) into the seminiferous tubules. (C) 5 weeks after transplantation teratocarcinoma tumors are formed in the transplanted testis, (D) Most of the neoplastic tissue fluorescent. Scale bars represent 50μm

Besides the similarities between EC and ES cells (Andrews et al., 2005), several studies have also shown that ES cells share many phenotypic and genetic similarities with the CIS of the testis (Almstrup et al., 2006; Rajpert-De Meyts, 2006; Looijenga, 2009). For example, it has been estimated that nearly 50% of the genes up-regulated in human ES cells are also expressed in CIS cells. Among these genes pluripotentiality-related and undifferentiation-related genes can be found. These observations led us to establish a TGCT model based in the transplantation of ES cells into the seminiferous tubules, with the belief that this model mimics more accurately than others the early stages of TGCT development (Silván et al., 2009a; Silván et al., 2010a). To follow the fate of the transplanted cells, we generated a stable GFP-transfected ES cell line, named AB1^{GFP}. Short after the transplantation ES cells can be seen in the lumen of the seminiferous tubules (Figure 3 A and B). Around 24 to 36 hours later, ES cells are found integrated in the seminiferous epithelium, close to the basal membrane. Interestingly, this localization is similar to that of the spontaneous CIS of the testis. Five weeks after the ES cell transplantation, mature teratocarcinoma are completely formed (Figure 3 C). Most of the structures of the formed tumors were found to be derived from the transplanted cells, as can be determined by their fluorescence (Figure 3 D).

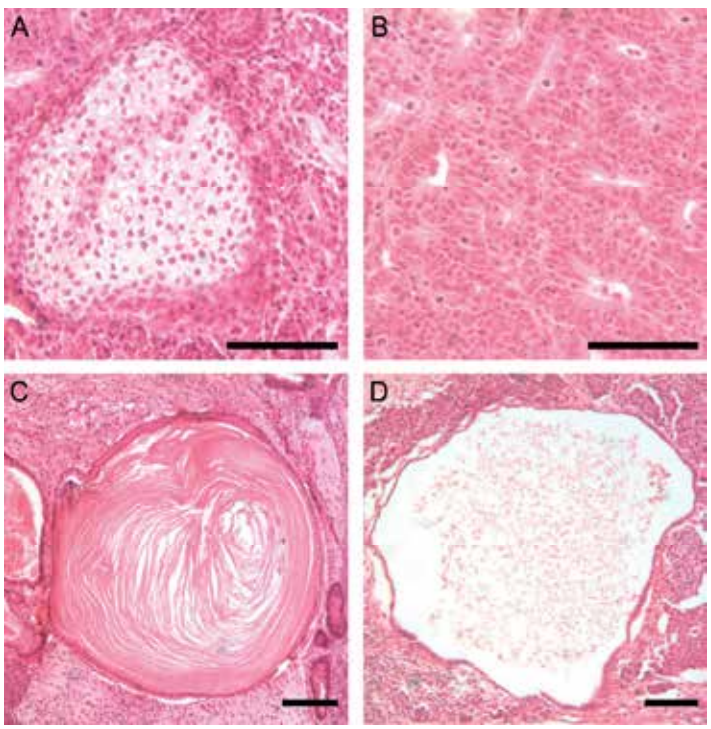

Fig. 4. Experimental teratocarcinomas showed different histological patterns of differentiation, such as cartilage (A), neural (B), epidermoid (C) and adenomatous areas (D). Scale bars represent 50µm

This transplantation procedure mimics better than others the early stages of TGCT development. At the histological level, experimental teratocarcinomas highly resembled the spontaneous TGCTs, showing structures derived from the three germ layers (Figure 4 A-D). In addition, regions with undifferentiated appearance (Figure 3C), which would be similar

to the EC component of spontaneous non-seminoma tumors, could as well be found (Silván et al., 2010a).
Although TGCT treatment has a high success rate (Gerl et al., 1996; Bosl, 1999), early diagnosis and identification of the causes of the high incidence among young men are still unknown. The experimental model that we have developed allows the study of the pre-invasive state of testicular teratocarcinomas and is potentially useful for the screening of novel therapeutic drugs, such as inhibitors of angiogenesis. Furthermore, prior to the transplantation procedure, donor cells can be modified, up- or down-regulating one or more genes, thereby providing a functional assay to evaluate the effect of these genes in EC transformation. Hence, we believe this model can help in the study of the cellular and molecular mechanisms involved in CSC establishment.

6. CSCs, ES cells and hypoxia

It is well known that adult tissues contain stem cell populations for cell renewal and an increasing number of evidences support that these cells are localized in specific *niches* characterized by low oxygen levels (Mazumdar et al., 2009). Since stem cells are the only cell lineage that is not replaced during the whole animal's life span, it is likely that the hypoxic microenvironment, where these cells are found, protects them from potential damages caused by oxygen (Diabira & Morandi, 2008). *In vitro* studies that tested the effect of oxygen showed a direct relationship between oxygen levels and the regulation of stem cell proliferation, differentiation and survival. More precisely, hypoxia has been reported to increase the proliferation of neural crest stem cells (Morrison et al., 2000), stimulate the survival of chondrocytes (Schipani et al., 2001) and disrupt adipocyte differentiation (Yun et al., 2002). Applying observations to the cancerous tissue, it is likely that CSCs are as well located in low oxygenated regions. In fact, due to their rapid growth and defective vascularization, solid tumors frequently present regions with reduced oxygen supply and necrosis. Consequently, tumor hypoxia has been correlated with bad prognosis factors, such as malignancy stage and resistance to radio- and chemotherapy (Jubb et al., 2010; Bertout et al., 2008).
Cellular response to hypoxia leads to several changes in gene expression triggered by a group of transcription factors known as Hypoxia Inducible Factors (HIFs). These factors, which belong to the basic helix-loop-helix (bHLH) and PAS (PER-ARNT-SIM) families, are composed by two subunits: one of them is variable (HIF-1α, -2α or -3α) and oxygen sensitive, but rapidly stabilized in response to hypoxic conditions. The other subunit, HIF-1β, also known as ARNT (aryl hydrocarbon receptor nuclear translocator), is constant and constitutively expressed (Wang & Semenza, 1995). When the cell is exposed to low oxygenation conditions, degradation of the alpha subunit is inhibited, binds the β subunit and the resulting complex translocates into the nucleus. There, it activates specific genes through the recognition of promoter regions known as hypoxia response elements (HREs). The up-regulation of these hypoxia-related genes mediates a number of changes at both cellular and systemic level.
Oxygen availability regulates several physiological processes, such as cell proliferation, differentiation and migration, being low oxygen levels, one of the main causes of cellular stress. The relationship between hypoxia and tumor growth was first reported as the radioprotective effect of anoxia in normal tissues was demonstrated using whole-body anoxia in newborn rodents (reviewed by Gray et al., 1953). Later on, the study of ex-utero

mouse embryo growth demonstrated that hypoxia is needed for a proper embryonic development (Morriss & New, 1979). In fact, during early development ES-precursor cells are exposed to a hypoxic microenvironment due to the absence of vasculature in early embryos (Mitchell & Yochim, 1968). Because of this common hypoxic environment, HIF factors play a critical role in normal development and tumor growth, invasion and metastasis (Harris, 2002).

The study of HIF knockout mice has demonstrated the key role of this transcription factors during the embryonic and fetal development. HIF-1α and HIF-1β null mice embryos die as the result of defects in vascular development (Ryan et al., 1998, Maltepe et al., 1997). The effects of HIF-2α disruption range from embryonic lethality due to aberrant vasculature (Peng et al., 2000) to postnatal lethality because of multiorgan failure and altered mitochondrial metabolism (Scortegagna et al., 2003). The relationship of hypoxia with solid tumor progression has been repeatedly reported in clinical studies that prove that those patients with hypoxic tumors have a significantly poorer clinical outcome (Vaupel, 2008; Liu et al., 2010; Jubb et al., 2010). The causes of this worst prognosis include an increased ability for tumor invasion and metastasis and a higher resistance to radio- and chemotherapy (Brizel et al., 1999; DeClerck & Elble, 2010) as the result of a hypoxia-dependent expression of drug-resistance genes (Wartenberg et al., 2003), the selection of apoptosis-resistant clones (Graeber et al., 1996) and the disruption of DNA repairing mechanisms (Chan et al., 2009).

The link between low oxygen levels and tumor progression, together with the identification of CSCs and the known role of hypoxia as a key component of the stem cell microenvironment has lead to the hypothesis that hypoxia maintains the undifferentiated state of CSCs and thus contributes to cancer growth, invasion and metastasis. Stem cell niches constitute the adequate environment for the maintenance of the undifferentiated state and for regulating stem cell differentiation into specific cell lineages (Moore & Lemischka, 2006). It has been recently speculated that hypoxia may regulate stem cell localization and maintenance inducing the expression of paracrine factors in a HIF-dependent manner. That is the case of endothelial progenitor cells. Hematopoietic stromal cells expressing the SDF-1 chemokine attract the stem cells expressing CXCR4 at low oxygenation conditions (Ceradini et al., 2004). This example illustrates the importance of stroma cells in the regulation of the stem cell microenvironment. The differentiation status of the stroma cells is crucial for the maintenance of stem cells. In bone marrow, for instance, when osteoblasts are removed during early differentiation, there is a critical reduction in bone marrow-derived hematopoietic stem cells (Visnjic et al., 2004). Nevertheless, this effect on hematopoiesis is significantly reduced when osteoblasts are removed at later stages of differentiation (Corral et al., 1998). Further evidences, such as the expression of hematopoietic stem cell maintaining factor angiopoietin-1 by undifferentiated CD146+ osteoprogenitor cells but not in differentiated cells, point to immature stroma cells as key supporters of the stem cell niche (Sacchetti et al., 2007). The crosstalk between tumor cells and the surrounding stroma cells has a crucial effect on tumor growth, invasion and metastasis (Diez-Torre et al., 2010). The importance of this interaction suggests that stroma cells may provide the adequate microenvironment to maintain CSCs. In fact, hypoxia may regulate CSC maintenance and differentiation through three different mechanisms and can act directly on CSCs inhibiting them to differentiate. Low oxygen levels can also induce or maintain an immature state in tumor stroma cells and, finally, it can up-regulate the expression of paracrine factors that mediate the interaction between tumor and stroma cells in a way that stimulates CSC homing, proliferation and invasion.

Karnoub and colleagues (2007) reported that mouse stroma cells surrounding human breast cancer xenografts contain mesenchymal stem cells able to form fibroblastoid colonies *in vitro* whereas this ability is absent in those cells obtained from adjacent normal stroma. The same study demonstrated that the presence of bone marrow-derived mesenchymal stem cells is sufficient to induce the acquisition of a highly aggressive phenotype by poorly metastatic human breast carcinoma cells when injected in host mice. Thus, immature stroma cells may be recruited by the tumor microenvironment, being these undifferentiated cells involved in the acquisition of an aggressive phenotype by breast carcinoma cells.

Surprisingly, it has been reported that brain CSCs are preferentially located in the proximity of tumor associated endothelial cells. Although this result may partially contradict the hypoxic niche-theory, *in vitro* co-culture experiments have shown that endothelial cells secrete paracrine factors that promote CSC growth and stemness. Thus, the role of endothelial cells within the tumor stem cell niche could be independent of their vascular function (Calabrese et al., 2007). This idea is in concordance with the well known abnormality of tumor-associated blood vessels that lead to the formation of hypoxic or anoxic regions into the solid tumors and the presence of endothelial precursors without vascular functionality near the tumor cells (Silván et al., 2010b). Further research on the interactions between CSCs and endothelial cells in tumor hypoxic regions could provide valuable information for a better understanding of the tumor stem cell niche.

The role of hypoxia in the induction and maintenance of CSC undifferentiation has been recently studied in two different tumor types, neuroblastoma and breast carcinoma (Axelson et al., 2005). In both tumors, a correlation between poorly differentiated regions and tumor aggressiveness has been observed. In the case of neuroblastoma, the analysis of gene expression profiles of cultured tumor cells and neuroblastoma xenografts has demonstrated that hypoxia down-regulates the expression of mature neuron marker genes whereas induces the expression of c-Kit and Notch, genes associated with a neural crest-like phenotype (Jögi et al., 2002). Similar effects have been reported in ductal breast carcinoma (Helczynska et al., 2003) and cultures of EC cell lines (Silván et al., 2009b). However, the role of hypoxia in tumor progression is not limited to the maintenance of the undifferentiated state of CSCs, it has also been shown that low oxygen availability increases tumor cell proliferation and apoptosis resistance in lung adenocarcinoma (Chen et al., 2007a), melanoma (Bedogni et al., 2008) and thymus lymphomas (Bertout et al., 2009) through the alteration of Notch1 signaling. The relationship between hypoxia and tumor progression is also mediated through the overlapping of HIFs and some oncogene signaling pathways, such as cMyc and p53 (Mazumdar et al., 2009).

All these observations point out that oxygenation level plays a critical role in the stem cell microenvironment, and that, together with other niche components such as stromal cell contacts, extracellular matrix proteins, growth factors and temperature, is directly involved in CSC behavior, and thus, cancer progression and metastasis.

7. Importance of CSCs for cancer therapy

Many of the currently used cancer therapies are directed against rapidly dividing cells, which represent most of the tumor cell population. However, in a significant number of cases these therapies fail to eliminate the stem cell fraction of the tumor, what leads to tumor relapse and the selection of a more aggressive and therapy-resistant cancer cells and thus implies a worst clinical outcome. The development of therapies that specifically target CSCs

seems to be needed in order to achieve the complete tumor remission and prevent metastasis. The identification of the different cancer cell populations through DNA and tissue microarray analyses, the study of *in vitro* obtained cancer spheroids or the selection of more aggressive and therapy-resistant tumor cell lines by successive xenograft transplantation, are some of the strategies that are being followed with the aim of identifying those agents that selectively eliminate CSCs (Ischenko et al., 2008). It has to be considered that CSCs share many features with normal adult stem cells, and therefore, it has to be checked that the new therapies are effective against CSCs without being harmful for healthy adult progenitor cells. The results obtained by recent research works support the feasibility of this objective.

As we have previously mentioned, recent evidence demonstrates that the stem cell fraction of several tumor types show a higher resistance to radio and chemotherapy (DeClerck & Elble, 2010; Elliot et al., 2010). Different mechanisms have been suggested to explain the acquisition of this resistance. One of these explanations is related to the slow proliferation rate shown by stem cells, which stay in the G_0 phase of the cell cycle during long periods of time. This feature protects them from drugs that target actively dividing cells, the so-called cell-cycle specific agents. Moreover, CSCs show an increased expression of adenosine triphosphate-binding cassette proteins, which are known to outflow chemotherapeutic drugs. In fact, one of these proteins, the breast cancer-resistance protein (ABCG2), has been used to identify and isolate the side population of breast and other cancer types by flow cytometry due to its ability to extract Hoechst dye from the cell (Kim et al., 2002).

Additional mechanisms used by CSCs to escape chemotherapy are the up-regulation of drug metabolizing enzymes, for example the ALDH1 enzyme that metabolizes cyclophosphamide (Smalley et al., 2005), and the over-expression of survival promoting factors, such as the apoptosis inhibitors Survivin and Bcl-2 family proteins (Litingtung et al., 1999). Obviously, some of these stem cell markers have a prognostic significance. The expression of ALDH1 in breast carcinoma, for example, has been associated with poor clinical outcome and an increased risk of recurrence (Ginestier et al., 2007)

The results of several studies on the resistance of CSCs to radio- and chemotherapy suggest that the limited success of the current therapies in some tumor types could be related to their inability to target the CSC population. Indeed, it has been recently reported that chemotherapy leads to an increase of the breast CSC population, characterized by a CD44+/CD24- phenotype (Yu et al., 2007; Li et al., 2008b). Interestingly, in one of these neoadjuvant therapeutic trials, it was shown that targeting Her-2 with *Lapatinib*® produces a reduction of the CSC population and that this effect leads to a significantly increased pathologic complete response rate (Li et al., 2008). Since Her-2 is a known stem cell regulator, this observation constitutes important evidence in favor of the CSC hypothesis and suggests that the effectiveness of Her-2 inhibitors, like *Trastuzumab®* and *Lapatinib®*, could be connected with its ability to target the CSC fraction.

The encouraging results obtained with Her-2 inhibitors point to other proteins and signaling pathways that regulate self-renewal of stem cells, such as Wnt, Notch, Bmp4 and Hh, as potential targets for future therapeutic compounds. In this regard, targeting of Hh signaling by *Cyclopamine®*, a steroid-like compound, has been shown to inhibit the growth of ovarian carcinoma (Chen et al., 2007b) and the EMT in pancreatic cancer cell lines, leading to a significant reduction of *in vitro* invasiveness and metastatic potential *in vivo* (Feldmann et al., 2007). Interestingly, a combined treatment with *Cyclopamine®* and EGFR inhibitor not only resulted in a reduction of tumor cell invasion *in vitro* but it also increased the rate of

apoptotic death in prostate carcinoma cells (Mimeault et al., 2006) and several human pancreatic cancer cell lines (Hu et al., 2007). Moreover, it has been reported that *Cyclopamine®* increases the cytotoxic effect of both radio- and chemotherapy in different pancreatic cancer cell lines (Shafaee et al., 2006). The inhibition of Hh signaling may have interesting applications in the treatment of glioma. This idea is supported by the fact that the treatment with this drug targets CSCs *in vitro* and leads to a reduction of glioma tumor formation *in vivo* (Clement et al., 2007). Another interesting effect caused by the blocking of the Hh pathway has been observed in multiple myeloma. In this poor prognosis tumor, the inhibition of Hh significantly reduced the myeloma stem cell clonal expansion and, additionally, stimulated the complete differentiation of these stem cells without affecting the growth of other plasma cells (Peacock et al., 2007).

Some promising results related to blocking of the Notch signaling pathway have been already obtained. The treatment of medulloblastoma cells with GSI-18, a inhibitor of Notch signaling, produces a remarkable reduction of the CD133+ stem cell population and the complete eradication of the side population detected by flow cytometry in medulloblastoma cell mass (Fan et al., 2006). Interestingly enough, the suppression of the stem cell fraction correlates with the loss of tumorigenic potential of the cell mass when transplanted into nude mice. Given that Notch signaling has been reported to participate in pancreatic differentiation, the targeting of this pathway might also be effective in the treatment of pancreatic cancer (Murtaugh et al., 2003).

There are several studies that emphasize the importance of Bmp4 has a potential target for anti-CSC therapies, this possibility is linked to the role of Bmp4 in the regulation of adult stem cell expansion (Hua et al., 2006). It has been reported, for example, that Bmp4 induces a decrease in CD133+ glioblastoma CSC population *in vitro* and *in vivo* (Piccirillo et al., 2006).

PTEN is another factor known to participate in the maintenance of stem cell populations and thus a potential target for cancer therapies. It is a tumor suppressor gene whose expression is usually down-regulated in many tumor types (Di Cristofano & Pandolfi, 2000). PTEN activity is mediated through the inhibition of PI3K/AKT signaling pathways, which include the downstream target mTOR, and in this way modulates several processes related to cell proliferation, growth and survival (Seeliger et al., 2007). A study performed with adult hematopoietic cells demonstrated that the conditional deletion of PTEN in these cells induces an expansion of leukemic cancer cells whereas significantly reduces the proportion of normal hematopoietic stem cells. Additionally, the treatment with *Rapamycin®*, an mTOR inhibitor, in order to overtake the PTEN deletion, leaded to the recovery of normal stem cell population and a reduction in CSCs (Yilmaz et al., 2006).

A different strategy to target CSCs based on the cytotoxic effect of IFN-a has been recently reported (Moserle et al., 2008). More precisely, it has been demonstrated that IFN-a presents a remarkable antiproliferative and pro-apoptotic activity on ovarian cancer cells containing a high proportion of side population cells. Similarly, *in vivo* gene therapy with human IFN-α leaded to a significant regression of large side population containing tumors, whereas not evident effects were observed in those tumors with a poor side population fraction. This result may have an important therapeutic significance since the IFN-a activity seems specifically target tumors that usually present a higher resistance to conventional treatments and, thus, with high number of CSCs.

It has also been reported that the resistance to radiation of glioblastoma CSCs correlates with an over-expression of DNA damage response genes (Bao et al., 2006). In fact, treatment

of this malignancy with radiotherapy leads to an increment of CD133+ glioblastoma cells. A similar observation has been reported for colorectal cancer, in which the CSC population is enriched after chemotherapy. Moreover, the CSCs that remain are responsible for tumor relapse and the acquisition of a more aggressive phenotype (Dylla et al., 2008). Colorectal CSC resistance to chemotherapy agents like 5-fluorouracil and *Oxaliplain®* seems to be mediated by CSC-derived IL-4, that acts as an autocrine apoptosis inhibitor (Todaro et al., 2007). Indeed, the pre-treatment of the colorectal tumor with IL-4 blocking antibodies results in a significant increase in the treatments effectiveness.

Finally, it is important to note that those CSCs present in the tumor at one particular time point, may change during the progression of the malignancy, resulting in a "moving target" (Clarke et al., 2006; Roesch et al., 2010). Thus, the adequate therapy of a neoplasia may depend of a balanced situation of CSCs rate of proliferation, stroma reaction and degree of differentiation of the tumor parenchyma. In conclusion, the development of novel therapies that target specifically the CSC population, may be effective for the treatment of those cancer types that can be interpreted according to the CSC theory.

8. Acknowledgements

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Genome Stability in Embryonic Stem Cells

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1. Introduction

The first embryonic stem cell (ESC) lines have been isolated at the beginning of the 1980s from the inner cell mass of mouse blastocysts (stage 5.5-7.5 days post-fertilization) with direct culture or immununosurgery by two groups of researchers working independently (mouse ESCs, mESCs, Evans & Kaufman, 1981; Martin 1981). It took more than a decade to obtain ESC lines from blastocysts of the primate rhesus monkey (Thomson et al., 1995), the common marmoset (*Callithrix jacchus*) (Thomson et al., 1996), human (hESCs; Thomson et al., 1998), dog (Hayes et al., 2008) and rat (Li et al., 2008; Buehr et al., 2008).
ESCs are undifferentiated, pluripotent and self-renewable cells that can be maintained *in vitro* in the same undifferentiated status over extended periods of culture. They grow in colonies and possess a high nucleus/cytoplasm ratio (Figure 1a). ESCs are characterized by the expression of specific transcription factors (OCT-4, SOX2 and NANOG), and surface markers (TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4 in hESCs, and also Ssea-1 in mESCs) (Figure 1b' and c'), by high telomerase expression and alkaline phosphatase activity (Figure 1d). If injected into a blastocyst, they are able to participate to foetal development and to the formation of the germ cell line; also, following their injection into immunodeficient mice, they form teratomas with derivatives of all three germ layers. Under appropriate *in vitro* culture conditions in suspension, ESCs form three-dimensional cell aggregates called embryoid bodies (EBs; Figure 1e), that differentiate into the three germ layers (ectoderm, mesoderm and endoderm; Figure 1f, g and h). Following the addition of bone morphogenetic protein 4 (BMP4) to the culture medium, it has been demonstrated that both hESCs (Xu et al. 2002) and mESCs (Hayashi et al., 2010) can differentiate into the trophoblast.

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Because of their plasticity and potential to differentiate in all the cell types, ESCs represent an important tool for investigating early development for the study of genetic disease and as a cellular *in vitro* model for screening the effects of new drugs or xenobiotics; and in regenerative medicine and tissue replacement after injury or disease. At this regard, many disorders such as blood and immune-system related genetic diseases, cancer diabetes, Parkinson's disease and spinal cord injuries could be potentially treated using a pluripotent stem cell therapy, even if technical problems of graft-*versus*-host disease associated with allogenic stem cell transplantation (histocompatibility problems) are not negligible (Guyette et al., 2010; Marr et al., 2010; Arenas, 2010). In 2006, a new type of mouse pluripotent cells, with characteristics very similar to ESCs, has been developed by the group of Yamanaka (Takahashi & Yamanaka, 2006). These cells, called induced pluripotent stem cells (iPSCs), are the result of genome reprogramming by the ectopic expression of four transcription factors (Oct-4, Sox2, c-Myc and Klf4) of differentiated fibroblasts. iPSCs exhibit ESCs morphology and growth properties; they are pluripotent, undifferentiated and express ESCs markers. iPSCs have also been subsequently generated from human, rhesus monkey and rat adult primary fibroblasts (Takahashi et al., 2007; Liu et al., 2008; Li et al., 2009) and, more recently, from human adult blood cells (Loh et al., 2009) and rat bone marrow (Liao et al., 2009).

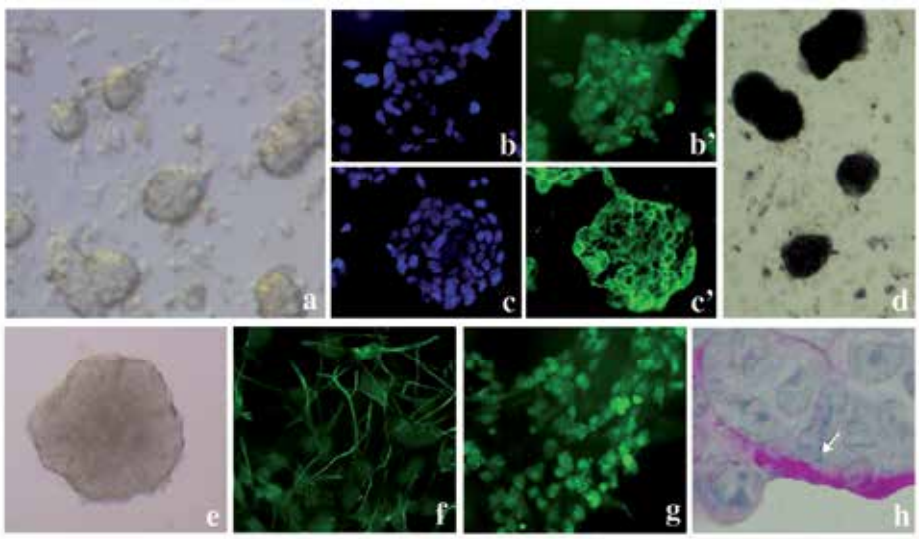

Fig. 1. Mouse embryonic stem cells and their derived embryoid bodies. Morfology of mESC colonies (a); immunocytochemical detection of Oct-4 (b') protein and Ssea-1 surface antigen (c') expression; alkaline phosphatase positive colonies (d); an embryoid body obtained after 5 days of mESCs differentiation (e); mESCs differentiated into cells of the ectoderm layer, expressing the Nestin marker (f); mESCs differentiated into cells of the mesoderm layer, expressing the Flk-1 marker (g); mESCs differentiated into cells of the endoderm layer, observed by histological examination of endodermal epithelial cells (arrow)

2. Loss of genome stability, the importance and consequences for ESCs

The mainteinance of the genomic stability is crucial for normal cell survival and cell growth. Genomic instability is a general term to describe the processes that can increase the rate of mutation of a population, enabling cells to develop new and aggressive phenotypes. Two are

the main mechanisms of instability: microsatellite and chromosomal instability (Lengauer et al., 1997). Microsatellite instability involves simple DNA base changes or tandemly repeated nucleotide sequences (microsatellite regions), whereas chromosomal instability involves whole chromosomes or large portions of them that are gained, lost or rearranged.
The maintenance of the correct chromosome complement is one of the most important necessity for ESCs, in particular for their possible therapeutical use. As other cell lines cultivated *in vitro*, ESCs are prone to accumulate karyotype abnormalities during long period of culture, although their mutation frequency is about 100 times lower when compared to differentiated cells, suggesting that ESCs have specialized mechanisms to preserve their genome integrity (Tichy & Stambrook, 2008).

3. Methods to study the chromosome complement

A chromosome aberration is either an incorrect number of chromosomes (that can occur as a consequence of an error during cell division) or a structural abnormality in one or more chromosomes. There are many types of chromosome anomalies, which can be organized into two groups: numerical or structural (Figure 2). An abnormal number of chromosomes is called aneuploidy and occurs when either one or more chromosomes are missing or gained. A structural abnormality is defined when the normal chromosome structure is altered (e.g., deletion, duplication, translocation etc.).

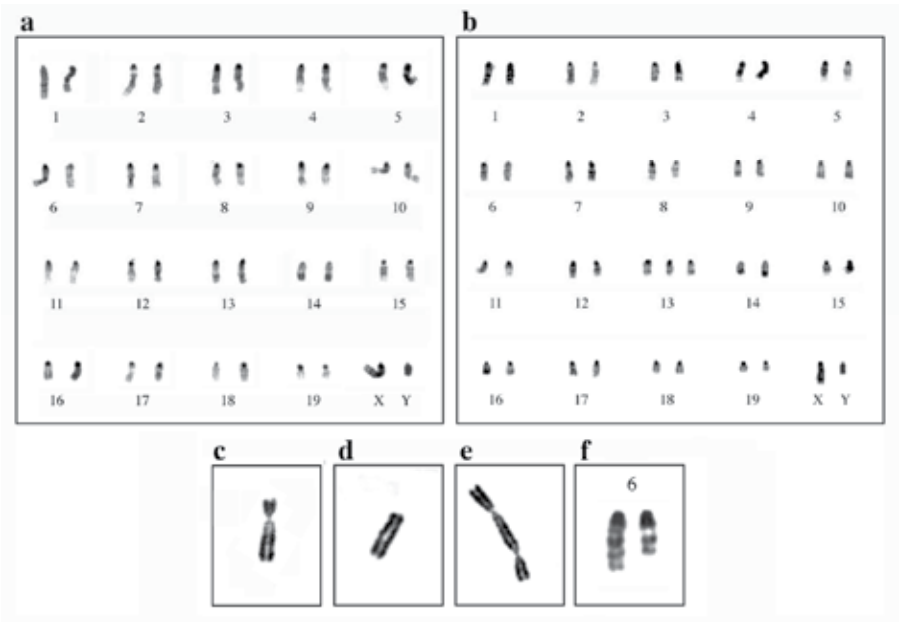

Fig. 2. Examples of numerical and structural chromosome abnormalities a mESC line. Reverted image of a DAPI-banded karyotype of a normal metaphase from a mESC line (a); numerical abnormal metaphase with a trisomy of chromosome 13 from a mESC line (b); structural chromosome abnormalities: metacentric chromosomes (c), chromosome fragment (d) dicentric chromosome (e), human chromosome 3 insertion (f), mouse chromosome 6 deletion (f)

Various types of methods are currently available to determine the chromosome complement and evaluate its integrity. Each technique has advantages and disadvantages in terms of

sensitivity, resolution and costs (Catalina et al., 2007). Classical simple banding techniques allow the regular check of the chromosome composition of the cell lines. For example, G-(Giemsa-stain) and DAPI-banding, providing 300-400 stained bands, permit both the identification of uncorrect chromosome numbers (aneuploidies) and structural chromosome abnormalities (e.g., translocations, deletions or insertions) of wide portions of chromosomes with a resolution of 5-10 Mb. Spectral karyotype (SKY technique) and multicolor fluorescent *in situ* hybridization (mFISH) represent an evolution of the conventional banding analysis (Schrock et al., 1996; Liyanage et al., 1996). Sky and mFISH allow the identification of each single chromosome with a higher resolution, approximately 1-2 Mb, when compared to classical cytogenetic methods and are usefull for the detection of submicroscopic deletions, insertions or DNA amplifications. However, to detect smaller genetic imbalances, the best techniques available at present are the array-based comparative genomic hybridization (array-CGH; Sanlaville et al., 2005) and the single nucleotide polymorphism array (SNP-array; Peiffer et al., 2006). The resolution of these techniques allows the detection of tiny aberrations (from 1 Mb to less than 100 kb) including homo- or hemizygous deletions, copy-neutral loss of heterozygosity, duplications and amplifications; however, these procedures are unable to evaluate the frequency within the cell population of a specific abnormality. Although these techniques shorten the whole screening procedure because they do not require cells blocked at metaphase, somehow, the costs of the equipment and consumables are an obstacle for their routinary use in the monitoring of chromosome stability.
In summary, the combination of both conventional and molecular cytogenetic technologies represents the best approach for the evaluation of the genomic integrity of a ESC lines.

4. Chromosome variation in human, primate and rodent ESCs

In the literature, a fine characterization of abnormalities and a potential explanation about their onset are present for both human and mouse ESCs. Some information is available also for iPSC cells, non-human primates and rat ESCs. A brief overview is reported below.
Human
hESCs can accumulate abnormalities when maintained in culture for few months. The chromosome changes observed affect more frequently chromosomes 12, 17, 20 and X. The reason why these chromosomes are more frequently involved is not clear, although it has been proposed that their alterations confer a selective and/or proliferative advantage to cells carring the mutations. The gain of part or of the entire chromosome 12 has been found in many hESC lines (i.e., BG01, BG02, BG03, H1, H7, H9, H14 and HS181) and observed in many indipendent laboratories (Brimble et al., 2001; Draper et al., 2004; Mitalipova et al., 2005; Ludwig et al., 2006; Imreh et al., 2006). The presence of an additional copy of chromosome 17 is another frequent abnormality found in hESCs (Brimble et al., 2001; Mitalipova et al., 2005). Sometimes associated with the gain of chromosome 12, the gain of the q arm of the chromosome 17 has been observed (Draper et al., 2004). Even if it is not really clear why these chromosomes are frequently involved in hESCs karyotypic changes, it has been suggested that the increased dosage of proteins coded by some genes located on chromosomes 12 and 17 could confer a selective advantage to cells carring these mutations. Human Stella-related (*STELLAR*), *NANOG*, the Growth differentiation factor-3 (*GDF3*) are stem cell pluripotency markers located on chromosome 12p (Clark et al., 2004) whose over-expression may participate to the maintainance of the pluripotent status (Spits et al., 2008). Similarly, the over-expression of *BIRC5* (that encodes for the anti-apoptotic survivin protein; Blum et al., 2009) or of hsa-mir-21 microRNA (involved in tumorigenesis, cancer

progression and a regulator of the anti-apoptotic *BCL2* gene; Caldas & Brenton, 2005), both located on chromosome 17, may confer a proliferation advantage.
The trisomy of chromosomes 12 and/or 17 is often associated with the X chromosome trisomy (Brimble et al., 2004; Inzunza et al., 2004; Mitalipova et al., 2005; Ludwig et al., 2006). Recently, Navarro and colleagues have demonstrated that in mESC the three pluripotency factors (Nanog, Oct4 and Sox2) bind and repress *Xist*, the master regulator of X inactivation, but it is not clear how the trisomy of this chromosome could confer a proliferative or selective advantage to cells (Navarro et al., 2008).
The gain of the entire or a part of chromosome 20 is an other typical chromosomal variation in hESCs (Rosler et al., 2004; Baker et al., 2007; Maitra et al., 2005; Spits et al., 2008; Lefort et al., 2008; Werbowetski-Ogilvie et al., 2009). It is known that the amplification of the region 20q11.2 is recurrent in many types of cancer (melanoma, Koynova et al., 2007; breast, Guan et al., 1996; lung, Tonon et al., 2005; bladder, Hurst et al., 2004) and the possible candidate genes that can increase cell proliferation, are *BCL2L1*, directly involved in cell death and proliferation, *DNMT3B*, important for the correct imprinting, and *POFUT1*, which is indispensable for *NOTCH* cascade signaling activation.
At present only a handful of papers has been published on the genomic integrity of human iPSCs. These pluripotent cells (derived from human adult fibroblasts; Takahashi et al., 2007; Lowry et al., 2008) usually own a normal karyotype during the early culture passages and they lack of hot spot instability regions. However, continuous passaging of iPSCs (e.g. derived from keratinocytes) resulted in the appearance of chromosomal abnormalities (46,XY,t(17;20)(p13;p11.2)) in 70% of the cells after 13 passages, involving the same chromosomes 17 and/or 20 frequently detected in hESCs (Aasen et al., 2008). Using human CGH Arrays, Chin and collaborators have observed few karyotypic alterations (the duplication of part of chromosome 8) in a late-passage (passage 44) in an iPSC line derived from a fibroblast line (Chin et al., 2009).

Non-human primates

Non-human primate ESC (nhpESC) lines are an important research tool for basic and applicative research. The rhesus macaque is physiologically and phylogenetically similar to human, and, therefore, it is a clinically relevant animal model for biomedical research. Even if a number of ESCs lines have been established from rhesus monkey (*Macaca mulatta*), common marmoset (*Callithrix jacchus*) and cynomolgus monkey (*Macaca fascicularis*) (Thomson et al., 1995; Thomsom et al., 1996; Nakatsuji and Suemori, 2002), very few studies have described their chromosome complement.
The little information available shows that using a serum-free medium and subculturing with trypsin, cynomolgus and rhesus monkey ESCs maintain a normal chromosome complement and pluripotency characterisitics even after over 1 year of continuous culture (Nakatsuji and Suemori, 2002). More recently, the cytogenetic analysis of 18 rhesus monkey ESC lines revealed that the majority (15) of them maintained a normal karyotype with a normal diploid chromosome number. The three unstable ESC lines (ORMES-1, -2, and -5) showed, even at low passages, structural abnormalities, such as translocations (t(11;16) and t(5;19) with der (18) t(1;18)), or invertions (inv (1)). It has been hypothesized that the collagenase-based dissociation technique, used for ORMES-1, -2, and -5, may have contributed to the onset of karyotypic abnormalities in these cell lines (Mitalipov et al., 2006).

Mouse

Unexpectedly, an accurate literature search showed that only a few papers described the genomic variation of mESCs during a long period of culture. In many mESC lines, no

recurrent chromosome abnormalities, but rather random alterations have been described by some laboratories (Longo et al., 1997; Guo et al., 2005; Sugawara et al. 2006; Rebuzzini et al., 2008a; Rebuzzini et al., 2008b).

The most complete analysis that has been made on mESCs was performed by Sugawara and colleagues (Sugawara et al., 2006). Following the observation of a total of 540 mESC lines, these authors showed that 66.5% of them presented a normal 2n=40 karyotype, whereas 15.9%, 9.1%, and 2.8% showed modal chromosomal numbers of 41, 42, and 39, respectively. Among 88 mESC lines, selected arbitrarily from the 540 lines, 60.2% showed a normal diploid karyotype, 51.4% showed a trisomy of chromosome 8, 14.3% had trisomy 8 in association with the loss of one sex chromosome, and 11.4% had trisomy 8 together with trisomy 11.

The chromosome complement of ESCs is important in contributing both to somatic cell chimaerism and to germ line transmission. Euploid mESCs cultured *in vitro* for up to 20 passages rapidly became severely aneuploid. Notably, when injected into the murine blastocyst, the percentage of euploid metaphases in the mESC clones correlates with the success obtained in the experiment: the more stable is the chromosome complement the higher is the number of chimeric embryos and pups obtained, and the higher is their the chimaerism. None of the mESC clones with more than 50% of chromosomally abnormal metaphases can be transmitted to the germline (Longo et al., 1997). Another confirmation that prolonged cell culture affects the normal diploid chromosomal composition of the population was reported by Guo and collaborators (Guo et al., 2005). Using mFISH analysis of four different mESC lines, they demonstrated that, although the morphology and the expression of stem markers appeared normal, two cell lines presented consistent numerical (41, 43, 44, sub- or tetraploid chromosome complement) and structural (trisomy of chromosomes 8, 12, 14 and 15, deletion of chromosome 6q and other aberrations with low frequency) aberrations (Guo et al., 2005). More recently, in our laboratory, we have analysed the chromosome complement of four indipendent mESC lines cultured for 3 months. In UPV04 mESC line about 60% of metaphases analysed were 2n=40 throughout the culture period. From passage 13, 50% of metaphases were euploid, with a correct chromosome complement and the remaining 50% showed gain or loss of entire chromosomes, both within the same passage and among different passages analysed. A very heterogeneous spectrum of abnormalites was described, indicating their continuous arising (Rebuzzini et al., 2008a). In other three mESC lines, named UPV02, UPV06 and UPV08, a progressive loss of euploid metaphases during culture has been observed and chromosome abnormalities, in particular metacentric chromosomes, accumulated at the latest passages analysed (passage 31, 29 and 22 for UPV02, UPV06 and UPV08, respectively). We observed that in coincidence with, or few passages after, the drop of euploidy, the alkaline phosphatase activity, one important ESC marker, was partially or totally lost (Rebuzzini et al., 2008b).

Rat

The rat ESCs (rESCs) are an important resource for the study of disease models, however, despite several temptatives (Brenin et al., 1997; Vassilieva et al., 2000) they have been derived only very recently (Buehr et al., 2008; Ueda et al., 2008; Zhao et al., 2010). In two cell lines derived by Buehr and collegues in 2008, a trisomy for chromosome 9 was described both by CGH and by FISH analysis. In two rESC lines, recently established from Wistar rat blastocysts, a normal number of chromosomes was observed at low passages (before passage 11, approximately 40% exhibit a normal karyotype), but a rapid accumulation of chromosomal abnormalities was described at later passages (up to 16 passages) (Ueda et al., 2008).

5. Possible causes of chromosome variations during culture

The variety of culture protocols applied in different laboratories working with ESCs may be the source of variations in cell differentiation and genome stability. Many papers published during the last decade described the presence of the feeder layer, the source of the serum (whether of animal or artificial origin) and the techniques used for cell passaging as the main and major factors affecting the maintainance of genome integrity during long culture periods. The majority of the data and information available on culture conditions are on human and mouse ESCs.

Generally, ESCs are derived and maintained *in vitro* with a co-culture protocol on a feeder layer of mitotically inactivated fibroblast cells (mouse embryonic or immortalized fibroblasts) or on defined supportive matrixes (i.e., gelatin, fibronectin or matrigel™). Whether using the former or the latter, genetic alterations were observed both in mESC and hESC lines (Cowan et al., 2004; Draper et al., 2004; Rosler et al., 2004; Mitalipova et al., 2005; Maitra et al., 2005; Guo et al., 2005; Longo et al., 2005; Imreh et al., 2006; Sugawara et al., 2006; Rebuzzini et al., 2008a; Rebuzzini et al., 2008b), suggesting that the presence or absence of a supporting cellular feeder layer can not exclude the onset of aberrations in the ESCs genome.

A fundamental component of the ESC medium is the serum of animal (calf or bovine) or artificial (knockout serum replacement of defined composition) origin. Despite the type of serum used, the genomic stability seems compromised. In a recent publication (Herszfeld et al., 2006) better results were obtained in the production of more stable hESCs when a serum replacement was used, likely because the use of artificial serum avoids the uncertainty of its composition which is frequently observed with animal-derived sera.

The technique used to detach ESCs for passaging seems to play a major role in the maintenance of their genomic stability. ESC colonies can be dissociated mechanically (i.e., pipetting in and out and flushing the medium until the colonies are detached and disgregated), enzymatically or by manual (i.e., colonies are cut and removed using a blade) dissection. The manual and mechanical dissection are preferentially used during hESCs subculturing, as, being less aggressive, they better preserve the genome integrity (Buzzard et al., 2004; Mitalipova et al., 2005). The manual dissection can introduce a bias due to the choice of the colonies and the skill of the researcher (Lefort et al., 2000). A modified enzymatic dissociation solution, consisting of 0.25% trypsin, 0.1% collagenase IV, 20% KSR, and 1 mM $CaCl_2$ in PBS, in combination with manual dissection for bulk passaging of hESCs has been proposed by Suemori and colleagues in 2006, demonstrating the maintenance of a normal chromosome complement after more than 100 passages in culture (Suemori et al., 2006).

6. Conclusions

Because of their characteristics, ESCs represent an important and unique biological resource for cell therapy and regenerative medicine, but also they are more and more envisioned as opening new routes for pharmacological research (Laustriat et al., 2010). As addressed in this review, the mainteinance of a correct chromosome complement is fundamental for the employment of these cells and a constant monitoring of their stability is required. We have produced an up-to-date summary of the litterature available on chromosome complement in ESCs of several different species, highlighting the need for world-wide guidelines that would restrict a rather fragmented and puzzled scenario. Given the actual culture

conditions used, the preservation of ESCs with a stable karyotype appears to be difficult. Clearly, a single culture protocol for all the species under study does not appear feasible; instead, each model animal will necessitate its own specific guidelines. Based on our own experience mutuated with that gathered from the litterature described above, following is a summary of some important start points that we believe should be taken on board when aiming to obtain an ESC line with low chromosomal variations: 1) use of a serum with a chemically defined composition; 2) manual dissection of ESC colonies; 3) routine monitoring of the chromosome complement throughout the culture period.

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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