**Stem Cells and Cancer Stem Cells 8**
Therapeutic Applications in Disease and Injury

M.A. Hayat
*Editor*

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## Volume 8
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## Therapeutic Applications in Disease and Injury

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*Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena.*

Richard J. Reed, MD

# One Point of View

All small tumors do not always keep growing, especially small breast tumors, testicular tumors, and prostate tumors. Some small tumors may even disappear without a treatment. Indeed, because prostate tumor grows slowly, it is not unusual that a patient may die at an advanced age of some other causes, but prostate tumor is discovered in an autopsy study. In some cases of prostate tumors, the patient should be offered the option of active surveillance followed by PSA test or biopsies. Similarly, every small kidney tumor may not change or may even regress. Another example of cancer or precancer reversal is cervical cancer. Precancerous cervical cells found with Pap test, may revert to normal cells. Tumor shrinkage, regression, dormancy, senescence, reversal, or stabilization is not impossible. Can prosenescence therapy be an efficient alternative strategy to standard therapies for cancer prevention and treatment?

Another known example of cancer regression is found in pediatric neuroblastoma patients. Neuroblastoma shows one of the highest rates of spontaneous regression among malignant tumors. In addition to the well-known spontaneous regression in stage 4S disease, the high incidence of neuroblastoma remnants found during autopsy of newborns suggest that localized lesions may undergo a similar regression (Guin et al. 1969). Later studies also indicate that spontaneous regression is regularly seen in infants with localized neuroblastoma and is not limited to the first year of life (Hero et al. 2008). These and other studies justify the “wait and see” strategy, avoiding chemotherapy and radiotherapy in infants with localized neuroblastoma, unless *MYCN* gene is amplified. Infants with nonamplified *MYCN* and hyperdiploidy can be effectively treated with less intensive therapy. Infants with disseminated disease without *MYCN* have excellent survival with minimal or no treatment. Another example of spontaneous shrinkage and loss of tumors without any treatment is an intradural lipoma (Endoh et al. 1998).

Although cancers grow progressively, various lesions such as cysts and thyroid adenomas show self-limiting growth. Probably, cellular senescence occurs in many organ types following initial mutations. Cellular senescence, the growth arrest seen in normal mammalian cells after a limited number of divisions, is controlled by tumor suppressors, including p53 and p16, and so this phenomenon is believed to be a crucial barrier to tumor development. It is well established that cell proliferation and transformation induced by oncogene activation are restrained by cellular senescence.

Metastasis is the main cause of death from cancer. Fortunately, metastasis is an inefficient process. Only a few of the many cancer cells detached from

the primary tumor succeed in forming secondary tumors. Metastatic inefficiency varies depending on the location within an organ, but the malignancy may continue to grow preferentially in a specific tissue environment. Some of the cancer cells shed from the primary tumor are lost in the circulation due to hemodynamic forces or the immune system, macrophages, and natural killer cells.

Periodic rejection of a drug by FDA, which was previously approved by the FDA, is not uncommon. Most recently, the FDA ruled that Avastin should not be used to treat advanced breast cancer, although it remains on the market to treat certain other cancers, including colon and lung malignancies. Side effects of Avastin include high blood pressure, massive bleeding, heart attack, and damage to the stomach and intestines.

Unwanted side effects of some drug excipients (e.g., propylene glycol, menthol) may also pose safety concerns in some patients. Excipients are defined as the constituents of the pharmaceutical formulation used to guarantee stability, and physicochemical, organoleptic, and biopharmaceutical properties. Excipients frequently make up the majority of the volume of oral and parenteral drugs. Not all excipients are inert from the biological point of view. Although adverse drug reactions caused by the excipients are a minority of all adverse effects of medicinal products, the lack of awareness of the possible risk from excipients should be a concern for regulatory agencies, physicians, and patients (Ursino et al. 2011). Knowledge of the potential side effects of excipients is important in clinical practice.

It is known that chemotherapy can cause very serious side effects. One most recent example of such side effects was reported by Rubsam et al. (2011). Advanced hepatocellular carcinoma (HCC) induced by hepatitis C virus was treated with Sorafenib. It is an oral multikinase inhibitor that interferes with the serine/threonine kinases RAF-1 and B-Raf and the receptor tyrosine kinases of the vascular endothelial growth factor receptors and the platelet-derived growth factor receptor-beta. Although sorafenib is effective in regressing HCC, it shows serious side effects including increasingly pruritic and painful skin changes (cutaneous eruption).

An example of unnecessary surgery is the removal of all the armpit lymph nodes after a biopsy when a sentinel node shows early stage breast cancer; removal of only the sentinel node may be needed. Limiting the surgery to the sentinel node avoids painful surgery of the armpit lymph nodes, which can have complications such as swelling and infection (such limited surgery is already being practiced at the Memorial Sloan-Kettering Cancer Research Center). Radiation-induced second cerebral tumors constitute a significant risk for persons undergoing radiotherapy for the management of cerebral neoplasms. High-grade gliomas are the most common radiation-induced tumors in children (Pettorini et al. 2008). The actual incidence of this complication is not known, although it is thought to be generally low.

## Medical Radiation

Chromosome aberrations induced by ionizing radiation are well-known. Medical radiation-induced tumors are well-documented. For example, several types of tumors (sarcomas, meningiomas) can develop in the CNS after

irradiation of the head and neck region (Parent 1990). Tumorigenic mechanisms underlying the radiation therapy of the CNS are discussed by Amirjamshidi and Abbassioun (2000) (See below).

Radiation therapy is commonly used to treat, for example, patients with primary and secondary brain tumors. Unfortunately, ionizing radiation has limited tissue specificity and tends to damage both neoplastic and normal brain tissues. Radiation-induced brain injury, in fact, is a potential, insidious later cerebral side effect of radiotherapy. Most commonly it consists of damage in small arteries and capillaries, resulting in secondary processes of ischemia.

After radiation therapy, imaging techniques (CT, MRI, SPECT) can be used to assess treatment response and detect radiation-induced lesions and recurrent tumors. Optical spectroscopy has also been used for detecting radiation damage (Lin et al. 2005). The $F_{500}$ nm spectral peak allows accurate selection of tissues for biopsy in evaluating patients with new, contrast enhancing lesions in the setting of previous irradiation. This peak is highly correlated with a histological pattern of radiation injury. Deep lesions require a stereotactic biopsy to be conclusive. Also, much of the radiation effect is mediated by acute and chronic inflammatory cellular reactions. Biopsy samples supplement pathological differentiation of radiation effect from tumor progression. It should be noted that most of the biopsies show radionecrosis as well as scattered tumor cells.

Women treated with therapeutic chest radiation may develop cancer. This possibility becomes exceedingly serious considering that 50,000–55,000 women in the United States have been treated with moderate to high-dose chest radiation (~20 Gy). This possibility is much more serious for pediatric or young adult cancer patients, because these women are at a significantly increased risk of breast cancer and breast cancer mortality following cure of their primary malignancy (Mertens et al. 2008). A recent study also indicates that such young women develop breast cancer at a young age, which does not appear to plateau (Henderson et al. 2010). In this high risk population, ironically there is a benefit associated with early detection. In other words, young women with early stage breast cancer following chest radiation have a high likelihood for favorable outcome, although a life-long surveillance is needed.

Presently, although approximately 80% of the children with cancer are cured, the curative therapy could damage a child's developing organ system; for example, cognitive deficits following cranial radiotherapy are well known. Childhood survivors of malignant diseases are also at an increased risk of primary thyroid cancer (Sigurdson et al. 2005). The risk of this cancer increases with radiation doses up to 20–29 Gy. In fact, exposure to radiation therapy is the most important risk factor for the development of a new CNS tumor in survivors of childhood cancer, including leukemia and brain tumors. The higher risk of subsequent glioma in children subjected to medical radiation at a very young age reflects greater susceptibility of the developing brain to radiation. The details of the dose–response relationships, the expression of excess risk over time, and the modifying effects of other host and treatment factors have not been well defined (Neglia et al. 2006).

A recent study indicates that childhood brain tumor survivors are at an increased risk of late endocrine effects, particularly the patients treated with cranial radiation and diagnosed at a younger age (Shalitin et al. 2011). Among

children with cancer, the application of radiotherapy, therefore, should not be taken lightly, and it should be administered only when absolutely necessary to successfully treat the primary tumor. When radiotherapy is administered, use of the minimum effective dose tends to minimize the risk of second CNS neoplasms (late effect). Prolonged follow-up of childhood cancer survivors (particularly those treated with radiation) is necessary because of the long period between treatment and the development of malignancy. This practice should be a part of the effective therapy of the primary disease.

It is well established that radiation doses are related to risk for subsequent malignant neoplasms in children with Hodgkin's disease. It has been reported that increasing radiation dose was associated with increasing standardized incidence ratio ($p=0.0085$) in survivors of childhood Hodgkin's disease (Constine et al. 2008). Approximately, 75% of subsequent malignancies occurred within the radiation field. Although subsequent malignancies occur, for example, in breast cancer survivors in the absence of radiotherapy, the rise increases with radiation dose.

The pertinent question is: Is it always necessary to practice tumor surgery, radiotherapy, chemotherapy, or hormonal therapy, or a combination of these therapies? Although the conventional belief is that cancer represents an "arrow that advances unidirectionally," it is becoming clear that for cancer to progress, it requires cooperative microenvironment (niche), including immune system and hormone levels. However, it is emphasized that advanced (malignant) cancers do not show regression, and require therapy. In the light of the inadequacy of standard treatments of malignancy, clinical applications of the stem cell technology need to be expedited.

## Prostate Cancer

There were an estimated 217,730 new cases of prostate cancer in the United States in 2010 with 32,050 deaths, making it the second leading cause of cancer deaths in men. Currently, there are more than 2,000,000 men in the United States who have had radical or partial prostate surgery performed. Considering this huge number of prostate surgeries and the absence of a cumulative outcome data, it seems appropriate to carefully examine the benefits of radical surgery, especially in younger men.

Clinical prostate cancer is very rare in men of the ages younger than 40 years. In this age group the frequency of prostate malignancy is 1 in 10,000 individuals. Unfortunately, the incidence of malignancy increases over the ensuing decades, that is, the chance of prostate malignancy may reach to 1 in 7 in men between the ages of 60 and 79 years. Reactive or aging-related alterations in the tumor microenvironment provide sufficient influence, promoting tumor cell invasion and metastasis. It has been shown that nontumorigenic prostate epithelial cells can become tumorigenic when cocultured with fibroblasts obtained from regions near tumors (Olumi et al. 1999).

Prostate cancer treatment is one of the worst examples of overtreatment. Serum prostate specific antigen (PSA) testing for the early detection of prostate cancer is in wide use. However, the benefit of this testing has become

controversial. The normal cut-off for serum levels of PSA is 4 ng/ml, so any man presenting a PSA above this level is likely to require rectal biopsy, but only 25% of men with serum levels of PSA between 4 ng and 10 ng/ml have cancer (Masters 2007). The PSA threshold being used for biopsy ranges between 2.5 ng/ml and 3.4 ng/ml. Up to 50% of men presenting with prostate cancer have PSA levels within the normal range. It is apparent that screening of prostate cancer using PSA has a low specificity, resulting in many unnecessary biopsies, particularly for gray zone values (4 ng–10 ng/ml). According to one point of view, the risks of prostate cancer overdetection are substantial. In this context, overdetection means treating a cancer that otherwise would not progress to clinically significant disease during the lifetime of the individual. Overdetection results in overtreatment. The advantages and limitations of PSA test in diagnosing prostate cancer were reviewed by Hayat (2005, 2008).

Androgen deprivation therapy (ADT) is an important treatment for patients with advanced stage prostate cancer. This therapy is carried out by blocking androgen receptor or medical or surgical castration. Although ADT is initially very effective, treated tumors inevitably progress to androgen-independent prostate cancer (AIPC), which is incurable. One possible mechanism responsible for the development of AIPC is modulation of the tissue microenvironment by neuroendocrine-like cancer cells, which emerge after ADT (Nelson et al. 2007).

Recently, Pernicova et al. (2011) have further clarified the role of androgen deprivation in promoting the clonal expansion of androgen-independent prostate cancer. They reported a novel linkage between the inhibition of the androgen receptor activity, down-regulation of S-phase kinase-associated protein 2, and the formation of secretory, senescent cells in prostate tumor cells. It is known that several components of the SASP secretome, such as IL-6, IL-8, KGH, and epidermal growth factor, are capable of transactivating androgen receptor under androgen-depleted conditions (Seaton et al. 2008). It needs to be pointed out that androgen deprivation therapy, used in high-risk patients with prostate cancer, may cause reduced libido, erectile dysfunction, fatigue, and muscle loss; osteoporosis is also a late complication. Therefore, periodic bone density scanning needs to be considered.

Recently, the FDA cleared the use of NADiA (nucleic acid detection immunoassay) ProsVue prognostic cancer test. This proprietary nucleic acid detection immunoassay technology identifies extremely low concentrations of proteins that have not been routinely used as a diagnostic or prognostic aid. It is an in vitro diagnostic assay for determining the rate of change of serum total PSA over a period of time. The assay can quantitate PSA at levels <1 ng/ml. This technique can be used as a prognostic marker in conjunction with clinical evaluation as an aid in identifying the patients at reduced risk for recurrence of prostate cancer for years following prostatectomy. It targets the early detection of proteins associated with cancer and infectious diseases. This technique combines immunoassay and real-time PCR methodologies with the potential to detect proteins with femtogram/ml sensitivity (10–15 g/ml). Additional clinical information is needed regarding its usefulness in predicting the recurrence.

A significant decrease in the risk of prostate cancer-specific mortality is observed in men with few or no comorbidities. Indeed, active surveillance in lieu of immediate treatment (surgery or radiation, or both) is gaining acceptance. Most men with prostate cancer, even those with high-risk disease, ultimately die as a result of other causes (Lu-Yao et al. 2009). Debate on this controversy is welcome, but narrow opinions and facile guidelines will not lead to facts and new information; men worldwide deserve it (Carroll et al. 2011). Automatic linking positive diagnosis with treatment, unfortunately, is a common clinical practice. Unfortunately, even men who are excellent candidates for active surveillance in the United States often undergo some treatment. Deferment of treatment is advised in men with low-risk disease, especially of a younger age.

Active surveillance is proposed for patients with low-risk prostate cancer in order to reduce the undesirable effects of overdiagnosis. Prostate specific antigen serum level lower than 10 ng/L and Gleason score lower than 7 are the main criteria to select patients for active surveillance. The correct use of these two criteria is essential to differentiate between aggressive and nonaggressive prostate cancer. Autopsy studies indicate that approximately one out of three men older than 50 years show histological evidence of prostate cancer (Klotz 2008). Thus, a large proportion of prostate cancers are latent, never destined to progress, and affect the life of the patient. It is estimated that the percentage of low-risk prostate cancer is between 50% and 60% of newly diagnosed cases. A large number of patients die having prostate cancer, but not because of this cancer (Filella et al. 2011).

First whole genome sequences of prostate tumors were recently published online in *Nature* journal (vol. 470: 214–220, 2011). This study revealed that rather than single spelling errors, the tumor has long "paragraphs" of DNA that seem to have broken off and moved to another part of the genome (rearrangement of genes), where they are most active. These portions of DNA contain genes that help drive cancer progression. The mutated genes involved include *PTEN*, *CADM2*, *MAG12*, *SPOP*, and *SPTA1*. This information may lead to the development of more efficient, less invasive ways to diagnose and treat this cancer. Such information, in addition, should lead to personalized therapeutics according to sequencing results of different gene mutations or chromosomal rearrangements. The urgent need of such studies becomes apparent considering the huge number of new cases of prostate problem reported every year.

In contrast to prostate cancer, cardiovascular disorders take the heavier toll of life. In other words, the risk of death for men in the United States between the ages of 55 and 74 years due to cardiovascular disease surpasses that of prostate cancer. Cardiovascular disease is the most common of the chronic noncommunicable diseases that impact global mortality. Approximately, 30% of all deaths worldwide and 10% of all healthy life lost to disease are accounted for by cardiovascular disease alone.

In conclusion, initial treatment with standard surgery, irradiation, chemotherapy, or hormonal therapy, or combination of these protocols can result in both local and systemic sequalae. Therefore, surveillance for late recurrence

and secondary primary malignancies is recommended for most cancer patients. Patients with breast, lung, prostate, colorectal, and head and neck cancers constitute the largest groups requiring long-term monitoring and follow-up care.

Eric Hayat

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# Preface and Introduction

Stem Cells are nature's indispensable gift to multicellular organisms, including humans.

This is volume 8 of the 14-volume series, *Stem Cells and Cancer Stem Cells: Therapeutic Applications in Disease and Tissue Injury*. A stem cell is defined as a cell that can self-renew and differentiate into one or more specialized cell types. A stem cell may be pluripotent, which is able to give rise to the endodermal, ectodermal, and mesodermal lineages; an example is embryonic stem cells. A stem cell may be multipotent, which is able to give rise to all cells in a particular lineage; examples are hematopoietic stem cells and neural stem cells. A stem cell may be unipotent, which is able to give rise to only one cell type; an example is keratinocytes.

A cancer stem cell is a cell type within a tumor that possesses the capacity of self-renewal and can give rise to the heterogeneous lineages of cancer cells that comprise the tumor. In other words, a cancer stem cell is a tumor-initiating cell. A unique feature of cancer stem cell is that although conventional chemotherapy will kill most cells in a tumor, cancer stem cells remain intact, resulting in the development of resistance of therapy. All of these types of stem cells are discussed in this series.

## Culture Conditions

Mesenchymal stem cells play a part not only in the maintenance of tissue homeostasis and tissue repair but also in acute response to injury. Because stem cells always balance between self-renewal and differentiation, stem cell cultures are always a mixture of cells including stem cells, progenitors, and cell progeny. Hence, a stem cell culture requires continuous monitoring and regulation of the cell culture conditions. Potential strategies to produce therapeutically-relevant, large number of high quality mesenchymal stem cells for use in clinical applications are discussed. The relative merits of these cells derived from different tissue resources as well as the development of defined culture conditions, in terms of oxygen levels, soluble media, and culture substrates from which high quality mesenchymal stem cells can be derived for clinical applications, are pointed out.

Traditional skeletal muscle culture systems consist of monolayer cultures growing in a two-dimensional architecture. Although these cultures generally are sufficient for studying simple cellular functions, most functional tissues

are characterized by complex intracellular relationships that are best studied only in three-dimensional systems. Three-dimensional methods facilitate stronger cell-cell contacts and interaction of cells with the extracellular matrix, allowing cells to adapt their native morphology, which may influence signaling activity. Muscle stem cells can be cultured as a multicellular myosphere. The method for retaining muscle stem cell properties after expansion in myosphere culture is explained.

## Bone Marrow Stem Cells

Reactive oxygen and oxidative stress have a significant impact on the regulation of stem cells, including bone marrow stem cells and bone marrow-derived progenitor cells. The effects of these stresses on the expression of stem cell marker Oct-4, self-renewal, mobilization, and differentiation are discussed. The role of reactive oxygen species generated from low-density lipoprotein in the formation of atherosclerotic plaques is explained. How solid tumors utilize bone marrow-derived hematopoietic and mesenchymal progenitor cells during tumor growth and tumor neovascularization is discussed. The role of various mechanisms of tumor angiogenesis and key cellular players is explained. Also, some of the controversies regarding the identification of bone marrow-derived endothelial progenitor cells in tumors are described.

Myelodysplastic syndromes are a heterogeneous group of neoplastic disorders of the hematopoietic tissue mainly in older adults. This disorder arises because of bone marrow failure due to the interaction between a clonal and deranged hematopoietic stem cell and a deregulated bone marrow microenvironment. The syndrome is characterized by dysplasia (abnormal tissue development), ineffective hematopoieses, peripheral cytopenias, and increased risk of overt acute leukemia. Gene expression profiling facilitates a clear discrimination between normal and myelodyplastic hematopoietic stem cells, which is discussed here. This technique also allows the identification of crucial genes and biologic pathways deranged in this syndrome.

## Mesenchymal Stem Cells

Mesenchymal stem cells are a population of stem cells that have been isolated from virtually every adult tissue. The intrinsic tissue forming capacity of mesenchymal stem cells has elevated them to the forefront of regenerative medicine applications and tissue engineering strategies. The multipotent differentiation capacity, relative ease of isolation, and *ex vivo* amplification have made mesenchymal stem cells attractive candidates for a broad range of cell-based therapies; however, challenges remain. One issue relates to the in vivo location of mesenchymal stem cells and how coresident tissue cells may influence their behavior. Mesenchymal stem cells have been described as being positive or negative for biomarkers: Positive biomarkers: CD166, CD105, CD90, CD73, CD44, CD146, CD271, and stro-1. Negative biomarkers: CD235a, CD144, CD79a, CD45, CD34, CD19, CD14, CD11a, and HLA-DR.

Other biomarkers have also been reported. Mesenchymal stem cells are associated with cells of the vascular system, which would permit a broad tissue distribution.

Mesenchymal stem cell-based therapy is a promising approach in regenerative medicine and antitumor strategies. After in vivo administration, these cells induce peripheral tolerance and migrate to sites of injury where they promote tissue repair and regeneration. The potential of mesenchymal stem cell-based strategies in the therapy of neurodegenerative diseases of the central nervous system is explained. Mesenchymal stem cells have therapeutic potential in graft-versus host disease, Crohn's disease, chronic obstructive pulmonary disease, and type-1 diabetes. Mechanisms of mesenchymal stem cell-induced immunomodulation as it relates to inflammation and tumors are described. Recruitment of these cells to sites of inflammation and their potential role in malignant tissue progression are discussed.

The HIV-1-infected population is prone to disorders not only of bone and fat but also to regenerative and ameliorative functions of mesenchymal stem cells, which contribute to a wide variety of HIV-1/HAART (highly active antiretroviral therapy)-associated toxicities. Administration of HAART significantly decreases the mortality of HIV-1 patients. This therapy consists of at least two-drug antiretroviral regimen: (1) HIV-1 protease inhibitor that prevents the production of the viral capsid, (2) HIV-1 reverse transcriptase inhibitor that prevents the production of viral DNA. Despite the success of this therapy, several clinical problems have emerged in the HIV-1-infected patients. These problems include lipoathrophy, dyslipidemia, increased cardiovascular risk, and reduced bone mineral density. In addition to these toxicities is the persistence of populations of latently-infected cells that allow the HIV-1 virus to survive the onslaught of HAART. In other words, HIV-1 therapy only succeeds in controlling rather than eradicating the virus. The putative role of mesenchymal stem cells in the bone and fat disorders associated with HIV-1 infection and treatment has begun to be deciphered. The assumption that HIV-1 and its treatment may induce a generalized impairment of mesenchymal stem cell function is discussed.

## Reprogramming and Differentiation of Stem Cells

A number of human embryonic stem cell lines have been developed and characterized and are discussed in this volume. The strategies used to direct tissue-specific differentiation of embryonic stem cells are explained. Embryonic stem cells have two defining properties: self-renewal and pluripotency, making them ideal for tissue engineering, regenerative therapies, and cell transplantation therapies. Oct 4, Nanog, and Sox2 are the main transcription factors in regulating the pluripotency of these cells. Information on the molecular and cellular mechanisms of stem cell self-renewal and pluripotency provides the necessary tools to harness the regenerative potential of embryonic stem cells for therapeutic purposes. It is explained that natriuretic peptide receptor A, a specific receptor for atrial and brain natriuretic peptide, is expressed in preimplantation embryos and in embryonic stem cells, and is functional in these cells.

Pluripotent stem cells established in vitro from mammalian embryos are essential tools to understand pluripotency. In the embryo, pluripotent cells are present only transiently, from the preimplantation to pregastrulation stage. Two types of stem cells can be derived from the mouse embryo, both being pluripotent: embryonic stem cells (ESCs) are derived from the pluripotent cells in the inner mass of the blastocyst, while epiblast stem cells (EpiSCs) are derived from the post-implantation late epiblast at the time of gastrulation. These two types of stem cells share some common properties, but each of them also display specific features that closely define two states of pluripotency. How these cells are obtained is described in this volume, and their molecular and functional characteristics are also explained. For example, reprogramming of somatic cells into EpiSCs through nuclear transfer and induction with transcription factors are included.

During stem cell differentiation, genes related to their unique characters are silenced and genes related to the target lineage are activated. The role of epigenetic modifiers is known to control gene expression, and changes of the epigenetic status during differentiation are also known. These modifiers have no differentiation ability by themselves but they enhance the differentiation upon culturing the stem cells in the relevant conditions. The role of such modifiers (e.g., DNA methylation modifiers) in gene expression is discussed here.

Steroid hormones are produced from cholesterol mainly in adrenal glands and gonads. These hormones play a crucial role in normal physiology including glucose metabolism, stress response, sex differentiation, and reproduction. Therefore, steroidogenic abnormality could be life threatening. Congenital adrenal hyperplasia is one of the most common disorders caused by deficiency of an enzyme involved in the adrenal gland. Replacement of deficient steroid hormones by hormone replacement therapy is necessary for patients with adrenal and gonadal failure. However, such therapies are associated with serious risks. Therefore, an alternative treatment involves the use of mesenchymal stem cells that can be differentiated into steroidogenic cells by stable expression of steroidogenic factor-1 and cAMP treatment, which is discussed here.

Stage-specific embryonic antigens (SSEA-3 and SSEA-4) are markers of human embryonic stem cells, while A2B5 and DG3 antigens are markers of neural lineage cells. Glycosphinolipids (GSLs) are components of cell membranes, which can act as mediators of cell adhesion and signal transduction, and possibly be used as cell type-specific markers. A systematic survey of expression profiles of GSLs in human embryonic stem cell lines has been carried out. In addition to SSEA-3 and SSEA-4 markers, several other markers are found in the undifferentiated human embryonic stem cells. When these cells differentiate into neural progenitors, changes occur in the core structure of the GSL. New insights into the unique stage-specific transition and mechanisms for alterations of GSL core structures during human embryonic stem cell differentiation are pointed out in this volume.

Diabetes is a chronic and debilitating disease that results from insufficient production of insulin from pancreatic β-cells. Islet cell translation can effectively treat this disease, but is severely limited by the reliance upon cadaveric donor tissue. Human embryonic stem cells are a promising alternative source of transplantable insulin-producing β-cells, as they are renewable in vitro and

have the inherent capacity to differentiate into pancreatic endocrine cells. Several strategies have been explored for differentiating human embryonic stem cells into pancreatic β-cells in vitro, with the most effective being mimicking the key signaling pathways required for pancreas development using small molecules. Although there are still numerous challenges to overcome, this technology demonstrates the feasibility of human embryonic stem cells as a potential alternative to cadaveric islets for treating patients with diabetes. These possible treatments are discussed here.

Vascular endothelial cells demonstrate remarkable plasticity and differentiate into other cell types during embryonic development and disease progression. This occurs through a process known as endothelial-mesenchymal transition (EMT) in which endothelial cells acquire properties of mesenchymal stem cells, including multipotency. The current evidence of EMT and its contribution to the generation of stem cell phenotype is explained here. Also, the role of EMT in generating fibroblasts or myofibroblasts in cardiac development, cancer, and fibrosis is discussed.

## Treatment

The development of strategies for cancer therapy has been one of the major challenges of the last decades. The identification of appropriate target antigens is the most critical step in the development of antigen-specific cancer immunotherapy. Conventional treatments for cancer such as chemotherapy and radiotherapy are able to shrink tumors, but fail to destroy cancer stem cells that maintain the tumoral growth and metastasis. The discovery of cancer testis antigen expression in cancer stem cells and in immunologically privileged tissues, such as germ cells of the testis, lead to the concept that these antigens can be used as potential targets for cancer treatment.

Colorectal cancer is a cancer of the colon, rectum, or vermiform appendix. It is the third most common cancer in women and the fourth common in men, taking 610,000 lives per year worldwide. Approximately, 72% of the disease arises in the colon and the remaining in the rectum. The metastasis of this cancer into the liver is the primary cause of death. Approximately, 101,340 new cases of colon cancer and 39,870 cases of rectal cancer and 49,380 deaths from this disease were reported in 2011 in the United States. Approximately, 50% of patients with this cancer show tumor recurrence. One of the main reasons for tumor recurrence is the presence of chemotherapy-resistant cancer stem cells that retain limitless potential to regenerate. Markers of these cells have been identified and they include CD133, CD44, CD166, ALDH1, Lgr5, and several other proteins. Some of these markers are discussed here. Signaling pathways regulating cancer stem cell functions are explained. Therapeutic strategies to target colorectal cancer stem cells are presented.

Stroke is the second leading cause of death in the world, including Japan, China, and Korea, and the third leading cause of death in the western world, and results in a drastic reduction in the quality of life. It is the common disease among older people, and is the leading cause of chronic disability. In the infracted brain lesion, multiple neuronal cell death occurs and results in

the distribution of the neuronal network, leading to neurological dysfunction. The number of individuals over the age of 65 is expected to double between 2005 and 2030. Furthermore, the risk of stroke is more than doubled for each decade of life over age 55, resulting in a huge burden for family and society. After acute stroke, the survivors still suffer from disability in most cases. Thus, substantial advances in the prevention and treatment of stroke are of paramount importance.

Therapeutic strategies for stroke have been focusing on two main aspects: restoration of cerebral flow and minimization of the deleterious effects of ischemia on neurons. Four potential therapeutic strategies are discussed in this volume, and they consist of enhancing neuronal plasticity, promoting endogenous neurogenesis, stem cell transplanting, and mobilizing endogenous endothelial progenitors to angiogenesis. However, current therapeutic protocols remain suboptimal and the development of more effective neuroprotectants is imperative. Four potential therapeutic strategies are discussed in this volume, and they consist of enhancing neuronal plasticity, promoting endogenous neurogenesis, stem cell transplanting, and mobilizing endogenous endothelial progenitors to angiogenesis. Grafting neural stem cells as cell replacement for neuronal loss due to ischemia has been demonstrated to improve neurological deficits after stroke. The treatment by using neural stem cells with overexpressed glial cell-derived neurotrophic factors *ex vivo* has been demonstrated to significantly improve therapeutic efficacy, suggesting that this approach may be a promising approach for neurodegenerative diseases including stroke. Therapeutic efficacy after transplantation of neural stem cells is discussed in this volume. The niche influencing the fate of grafted neural stem cells is also explained. It should be noted that even the adult brain has the capacity to regenerate the vascular components to the same extent.

Germ cells are unique in that they feel responsible for passing genetic information to the next generation. Pluripotent stem cells can differentiate into germ cells and even gametes. Method to produce germ cells is detailed, and differentiation of these pluripotent stem cells into germ cells is also included in this volume.

In addition to the role of purinergic receptors in the neurotransmission, they are involved in proliferation, differentiation, cell death, and successful engraftment of stem cells originated from diverse origins. Besides providing promising targets for cell regeneration therapy, purinergic receptors have implications in cancer stem cell growth and differentiation, providing novel mechanisms for therapeutic intervention. Regulation of proliferation and differentiation by these receptors are explained in this volume, and the involvement of purinergic signaling in cancer stem cells is also discussed.

Parthenogenesis is a type of asexual reproduction in which the offspring develops without genomic contribution from sperm. It occurs naturally in some invertebrate animal species but not in mammals. Parthenogenesis can be induced by artificial activation of an egg in mammals, but such embryos fail to develop past mid-gestation. Parthenogenetic embryonic stem cells (PESCs) can be generated from parthenogenetic embryos. The limited availability of human embryonic stem cells and the ethical issues raised by their derivation from potentially viable human embryos constrain their utilization.

Parthenogenetic embryonic stem cells, on the other hand, are derived from artificially activated eggs, so they generate less controversy, yet they exhibit pluripotency similar to embryonic stem cells. PESCs, therefore, provide a promising option for stem cell therapeutics. Methods for artificial activation of oocytes and generation of pluripotent stem cells from such oocytes are presented here.

Head and neck cancer is the fifth most common cancer with 49,260 estimated new cases in 2010 in the United States. Radiotherapy is the most common form of treatment for this cancer, but nondiseased salivary glands are often exposed to radiation. Due to intense radiosensitivity of salivary glands, irreversible hyposalivation is common in the head and neck cancer survivors. Current treatments such as artificial saliva and saliva secretion stimulatory can only temporarily relieve the symptoms. Transient activation of Wnt and Hedgehog pathways prevent or rescue radiation-induced hyposalivation, respectively, through inhibition of apoptosis or maintenance of salivary stem/progenitor cell populations and their preservation of functional differentiation. The association of Wnt/β- catenin pathway and Hedgehog pathway with regeneration of salivary glands is explained here.

Neural stem cell migration is a key component of their developmental function and therapeutic potential. These cells are among the potential tools for cell-based therapies directed at repairing the central nervous system. Neural stem cell migration in vitro can be precisely controlled by the application of an external electric field. Electric fields have been widely studied as directional cues in vitro, and their application to control cell migration in vivo as well as their use in clinical settings are beginning to be developed. Controlling neural stem cell migration by using diverse directional cues, among them external electric fields, will contribute to their use as therapeutic tools. Use of directional cues for neural stem cell migration in brain repair is discussed in this volume.

Chronic myeloid leukemia (CML), characterized by a clonal proliferation of myeloid progenitors originating from a single malignant stem cell, represents the model of myeloproliferative neoplasms. During the last three decades, significant improvements have been achieved in the therapy of CML. Allogeneic stem cell transplantation was the first potentially curative treatment for patients in chronic phase. Interferon-alpha alone or in combination with cytarabine displayed real efficiency in a small number of patients, allowing durable complete cytogenetic remissions in patients responding to the treatment. More recently, tyrosine-kinase inhibitors (TKIs) have been developed to target the BCR-ABL oncoprotein through its enzymatic activity. The introduction of imatinib mesylate has dramatically improved patient survival. Nevertheless, a small proportion of patients develop resistance toward target therapies. It is explained in this volume that missense mutations located within the kinase domain of the BCR-ABL oncogene are the most common mechanisms of resistance. ABL-kinase mutations occur in hematopoietic progenitors and stem cells expressing the BCR-ABL oncogene.

## Transplantation

Prostate cancer is the most commonly diagnosed and treated solid malignancy among males. It has a significant impact on men's health, with 217,730 new cases diagnosed each year, and 32,050 annual deaths attributed to this disease. The lifetime probability of developing prostate cancer is estimated to be 20%. Although several treatment options are available for prostate cancer, radical prostatectomy is the common therapy for most cases of organ-confined disease. The incidence of erectile dysfunction in men following radical prostatectomy ranges from 16% to 82% depending on a number of factors including cancer stage and grade of prostate cancer, patient age, comorbidities, preoperative erectile function, and degree of cavernous nerve preservation during radical prostatectomy. In spite of the fact that mesenchymal stem cell therapy has shown efficacy in animal models of cavernous nerve injury that reflects the nerve injury following radical prostatectomy and other pelvic surgeries, further research is warranted to overcome a number of translational issues on the path toward clinical application of mesenchymal stem cells for the treatment of post-prostatectomy erectile dysfunction.

Mobilized peripheral blood stem cells are being increasingly used in allogeneic stem cell transplantation due to their relative easiness to be collected compared with those from bone marrow. These cells are mobilized from bone marrow to the peripheral blood with growth factors, usually granulocyte colony-stimulating factor (GCSF). By using this method, higher amounts of CD34 cells are collected, resulting in faster engraftment. Another advantage of this method is that it is less invasive in comparison with bone marrow collections. In some cases it is not possible to find a matching sibling/related donor. To circumvent this problem, International registries play an essential role in allogeneic transplantation by recruiting donors from all over the world and by keeping records of HLA typing for all of them. The safety and efficiency of this collection procedure are explained here.

Tobacco use is the leading preventable cause of death in the United States. Smoking is associated with multiple adverse hematopoietic stem cell transplantation outcomes, including shorter duration of disease-free and overall survival, higher treatment related mortality, higher pulmonary infection and respiratory failure rates, higher rates of disease recurrence, increased risk for cardiovascular events, and longer duration of hospitalization. It is pointed out that head and neck cancer patients are highly correlated with tobacco use.

Thrombopoietin (TPO) is much more than a lineage-specific growth factor; it is also an important growth factor of hematopoietic stem cells. This property of TPO qualified it for *ex vivo* expansion procedures aimed to amplify committed progenitors and stem cells or at least to maintain stem cells. The expansion procedures facilitated the acceleration of hematopoietic reconstitution after transplantation. All successful procedures are based on a cytokine cocktail containing TPO among other cytokines. It is also pointed out that the action of TPO on stem cells is related to its hypoxia-mimicking properties.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against

terrible human disease and injury. It is difficult for a single author to discuss effectively the complexity of diagnosis, therapy, including tissue regeneration. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer cure and tissue regeneration. I hope these goals will be fulfilled in this and other volumes of the series. This volume was written by 85 contributors representing 14 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in these important areas of disease and injury. I respect and appreciate the hard work and exceptional insight into the nature of cancer and other diseases provided by these contributors. The contents of the volume are divided into six parts: Stem Cell Culture, Bone Marrow Stem Cells, Mesenchymal Stem Cells, Reprogramming and Differentiation of Stem Cells, Treatment, and Transplantation for the convenience of the readers.

It is my hope that subsequent volumes of the series will join this volume in assisting in the more complete understanding of the causes, diagnosis, and cell-based treatment of major human diseases and debilitating tissue/organ injuries. There exists a tremendous, urgent demand by the public and the scientific community to address to cancer diagnosis, treatment, cure, and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating deadly malignancies over military superiority.

I am thankful to Jennifer Russo for her help in many ways in completing this project.

M.A. Hayat

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# Part I

# Stem Cell Culture

# 1 Stem Cell Culture: Optimizing Amidst the Complexity

Blake W. Axelrod and Didier Wion

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**Abstract**

Stem cell cultures are presently necessary to investigate the cellular and molecular mechanisms of stem cell biology, to perform pharmacology and toxicology screenings and to provide the material required for regenerative therapies. Hence, optimizing stem cell culture conditions is currently a major challenge in stem cell research. Stem cell culture conditions will never capture the extraordinary complexity of the stem cell niche. Stem cell culture must be viewed as a tool for which the living cell is the material. However, we must also keep in mind that cell culture converts stem cells themselves into tools for basic research or regenerative therapies. Therefore, optimal stem cell culture conditions must be defined according to the endpoint of the culture. Instructing stem cells not necessarily by mimicking the stem cell niche biology but by using artificial, well-controlled and reproducible devices is a realistic aim for the cell culturist. This in turn requires well-defined experimental conditions and real-time probing of the cultured cell environment. Suspension culture in controllable bioreactors is the method of choice for stem cell cultures intended for a final clinical or industrial use whereas microfluidic systems are better designed for dissecting the molecular mechanisms founding stemness. Not only the cell culture medium but also the chemistry, the physical properties and the topography of cell culture substrates are able to modulate stem cell self-renewal and to

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control stem cell fate. Hence, the design of surface-engineered substrates is the subject of intense and fruitful research. Finally, not only preserving the self-renewal and differentiation potential of stem cells in culture but also preserving their genetic integrity is mandatory.

## Introduction

Most of the concerns regarding the optimization of stem cell culture conditions find their origin in the unique properties of these cells and in their applications ranging from basic research to regenerative therapies. Stem cells are defined by their self-renewal and differentiation potentials. It is notable that self-renewal and differentiation can be viewed as two antagonistic forces. A challenge for stem cell culturists is to control these two opposed potentials. Because stem cells always balance between self renewal and differentiation, stem cell cultures are always a mix of cells including stem cells, progenitors and cell progeny. Hence, a stem cell culture must be viewed as a dynamic process that requires continuous monitoring and regulation of the cell culture conditions.

In vivo the balance between adult stem cell quiescence, self-renewal and differentiation is regulated by dynamic interactions between stem cells and a specialized microenvironment referred as the stem cell niche. Although several signaling pathways such as Wnt, BMP and Hedgehog pathways have already been shown to regulate stemness, we are far from a complete knowledge of the stem cell niche's characteristics. In addition, stem cell niches vary widely between species and in the same specie between different organs or tissues. This complexity is further increased by the fact that the stem cell niche microenvironment is dynamic. It instructs stem cells for quiescence, self-renewal and migration depending on the tissue repair or regeneration requirement. This is to say that reproducing the stem cell niche microenvironment is not presently achievable. Instructing stem cells without necessarily trying to mimic the stem cell niche is likely a fruitful philosophy for the stem cell culturist.

Another confounding point for the stem cell culturist is how to control the purity of the stem cell culture. Stem cells can be defined either as cell entities or on the basis of their function. Testing stem cells on the basis of their functional properties means testing their potency, which is the ability to generate different cell types. This leads to the loss of the stem cell phenotype. Alternatively a stem cell can be considered as an entity defined by specific markers. Cell sorting on the basis of panels of stem cell markers by flow cytometry is a way to obtain stem cells or to control the purity of the culture. However this process can be stressful for cells and might alter their properties or phenotype. Moreover, cell profiling does not always correspond to an obvious discrete population but may exist as a continuum of cells labeled with low to high intensities. Thus, the stem cell sorting process by flow cytometry should be better viewed as an enriching rather than a purifying step.

Optimizing cell culture conditions also depends on the ultimate goal of the culture. Mass production of specific stem cells for regenerative therapies does not necessarily require the optimization of the same parameters as stem cell cultures for basic stem cell research. "Optimal" stem cell culture conditions for lab-on-a-chip miniaturized platforms designed for single-cell experimentation may differ from large scale stem cell cultures in a bioreactor. Likewise cell culture conditions may differ when stem cells need to undergo directed differentiation in culture before their use in regenerative medicine. Because the endpoints of stem cell cultures may vary and because different types of stem cell exist (embryonic, adult, induced stem cells), determining what the universal stem cell culture conditions must be seems to be a never ending quest. A way to grasp this problem is to consider that stem cell culture optimization requires a similar maturation process as any technology. This includes for instance, efficiency, reproducibility and safety. With these considerations in mind several guidelines and prospects can be drawn for optimizing stem cell cultures. Basically, cell culture parameters amenable to optimization are the medium components, the atmosphere, the temperature, the extracellular

matrix and cell-cell interactions. A notable point is that, as a consequence of cell metabolism and cell growth, most of these different cell culture parameters are subject to variations during the course of the culture. A dynamic reciprocity occurs during the course of cell culture between the components of the culture environment artificially provided and those produced by cells in culture such as secreted growth factors. Hence, what the optimal stem cell culture conditions should be not only includes the determination of the initial cell culture conditions (composition of culture medium, of the cell substrate…), but also the on-line monitoring and control of cell culture parameters. With this in mind several points have proved critical for optimizing stem cell culture and are an actively growing field of investigation.

## The Medium

We are far from having developed a fully defined generic cell culture medium for stem cell culture. This comes from the fact that stem cell requirements not only depend on the type of stem cell cultured (embryonic stem cell, adult stem cell, induced pluripotent stem cell) but also on the species they originate from. For example mESC and hESC do not have the same dependency on LIF, bFGF and PDGF. Significant effort has been made to formulate chemically defined, serum-free media. As a consequence culturing cells on irradiated or mitomycin-treated feeder cells such as fibroblasts is progressively phasing out. Currently, the base medium used is usually DMEM or a DMEM/F12 mix to which different components are added. The optimal stem cell culture medium not only needs to nurture stem cells but also must be formulated to either prevent or direct the differentiation of stem cells. Hence, depending on the type of the stem cell cultured and on the goal of the culture these supplements vary. bFGF is probably the most common growth factor added to stem cell cultures. Other supplements used are commercially defined serum-free formulation such as N2, B27 or the KnockOut™ serum replacement, individually added proteins, hormones or drugs. For example, cultured conditions for hematopoietic stem cell (HSC) expansion require supplements such as thrombopoietin, stem cell factor, flt3 ligand, interleukin 6 and the aryl hydrocarbon receptor antagonist SR1 (Boitano et al. 2010). On the other hand, human pluripotent stem cells (hPSCs) medium supplementation with the Rho kinase inhibitor Y-27632 (Watanabe et al. 2007), or the full length IL6RIL6 chimera (Amit et al. 2011) is effective for maintaining proliferation in an undifferentiated state. Indeed it is anticipated that the list of drugs able to control stem cell fate by activating or inhibiting relevant signaling pathways will exponentially increase in the next few years (Xu et al. 2008; Ding et al. 2003; Yao et al. 2006). Wnt signalling is also essential for maintaining embryonic stem cell (ESC) self-renewal and although Wnts are produced by ESCs themselves, their concentration in the medium is generally insufficient. Thus, adding Wnt3a to LIF is sufficient to prevent mouse embryonic stem cell (mESC) differentiation in the absence of feeder cells (Ten Berge et al. 2011). Interestingly, $O_2$ modulates Wnt/β-catenin signaling in undifferentiated stem cells (Mazumdar et al. 2010). This recent finding is of paramount importance. It establishes a relationship between $O_2$, Wnt/β-catenin and the stem cell undifferentiated state that could explain why controlling oxygen tension in stem cell culture is so critical.

## Oxygen, the Other Cell Culture Medium Soluble Factor

Cell cultures have for many years been performed under atmospheric oxygen which is around 20% $O_2$, however, it has long been known that the physiological oxygen levels experienced by most cells in the organism are lower, ranging from 10% $O_2$ to 3% $O_2$. This point is critical. Oxygen gradients that occur in the organism are now considered signaling pathways which control gene expression through, for example, the HIF family of transcription factors. Indeed, the role of these oxygen gradients during embryogenesis has led to the categorization of oxygen as a morphogen (Simon and Keith 2008). It is therefore not

surprising that accumulated data demonstrates the critical role played by oxygen in stem cell proliferation and differentiation. Indeed, stem cells in vivo are often located in niches where they experience oxygen tension lower than 3% $O_2$. Therefore, oxygen is now considered as a critical soluble factor just as cytokines or growth factors are. Hence, culturing stem cells under low oxygen tension around 3–5% $O_2$ is becoming the rule (Csete 2005; Wion et al. 2009). However, controlling $pO_2$ in the gas phase of the incubator does not sufficiently regulate $pO_2$ at the pericellular level. From a biological stand point only the pericellular $pO_2$ experienced by cells is relevant. Unfortunately, controlling the $pO_2$ in the gas phase of the incubator does not achieve this goal. Oxygen in the gas phase needs to diffuse into the medium. This can take, in the absence of stirring and depending of the depth of the medium, several hours. Moreover, measuring $pO_2$ in the bulk of a culture medium only approximates pericellular $pO_2$. This point is highly relevant when cells are grown as spheroids or on a feeder layer of confluent cells since cell oxygen consumption and limited oxygen diffusion generate oxygen gradients. Indeed, the pericellular $pO_2$ of a confluent layer of cells cultured under a gas phase at 20% $O_2$ can be as low as 1% $O_2$ due to cell oxygen consumption. Interestingly, this suggests that one of the effects of feeder cell layers could be to locally decrease the pericellular $pO_2$ experienced by stem cells.

## Temperature, the Forgotten Parameter

Stem cells are commonly cultured at 37°C and the effect of temperature on stem cell cultures does not seem to have been thoroughly investigated. However, keeping in mind the widespread but mistaken use of atmospheric oxygen as a cell culture standard instead of low oxygen conditions, it may be worth considering what would be the effects of changing the 37°C standard cell culture temperature. Even if the fact that lowering temperature of human spermatogonia from 37 to 31°C increases DNA synthesis (Nakamura et al. 1987) is probably an exception in stem cell physiology due to the external location of testis in some species, it should be noted that bone marrow, another location where stem cells reside, has a lower temperature (1.6–4.8°C below) than normal body temperature (Petrakis 1952). Determining the effects of temperature variation on stem cell behavior or on stem cell chromosomal abnormality frequency is certainly a point that warrants further studies.

## Cell Interactions

In vivo, stem cells are not usually floating cells. They interact with a three dimensional dynamic environment made up of other cells and of an extracellular matrix. These interactions provide critical cues for stem cell fate in vivo and can be reproduced and engineered in vitro with synthetic materials for controlling stem cell fate. In addition physical homotypic and heterotypic cell-cell interactions are also likely to play a critical role in stem cell renewal and differentiation. This complex interacting network of connections also occurs in cell culture. Again, the challenge for stem cell culture is not necessarily to reproduce the in vivo conditions but rather to use some of the features of this network of interactions to control the stem cell culture fate.

Just as a plant efficiently exploits the soil for nutrients and anchorage, cells sense the substrate on which they are growing. In vivo, proteoglycans, hyaluronic acid, and proteins such as collagens, elastin and fibronectin, are the basic components of ECM. Cell surface integrins are the best characterized family of receptors interacting with ECM components such as laminins, collagens, fibronectins, vitronectin and tenascin to transduce biological signals (Prowse et al. 2011). Cell-ECM interactions through integrin signaling control many cellular functions such as cell motility, survival, differentiation and proliferation. The ECM also sequesters and releases growth factors and cytokines. Thus the cues provided by the ECM to cells include both biophysical and biochemical signals which are then translated into signaling cascades by the cell. Cells integrate

physical signals, such as stiffness, roughness, topography of ECM, by translating mechanic forces and deformation into biochemical signals.

Cellular mechanotransduction is the process by which cells transduce physical signals from their microenvironment into biochemical responses. Mechanotransduction recruits specific cell surface adhesion proteins such as the members of the integrin family which interact with different components of the ECM such as vitronectin, fibronectin, laminin and collagen. These interactions in turn activate mechanoresponsive pathways triggering cell biological response and determining cell fate (Discher et al. 2009). For example, using human recombinant laminin-511 as a substrate coating enabled the long-term self-renewal of hES and iPS (Rodin et al. 2010). Matrices have also been engineered to present receptor ligands to cells either as linked to the solid phase or as locally released soluble factors (Pompe et al. 2010).

Matrix elasticity is also a critical factor. It has been shown that ECM elasticity drives human mesenchymal stem cells (hMSCs) to phenotypes that are compliance matched to the ECM. Thus, soft brain-like matrices yield neurogenic cells, stiffer muscle-like matrices yield myogenic cells and rigid bone-like matrices yield osteogenic cells (Engler et al. 2006). Components of the mechanotransduction process are being discovered. They include the integrin-based focal adhesion stress signaling and the transcriptional regulators YAP and TAZ (Dupont et al. 2011).

Currently, a major challenge is to control cell mechanoresponsiveness to optimize stem cell culture. Hence, engineering cellular matrices for producing specific stem cell response is an active field of investigation. Modulating the rigidity and topography of synthetic matrices can promote stem cell renewal or induce differentiation. For example the geometric cues generated by 100 nm diameter titanium oxide nanotubes induce the differentiation of hMSC into osteoblast-like cells (Oh et al. 2009). Other studies show that hMSCs cultured on nanogratings with 350 nm linewidth up-regulate markers of neuronal lineage (Yim et al. 2007). Likewise, a matrix with disordered square array of dots displaced randomly by up to 50 nm on both axes from their position in a true square induces the expression of osteogenic markers in cultured hMSCs (Dalby et al. 2007). These results illustrate that stem cell culture conditions are at the dawn of a new era. After much effort has gone into formulating synthetic chemically well-defined growth medium, we are doing the same thing for the cell culture substrate. The combinations of substrate elasticity control, micropatterning, nanotopography and tethering of proteins or bioactive peptides will soon generate standardized bioinformative matrices with special specificities depending on the type of stem cell cultured and on the purpose of the culture. However we must keep in mind that stem cells in culture do not only interact with the medium and the underlying substrate. Cell-cell interactions are also critical. The importance of heterotypic cell interactions is illustrated by the use of feeder cell layers in the first stem cell cultures. However, interactions between stem cells are also critical. Our knowledge of how these homotypic interactions modulate stem cell behavior in culture is still in its infancy. This field of investigation is very promising as cell-cell interactions are not limited to cell-cell contact but also include the release by cultured stem cells of soluble factors as well as components of the ECM. This illustrates the extraordinary complexity of the stem cell culture system in which the stem cell is not only the product of the system but one of its dynamic components. Therefore, the on line monitoring and control of stem cell cultures parameters is critical.

## The Need to Monitor and Control Stem Cell Culture

As for any manufacturing process, the on-line monitoring and control of stem cell cultures is critical. It is clear that stem cell culture conditions must be monitored and controlled at the pericellular level. For many parameters this means sensing/measuring/assaying the conditions at the pericellular level and then using feedback to maintain those conditions within a proper range. For temperature, oxygen and certain critical components of media technologies currently

exist that can achieve these goals. Most of these technologies have been developed for in vivo measurement and imaging, thus effort is needed to adapt the technologies to the specific task of monitoring and maintaining stem cell culture and to standardize the techniques across labs.

## Sensors for Pericellular Monitoring

In cell culture, three obvious parameters to measure and control at the pericellular level are $pO_2$, pH and glucose. Both optical and electrical probes have been developed that enable local sensing of each. Optical probes measure fluorescence characteristics of an excited dye. Either fluorescence intensity or lifetime are measured; lifetime measurements are more stable and have lower background but require modulating the light source, measuring the time evolution of the output and fitting the data. Intensity measurements require simpler optics and analysis but are best done utilizing dual-excitation or dual-emission ratiometric techniques that reduce errors caused by variations in concentration, optical path length, and photobleaching. In general, the use of fluorescence probes requires incubation on a microscope system with a fluorescent light source, filters and quantitative camera. Such systems, while expensive, are available commercially and are in use in many labs, though not generally for tissue culture. Low cost LED and RGB CMOS based systems have been demonstrated and indicate a low cost path for integrating optical monitoring of media components. The principle downside to fluorescence sensors is the damage or poisoning of cells by intense UV light. This can be mitigated by long wavelength fluorophores or two-photo excitation, however two photon probes are in earlier phases of development and the TiSaphire lasers used in two-photon systems are quite expensive. The use of multiple fluorescent probes is also limited by the available bandwidth to a maximum of four or five separate fluorophores in a single system.

Fluorescent pH indicators are commercially available in both cell permeable and cell impermeable variants. Fluorescein and related pH sensitive dyes exhibit multiple, pH dependent ionic equilibria. At pH 9 Fluorescein is almost entirely a fluorescent dianon, as the pH decreases fluorescein protonates to form an anion (pKa ~6.4) with similar emission spectra but lower quantum yield and ultimately a nonfluorescent neutral species (pKa <5). Thus the fluorescent intensity is dramatically reduced at acidic pH with a nearly linear decrease from pH 7.5 to 6.5. Fluorescein based BCECF is the most widespread pH probe, a dual excitation ratiometric measurement is used, excited at 440 nm (CFP excitation) and 490 nm (YFP excitation) and detected at 535 nm (YFP emission). Two-photon excitation has also been used with BCECF which is advantageous in that it is far less damaging to cells than UV and visible excitation. SNARF (Seminaphthorhodafluors) pH indicators are dual excitation dyes that offer similarly good optical excitation and are available in cell permeable and impermeable variants. Normal cell metabolism will shift pH down, which is a sign that media exchange or increased perfusion is necessary to correct the pH drift.

Oxygen is an efficient quencher of excited dye molecules. Most optical oxygen sensors operate on the principle of reversible luminescence quenching of the intensity or excited-state lifetime of a luminescent indicator dye. This process occurs when the excited state energy of a fluorescent or phosphorescent indicator molecule is transferred to an oxygen molecule rather than being emitted in the form of a luminescence photon. The dyes can operate in solution or immobilized in particles or membranes. The development of fluorescent oxygen sensors is an active and productive area of research that is presently focused on refining dual emission sensors for incorporation into polystyrene and other biocompatible polymer materials. Examples include fluorescine based FITC, metalo-porphyrin compounds and boron based nanoparticles (difluoroboron β-diketonate photoluminescent dye immobilized in poly (lactic acid)).

The Wolfbeis lab has demonstrated a noteworthy dual immobilized sensor system for simultaneously monitoring pH and $pO_2$ (Meier et al. 2011). They utilize three fluorescent reporters – FITC for pH, Pt-porphyrin for $pO_2$ and DPA

as a constant reference – that are immobilized first in microbreads and then in a membrane to increase specificity, avoid uneven distribution and toxicity/uptake of soluble reporters. The response time is 9 s for pH and 25 s for $pO_2$. The choice of fluorophores allows the use a low cost LED excitation source and of the red, green and blue filters built into conventional digital camera CMOS chips to simultaneously image the output signal from all three fluorophores. The RGB method of sensing is of particular note for devising cell culture systems at a reasonable cost.

Controlling $pO_2$ at the pericellular level is possibly a greater challenge than measuring $pO_2$. Oxygen diffusivity through water and PDMS ($\sim 5 \times 10^{-5}$ cm$^2$/s for both) is slow and thus it takes hours for changes in atmospheric oxygen levels to equilibrate at the bottom of a petri dish. Oxygen control systems for tissue culture with equilibration times on the order of a few minutes have been achieved in multiwell plates by bubbling the oxygen gas mixture directly in the well and by flowing oxygen through a thin PDMS insert in the well that avoids bubbles in the media (Oppegard et al. 2009). A similar oxygen control system has been implemented in a PDMS chip by integrating gas flow lines into the chip within ~20 μm of the cell chambers and utilizing a soluble metaloporphyrin oxygen sensor with low cost LED excitation and photodiode detection to monitor the pericellular oxygen level (Lam et al. 2009).

Extensive work has gone into developing glucose sensors for monitoring diabetes. Optical glucose sensors generally fall into two categories (Steiner et al. 2011). Most measure the consumption of oxygen by glucose oxidase with fluorescent probes that are quenched by oxygen. This presents a number of problems for tissue culture: first, glucose is consumed in the sensing process and second, the low oxygen conditions of tissue culture, particularly stem cell tissue culture, make calibration difficult. At the low $pO_2$ levels of tissue normoxia the oxygen level must be measured independently of the glucose oxidase to calibrate the glucose oxidase activity to the local $pO_2$ levels. The second category of glucose sensors are FRET and boronic acid based sensors that change their emission characteristics upon binding glucose thereby avoiding the oxygen dependence issues. However, binding glucose effectively sequesters and buffers the glucose in the media.

Electronic sensors are typically built around three electrode electrochemical cells with a (usually) Ag/AgCl reference electrode, Pt or Au counter electrode and a working electrode that has been functionalized with an analyte specific redox active compound, typically a protein. Alternately when monitoring redox active analytes a selective barrier that effectively concentrates and/or isolates the analyte of interest is necessary. The electrodes, including Ag/AgCl reference electrodes, can be built into PDMS microfluidic systems with basic microfabrication techniques (Polk et al. 2006). One can envision a system consisting of a Ag/AgCl reference electrode, Au or Pt counter electrode and an array of working electrodes each biofunctionalized to measure a different component of the media – e.g. $pO_2$, pH and glucose. The advantages of electronic sensors are the long and short measurement times (good resolution and dynamic range in time) and the ability to measure without damaging or labeling cells. The principle difficulties are addressing/multiplexing large arrays of electrodes to properly functionalize each working electrode, loss of signal when scaling down the electrode size, and degradation/biofouling over the course of long measurements. In particular there is a clear tradeoff between signal magnitude and unwanted reaction byproducts (e.g. hydroxide ion production or glucose consumption) that can be tuned by optimizing the sensor size.

Electrical pH sensors include electrochemical sensors with a hydrogen ion sensitive membrane, typically glass, coating the working electrode and ion sensitive field effect transistors (ISFETs) which are MOSFETs where the gate electrode has been replaced by the ionic solution. ISFETs are more sensitive than the electrochemical sensors however transistor fabrication is significantly more complex and expensive than membrane coated metal electrodes. Additionally, silicon is opaque and thus not an optimal substrate for tissue culture.

The standard and widely used electrochemical oxygen sensor is the Clark sensor which consists

of two electrodes (Pt & Ag/AgCl) encased in an oxygen permeable membrane (typically PDMS) and operated at an 800 mV bias that reduces oxygen to hydroxide ions thus generating a current that is linearly dependent upon the oxygen concentration. Clark sensors are fairly easy to fabricate on glass slides and to integrate into microfluidic systems and show response times of ~15 s (Wu et al. 2010). The creation of hydroxide ions will make the local media more basic, which can be mitigated by scaling the size of the sensor down at the expense of signal resolution. There is a commercial tissue culture system from Bionas Gmbh focused on cytotoxicity and cancer drug response that incorporates an ISFET pH sensor and a Clark $pO_2$ sensor, however the tissue culture wells are fabricated on silicon to accommodate the ISFET which prevents transmitted light microscopy thus limiting the utility of the system.

Electrical glucose sensors are typically electrochemical oxygen sensors coupled to the glucose oxidase enzyme, which consumes oxygen at a rate dependent upon the glucose concentration (Heller and Feldman 2008). These sensors suffer from the same difficulties as optical glucose oxidase based sensors – consumption of glucose and dependence on $pO_2$ at low oxygen levels. To address these issues the glucose sensor should be paired with an oxygen sensor and the sensor sizes must be optimized to minimize glucose consumption and hydroxide production without overly compromising sensitivity. Rodrigues et al. present a microfluidic chip with integrated electrochemical glucose and oxygen sensors located upstream and downstream of a cell culture chamber for measuring oxygen and glucose metabolism by the cells (Rodrigues et al. 2008).

In the near future, monitoring should extend to specific growth factors or signaling molecules directly involved in controlling stem cell fate. Electrochemistry based biosensors have been used to monitor a larger variety of different biomolecules including lactate, reactive oxygen, reactive nitrogen, glutamate, pyruvate, acetylcholine, choline, dopamine and ammonia (Wilson and Gifford 2005).

## Microfluidics for Pericellular Control

Cell-matrix conditions still require basic research both in the cellular biology to define and understand the key variables and in the sensor and control technology to measure and control with feedback the critical cell-matrix variables. Toward this end, there is a significant role for microfluidic systems in developing stem cell tissue culture. With their small dimensions, precise flow control and flexible geometry microfluidic systems can better emulate and control the 3D confinement, effective circulation, shear forces, matrix elasticity and topology of the niche than traditional culture techniques. For example, a microfluidic chip was recently used to identify a role for cell secreted ECM remodeling proteins in mouse ESC self-renewal (Przybyla and Voldman 2012). Furthermore, microfluidic systems are particularly amenable to large arrays and automation (Thorsen et al. 2002), which is critical for performing the matrix experiments necessary to compare multiple cell culture variables as well as coping with the heterogeneity in cell, stem cell and particularly cancer stem cell populations. Microfluidic systems are typically made from compliant hydrogels or elastomers that are quickly and inexpensively fabricated by soft lithography. Material properties are critically important not the least because of the enormous material volume to fluidic chamber volume ratio, on the order of $10^6$ in a typical microfluidic system. Hydrogels are made from hydrophilic polymers that, like living tissue, absorb water and are highly hydrated. Hydrogels have the advantage of being media permeable, biomimetic/ECM like in chemical composition, cell permeable thus enabling cells to grow in 3D, the compliance can be tuned by controlling mix ratios and they can be molded over simple structures. They are thus well suited for basic microfluidic tissue culture applications. The primary disadvantage of hydrogels is that they have not been made into the multi-layer structures necessary to fabricate complex microfluidics with valves that enable arrays and automated cell culture micro chambers.

Poly dimethyl siloxane (PDMS) is a hydrophobic, silicone-based elastomer that has been used extensively in many microfluidic systems. PDMS is easily molded by soft lithography, can be bonded to glass, polystyrene as well as other PDMS layers and has thus been used to make microfluidic chips incorporating active pneumatic valves that enable complex fluid manipulations, a high degree of automation and scalability. PDMS is frequently described in the literature as "biocompatible" and has been used to fabricate a number of devices for microfluidic cell culture. However, there is a growing body of evidence that PDMS is not biocompatible and causes significant problems with cell culture, particularly in microfluidic devices where the PDMS to media volume ratios are so high. Principally, PDMS is hydrophobic and absorbs small hydrophobic molecules including ions and growth factors such as estrogen (Regehr et al. 2009). This probably explains the low growth rates and high perfusion and media exchange rates needed in most chip based cell culture systems. The Hansen group has employed an iso-osmotic bath that displaces much of the PDMS volume with media and saturates the remaining PDMS with media, thereby relaxing the gradients that drive the absorption and achieving robust growth curves (Lecault et al. 2011). PDMS also does not fully polymerize under normal processing conditions, it is necessary to boil the PDMS to drive the polymerization as far as possible and a further solvent extraction or dialysis step is necessary to remove the remaining unpolymerized PDMS (Lee et al. 2004). Thus there are significant downside bio-incompatibilities to using PDMS but they can be addressed with the appropriate processing and design considerations, which we believe are worth the trouble in order to capitalize on the complexity and scalability that PDMS valves allow.

Moreover, microfluidic systems can bring control of the media down to the pericellular level by enabling precise media exchange and rapid gas equilibration in chambers that are small enough to prevent the establishment of gradients in rapidly diffusing species such as ions and small molecules. Cell culture systems that combine pericellular sensors with microfluidic control create the high fidelity feedback loops necessary to continuously maintain each cell culture parameter within its proper range.

In conclusion, at its beginning stem cell culture used the same procedures and equipment as those used for other cells. At that time, most of the cell culture conditions had been empirically determined on the basis of cell survival and proliferation. However, because of the differentiation potential of stem cells and their interest in regenerative therapy, the limits of this empirical approach rapidly became evident. Thus began an intensive and ongoing effort to identify, understand and control the myriad variables that influence stem cell culture. In a sense, the current progress in the stem cell culture field is reminiscent of that which occurred during the transition from alchemy to chemistry. Could the culture substrate, the medium, the $pO_{2,}$ and the temperature, be viewed as the respective counterparts of the four alchemical elements Earth, Water, Air and Fire, while cell-cell interactions might be Ether? Is the definition of the optimal stem cell culture condition an endless quest, always opening new fascinating possibilities? Regardless, for the cell culturist controlling stem cell fate holds the same promise as the philosopher's stone: regeneration and wealth.

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# 2 Development of Defined Culture Conditions for Expansion of Human Mesenchymal Stromal Cells for Clinical Applications

Jessica E. Frith, Pamela Jaramillo-Ferrada, Richard J. Mills, Andrew R. Cameron, Drew M. Titmarsh, Ernst J. Wolvetang, and Justin J. Cooper-White

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**Abstract**

Mesenchymal stromal cells (MSCs) are of clinical interest due to their ability to self-renew and differentiate into multiple tissue types. Unfortunately, both the number of MSCs within tissues and their reparative potential decreases with age, resulting in a decreased capability for tissue repair. The ability to isolate MSCs from a multitude of tissue sources and expand and differentiate them *in vitro* has made them excellent candidates for a range of therapeutic applications. However, a number of existing limitations in terms of their culture must first be addressed prior to them being widely available for use in the clinic. In this chapter we discuss potential strategies to produce therapeutically-relevant numbers of high-quality MSCs for use in clinical applications. The relative merits of MSCs derived from different tissue sources and the development of defined culture conditions, in terms of oxygen levels, soluble media and culture substrates from which high-quality MSCs may, in future be derived for clinical applications, are described.


## Introduction

Mesenchymal stromal cells (MSCs) are multipotent cells that can self-renew and differentiate into multiple lineages (Pittenger et al. 1999). In vivo, MSCs are known to reside in most tissues throughout the lifespan of humans and are involved


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in promoting cellular homeostasis of connective tissue and thus, are present as reservoirs of reparative cells. Unfortunately, both the number of MSCs within tissues (which varies depending on the tissue but even in early life may be as low as 1 cell in every 100/1,000 mononuclear cells), and their reparative potential decreases with age, resulting in a decreased capability for tissue repair (Roobrouck et al. 2008).

The ability to isolate MSCs from a multitude of tissue sources and expand and differentiate them *in vitro* has made them excellent candidates for a range of therapeutic applications, including their use in tissue engineering strategies for bone, cartilage, tendon, ligament and intervertebral disc. MSCs have low overall immunogenicity, which suggests that MSCs from one individual can be used as an off-the-shelf product to treat many individuals without long term problems of immune rejection. They have also been shown to be immuno-modulatory post implantation in a number of therapeutic applications, including in the treatment of graft-vs-host disease (GVHD) to improve engraftment of haematopoietic cell transplants, and after myocardial infarction. Overall, MSCs have significant clinical potential for the treatment of damaged or diseased tissues and as well as a number of other therapeutic applications. However, a number of existing limitations in terms of their culture must first be addressed prior to them being widely available for use in the clinic.

Within research laboratory environments, MSCs are routinely cultured in tissue culture flasks in the presence of media containing fetal bovine serum (FBS). While these methods are adequate for MSC expansion, there are numerous reports that describe a loss of replicative ability, colony-forming efficiency, and differentiation capacity of MSCs with extended time in culture (Banfi et al. 2000). The numbers of cells obtained from tissue extraction protocols are inherently low (0.01–0.001% of mononuclear cells in the bone marrow) and extensive expansion of this initial population must be achieved to reach appropriate cell numbers for clinical doses (for example, up to 20 million cells per dose are often required) highlighting the requirement for improved methods for the *in vitro* culture of MSCs to achieve large numbers of cells of high potentiality that are genetically and epigenetically stable. In addition, any therapeutic application requires the imposition of strict product quality controls. An important aspect of this is the removal of any factors with the potential for contamination or transfer of pathogens. Culture conditions that ensure a quality MSC-source for clinical applications that utilize fully defined and xenofree media and substrates for their expansion and differentiation are in various stages of development.

In this chapter we will discuss potential strategies to produce therapeutically-relevant numbers of high-quality MSCs for use in clinical applications. We will discuss the relative merits of MSCs derived from different tissue sources as well as the development of defined culture conditions, in terms of oxygen levels, soluble media and culture substrates from which high-quality MSCs may in future be derived for clinical applications (Fig. 2.1).

## Tissue Sources of Mesenchymal Stem Cells (MSCs): Cellular Composition, Ontological Origin, Tissue Forming Potential and Clinical Applications

### MSCs from Different Tissue Sources

Mesenchymal stromal cells (MSCs) were initially isolated from the bone marrow (BM) stroma over 30 years ago. However, it is now widely acknowledged that MSC-like cells can also be isolated from almost every postnatal tissue, as well as from gestational or fetal tissue sources. Adult tissue sources include adipose tissue, periodontium, synovial membrane, skin, eye limbus, trabecular bone, thymus, spleen, lung, anterior cruciate ligament, tendon, cartilage and meniscus (Augello et al. 2010). Gestational sources of MSCs include placenta (amnion, chorion and deciduas basalis), chorionic villi, Wharton's jelly of the umbilical cord, amniotic fluid, umbilical cord and umbilical cord blood, and fetal-derived blood, liver, yolk sac and bone-marrow (Pappa and Anagnou 2009) (Fig. 2.2a).

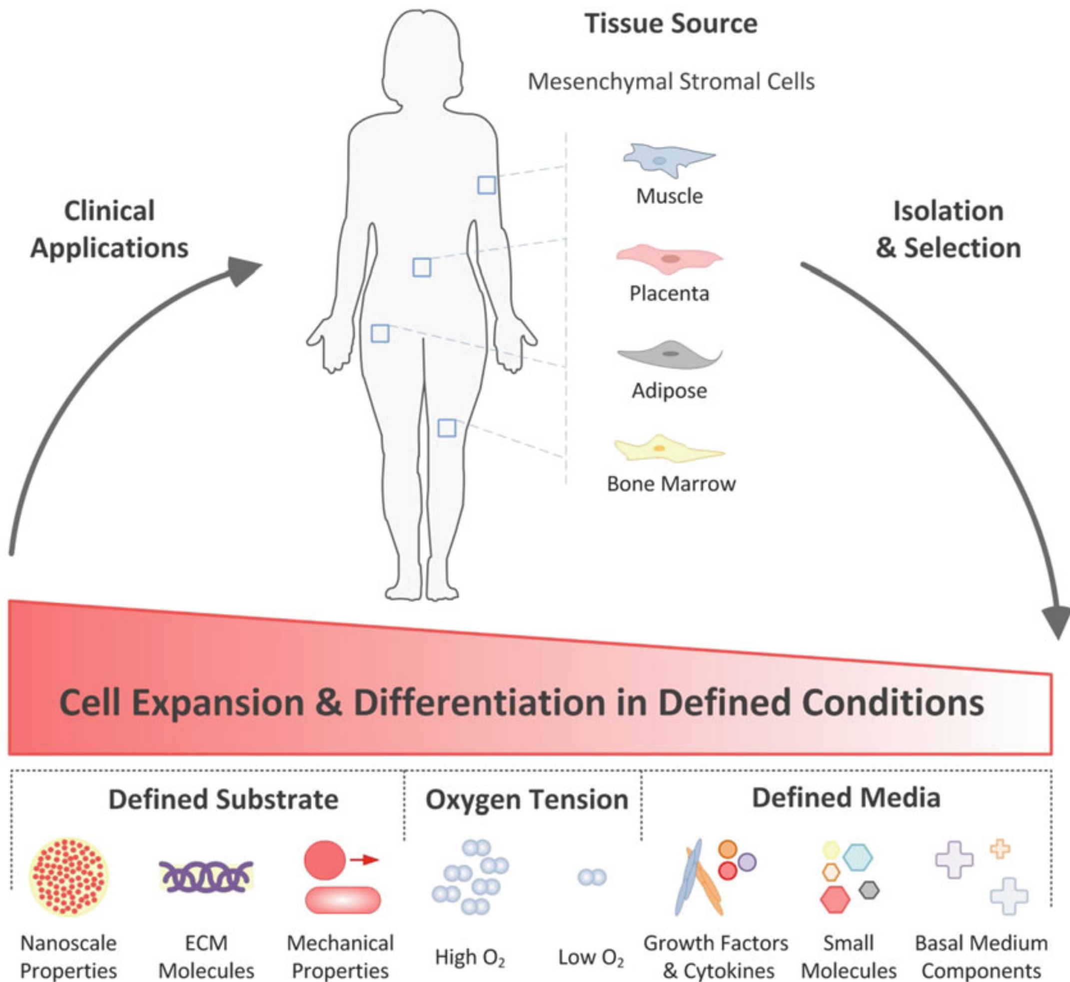


**Fig. 2.1** Schematic outlining the factors to be considered in the development of clinical therapies using mesenchymal stromal cells

To be considered a true "mesenchymal stromal cell" population, cell preparations should meet the minimum criteria set by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular therapy (ISCT) which includes: (Fig. 2.2b)

- Plastic adherence in standard culture conditions
- Positive expression (>95%) of CD73, CD90, CD105 and negative expression (<2%) of CD34, CD14, CD45, CD19 and HLA-DR.
- *In vitro* tri-linage differentiation towards osteogenic, chondrogenic and adipogenic lineages.

Of all adult tissue sources of MSCs, BM-MSCs represent the most extensively studied and accepted gold-standard source of MSCs. However, more recently adipose-derived MSCs (AD-MSCs) have received increased attention as a clinical source of MSCs. They are commonly isolated from biological remnants generated following liposuction, lipoplasty, or lipectomy surgeries. AD-MSCs are easily *ex vivo* expanded, display similar immunophenotype and immunomodulatory characteristics to BM-MSCs and have higher proliferation rates (Zuk et al. 2002), although some studies have

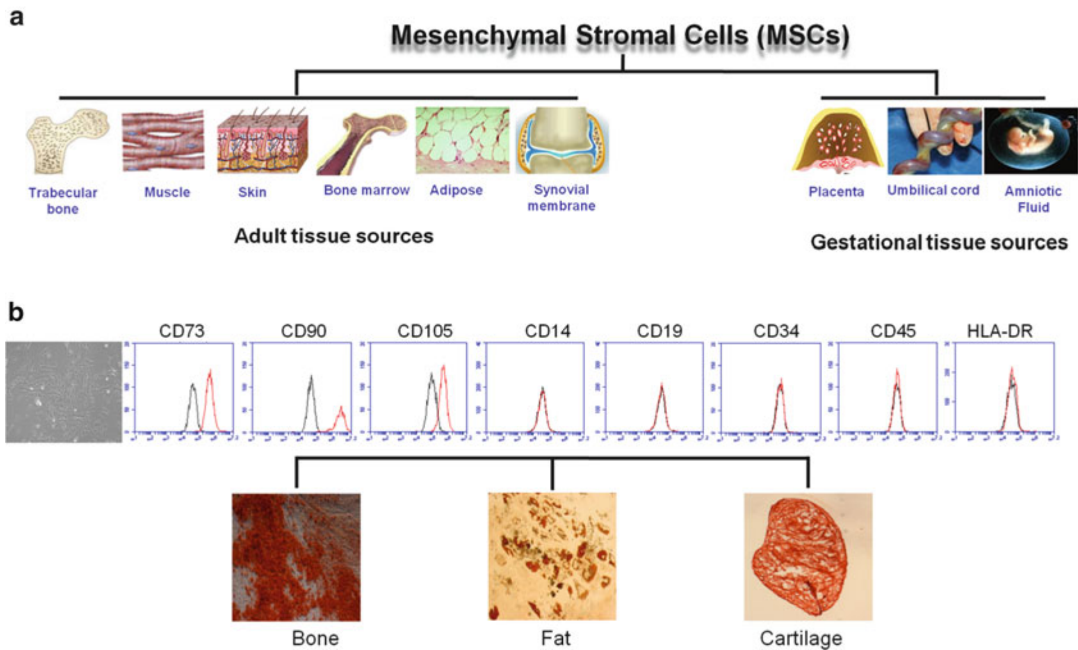


**Fig. 2.2** Tissue sources, immunophenotype and differentiation potential of mesenchymal stromal cells (MSCs)

reported an inferior potential for osteogenic differentiation and increased adipogenic conversion, which may limit their potential for the treatment of bone-related disorders (Zhang et al. 2009).

## Different Ontological Sources of MSCs

There is also increasing interest in the use of MSCs originating from different ontological sources. In general, MSCs derived from gestational and fetal tissues, display a more primitive/early developmental cellular status compared to their adult counterparts, resulting in enhanced differentiation potential in most cases. For example, with regards to bone forming capacity, first trimester human fetal BM-MSCs have shown enhanced osteogenic potential when compared to postnatal BM-MSC (Zhang et al. 2009). The chondrogenic potential of placenta-derived MSCs has also been shown to be higher than that of adult BM and AD- MSCs (Hsu et al. 2011).

Perinatal sources seem attractive with greater frequency and higher proliferative-potential MSC, but a major limitation is that MSC can typically only be isolated from around a third of umbilical cord blood specimens (Bieback et al. 2008). Earlier fetal, placental and amniotic sources yield abundant primitive MSC with greater differentiative ability than later source, but require invasive procedures and/or access to abortal tissue with its attendant ethical issues. There is thus a major unmet need for new sources of MSC for clinical application. In an attempt to address this need, several groups have reported the derivation of MSC from pluripotent cells, although this required cumbersome or untranslatable techniques such as murine co-culture, physical manipulation, sorting or viral transduction.

Boyd et al. (2009) used a 30 day epithelial culture to differentiate manually-dissociated hESC, which after subsequent passaging underwent an apparent spontaneous EMT, generating cells with decreased pluripotency and increased mesodermal/MSC marker expression, and bi-lineage mesenchymal differentiation potential. We recently devised a single-step method to direct mesengenic differentiation of human embryonic stem

cells (ESC) and iPSC using a small molecule inhibitor, SB431542 (Chen et al. 2012). They concluded that treatment with SB431542 in 2-dimensional cultures followed by culture-induced EMT leads to rapid and uniform MSC conversion of human pluripotent cells without the need for embryoid body formation or feeder cell co-culture, providing a robust, clinically-applicable and efficient system for generating MSC from human iPSC.

## Requirement for Selection Methods for MSC Purification

It is important to recognise that tissue source, as well as ontological age influence culture outcomes. For example, in a study where the chondrogenic potential of MSCs isolated from amniotic fluid, neonatal bone marrow, and preterm umbilical cord blood was assessed, all perinatal MSC sources were able to undergo chondrogenesis, but the specific ECM composition of the cartilage matrix formed, was highly dependent on the particular cell source used (Kunisaki et al. 2007). In addition, the osteochondral potential of MSC preparations isolated from BM, adipose tissue and synovial membrane has shown to differ considerably (Ronziere et al. 2010).

Further, irrespective of their tissue of origin, *ex vivo* expanded MSC cultures are heterogeneous in terms of cell size, morphology, proliferation and differentiation potential. Clonal analyses have shown that BM-MSC populations are comprised of a mixture of multi- and bi-potent progenitors, as well as lineage-restricted precursors and a small percentage of fibroblasts that lack any capacity to differentiate into connective tissue lineages. Recent reports have described the expression of some lineage-specific markers in undifferentiated MSCs at both the mRNA and protein level (Woodbury et al. 2002). This is consistent with the hypothesis that *ex vivo* expanded MSCs harbour subsets of lineage-committed precursors and suggests that MSC differentiation may involve up or down-regulation of marker expression and activity, rather than complete on/off activation.

This heterogeneity has shown to be strongly influenced by the anatomical site of cell harvest and ontological origin of the tissue source. This phenomenon may be attributed, at least in part, to a differential responsiveness of the cells to differentiation cues due to epigenetic programming, or a differential expression/activation/availability of growth factor receptors, co-receptors, cytokines, and/or intracellular mediators that are necessary to drive and sustain particular differentiation pathways. Alternatively, the presence of distinctive subsets of tissue-committed precursors in each mesenchymal tissue at particular developmental age and anatomical site, might account for this differential potential. These differences in differentiation potential associated with the age of the donor and tissue source have important consequences for the clinical application of these cells, implying that MSC preparations from different tissues and ontological sources should be carefully characterized and, potentially, differentially used for particular tissue regeneration approaches.

Alternatively, in an effort to overcome some of these issues, there is increasing interest in the development of protocols using markers to select and purify MSC populations prior to use in the clinic. For this reason, a single definitive marker of MSCs has long been sought with suggested candidates including CD49a (Integrin $\alpha$1), CD106 (VCAM-1), CD166 (ALCAM), CD271 (low-affinity nerve growth factor) and Stro-1. Of these Stro-1 is perhaps the most accepted and widely used marker and Stro-1-enriched MSCs indeed demonstrate greatly increased proliferative and multilineage differentiation capacities (Psaltis et al. 2010).

## Clinical Applications and Cell Therapy Companies Based on MSCs from Different Sources

Mesoblast Ltd is currently evaluating the applicability of using allogenic mesenchymal progenitor cells (MPCs) isolated from bone marrow to treat posterolateral lumbar fusion and degenerative disc disease. Its partner company, Angioblast Systems,

US., is also currently assessing the feasibility of using MPCs to treat acute myocardial infarction. The MPCs used in these treatments are an enriched population of cells expressing high levels of Stro1 together with CD106 (VCAM-1).

The US-based company, Osiris, is also investigating the therapeutic applicability of MSCs. Their product, Prochymal, consists of adult BM-MSCs and is currently being assessed in phase III trials to induce remission of Crohn's disease, as well as treating acute and refractory graft versus host disease. Similarly, Athersys, Inc., is currently assessing the applicability of autologous MSCs (selected by depletion of CD45+/glycoporin A+ cells from the population) (MultiStem®) in phase I trials for treating ischemic injury and autoimmune conditions.

With regards to the clinical use of AD-MSCs, Cellerix S.A. (Spain) is a company that focuses on using culture expanded stem cells from adipose tissue. Recently, the company reported the successful completion of a phase IIa clinical trial using allogenic AD-MSCs for the local treatment of complex perianal fistulas in Crohn's patients. The trial showed the safety and efficacy of the treatment, both in terms of closure and reduction of draining fistulas. In addition, low immunogenicity from the allogenic expanded stem cells obtained from adipose tissue was reported, thus validating the viability of this allogeneic source of cells.

## Development of Defined Culture Conditions

### Defined Media

Current expansion techniques for MSCs rely on media supplemented with fetal bovine serum (FBS). However, supplementing media with FBS to expand MSCs for clinical applications carries the substantial risk of transmitting known and unknown xeno-pathogens, along with xeno-immunisation. Furthermore, FBS has considerable batch-to-batch variability and can result in significant variations in experimental results and MSC properties, hindering the standardisation of MSCs that is required for regulatory approval and broad clinical adoption (Schallmoser et al. 2007). Several strategies have been proposed to reduce or remove reliance on FBS as a medium supplement. These include using human platelet lysates, human serum and chemically defined media. Human platelet lysates (Schallmoser et al. 2007) and serum products (Turnovcova et al. 2009) have both been shown to successfully expand MSCs and furthermore enhance cell expansion compared to FBS medium supplementation. However, both of these replacements still suffer from batch-to-batch variability and introduce the possibility of disease transmission between donor and patient (unless an autologous source is used).

There is thus a strong motivation for the development of a fully defined media for the expansion of MSCs. The majority of research has focussed on growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β) and epidermal growth factor (EGF), which have been shown to be essential for the formation of colonies from Stro-1 selected hMSCs when cultured on fibronectin (Gronthos and Simmons 1995). However, when MSCs are expanded in a base medium with these growth factors in combination with 2% FBS they have been shown to spontaneously differentiate toward the osteogenic lineage during culture (Apel et al. 2009). Therefore, a formulation containing these two growth factors alone may not be a suitable alternative to FBS and demonstrates that it is not only essential for a serum-free replacement media to support expansion but to do so without influencing their differentiation potential.

There are several commercial serum-free media formulations (Table 2.1) available for the expansion of MSCs, which claim to maintain tri-lineage differentiation potential. This includes Mesencult® from Stem Cell Technologies and TheraPEAK™ MSCGM-CD™ from Lonza. However the composition of these media, and associated ECM substrate upon which the cells are cultured, are unfortunately confidential. Whilst, StemPro MSC SFM®, a serum free media from Invitrogen, is known to be supplemented with fibroblast growth factor (FGF-2), transforming growth factor-beta (TGF-β), PDGF-BB and insulin, its base media

**Table 2.1** Serum free media for MSC culture

| Company/literature reference | Cell source | Medium | Recommended ECM | Formulations disclosed | Expansion from isolation | Outcomes/notes |
|---|---|---|---|---|---|---|
| Invitrogen/Chase et al. (2010) | BM-MSC | Stem Pro MSC SFM® | CELLstart™ or Human Fibronectin | No | No | Based on PDGF-BB, TGF-β1, b-FGF |
| Lonza | – | TheraPEAK™ MSCGM-CD™ Mesenchymal Stem Cell Growth Medium | N/A | No | No | |
| StemCell Technologies | – | MesenCult®-XF Medium | MesenCult®-XF Attachment Substrate | No | Yes | Used for isolation of amniotic fluid MSCs (ref Sessarego) |
| Hudson et al. (2011) | BM-MSC | mTeSR®1 (StemCell Technologies) | Human Fibronectin (or Collagen I, Collagen IV, Laminin) | Yes | No | Enrichment of osteo/chondro potential |
| Platelet Lysates Lange et al. (2007) | | | | | | |
| Liu et al. (2007) | CB-MSC | IMDM, 17.91 ng/mL bFGF, 2.80 mg/mL human albumin, 27.65 μM hydrocortisone, 1.18% SITE | N/A | Yes | No | |
| Meuleman et al. (2006) | BM-MSC | Ultra-Culture medium (Cambrex), 2% Ultroser G serum substitute (Pall BioSepra), 2 mm L-glutamine (Cambrex) | N/A | No | No | Semi-defined composition |

Notes
*BM-MSC* Human bone marrow-derived mesenchymal stromal cells
*CB-MSC* Human cord blood-derived mesenchymal stromal cells
*SITE* 0.5 μg/mL sodium selenite, 1.0 mg/mL bovine insulin, 0.55 mg/mL human transferrin and 0.2 mg/mL ethanolamine

are also of confidential composition. Ideally to optimise expansion conditions and study the biology of hMSCs in serum-free conditions, a defined media with known composition (including substrate) is required. Furthermore, another issue that remains is the direct isolation of MSCs in serum-free conditions. Mesencult® has reportedly been utilised for isolation of amniotic fluid MSCs (Sessarego et al. 2008), however the applicability of this approach for other tissue sources has not been explored in depth.

Recently, a study by Hudson et al. (2011) demonstrated the use of a defined embryonic stem cell expansion media, mTeSR (Stem Cell Technologies), for the expansion of human bone marrow derived MSCs. Unlike currently available commercial defined MSC media, the constituents of mTeSR are fully disclosed. Furthermore, growth-factor free, custom formulations are available from StemCell Technologies. This makes it straightforward to study MSC biology or make modifications to the medium and substrate based on experimental data. Such a defined culture combination is seen as a valuable research tool for the study of MSCs and provides a key step towards generating a clinical grade media for expansion of MSCs for clinical applications.

## Oxygen Tension

The potential of MSCs for clinical applications depends largely on being able to retain their beneficial properties during isolation and expansion procedures and in uniformly directing the cells to develop into specific tissue types. To achieve this, it is commonly accepted that the *in vitro* environment must be made to mimic the conditions provided *in vivo* as closely as possible. *In vitro*, MSCs are routinely cultured at atmospheric levels of $O_2$ (21%) with 5% $CO_2$. However, physiological oxygen ($O_2$) tensions are much lower than this, ranging from 12% in blood to as low as 1% in the deep zones of cartilage. It is therefore unlikely that atmospheric levels represent the optimum oxygen tension for MSC culture.

Studies investigating the effects of hypoxia on MSC properties have determined that under conditions of low oxygen tension, MSCs retain their mesenchymal marker expression profile, show enhanced proliferative potential and decreased senescence. In addition, the expression of genes associated with ‘stemness’ (eg. Oct4, Nanog and Klf4) is increased (Hung et al. 2012). However, investigations into the effects of hypoxia upon MSC differentiation have shown that low oxygen can both enhance and diminish differentiation. A potential explanation for these diverse outcomes may lie in the conditions the MSCs were cultured in prior to initiating differentiation, as a study by Volkmer at el. has shown that the negative influence of hypoxia on osteogenic specification could be reversed by pre-conditioning the cells under 2% oxygen for 3 days (Volkmer et al. 2010). The outcome of this study has particular clinical relevance, as it is likely that MSCs introduced to the body for a therapeutic application will be delivered to sites with well below atmospheric oxygen levels. Therefore, to achieve the optimum therapeutic benefit, it may be beneficial to precondition the MSCs to low oxygen levels prior to introduction into the body.

## Defined Substrates

In addition to the development of defined media and controlled oxygen tension for MSC culture, the development of defined substrates is also receiving much attention due to the increasing recognition of the role that the physical environment has on MSC behaviours. As well as using such substrates to facilitate cell attachment in the absence of serum proteins, it is also hoped that defined substrates with specific properties may be used to *direct* MSC activities, for example, through such processes like mechanotransduction (Ingber 2003).

MSCs interact with the physical environment via integrins, transmembrane receptors that bind to specific peptide motifs within the surrounding extracellular matrix (ECM). MSCs express a wide range of both alpha and beta integrin subunits, which bind to their ligand as a heterodimer.

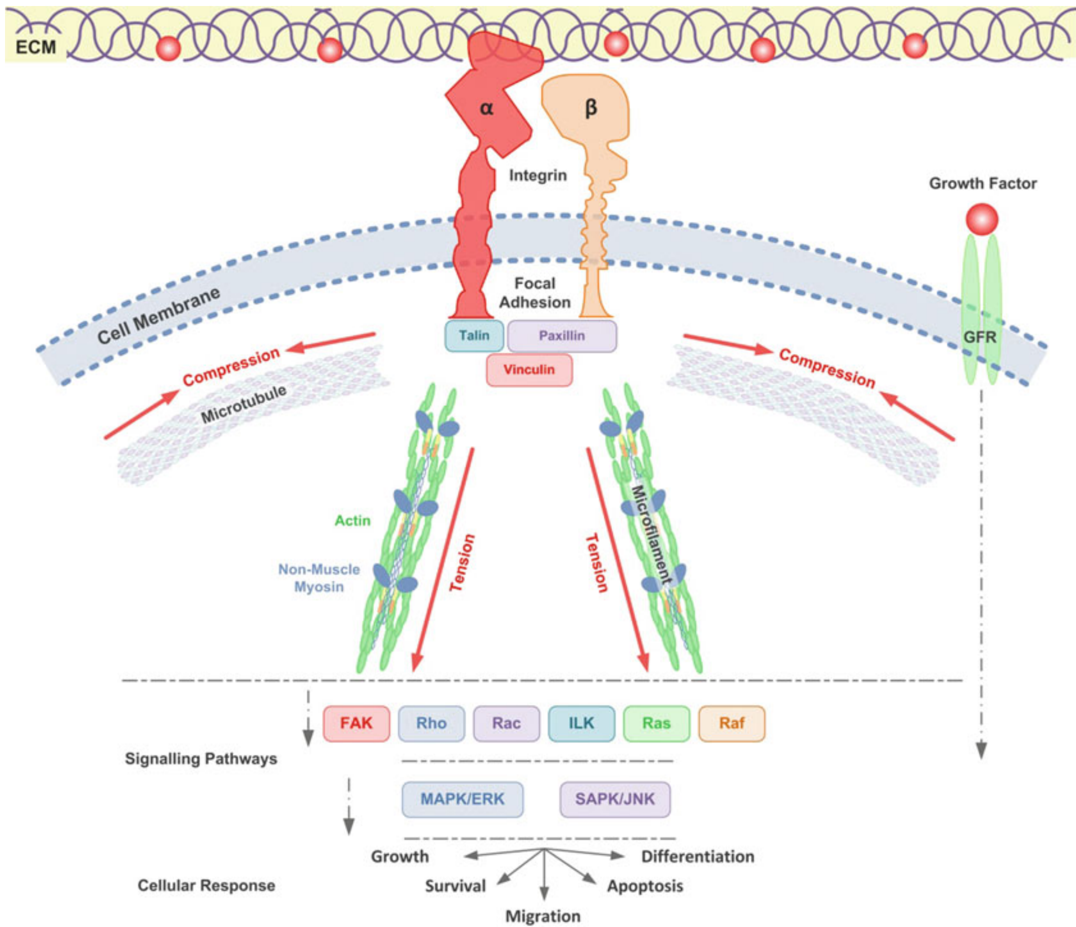


**Fig. 2.3** Interactions of mesenchymal stromal cells with the extracellular environment

There are 18 alpha and eight beta integrins and it is through the varying combinations of these subunits that specificity for different ECM molecules is achieved. Upon binding of integrins to the ECM, the intracellular domain of the integrins undergoes a conformational change and induces integrin clustering. Intracellular proteins are recruited, forming a multiprotein complex or focal adhesion (FA), dynamic assemblies comprised of a large and diverse array of proteins that are key centres for anchorage and organization of the actin cytoskeleton. Initial integrin clustering initiates the recruitment of proteins such as talin, vinculin and α-actinin which ultimately link the integrins to the actin cytoskeleton. FAs therefore fulfil a mechanical role by their presence as an anchor point from which the cytoskeleton can exert tension but also act as signaling centres by linking mechanical cues to intracellular signaling pathways such as Rho and Rac (Fig. 2.3), (reviewed by Critchley 2000).

The ECM that surrounds cells is structurally complex. Different tissues are not only comprised of different molecules but these can also be present in varying proportions and presented in different ways. ECM proteins themselves are large and contain multiple repeating units and cell adhesion motifs, whose presentation can greatly affect cell outcomes. For example, native and denatured conformations of collagen-I and collagen-IV (displaying different cell-binding motifs) and different domains of Fibronectin

have all been shown to exert different influences on the osteo- and adipogenic differentiation of MSCs (Martino et al. 2009)

Demonstrating the complexity of this system, a number of studies have isolated the roles that different features of the extracellular environment have to play in determining MSC behaviour and shown that MSC properties are influenced by substrate elasticity (Engler et al. 2006), viscosity (Cameron et al. 2011), ECM composition (Salasznyk et al. 2004), topography and ligand presentation (Frith et al. 2011). Given the influence of so many aspects of the extracellular environment, the use of such defined substrates therefore presents a great opportunity to exert control over MSC behaviour for therapeutic applications. Options range from simple physisorption of ECM in tissue culture dishes, to engineered systems providing precise control over these different aspects of the environment. Here, we discuss a number of systems offering different properties.

### Presentation of Desired Extracellular Matrix Components

In the absence of serum, the simplest substrate on which MSCs can be cultured is tissue culture plastic (TCP), to which ECM proteins have been adsorbed. Using this method MSCs have been cultured on a variety of ECM molecules, including Fibronectin, Collagen and Laminin. Indeed, studies have shown that MSCs bind with decreasing affinity to Fibronectin, Collagen-I, Collagen-IV and Laminin respectively, and suggest that these different substrates have varying abilities to support osteogenic differentiation (Salasznyk et al. 2004). A major problem with this approach is that the substrate is not defined over longer periods of time due to the secretion and competitive adsorption of ECM secreted by the cells and resultant desorption of original adsorbed proteins. For this reason, it is often preferred to have a non-fouling substrate upon which ECM molecules can be presented. One method uses the generation of a multilayered hyaluronic/chitosan (HA/CHI) surface that can subsequently be functionalised with ECM to facilitate cell attachment (Doran et al. 2010). Using this substrate it was shown that Collagen-I and –IV were more effective in supporting osteogenic differentiation of MSCs than Fibronectin, an effect that was unable to be discriminated when MSCs were cultured on these same ECM molecules that had simply been physisorbed to TCP, highlighting that the presentation of such ligands is critical and affects differentiation outcomes.

Another consideration, however, is that ECM constituents are often prepared by secretion from a cell line (as is the case for Matrigel from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells) and therefore are impure, containing a complex mixture of ECM components and bound growth factors. To overcome this, recombinant ECM proteins or domains may be used, or even short peptide fragments containing just the cell adhesion motif. Examples of such motifs that have been used to facilitate cell attachment are the RGD from Fibronectin and the Laminin-derived IKVAV sequence. The use of such short sequences is advantageous in that it allows the composition of the substrate to be fully defined. However, ECM proteins are complex and cell attachment is often facilitated by interactions with multiple motifs (for example PHSRN the synergy sequence that cells bind in addition to RGD), which may themselves convey important information to the cell. These factors must be taken into consideration when choosing a defined matrix for MSC attachment and are an area of ongoing development.

### Tailoring Elastic and Viscous Properties

Most polymer substrates, synthetic or natural, are viscoelastic materials with a broad spectrum of both elastic and viscous behaviours. Studies have shown at all levels of organisation that tissues in the body behave in a viscoelastic manner. Given the role that mechanical cues can have in cell signalling, it is useful to consider how these elastic and viscous properties may be controlled to direct MSC behaviour.

With respect to substrate elastic properties, many studies have analysed the effect of systematically modifying substrate compressive modulus (rigidity) on various cell behaviours. In line with the cellular tensegrity model (Ingber 2003), substrate rigidity has been shown to affect

cytoskeletal organisation as well as other cell behaviours including cell spreading, attachment (i.e. integrin expression) and the size of focal adhesions, proliferation and apoptosis. Signalling molecules related to cytoskeletal and focal adhesion organisation, such as Rho-kinase (ROCK) and focal adhesion kinase (FAK), have been implicated in affecting these responses to variations in substrate rigidity (Engler et al. 2006).

Substrate rigidity has been shown to affect the differentiation of MSCs. Human MSCs that were seeded onto chemically uniform substrates with rigidities that mimicked those of various native tissues differentiated towards cell types relevant to those tissues (Engler et al. 2006). For example, MSCs on rigid substrates (~40 kPa) had increased expression of osteogenic specific genes while those on substrates with a similar rigidity to muscle (~10 kPa) upregulated myogenic specific markers. This demonstrated the ability for substrate rigidity to direct MSC behaviour in terms of lineage specification. Substrate rigidity has also been shown to affect MSC quiescence as MSCs plated sparsely onto 250 Pa gels halted their progression through the cell cycle. These cells were shown to retain their multilineage differentiation potential and could be induced to proliferate by replating them on stiff substrates (Winer et al. 2009).

A recent study has also investigated the effect of the viscous component of a substrate on MSC behaviour (Cameron et al. 2011). Variations in viscous mechanical properties, specifically loss modulus and consequently creep, were shown to have a substantial influence on the morphology, FA size and motility. MSCs were also shown to proliferate more rapidly when attached to substrates with a high loss modulus (creep). Furthermore, increases in the creep of substrates were shown to enhance the differentiation potential of MSCs towards multiple lineages when in the presence of soluble induction cues. In this manner, the effects of viscous substrate properties appear to be different to those of elastic properties in that they enhance MSC differentiation (in response to stimulation from soluble induction cues) rather than specifically directing lineage specification.

The ability for substrate mechanical properties to influence and direct multiple MSC behaviours has significant implications on the optimisation of defined culture conditions for clinical applications. Most culture vessels currently used are made from plastics which have mechanical properties far outside the physiologically relevant range and this can have a significant and detrimental effect on the behaviour of cells. However, the commercial potential for developing mechanically defined culture conditions is already being realised with the emergence of companies such as ExCellness Biotech (Switzerland) and Matrigen (U.S.A.), which specialise in producing tissue culture vessels with mechanical properties representing those of natural tissues. When defining culture conditions, the optimisation of different substrate mechanical properties for specific applications, in combination with selection of appropriate surface attachment ligands, provides great potential for improving the prospects of attaining successful clinical outcomes.

### Nanoscale Engineering of Surfaces for MSC Expansion and Differentiation

Other recent advances have allowed the engineering of substrate properties at the nanoscale in order to influence cellular properties. Through the use of electron beam lithography, substrates were produced with nanopits in varying patterns and when MSCs were cultured on these substrates, it was shown that that differences in nanotopography influenced the differentiation of MSCs into osteoblasts (Dalby et al. 2007). Substrates presenting a disordered topography induced osteogenic induction of the MSCs, even in the absence of soluble cues. More recently, substrates with nanopits have been shown to maintain MSC multilineage potential during expansion over an 8 weeks culture period (McMurray et al. 2011).

A number of alternate systems have also been developed which allow spatial control of ligand presentation at the nanoscale. In one such system (George et al. 2009), adhesive peptides (RGD) were presented on nano-islands formed from the self-assembly of poly(styrene-block-ethylene oxide) (PS–PEO) block copolymers. Micro-phase separation of the PS-PEO resulted in separated, vertically oriented cylinders of PEO chains (to which the adhesive peptides were attached)

in a matrix of PS (Fig. 2.3). Experiments using this system demonstrated that by altering the spacing between these functionalized nano-islands (by blending the PS-PEO in different ratios with PS homopolymer), the cell spread area, cytoskeletal morphology and migration rate were altered (Frith et al. 2011). There were no indications of spontaneous differentiation on the different substrates, however there were differing abilities of the cells to respond to inductive osteo- and adipogenic media depending on the spacing of the presented RGD peptide. The effects were shown not to be caused by differences in the overall concentration of the presented RGD peptide, and implicate the spatial presentation of ligand as an additional parameter that can be controlled to efficiently expand MSCs cultured in basal media or enhance the differentiation of MSCs in the presence of induction media.

Many of the substrates outlined above result in differences in cell spread area and cytoskeletal morphology, suggesting that mechanisms controlling MSC fate are intrinsically linked to mechanotransduction and highlighting the importance of the substrate when designing culture methods for MSC expansion and differentiation. The diversity of parameters affected by these different systems accentuates the complexity of the physical extracellular environment and the many factors that must be optimised in order to fully control MSC behaviour. Whilst much has so far been learned using these defined substrates, finding and reproducibly presenting the optimal combination of extracellular cues for cell expansion and tissue development remains a major challenge for the future development of clinical protocols for MSC culture.

### Current Commercially Available Culture Platforms

The examples mentioned previously highlight the complexity of the extracellular environment and the important role that substrate has when culturing MSCs for clinical applications. Together they show that the mechanical properties, ECM specificity and the way in which the ECM is presented to MSCs (even down to the nanoscale) all have an influence on MSC behaviour and that this is mediated by changes in integrin-binding, cytoskeletal assembly and subsequent intracellular signalling cascades. However, many of these systems, whilst being crucial in elucidating how different features of the substrate influence different MSC properties, are so far limited to use in the laboratory and yet to be made available commercially.

Current commercially available substrates include the purified ECM components (Collagen, Fibronectin, Matrigel) that may be adsorbed 'in-house' to provide a specific surface. Alternatively, a collaboration between Corning Life Sciences and Geron Corporation has developed cell-culture plates which consist of synthetic acrylate surfaces to which peptides derived from bone sialoprotein and vitronectin are covalently linked. These have been shown to support MSC expansion in defined, serum-free media (including STEMPRO® MSC SFM, and MesenCult®-ACF). Invitrogen also offer a xeno-free substrate for defined MSC culture called CELLstart™ that is said to maintain the multi-lineage differentiation potential of MSCs expanded on it. However, the composition of this substrate is not readily available. In terms of offering substrates presenting properties other than a change in ECM composition, mechanically defined culture plates such as those produced by ExCellness Biotech and Matrigen (mentioned earlier) provide a means of specifying the elastic modulus of the underlying substrate, whilst the new 3D cell culture technology from AMSBIO, alvetex®, is made from the standard treated polystyrene used in culture flasks but presented as a porous 3D scaffold, thereby changing the geometry of the substrate. As these examples do not yet cover the full range of substrate stimuli that are known to influence MSCs, additional systems will undoubtedly be released in the future.

### Culture in the Absence of a Substrate (Multi-cellular MSC Spheroids)

A final option is not to provide a substrate at all. There has been increasing interest in culturing MSCs as multicellular spheroids in which the cells interact with each other but do not adhere to any external surface. Such MSC spheroids can be cultured statically, for example in low-attachment plates but also have the advantage of being able to be cultured as a suspension in bioreactors, which have the potential to be readily scaled up to produce the cell

numbers required for a therapeutic application (Frith et al. 2010). Using such methods it has been shown that, as compared to MSCs cultured as monolayers on TCP, MSCs from spheroid culture have altered expression of cell surface antigens and an increased ability to differentiate along the osteogenic and adipogenic lineages. Gene expression analysis also determined a number of changes, such as increased expression of CXCR4 (which is involved in MSC homing in the body) and IL24 (an anti-cancer factor) which suggest further applications of this method for MSC expansion for therapeutic applications (Frith et al. 2010).

## Summary and Future Perspective

The current 'gold standard' conditions for MSC expansion (and differentiation), regardless of the tissue source, are largely undefined in terms of media composition and the ligand composition being presented on the culture substrate, and performed under normoxic (20% $O_2$) and under static (no flow) conditions. There is however growing evidence that improved culture outcomes can be achieved through the use of defined media, physiologically-relevant oxygen tensions, substrates of varying mechanical property slate, controlled presentation of ligands for cell attachment and tailored receptor binding, and 3D culture protocols.

The field is yet to combine all of these microenvironmental facets to produce an optimised (largely) synthetic platform for MSC expansion that is not only more efficient than current standard protocols, but that also produces a higher quality product (every time) and that is more cost effective. Major cost factors to production for cellular therapy companies across the globe would be alleviated if the cost of media, associated quality control of the cellular product and culture ware consumables could be reduced. Bioreactor methodologies that are often used for protein production from mammalian cells (e.g. Chinese Hamser Overy (CHO) cells) are being investigated as viable alternatives to standard MSC culture methods, but even here, optimisation of the same microenvironmenal facets as listed above are required and this remains to be achieved. These reductions would result in lower cost MSC products for clinical application, resulting in broader uptake and impact to the global health system. There are substantial efforts worldwide attempting to broach these challenges, with progress in both our fundamental understanding of MSC biology, cell-substrate interactions, and bioprocessing impacts of cell behaviour rapidly pushing the field towards sensible and cost effective solutions.

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# Retention of Stem Cell Properties Post-expansion in Myosphere Culture

3

Yan Wei, Andreas M. Kaufmann, and Andreas E. Albers

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**Abstract**

Skeletal muscle has a prominent potential to regenerate after repeated trauma due to a pool of stem cells that reside in skeletal muscle. Satellite cells (SCs), as the primary source of this capability, originate in the embryonic dermamyotome and are located between the muscular basal membrane and the sarcolemma of mature adult skeletal muscle and compose the tissue-specific stem cell population. In response to muscle damage, SCs proliferation is triggered and they either differentiate and fuse to form new muscle fibers or alternatively self-renew to maintain the stem cell pool.

To facilitate the use of SCs in clinical scenarios, we need to explore the biology of these cells in greater detail. One clear goal is to be able to definitively identify and purify SCs. The myosphere forming assay is robust and reflects the behavior of SCs. Clonal analysis where single cells give rise to myospheres need to be used to follow the self-renewal and multipotency characteristics of SCs. Myosphere formation in combination with other markers of SCs behavior represents the state of the art to follow these cells. Remarkably, myospheres-derived progenitor cells (MDPCs) are not only able to differentiate into mesodermal cell types including the myogenic, adipogenic, osteogenic, cardiacogenic, and hematopoietic lineages, but also possess the potential to break germ layer commitment and differentiate into ectodermal lineages including neuron-like cells under certain conditions.

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## Introduction

Adult stem cells are undifferentiated cells that are found throughout the body after conclusion of embryonic development. Stem-cell based therapies for the repair and regeneration of various tissues and organs offer a paradigm shift that may provide alternative therapeutic solutions for a number of diseases, including musculoskeletal, hematopoietic, neurological and cardiovascular diseases (Bosi and Bartolozzi 2010; Chojnacki and Weiss 2008; Khan et al. 2010; Messina et al. 2004; Slorach et al. 1999). Skeletal muscle is an accessible adult stem-cell compartment in which differentiated myofibres are maintained and repaired by a self-renewing stem-cell pool. These resident stem cells, which are known as satellite cells (Mauro 1961), lie on the surface of the muscle fiber, between the plasmalemma and overlying basal lamina. Skeletal muscle is a dynamic tissue that is capable of responding to exercise-, immobilization-, chemically-induced damage, physiological stimuli, or a severe injury by mounting a well-orchestrated regenerative response that restores the cytoarchitecture (Cossu and Biressi 2005; Dhawan and Rando 2005; Hawke and Garry 2001; Holterman and Rudnicki 2005). As the largest organ in the body, muscle tissue represents an abundant, accessible, and replenishable source of stem cells. However, due to the lack of specific molecular surface markers for muscle stem cells, it was a challenge to identify and characterize muscle stem cells. The ideal stem cell for regenerative medicinal applications should meet the following criteria (Wu et al. 2010):

1. be found in abundant quantities (millions to billions of cells)
2. be harvested by a minimally invasive procedure
3. be differentiated along multiple cell lineage pathways in a regulatable and reproducible manner.
4. be safely and effectively transplanted to either an autologous or allogeneic host
5. be manufactured in accordance with current Good Manufacturing Practice guidelines

This article reviews the current preclinical studies and potential clinical applications of MDPCs mediated tissue-engineering and methods for MDSC isolation, differentiation, and cellular characterization.

## Identification of Myospheres

SCs are the resident stem cells of embryo- and adult skeletal muscle and represent a promising source for cell-based therapies of musculoskeletal disorders. It is essential to define a specific molecular signature for SCs, in order to identify and manipulate them efficiently. Therapeutic approaches for skeletal muscle degeneration by cell transplantation have been hindered by poor cellular survival rates and the limited spread of the injected cells (Cerletti et al. 2008). Major efforts were made to identify the most suitable cells for transplantation. One of the greatest challenges in using cell therapy for the treatment of muscle disease is the ability to isolate, expand, and deliver suitable donor cells needed for transplantation. Properties considered to be important include the ease of isolation, the ability to be maintained and expanded in culture without loss of “stemness”.

Recently, there has been some reports focussing on the isolation of potential stem cells for muscle regeneration (Nomura et al. 2007; Ogata et al. 2007; Wei et al. 2011). Traditional skeletal muscle stem cell culture systems consist of monolayer cultures growing in a two dimensional (2D)-architecture. While 2D-culture conditions are generally sufficient for investigations of simple cellular functions and/or activity of epithelial tissues or endothelia, most functional tissues are characterized by complex intercellular relationships that are best studied only in three-dimensional (3D) systems. The application of 3D cell culture techniques is receiving increased interest with evidence showing significant differences between the cellular phenotype and biological response of cells cultured in monolayer and 3D (Frith et al. 2010). 3D methods facilitate stronger cell-cell contacts and interactions of cells with the extracellular matrix (ECM), allowing

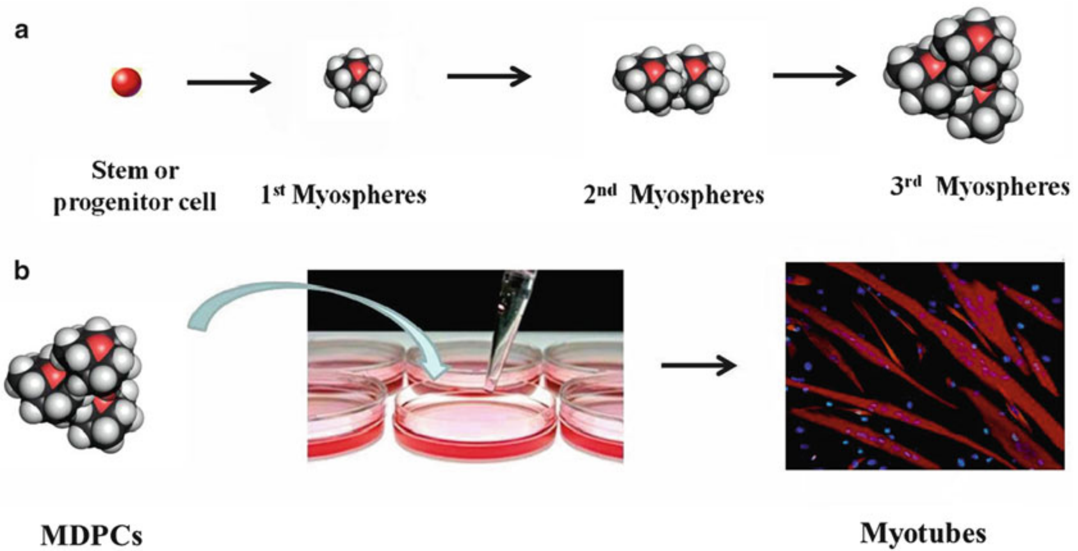


**Fig. 3.1** Experimental approaches for stem cell use in regenerative medicine. (**a**): The non-adherent sphere assay predicts that a muscle stem cell can be serially passaged for many cycles and that it generates a myosphere resembling the primary sphere in each case. (**b**): Muscle cells may be expanded directly on plastic or embedded in collagen. Each colony-forming assay represents a progenitor cell activity

cells to adapt their native morphology, which may influence signaling activity. As a result, it is becoming increasingly accepted that 3D culture methods provide a cellular environment more consistent with that *in vivo* (Fig. 3.1a).

The 3D-spheroids of muscle cells called "myospheres" resemble spheres formed by neural stem cells (Deleyrolle and Reynolds 2009; Ahmed 2009) and cardiac stem cells (Messina et al. 2004). In serum-free medium and in the presence of mitogens such as epidermal growth factor (EGF) and/or fibroblast growth factor-2 (basic FGF), muscle stem cells can be cultured on nonadhesive surfaces as multicellular myospheres (Poulet et al. 2011; Sarig et al. 2006; Westerman et al. 2010). In the primary culture, differentiating/differentiated cells rapidly die, whereas muscle stem cells proliferate, giving rise to secondary spheres that can then be further subcultured. Importantly, myospheres can be serially subcultured by trypsinisation and by replating under the same *in vitro* conditions (Fig. 3.1b). This procedure can be sequentially repeated several times, and each stem cell gives rise to many stem cells by the time a sphere is formed. This is allowing for the progressive enrichment and continuous expansion of stem cells in culture (Wei et al. 2011).

The extensive proliferation of myosphere-forming cells has been used as an indicator of stem cell function based on the assumption that each myosphere is derived from a stem cell. These myospheres could be serially passaged to form more myospheres, or be differentiated into skeletal muscle cell types in serum-containing medium and by plating the cells on an adhesive substrate (Fig. 3.1b). Using the myosphere assay, we and other groups have demonstrated that a population of cells exist in the fetal through to the adult mammalian skeletal muscle that can be isolated in cultures and will exhibit the critical stem cell attributes of proliferation, self-renewal, and the ability to give rise to a number of differentiated, functional progeny (Nomura et al. 2007; Poulet et al. 2011; Sarig et al. 2006; Wei et al. 2011; Westerman et al. 2010). The gold-standard for evaluating the presence of MDPCs is orthotopic transplantation of sorted human subpopulations into an immunocompromised mouse (Alessandri et al. 2004).

**Table 3.1** Immunophenotype of passaged human and murine MDPCs isolated from skeletal muscle tissue

| Species | Marker type | Surface antigen | References |
|---|---|---|---|
| Human | Positive | Desmin, vimentin, CD105 | Messina et al. (2004) |
| | | CD56 | Wei et al. (2011) |
| | Negative | CD34, CD45, CD31, FLK-1 | Messina et al. (2004) |
| | | CD34, CD45 | Wei et al. (2011) |
| Murine | Positive | CD29, CD44 | Nomura et al. (2007) |
| | | Sca-1 | Sarig et al. (2006) |
| | | Sca-1, CD29, CD34 | Westerman et al. (2010) |
| | Negative | CD45 | Sarig et al. (2006) |
| | | CD45, CD31 | Nomura et al. (2007) |

## Characterization of Myospheres-Derived Progenitor Cells (MDPCs)

Skeletal muscles are highly vascularised and contain a variety of mononuclear cells, including SCs, myoblasts, endothelial cells, immune cells, fibroblasts, and progenitor cells. Furthermore, MyoD and/or myogenin expressing myoblasts that exist in the interstitial spaces in the growing animal will also be extracted during the isolation procedure. When isolated by using the myosphere technique, myosphere-derived progenitor cells (MDPCs) remain a heterogeneous mixture of cells with variable proliferation and differentiation potentials. Although potentially acceptable for cell-based therapeutic applications, a deep understanding of the composition of MDPCs requires a better investigation of MDPCs. The expression of specific surface antigens has been proposed to define MDPCs. Table 3.1 provides information on the surface immunophenotype reported in several studies. All studies focused on MDPCs from human and murine skeletal muscle tissues. Currently, no standard marker has been identified for muscle stem cells. Further studies are required that should aim to define differences and similarities between the various populations.

## Differentiation Potential of MDPCs

Myosphere-derived cells were also shown to have pluripotent capability, giving rise to cells from all three germ layers, and participate in skeletal muscle and hematopoietic regeneration (Messina et al. 2004; Sarig et al. 2006). *In vitro*, when cultivated in basic medium, MDPCs can spontaneously differentiate into myotubes, and when stimulated with inductive factors, MDPCs display an impressive capacity for differentiation into cell types from ectodermal and mesodermal tissues, including neurogenic, hematopoietic, osteogenic, adipogenic, and chondrogenic cells. When MDPCs were grafted into d-sarcoglycan knockdown hearts that served as a model of cardiomyopathy, neoangiogenesis was enhanced and cardiac function was improved (Nomura et al. 2007). MDPCs display several electrophysiological properties similar to those of cardiomyocytes by culture conditions (Poulet et al. 2011). Alessandri et al. (2004) have induced MDPCs obtained from human muscle samples to make several molecules (including GFAP and beta-tubulin III) that are frequently used to identify astrocytes and neurons. The findings indicate that MDPCs are potentially useful for reconstitution therapy of the nervous systems and provide new insights into MDPCs with regard to neurogenesis. The evidence of MDPCs multi-lineage differentiation *in vitro* has stimulated investigators to seek rapid clinical translations and also raises hope of tissue repair by stem cell transplantation in the near future. However, the mechanisms involved in differentiation and plasticity are not well understood yet and require further investigation and confirmation in suitable animal models before application in regenerative medicine.

In conclusion, adult MDPCs are a potentially powerful candidate cell type for regenerative medicine. At present, cell and gene therapy in

selected experimental models are stimulating investigators to seek rapid clinical translation. However, this translation will require a complete understanding of the regulation of the maintenance and differentiation of MDPCs both *in vivo* and *in vitro*. Clearly, a number of challenges remain before MDPCs can be used in everyday clinical practice.

In summary, myospheres can be easily isolated and propagated from individuals of all ages. These myospheres represent an important new option to isolate and maintain muscle stem cells in culture, which in turn could extend future possibilities of cell-based therapies for muscular diseases.

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# Part II

# Bone Marrow Stem Cells

# 4 Role of Reactive Oxygen Species Formation from Oxidized Low Density Lipoprotein in Bone Marrow Stem Cells

Zhenguo Liu, Tiewei Lu, Sampath Parthasarathy, Hong Hao, and Chandan K. Sen

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**Abstract**

Reactive oxygen species (ROS) are a group of small molecules that regulate tissue redox status and oxidative stress. Redox regulation is an important determinant for cell development, activities, and function. ROS and oxidative stress also have a significant impact on the regulation of stem cells including bone marrow stem cells (BMSCs) and bone marrow-derived progenitor cells. ROS could regulate the self-renewal or proliferation of BMSCs, their mobilization, homing, migration, differentiation, and apoptosis or senescence. The effects of ROS and oxidative stress on BMSCs are complex and closely related to the ROS levels in the intracellular compartment and in the extracellular microenvironment. Oxidized low density lipoprotein (ox-LDL), a key contributor to the development of atherosclerosis in hyperlipidemic state, produces a significant amount of ROS spontaneously *in vitro* at clinically relevant concentrations. In the present review, the effects of ROS and oxidative stress on BMSCs and bone marrow-derived progenitor cells and related mechanisms are highlighted and discussed especially on the expression of stem cell marker Oct-4, self-renewal, mobilization, and differentiation. Specific efforts are made to discuss the role of ROS generated from ox-LDL in mediating the actions of ox-LDL on BMSCs and the potential impact on cell therapy with BMSCs.

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## Introduction

Cell transplantation into damaged organs or tissues like myocardium remains a viable and attractive option for tissue repair and regeneration after injuries including myocardial infarction. Bone marrow mesenchymal stem cells (MSCs) are one of the main sources for cell therapy for cardiac repair and regeneration since these cells are easily obtainable without ethical concerns, and have little (if any) inherent immunogenicity for adverse immune reactions. The documented beneficial effects of MSCs and other bone marrow-derived progenitor cells including multipotent adult progenitor cells (MAPCs) on cardiac repair include enhanced myogenesis, angiogenesis and vascularity, promotion of collateral development, and improvement in cardiac and limb function in the setting of ischemia (Williams and Hare 2011). Bone marrow-derived endothelial progenitor cells (EPCs) also make significant contribution to re-endothelialization of injured blood vessels and formation of new vasculature (angiogenesis), as well as in the prevention of neointima formation after vascular injury (Alev et al. 2011). On the other hand, cardiovascular risk factors such as hypercholesterolemia and diabetes mellitus attenuate native collateral development and associated with incomplete revascularization and decreased re-endothelialization after arterial injury in both human and animals (Kinnaird et al. 2008). The mechanisms for the impaired outcome in the patients with these risk factors have not been well defined.

Hyperlipidemia is a major risk factor for cardiovascular diseases including coronary artery disease and stroke. It is well established that atherosclerosis is one of the principal contributors to the development of cardiovascular diseases in hyperlipidemic patients. The pathogenesis of atherosclerosis is very complex and multifactorial. Although elevated level of low-density lipoprotein (LDL) is related to the formation of atherosclerotic plaques, LDL itself is not atherogenic in nature. It is widely accepted that oxidative modification of LDL with the formation of oxidized LDL (ox-LDL) renders LDL atherogenic.

There are extensive interactions between ox-LDL and a variety of cells through multiple mechanisms. The biological effects of ox-LDL on its target cells are highly variable and complex, depending on the individual cell type. Ox-LDL is reported to promote cell proliferation of macrophage and vascular smooth muscle cells, and inhibit apoptosis of macrophages and monocytes through activation of PI-3 kinase/PKB pathway and mitogen-activated protein kinase (MAPK) (Matsumura et al. 1997). However, some studies show that ox-LDL inhibits proliferation and promotes apoptosis of vascular endothelial cells (Chen et al. 2007). In some cases, ox-LDL exhibits biphasic effects on cultured endothelial cells. It induces vascular endothelial cell proliferation at low concentration with short exposure time; while it promotes cell apoptosis and even necrosis of endothelial cells at high concentration with prolonged exposure time (Chen et al. 2007; Galle et al. 2001). Ox-LDL is also reported to inhibit EPC proliferation and differentiation, and suppress EPC function including inhibition of cell migration, adhesion and in vitro vasculogenesis, as well as ischemia-induced neovascularization in vivo (Imanishi et al. 2004; Zhou et al. 2007). Ox-LDL interferes with the function of EPCs through multiple mechanisms including inhibition of endothelial nitric oxide synthase (eNOS), downregulation of E-selectin and integrin alpha(v)beta(5) expression, inactivation of telomerase, and acceleration of cell senescence (Di Santo et al. 2008; Imanishi et al. 2004). In this focused review, efforts will be directed to summarize the role of reactive oxygen species (ROS) in bone marrow stem cells (BMSCs) with special emphasis on the role of ROS generated from ox-LDL in mediating the actions of ox-LDL on BMSCs.

## Spontaneous Production of Reactive Oxygen Species from Oxidized Low Density Lipoprotein

It is reported that the rate of ROS generation in the peripheral blood monocytes is increased in hyperlipidemic patients along with elevated plasma

ox-LDL level (Vasconcelos et al. 2009), and ox-LDL increases the intracellular formation of ROS in cultured endothelial cells (Cominacini et al. 1998). We quantitatively determined ROS production from different concentrations of ox-LDL (from 0 ox-LDL from 0 to 20 µg/ml) in a total volume of 100 µl detection solution in vitro using electron paramagnetic resonance (EPR) spectroscopy. We observed that a significant amount of ROS was generated from ox-LDL in the culture system within a few seconds after addition of ox-LDL in a dose- and time-dependent manner. ROS generation increased over time, and reached the maximum by 80 s, and stayed at high level during the measuring period for up to 30 min. No difference in the peak level of ROS formation was observed in the presence or absence of BMSCs. Interestingly, ROS formation from ox-LDL in the culture system was completely prevented when the antioxidant N-acetylcysteine (NAC) was present (Lu et al. 2010). These data suggest that ROS production from ox-LDL is spontaneous and independent from the cells.

## Oxidative Stress Regulates the Expression and Function of Stem Cell Marker Oct-4

ROS are highly reactive oxygen-containing molecules, such as oxygen ions and peroxides, due to the presence of unpaired valence shell electrons. These reactive species are generated as natural byproducts of normal oxygen metabolism, and have important roles in cell signaling and homeostasis in normal conditions. However, at times during disease states (like inflammation or infection) and environmental stress (e.g., UV or heat exposure) or ionizing radiation, ROS levels could increase dramatically, and may result in significant damage to cellular structures (Devasagayam et al. 2004). This cumulates into a situation known as oxidative stress.

Stem cells exhibit unique characteristics including specific cell markers and gene expression such as the transcription factor Oct-4, stage-specific embryonic antigen-1 (SSEA-1), and Rex-1 gene which encodes an acidic zinc finger protein. They are expressed in the embryonic stem cells, and are closely related to each other. Oct-4 is one of the transcription factors of the Pit-oct-Unc (POU) family, and is expressed at high level in embryonic stem cells and other pluripotent cells. It plays a critical role in the functionality of stem cells including maintaining their pluripotency, self-renewal, and differentiation. The precise regulation of Oct-4 expression is of great importance to the fate of stem cells (Niwa et al. 2000), and is a complex and dynamic process that involves a variety of factors through an extensive network of signaling pathways including histone modifications and STAT3 signaling. To investigate the effect of oxidative stress on Oct-4 expression in BMSCs, the cells were cultured in the presence of different levels of $O_2$. We observed that a significant level of Oct-4 was expressed in the cells when cultured at the $O_2$ level of 5.5%. Exposure of BMSCs to oxidative stress using increased $O_2$ levels for 24 h resulted in a concentration-dependent decrease in Oct-4 expression in the cells. When the $O_2$ level was increased to 50% or above, no detectable level of Oct-4 was present in the cells (Fig. 4.1). Oxidative stress by exposing the cells (mouse embryonic fibroblasts and HeLa cells) to ionizing radiation or $H_2O_2$ also regulates the selectivity of Oct-4 protein binding to specific DNA sequences (Kang et al. 2009). These observations indicate that Oct-4 expression and function are sensitive to oxidative stress in stem cells.

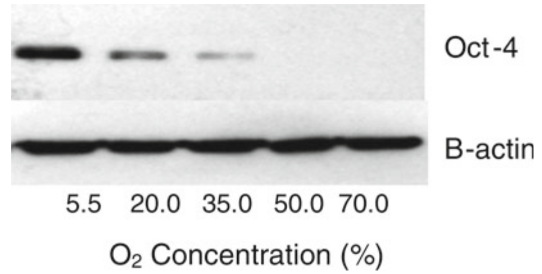


**Fig. 4.1** Oct-4 expression was concentration-dependently decreased in the cells exposed to elevated $O_2$ levels for 24 h with decreased levels of protein as analyzed by Western blot

## Role of Reactive Oxygen Species Formation from Oxidized Low Density Lipoprotein in the Expression of Oct-4 in Bone Marrow Stem Cells

Bone marrow multipotent adult progenitor cells (**MAPCs**) are clonally isolated, purified, and well characterized non-hematopoietic bone marrow **MSC** subpopulation from different species including human, rat and mouse. These cells express high level of Oct-4, exhibit extensive expansion capability, and are able to differentiate at the single-cell level into functional multiple cell lineages of all three germinal layers including endothelial cells, hepatocytes, myocytes, and neurons both in vitro and in vivo (Ulloa-Montoya et al. 2007).

When MAPCs were exposed to ox-LDL for 24–48 h, Oct-4 transcriptional expression was significantly decreased with a reduction of mRNA level in a concentration-dependent manner. Western-blot analysis demonstrated that Oct-4 protein content in MAPCs was also substantially decreased in the cells when incubated with ox-LDL for 24–48 h (Chu et al. 2011; Lu et al. 2010). Immunofluorescence staining showed that both Oct-4 expression level in the individual cells and Oct-4 positive cell population were decreased in ox-LDL-treated group. However, when the cells were pre-treated with the antioxidant N-acetylcysteine (NAC), ROS production from ox-LDL in the culture system was completely prevented. NAC treatment also significantly, but not completely, reversed the inhibitory effect of ox-LDL on Oct-4 expression in MAPCs. These data demonstrate that ox-LDL down-regulates Oct-4 expression in BMSCs largely, but not entirely, through ROS formation in vitro.

## Reactive Oxygen Species Regulate the Functionality and Fate of Bone Marrow-Derived Stem Cells

ROS formation is a dynamic and tightly-regulated process in normal situation. Redox regulation is an important determinant for normal cell development, activities, and function (Ogasawara and Zhang 2009; Pervaiz et al. 2009). ROS and oxidative stress also have a significant impact on the regulation of stem cells including bone marrow stem cells.

## Reactive Oxygen Species Regulate the Self-Renewal or Proliferation of Bone Marrow-Derived Stem Cells

Self-renewal, a process that produces two identical daughter cells, is one of the key characteristics of stem cells. Self-renewal and differentiation of hematopoietic stem cells (HSCs) are critically dependent upon the levels of local oxygen and intracellular ROS, ROS associated redox regulation, and niche microenvironments (Grek et al. 2011). It is known that HSCs are mainly located in the bone marrow at the lowest end of an oxygen gradient (hypoxic environment) (Eliasson and Jönsson 2010). In human bone marrow-derived adult hematopoietic stem/progenitor cells, there are a group of constitutively active NADPH-oxidases (NOXs) including the cell membrane-localized catalytic subunits of the NOX1, NOX2 and NOX4 isoforms that are involved in ROS production in the cells. A low level of ROS generated from the activities of these NOX isoforms could function as second messengers, and enable these cells to be more responsive to proliferative/differentiative stimuli (Piccoli et al. 2007). A recent interesting study shows that, based on the intracellular ROS content using 20-70-dichlorofluorescene diacetate staining, mouse HSCs could be separated into two groups: HSCs with low ROS and ROS with high HSCs. The cells in the two populations are identical phenotypically including cell surface markers ($CD34^-$, $Sca\text{-}1^+$, and $c\text{-}Kit^+$). However, the cells with low ROS level are able to maintain a higher long-term self-renewal potential throughout serial transplantation assays. In contrast, significant HSC exhaustion is observed in the cell population with high ROS level following serial transplantation with decreased adherence to the specific niche components. Treatment with the anti-oxidant NAC (by scavenging ROS) is able to restore the self-renewal potential and functional activity of these cells with high ROS level (Pervaiz et al. 2009). Thus, intracellular ROS levels are the key factors that control the long-term self-renewal capacity of HSCs.

Ataxia telangiectasia mutated (ATM) gene is involved in DNA damage checkpoint and responsible for genomic stability. It is shown to be essential for HSC self-renewal. ATM deficiency results in bone marrow failure at 24 weeks of age in mice due to a functional decline in HSCs second to elevated levels of ROS (Ergen and Goodell 2010). Increased ROS levels lead to activation of p38 mitogen activated protein kinase (MAPK), which in turn up-regulates the expression of the cyclin-dependent kinase (CDK) inhibitors p16Ink4a and p19Arf. These CDK inhibitors could inhibit cell division required for self-renewal of HSCs. The regulatory control of self-renewal or proliferation of mouse HSCs and neural stem cells by ROS that involve ATM gene is also related to Akt and Erk1/2 signaling pathways (Pervaiz et al. 2009).

Another important mediator for the effect of ROS and oxidative stress on HSC self-renewal is FoxO transcription factors especially $FoxO_3$. The bone marrow in the mice with triple conditional deletions of Foxo1, Foxo3a, and Foxo4 genes has defective long-term repopulating activity with increased cell cycling and apoptosis of HSCs. There is a significant decrease in the cell population of HSCs in the bone marrow, and a marked increase in ROS level in HSCs isolated from FoxO-deficient mice in association with changes in the expression of genes that regulate ROS (decreased expression for superoxide dismutase genes SOD1 and SOD3). In vivo treatment with the antioxidant NAC is able to reverse the changes in the FoxO-deficient HSC phenotype. Foxo3a single knockout mice have defective HSC self-renewal and decreased expression of p27Kip1 and p57Kip2 (Ergen and Goodell 2010; Pervaiz et al. 2009).

Of note, there is a close relationship between FoxO and ATM. FoxO is essential for normal ATM expression in HSCs. It is reported that FoxO3 suppression of ROS production in HSCs is mediated partly by regulation of ATM expression. FoxO3a also directly interacts with ATM to enhance the phosphorylation of ATM at Ser 1981 and prompt its downstream mediators to form nuclear foci in response to DNA damage. Both FoxO and ATM have extensive interactions with a variety of pathways including p53-mediated signaling (Pervaiz et al. 2009). Obviously, FoxO and ATM function closely together on the regulation of ROS generation and in response to oxidative stress in stem cells especially HSCs.

## Reactive Oxygen Species Regulate the Viability and Activity of Bone Marrow-Derived Stem Cells

ROS accumulation and oxidative stress have significant impact on cell viability and function including stem cells. Recent studies have suggested that ROS cause residual bone marrow injury by selectively inducing HSC senescence through redox-dependent activation of the p38 MAPK (p38)-p16Ink4a (p16) pathway (Grek et al. 2011). Exposure of rat MSCs to hydrogen peroxide leads to a concentration-dependent decrease in cell viability (Wang et al. 2010). When the activity of antioxidative selenoenzymes is impaired in human MSC and BMSCs, the superoxide anion processing enzyme SOD1 is not sufficiently stimulated with low level of ROS load, leading to oxidative damage with generation of micronuclei in BMSCs (Ebert et al. 2006). In vivo experiment shows that exposure of mice to a sub-lethal dose of total body irradiation induces a persistent increase in ROS production, leading to the induction of hematopoietic stem cell senescence (Wang et al. 2009). Hosokawa and colleagues (Hosokawa et al. 2007) have observed that ROS reduce N-cadherin-mediated cell adhesion, and induce the exit of hematopoietic stem cells (HSCs) from the niche by suppressing N-cadherin expression. Activation of ROS-p38 mediated pathway by advanced glycation products and related chemokines/cytokines inhibits the growth and migration of rat MSCs (Yang et al. 2010).

On the other hand, ROS could have beneficial effects on BMSCs as well including mobilization, homing, migration, self-renewal, and differentiation. It is reported that human HSCs constitutively generate low levels of hydrogen peroxide that in turn participate in the redox-mediated signaling for the growth and differentiation of HSCs (Piccoli et al. 2007). Recently, Busletta and colleagues (Busletta et al. 2011) demonstrate that intracellular generation of ROS during the early phase of hypoxia (15 min), through the activation

of extracellular regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal protein kinase 1/2 pathways, is a critical event for hypoxia-dependent migration of human bone marrow MSCs. ROS derived from Nox2-based NADPH oxidase in bone marrow play a critical role in mobilization, homing, and angiogenic capacity of EPCs and bone marrow stem/progenitor cells, thus promoting revascularization of ischemic tissue. Increased ROS beyond their basal level promotes haematopoietic progenitors to differentiate into mature blood cells in Drosophila (Owusu-Ansah and Banerjee 2009). Oxidative preconditioning with H(2)O(2) could increase MSCs migration in association with enhanced expression of CXCR4, and protect MSCs against apoptosis induced by hydrogen peroxide-related oxidative stress. It is reported that T-cell-produced IL-17 regulates the recruitment, proliferation, motility, and differentiation of human MSCs in a manner dependent on the generation of ROS (Huang et al. 2009). Reconstitution of a native extracellular matrix made by human marrow cells ex vivo strongly promotes the proliferation of MSCs, retains their stem cell properties with a low level of ROS, and substantially increases their response to BMP-2 (Lai et al. 2010). Cellular ROS is believed to play an important role in mediating the functionality (including proliferation and pathogenic stimuli like lipopolysaccharide and cytokines) and development of dendritic cells from hematopoietic progenitor cells during inflammation (Sheng et al. 2010).

The mechanisms for the complex actions, and yet sometimes seemingly paradoxical, of ROS and oxidative stress as well as related redox signaling on BMSCs or bone marrow-derived progenitor cells remain to be fully defined. Intracellular redox state is shown to be a critical and sufficient factor to maintain the balance between self-renewal and differentiation in the dividing oligodendrocyte-type-2 astrocyte progenitor cells (Noble et al. 2005). Redox state itself is a tightly regulated and balanced process in normal situation by a variety of cell-extrinsic signaling molecules such as NADPH oxidase complex, glutathione reductase, SOD, and catalase (Pervaiz et al. 2009). It is observed that growth factors or exogenous signaling molecules or pharmacological agents (like NAC) that promote self-renewal make the progenitor cells to become more reduced, while signaling molecules that enhance differentiation prime the cells to become more oxidized, then modifying the responses of the cells to extracellular signaling molecules. Thus, it is proposed that the cells that are slightly more reduced are more responsive to inducers of cell survival and division and less responsive to inducers of differentiation or cell death. On the other hand, the cells that are slightly more oxidized exhibit a greater response to inducers of differentiation or cell death, but less response to inducers of proliferation or survival (Noble et al. 2005). Clearly, other signaling mechanisms like FoxO and ATM-mediated pathways, p38 MAPK, ERK1/2, and Akt signaling are critically involved in the diverse actions of ROS on BMSCs. Further mechanistic investigations are needed to provide the in-depth insights of considerable relevance to the understanding of ROS on regulating the functionality and characteristics of BMSCs.

## Effect of Oxidized Low Density Lipoprotein on Bone Marrow Stem Cells

Ox-LDL has significant direct effect on a variety of cells including endothelial cells, EPCs, and smooth muscle cells. It is well known that Ox-LDL inhibits the proliferation and differentiation of bone marrow-derived EPCs, and suppresses their function including inhibition of cell migration, adhesion and in vitro vasculogenesis, as well as in vivo ischemia-induced neovascularization (Imanishi et al. 2004; Zhou et al. 2007). Ox-LDL negatively impairs the function of EPCs through multiple mechanisms including inhibition of eNOS, downregulation of E-selectin and integrin alpha(v)beta(5) expression, inactivation of telomerase, acceleration of cell senescence, enhanced expression of adhesion molecules, excessive

production of ROS, and dephosphorylation of Akt (Di Santo et al. 2008; Imanishi et al. 2004). A recent report shows that ox-LDL induces migration and recruitment of human bone-marrow derived stem cells in vitro through mechanisms involving platelet-activating factor (PAF)-receptor-dependent activation of MAPKs (Shin et al. 2010). It is reported that ox-LDL increases the mRNA and protein expression of chemokine stromal cell-derived factor 1 (SDF-1alpha) in human umbilical vein endothelial cells (HUVECs), and enhance the migratory and adhesion response of CXCR4(+)BMSCs (Li et al. 2010). For the past few years, we have been investigating the effect of ox-LDL on bone marrow stem cells, and observed that ox-LDL decreased bone marrow stem cell population in culture system in a concentration-dependent manner. The ox-LDL-induced reduction of cell population was due to the combined outcome of decreased cell proliferation and increased apoptosis. Treatment of the cells with ox-LDL also significantly inhibited their endothelial differentiation via suppression of Akt signaling in vitro. At high concentrations, ox-LDL was toxic to the cells and caused cell deaths within 24–48 h (Chu et al. 2011).

## Role of ROS Formation from Oxidized Low Density Lipoprotein in the Regulation of Bone Marrow Stem Cells

An important mechanism for the actions of ox-LDL is ROS formation and oxidative stress. Ox-LDL is a rich source of ROS that are directly related to tissue oxidative stress, and the development and progression of cardiovascular diseases like hypertension and atherosclerosis (Pervaiz et al. 2009; Vasconcelos et al. 2009; Wang et al. 2009). ROS is released spontaneously from ox-LDL in a concentration-dependent manner. Further studies demonstrated that the antioxidant NAC completely blocked ROS formation from ox-LDL at a concentration of up to 20 μg/ml, and restored the endothelial differentiation potential of MAPCs. However, NAC only reversed the inhibitory effect of ox-LDL at low concentration (5 μg/ml) on the proliferation of MAPCs without protective effect on the cells against higher concentrations of ox-LDL (over 5 μg/ml) (Lu et al. 2010), suggesting that other mechanism(s) were also important in mediating the effects of ox-LDL on these cells. These data are important since the ox-LDL concentrations used in the studies are clinically relevant. Based on recently published human data, the blood serum ox-LDL concentration is estimated to be 0.7 mg/dL (7 μg/ml) in healthy individuals. The serum ox-LDL level is 1.72 mg/dL (17.2 μg/ml), and 2.36 mg/dL (23.6 μg/ml) for the patients with stable coronary artery disease (with an average LDL of 125.8 mg/dL) and acute coronary syndrome (with an average LDL of 143.0 mg/dL), respectively (Imazu et al. 2008). However, future studies are warranted to investigate the effect of ROS from ox-LDL on BMSCs and their endothelial differentiation in vivo.

Apoptosis is involved in the toxic effects of ox-LDL on various types of cells including endothelial cells and the development of atherosclerosis (Wang et al. 2010). In fact, we recently observed that impaired Akt signaling and apoptosis were involved in the actions of ox-LDL on MAPCs. Ox-LDL-induced apoptosis of MAPCs was effectively prevented by over-expression of constitutively active Akt, suggesting that the apoptotic event was due to impaired Akt signaling (Chu et al. 2011). Interestingly, ox-LDL-impaired Akt phosphorylation was only slightly restored in the cells when treated with NAC. These data strongly suggest the concept that ox-LDL impairs the proliferation and function of BMSCs like MAPCs through multiple mechanisms including oxidative stress, inhibition of Akt signaling, and apoptosis. It is certainly possible that these mechanisms play a different role in the actions of ox-LDL at different concentrations on BMSCs like MAPCs. It appears that both oxidative stress and impaired Akt signaling are important when ox-LDL concentrations are low (5 μg/ml or less), while suppressed Akt signaling seems to be the dominant mechanism when ox-LDL concentrations is above 5 μg/ml. Further investigation is needed to define the relationship between these mechanisms in mediating the effects of ox-LDL on BMSCs including MAPCs.

## Effect of Antioxidants on BMSC and Clinical Implications

As discussed above, ROS formation and oxidative stress have both beneficial and adverse effects on BMSCs. It could be interesting to know how antioxidants like NAC affect the functional status of BMSCs. It is shown that supplementation of selenite restores the basal activity of antioxidative selenoenzymes, reduces ROS accumulation in human MSCs, and attenuates oxidative cell damage in BMSCs in vitro (Ebert et al. 2006). Treatment of MSCs with berberine that could scavenger ROS protects the cells against ROS-induced apoptosis in vitro. Overexpression of Hsp20 protects MSCs against cell death triggered by oxidative stress in vitro in association with enhanced Akt activation and increased secretion of growth factors (VEGF, FGF-2, and IGF-1) (Wang et al. 2009). Ohshima and colleagues (Ohshima et al. 2009) delineate that antioxidant therapy with daily subcutaneous injection of superoxide dismutase-mimic for 4 weeks significantly decreases the intracellular ROS level in bone marrow mononuclear cells (BM-MNCs) in the diabetic mice, and increases the percentage of EPCs and their potency of differentiation into endothelial cells. Treatment of the mice with total body irradiation with Mn(III) meso-tetrakis-(N-ethylpyridinium-2-yl) porphyrin (MnTE), a superoxide dismutase mimetic and potent antioxidant, significantly inhibits the increases in ROS production and DNA damage and cell senescence in HSCs in the bone marrow (Li et al. 2011). Treatment with the antioxidant NAC (by scavenging ROS) is able to restore the impaired self-renewal potential and functional activity of HSCs with high ROS level (Pervaiz et al. 2009). NAC treatment also protects MAPCs against the toxic effect of low concentration ox-LDL, and restores their endothelial differentiation potential impaired by ox-LDL (Lu et al. 2010). However, NAC treatment has no effect on the proliferation of MAPCs and their endothelial differentiation under normal culture condition with 5% $O_2$ in the absence of ox-LDL. These data suggest that antioxidants could have beneficial effects on BMSCs when excessive oxidative stress is present. However, clinical studies are needed to demonstrate the potential favorable clinical outcomes on BMSCs especially in disease states like hyperlipidemia, and cell therapy with BMSCs with antioxidants.

In conclusion, the effects of ROS formation and oxidative stress on BMSCs are significant and complex, and involve a variety of mechanisms and signaling pathways. The impact of ROS on BMSCs is broad and comprehensive including Oct-4 expression, self-renewal, mobilization, adhesion, recruitment, paracrine function, differentiation, and apoptosis. The outcome of the interactions between ROS and BMSCs appears to be related to the levels of ROS intracellular compartment and in the extracellular microenvironment. These data may have important clinical impact on the treatment of hyperlipidemic patients, and on patient selection for cell therapy with BMSCs especially for those with poorly controlled hyperlipidemia (for both recipients and donors). Future in vivo studies are warranted to investigate the effect of ROS generated from ox-LDL on BMSCs especially their self-renewal, mobilization, and differentiation, as well as related mechanisms(s).

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# The Role of Bone Marrow-Derived Progenitor Cells in Tumor Growth and Angiogenesis

# 5

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**Abstract**

As in normal tissues, solid tumors require vascular structures to form conduits for blood, oxygen, and nutrients. Tumors induce sprouting of new blood vessels (angiogenesis) or co-opt preexisting ones. Tumor blood vessels are dysfunctional and characterized by excessive branching and sprouting, leakiness, and inflammation. These abnormalities can contribute to tumor progression. For example, in a process resembling healing wounds, tumor cells and tumor blood vessels secrete factors that mobilize pro-inflammatory, hematopoietic progenitor cells from the bone marrow. These hematopoietic cells are, generally, proangiogenic, and their activity may be partially responsible for the dysfunctional features of tumor blood vessels. Thus, identifying key pathways controlling communication between tumors and bone marrow-derived pro-inflammatory cells will improve our understanding of vascular dysfunction in tumors and contribute to the identification of new therapeutic targets for anti-angiogenesis strategies.

## Introduction: Tumors Resemble Dysfunctional "Organs" of Malignant Cells and Stromal Cells

The concept that solid tumors are comprised of malignant cells and a heterogeneous population of infiltrating stromal cells has changed our understanding of how cancer develops and might

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be treated clinically. Fibroblasts, endothelial cells, smooth muscle cells, hematopoietic, and mesenchymal progenitor cells are typically found within the tumor stroma. Some of these stromal cells are co-opted from nearby tissue; others are mobilized from the bone marrow. Far from innocent bystanders, some tumor stromal cells function as "co-conspirators" altered in ways that contribute to tumor progression and metastasis. Viewing tumors as dysfunctional "organs" where all of the cellular constituents (both malignant cells and stromal cells) are potential therapeutic targets has opened the door to novel anti-cancer strategies (Egeblad et al. 2010). For example, anti-angiogenic therapies, pioneered by Folkman (1972) are designed to selectively eliminate the blood vessels feeding growing tumors with blood, oxygen and nutrients.

At the earliest stages of tumor development, when only a few malignant cells are present, stromal cells may actively restrict tumor cell growth. For example, incipient tumors that have not escaped the basement membrane may be encapsulated by activated, $\alpha$-SMA$^+$ fibroblasts that attempt to "wall-off" the burgeoning malignancy. Endothelial cells and fibroblasts elicit danger or stress signals which mobilize cells of the adaptive immune system, CD4$^+$ and CD8$^+$ T lymphocytes and natural killer cells, which identify and eliminate some cancer cells through perforin and granzyme-mediated apoptosis. Cells of the innate immune system including macrophages and neutrophils are also mobilized. Initially, macrophages may contribute to tumor rejection through type I cytokine production and antigen presentation. Later, tumor cells overcome the inhibitory effect of the stroma and escape immune destruction, while macrophages are polarized to stimulate angiogenesis, tissue remodeling, and tumor growth. Tumor neovascularization, the end-product of a complex collaboration between multiple stromal cells found in solid tumors, is a critical determinant of the growth, metastatic seeding, and escape from dormancy of malignant cells.

In this chapter, we will explore how solid tumors utilize bone marrow-derived hematopoietic and mesenchymal progenitor cells during tumor growth and tumor neovascularization. We will begin by discussing the various mechanisms of tumor angiogenesis and defining the key cellular players. We will explain some of the controversies surrounding the identification of bone marrow-derived endothelial progenitor cells (EPCs) in tumors. We will then describe the two major bone marrow-derived populations found in solid tumors: hematopoietic and mesenchymal progenitor cells and discuss their specific roles during tumor progression and tumor neovascularization. Our goal is to clearly define the different types of marrow-derived progenitor cells in tumors and to describe their functional contributions to tumor growth and angiogenesis.

## Angiogenesis and Vasculogenesis

During embryogenesis, blood vessels are first formed *de novo* by the patterned assembly of angioblasts in a process termed "vasculogenesis." In adults, the formation of new blood vessels occurs primarily through sprouting of endothelial cells (ECs) from preexisting vasculature (angiogenesis). Virtually all tissues in the body are vascularized and every cell is found within 100–200 μm of a capillary, the approximate diffusion limit for $O_2$.

As in normal tissues, tumors require their own vascular networks for oxygen and nutrients. For many years, the accepted paradigm was that the formation of new blood vessels in tumors occurred exclusively through angiogenesis. Thus, angiogenesis in tumors was a local phenomenon and new ECs originated from preexisting vasculature recruited from nearby tissues. Today, angiogenesis is an undisputed mechanism of tumor blood vessel formation. But the unequivocal evidence for tumor angiogenesis does not preclude the existence of alternative and complementary means by which tumors may become vascularized. For example, the concept that ischemic tissues may form new vessels by a mechanism of "postnatal vasculogenesis" was first proposed in 1997 with the identification of circulating, putative EPCs in peripheral blood (Asahara et al. 1997). Since then, postnatal vasculogenesis has been widely promulgated as a mechanism underlying

tumor neovascularization. By this model, some tumor vessels are formed by a systemic process involving the mobilization and collaboration between EPCs and an additional, heterogeneous population of bone marrow-derived hematopoietic cells. Recruited EPCs would ultimately contribute to new endothelium by lining tumor vessel lumens, while hematopoietic cells would support vessel formation through secretion of cytokines, growth factors, and remodeling of the extracellular matrix (ECM).

The concept of postnatal vasculogenesis has important implications in our understanding of tumor vascularization, and could be a major factor underlying tumor progression, dormancy, and metastatic potential. Furthermore, distinct mechanisms may control both angiogenesis and vasculogenesis in tumors, and these two different modes of blood vessel formation may respond differently to anti-angiogenic therapies. However, while the evidence for angiogenesis as a mechanism of vascularization in tumors is well documented, evidence for the incorporation of *bona fide*, circulating ECs into the lining of tumor blood vessels is equivocal and highly controversial. One of the main difficulties in understanding the role of EPCs in tumor neovascularization is lack of consensus regarding the definition, origin and function of these cells in humans and in animal models of disease. In the next section, we will define what constitutes an EPC and describe the controversy and confusion surrounding the identification of EPCs in solid tumors.

## Endothelial Progenitor Cells – Principals and Controversies

EPCs are defined as circulating precursor cells with the ability to differentiate into EC and form functional blood vessels at sites of neovascularization. However, the process for obtaining blood-derived EPCs with the ability to form blood vessels *in vivo* has not been straightforward. Most of the original studies identified putative, circulating EPCs based on expression of CD34, CD133, and VEGF-R2 (Asahara et al. 1997). However, this phenotypic definition was soon realized insufficient because these cellular markers are shared by hematopoietic cells which can also be mobilized into circulation and home to sites of neovascularization, particularly during injury or stress. Despite this ambiguity, numerous studies at the time continued to refer to blood- or bone marrow–derived $CD34^+/CD133^+/VEGF\text{-}R2^+$ plastic-adherent cells as EPCs. These ambiguities were paralleled by lack of consensus regarding cellular nomenclature. For example, hematopoietic cells with angiogenic potential have been referred to as colony-forming unit-ECs (CFU-ECs), circulating angiogenic EPCs, early EPCs and colony-forming units-Hill (CFU-Hill). On the other hand, cells which physically line blood vessel lumens have been referred to as outgrowth ECs, EPCs, late EPCs and endothelial colony-forming cells (ECFCs).

Taken together, the term EPCs has been routinely applied to blood or bone marrow-derived cells with hematopoietic and endothelial characteristics. Despite the often-confusing terminology, the functional distinction between these two, very different types of cells is coming into focus. Yoder et al. 2007 demonstrated that most of the cells long referred to as EPCs are in fact descendants of hematopoietic stem cells (HSCs); these cells are of the myeloid-lineage and assist during nascent vessel formation, but they have no known ability to differentiate into functional ECs and form vessel lumens *in vivo*. Cells with *bona fide* blood vessel–forming ability (referred to as ECFCs hereafter), do not display hematopoietic features such as the pan leukocyte marker CD45 (even at the mRNA level) and they form vessel lumens that carry blood when injected into mice.

## Endothelial Colony Forming Cells in Solid Tumors

The low frequency of ECFCs in circulation and lack of a unique set of distinctive cellular markers have prevented unequivocal identification of ECFCs in the tumor vasculature. Thus, the suggestion that *bona fide* ECFCs contribute at all to the vasculature in solid tumors is controversial and has been technically difficult to prove.

In humans, ECFCs comprise a very small percentage of all circulating cells in blood. ECFCs are found at a concentration of ~2–5 cells/mL in umbilical cord blood and at a concentration of ~0.05–0.2 cells/mL in adult peripheral blood (Ingram et al. 2004). Thus, the isolation of ECFCs by flow cytometry or other immunological techniques is challenging. As a result, most isolations of circulating ECFCs are based on methods similar to those originally reported for endothelial outgrowth colonies from peripheral blood (Lin et al. 2000). In this method, blood mononuclear cells (MNCs) are collected and plated onto collagen-coated plates in endothelial-specific growth media. Non-adherent cells are discarded, and EC-like colonies emerge from the adherent cell population 1–4 weeks after plating. The colonies display a cobblestone appearance typical of ECs and can be plated as single cells or routinely expanded for over 60 population doublings. In addition, the colonies possess *de novo* vessel-forming ability *in vivo*. ECFCs obtained by this methodology are phenotypically indistinguishable from cultured mature ECs in terms of morphology and expression of cellular markers.

To tackle the question of ECFC origin(s) in human tumors, researchers have designed experiments based on the premise that ECFC originate in the bone marrow, a hypothesis that was put forth in the early years of ECFC identification (Asahara et al. 1997; Lin et al. 2000). This idea has also been tested experimentally in rodent tumor models by many laboratories, including our own (Fig. 5.1). Peters et al. (2005) conducted a study in six individuals who developed different cancers after bone marrow transplantation with donor cells derived from individuals of the opposite sex. By performing fluorescence in situ hybridization (FISH) with sex-chromosome-specific probes colocalized with the EC marker vWF, the authors found that donor cells indeed contributed to tumor endothelium, although at low levels (a range of 1–12% depending on tumor type). Therefore, it was suggested that bone marrow derived cells, presumably ECFCs, definitively contribute to tumor endothelium.

In recent years, however, sites other than the bone marrow have been proposed as a source for ECFC. Ingram et al. (2004) demonstrated that the vessel wall of established vasculature contains a fraction of ECs with characteristics similar to blood-derived ECFCs. Therefore, the vessel wall itself may be a source for some circulating ECFCs. Notably, a preparation of bone marrow cells for transplantation, similar to the human study cited above, potentially includes capillary ECs from the marrow vasculature as well as circulating ECFCs from donor blood. Thus, whether multiple donor sources of ECFCs contribute, after transplantation, to host tumor endothelium remains an unresolved question. Definitive proof for the contribution of ECFCs to tumor endothelium is contingent on the identification of a unique marker that can distinguish these cells from mature vascular endothelial cells.

The identification of ECFC using tumor models in mice has been equally controversial and confusing. In mice, well developed imaging and lineage-restricted reporter systems should allow, in principle, an ability to identify whether circulating ECFCs are derived from the resident endothelium or the bone marrow. Though ECFCs have been found in the peripheral blood of several mammalian species, including dogs, pigs, sheep, and humans, they are exceedingly rare in rodents. Therefore, the published data regarding the contribution of murine bone marrow-derived ECFCs to the tumor endothelium are mostly inconclusive. The absolute numbers of ECFCs differ dramatically depending on the tumor site, mouse strain, stage of tumor progression, and method of quantification. Gao et al. (2008) suggest that ECFCs facilitate metastasis as their genetic ablation using ID1 shRNA mitigated pulmonary micro-metastasis and impaired angiogenesis. Also, some chemotherapies are reported to increase ECFCs in circulation and their homing to tumors following vascular damage (Shaked et al. 2006). Other studies report minimal contribution of bone marrow ECFCs to the tumor vasculature and question the importance of these cells during tumor growth (Purhonen et al. 2008). In this context, we recently showed that the

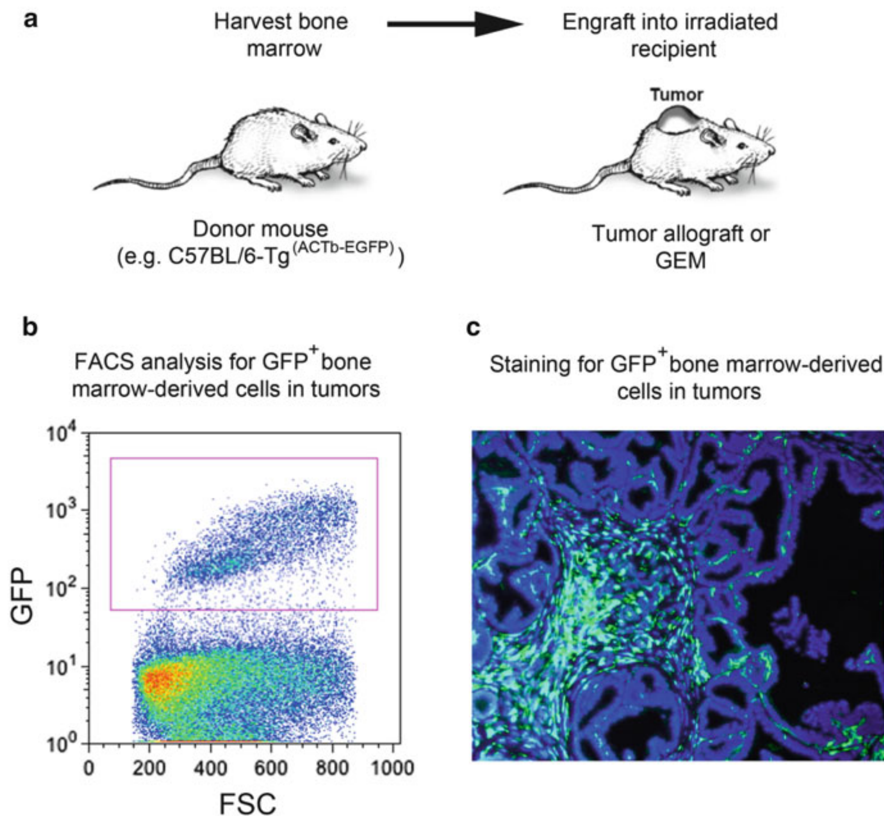


**Fig. 5.1 General strategy for tracking the mobilization of bone marrow derived cells in solid tumors.** (**a**) Transgenic C57BL/6-Tg$^{(ACTb\text{-}EGFP)}$ mice where GFP is under control of the β-actin promoter have made the routine tracking of bone marrow-derived cells in mouse tumor models feasible. More sophisticated transgenic mouse strains, where specific bone marrow-derived cells are genetically marked, are also routinely used. Typically, bone marrow is harvested from $GFP^+$ donor mice which is then engrafted into lethally irradiated recipient mice. The recipient mice can then receive allografts of murine tumor cells lines. Alternatively, $GFP^+$ donor marrow can be transferred to a genetically engineered mouse (*GEM*) that will later develop spontaneous tumors. The advantage of using a GEM is that marrow-derived cells can be monitored at different stages of tumor progression and in age-matched tissues from normal mice for comparison. (**b**) The numbers of $GFP^+$ bone marrow derived cells can easily by quantified using flow cytometry of collagenase-digested tumor extracts. The $GFP^+$ population is *boxed* within the gate. Furthermore, multi-color antibody labeling may be used in combination with GFP to identify specific bone marrow populations. (**c**) Immunohistochemistry using α-GFP antibodies clearly marks bone marrow-derived cells in tumors. Here, staining was carried out on a prostate tumor from a TRAMP mouse (transgenic adenocarcinoma of the mouse prostate) that was irradiated and then engrafted with bone marrow from a $GFP^+$ donor. Cell nuclei are labeled *blue* with DAPI. For more details see Dudley et al. 2010

endothelium in spontaneous prostate tumors was rarely, if ever, derived from the bone marrow and instead most likely recruited from nearby tissue (Dudley et al. 2010).

Taken together, the ability of tumors to form new blood vessels is a rate-limiting step which allows tumors to escape from dormancy and metastasize. Most of the *bona fide* ECs that actually line tumor vessels are likely co-opted from tissue nearby; but, as we will discuss in the next section, bone marrow-derived cells, especially myelomonocytic cells, do play an important catalytic role during tumor angiogenesis by supporting the establishment of nascent vascular structures through multiple mechanisms. The next section will describe how two distinct bone-marrow-derived

populations, hematopoietic and mesenchymal progenitor cells, specifically contribute to tumor development and angiogenesis.

## Other Bone Marrow-Derived Progenitor Cells in Tumors and Their Functions

### Hematopoietic Progenitors

Even physiological angiogenesis is dependent on cross-talk between the remodeling endothelium and cells of hematopoietic origin. One of the first clues for this dependency came from studies in the developing embryo. For example, AML1-deficient embryos, which lack definitive hematopoiesis, showed impaired angiogenesis in the head and pericardium that was rescued by addition of hematopoietic progenitor cells (HPC) expressing ANG-1 (Takakura et al. 2000). This dependency is also present during postnatal processes of blood vessel formation. For instance, we showed that “engineered” blood vessels in the adult mouse are dependent on hematopoietic cell recruitment, as selective elimination of GR-1$^{+}$ circulating myeloid-lineage cells impaired blood vessel development and anastomosis with host vasculature (Melero-Martin et al. 2010).

The participation of bone marrow-derived HPC in tumor vascularization has been a topic of intense investigation. Angiogenesis in solid tumors is characterized by recruitment of an array of pro-inflammatory, myelomonocytic cells with overlapping phenotypes and functions. There are numerous clinical and experimental studies demonstrating a link between inflammation and cancer onset, with infiltrated myeloid-lineage cells such as monocyte/macrophages, neutrophils, eosinophils, mast cells, and dendritic cells actively contributing to tumor progression (Murdoch et al. 2008). The elimination of any one of these cell types using blocking antibodies, pharmacological inhibitors or genetically engineered mice with specific myeloid-lineage cells ablated can generally decelerate tumor growth. Although direct stimulation of malignant cells by HPC is possible, the tumor-promoting abilities of the recruited myelomonocytic pool is largely related to their secretion of pro-angiogenic factors and ability to orchestrate the formation of new blood vessels.

The recruitment of myeloid cells into tumors is induced by an array of chemo-attractants released by both malignant cells and stromal cells. Hypoxic regions in necrotic areas of solid tumors and inflammation may be a potent stimulus. For example, the hypoxia-inducible HIF1-$\alpha$/SDF-1 axis was shown to mobilize pro-angiogenic monocytes from bone marrow. These monocytes control a postulated angiogenic switch in tumors (Du et al. 2008). Once recruited, the local cytokine milieu present within the tumor microenvironment impedes the potential anti-tumor functions of HPC while polarizing them to stimulate angiogenesis, tissue remodeling, and tumor progression (Du et al. 2008). A good example is the increased protumorigenic and proangiogenic activity of tumor-associated macrophages (TAM) as tumors become more vascular and invasive (Biswas et al. 2008). Also, marrow-derived HPC are important mediators of resistance to anti-VEGF therapies (Shojaei et al. 2007) and angiogenesis rebound following radiotherapy (Ahn et al. 2010). Thus, depletion of specific populations of HPC and their progeny can have a favorable anti-tumor response, perhaps by indirectly controlling the rate of angiogenesis.

The function of recruited myeloid cells in tumors includes the production of pro-angiogenic growth factors (vascular endothelial growth factor, VEGF) and vascular-modulating enzymes (matrix metalloproteinases, MMPs) (Murdoch et al. 2008). Myeloid-lineage cells are a rich source of MMPs which are fundamentally necessary for extracellular matrix remodeling and the formation of stabilized blood vessels. For example, numerous studies point to the critical function of myeloid cell-derived MMP9 at sites of tumor development (Coussens et al. 2000). Infiltrating MMP-9-expressing neutrophils were shown to play a crucial role in activating angiogenesis in previously quiescent tissue vasculature during the early stages of tumor initiation (Bergers et al. 2000; Nozawa et al. 2006; Du et al. 2008). VEGF is absolutely necessary for EC survival and is a potent stimulus for the homing of marrow-derived

HPC. Tumor and stromal cell-derived SDF-1 traps and retains HPC at the periphery of vessels where they can stimulate angiogenesis (Grunewald et al. 2006).

In addition to their paracrine functions, myeloid cells may act as cellular "bridges" during angiogenesis by guiding and connecting developing blood vessels. For example, TIE-2-expressing macrophages were recently shown to guide tip-cell fusion, a critical step during blood vessel anastomosis (Fantin et al. 2010). The ability of myeloid-lineage cells to directly form endothelium is also possible. Trans-differentiation of $CD45^+$ myeloid cells into endothelial-like cells was described by Bailey et al. (2006). Also, Yang et al. (2008) found that $Gr\text{-}1^+/CD11b^+$ cells acquired properties of endothelial cells in tumors, including gained expression of VE-cadherin and ability to line blood vessel lumens.

The contribution of myeloid cells to tumor angiogenesis and tumor growth has been routinely demonstrated by blocking their mobilization and/or retention at the tumor site. For example, the use of neutralizing antibodies to specific molecules such as colony stimulating factor 1 (CSF1), PlGF and the chemokine CCL2 markedly reduced the number of tumor-associated macrophages, tumor growth, angiogenesis, and the number of metastasic colonies (Qian et al. 2011). Notably, the functional importance of myeloid cells has been established in other disease models characterized by uncontrolled neovascularization and inflammation – these include models of wound healing, arthritis, atherosclerosis, and choroidal angiogenesis. The efficacy of some anti-inflammatory drugs may relate to their ability to inhibit pro-inflammatory cell recruitment and as a by-product, angiogenesis. For example, two mechanistically distinct anti-inflammatory drugs, cortisone and ibuprofen, are known to inhibit tumor angiogenesis. The well-known chemoprotective effect of non-steroidal anti-inflammatory drugs (NSAIDs) in cancer may be related to their ability to dampen inflammation which impedes the mobilization and recruitment of the pro-inflammatory cells that further fuel tumor growth and angiogenesis.

## Mesenchymal Progenitors

Mesenchymal progenitor cells (MPCs) are multipotent cells that reside in almost all postnatal organs of the body but are found primarily in the bone marrow. The key function of MPCs is the maintenance and regeneration of damaged tissues following injury or inflammation. Although a clearly defined role for MPCs in tumor development is still debated, multiple studies have now suggested that (i) MPC are mobilized to tumors from the bone marrow or nearby tissue and (ii) MPC enable metastasis, angiogenesis, and resistance to chemotherapy. However, a clearly defined role for MPC in tumors has been hampered by difficulty in characterizing these cells using standard cell surface markers. MPCs share many characteristics with other cells of mesodermal origin (e.g. fibroblasts); thus, identifying these specific cells in solid tumors has been challenging. A unique cell surface marker or genetically modified mouse model for lineage tracing studies would greatly facilitate the ability to specifically track the mobilization, recruitment, and trapping of MPCs in solid malignancies.

Despite the challenges identifying MPC in tumors, *in vitro* and *in vivo* evidence suggests that tumor cells have the ability to mobilize or recruit MPCs or similar cells with MPC characteristics. Trans-well migration assays have shown that tumor cell-conditioned media can directly stimulate MPC migration *in vitro*. Tumor-derived chemokines and inflammatory factors including IL-6 and SDF-1 will potently mobilize MPC (Bergfeld and Declerck 2010). To track MSCs *in vivo*, they are typically labeled with GFP and then introduced into the circulation of tumor-bearing mice. Although this is an artificial system that cannot fully recapitulate the "natural" process of MPC mobilization from bone marrow to the tumor site, these studies routinely demonstrate selective engraftment and tropism of MPCs in multiple tumor types (Kidd et al. 2009). Similar to hematopoietic cells, MPCs are mobilized to sites of injury, hypoxia or inflammation – all cardinal features of the tumor microenvironment. The ability of MPCs to "home" to these sites of injury has also been exploited as a strategy to deliver chemotherapeutic drugs.

The functional roles of MPCs in tumors are becoming clear. Karnoub et al. (2007) demonstrated that MPCs enhance breast carcinoma metastases through a CCL5/CCR5 axis. MPCs may also secrete factors that enhance the multidrug resistance properties of tumor cells. For example, Roodhart et al. (2011) recently demonstrated that upon treatment with platinum-derived chemotherapy, MPCs secrete polyunsaturated fatty acids that protect cancer cells and render them drug-resistant. This study nicely exemplifies a novel, MPC-mediated modulation of tumor response to chemotherapy. MPC (and other cells of mesenchymal origin) could also mediate drug resistance in tumors by directly impairing drug delivery. Particularly in pancreatic adenocarcinoma, one of the most intractable malignancies in humans, a desmoplastic stroma of α-SMA$^{+}$ carcinoma associated fibroblasts (CAFs) creates an impenetrable "fortress" which impedes delivery of chemotherapy (Olive et al. 2009). Notably, some CAFs may be derived directly from MPCs. For example, a significant proportion of CAFs in inflammation-associated gastric cancer were of bone marrow origin, were recruited to tumors at the earliest stages of development and could sustain tumor-associated inflammation by relocating the bone marrow niche to the tumor microenvironment (Quante et al. 2011).

MPC differentiation is controlled by the local production of cytokines and differentiation factors; thus, MPC usually differentiate into the type of tissue they engraft. The multi-potent abilities of MPCs, coupled with aberrant expression of tumor-derived growth/differentiation factors, could result in unexpected patterns of differentiation within the tumor stroma. For example, ectopic calcified nodules (hydroxyapatite) in breast, prostate, and lung tumors may be linked to tumor-mediated MPC differentiation. Wang et al. (2009) demonstrated that intravenously injected MPC localized to lung tumor nodules and formed bone whereas MPC at the primary tumor site (subcutaneous) formed fat. These tumor-driven differentiation programs in the MPC pool may compliment and support growth; for example, tumor-associated adipocytes may secrete factors (not normally expressed by undifferentiated MPC) that enable the growth and survival of malignant cells. The ability of MPC to form pericytes is a direct link to tumor angiogenesis. Bone marrow-derived MPC may differentiate into pericytes or reside in a perivascular position as mesodermal precursors that support endothelial cell survival by secreting high concentrations of VEGF (Crisan et al. 2008).

## Perspective

The field of cancer research has experienced many revolutions over the years. The "-omics" approach to classifying tumors by genetic makeup, metabolic profile or intrinsic drug-resistance properties comes to mind. Each of these approaches promises to advance our understanding of tumor formation and likelihood for tumor progression in patients while identifying the best modalities for therapy. The realization that tumors are more than homogeneous "spheres" of malignant cells and instead are heterogeneous mixtures of malignant cells and tumor-supporting stromal cells is another revolution in the field. This fact has opened the door to novel treatment options (anti-angiogenesis) and unexpected cellular targets (hematopoietic cells) as alternative antitumor strategies. One surprising finding is the complex cross-talk that occurs between tumor cells and stromal cells nearby; moreover, tumors can communicate "at a distance" with the bone marrow and perhaps other tissues outside of the primary tumor site. In a nice example, tumors may prepare the vascular bed of future metastatic sites before the first colonizing tumor cell ever arrives (Kaplan et al. 2005). Viewing cancer as a systemic disease whereby malignant cells utilize multiple, overlapping cell types from nearby tissue and from the bone marrow will identify new therapeutic options and increase our understanding of how tumors develop, how they create new blood vessels and how they metastasize.

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# Myelodysplastic Stem Cells: Gene Expression Profiling

6

Emiliano Fabiani, Francesco D'Alò, Giuseppe Leone, and Maria Teresa Voso

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**Abstract**

Myelodysplastic syndromes (MDS) represent a group of diseases associated with bone marrow failure arising from the interaction between a clonal and deranged hematopoietic stem cell (HSC) and a deregulated bone marrow microenvironment. Several abnormalities have been described in the development of myelodysplastic stem cells which give origin to dysplastic hematopoiesis, including increased apoptosis, decreased survival, depletion of early hematopoietic cells and abnormal differentiation. Gene expression profiling (GEP) studies have allowed a clear discrimination between normal and myelodysplastic HSCs. Moreover, distinct gene expression signatures have been associated with disease stage (early versus advanced), prognosis, morphology and presence of recurrent chromosomal abnormalities, such as monosomy 7/deletion 7q, trisomy 8 and deletion 5q. Most interestingly, GEP has allowed the identification of crucial genes and biologic pathways deranged in MDS including among all interferon signaling, ribosomal protein biogenesis, immune response, Wnt/ β-catenin signalling, cell cycle control and DNA damage response. These pathways beside shedding light on the comprehension of MDS pathobiology, may represent the basis for further investigations and realization of a more targeted approach in the therapeutic management of these diseases. Strictly correlated to GEP, miRNA profiling identified distinct miRNAs deregulated in

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MDS, which can significantly influence expression of coding transcripts in myelodysplastic stem cells.

## Introduction

Myelodysplastic Syndromes (MDS) are a heterogeneous group of neoplastic disorders of the hematopoietic tissue mainly occurring in older adults. They are characterized by uni- or multilineage dysplasia, ineffective hematopoiesis, peripheral cytopenias and increased risk of evolution into overt acute myeloid leukemia (AML). MDS classification takes into account morphological and cytogenetic criteria including number of cytopenias and dysplastic lineages, blast percentage in bone marrow and peripheral blood, and presence of recurrent cytogenetic abnormalities. According to the World Health Organization (WHO) classification system, seven nosographic entities have been identified: Refractory Cytopenias with Unilineage Dysplasia (RCUD), including Refractory Anemia (RA), Refractory Thrombocytopenia (RT) and Refractory Neutropenia (RN); Refractory Anemia with Ringed Syderoblasts (RARS); Refractory Cytopenia with Multilineage Dysplasia (RCMD); Refractory Anemia with Excess Blast-1 (RAEB-1); Refractory Anemia with Excess Blast-2 (RAEB-2); Myelodysplastic Syndromes unclassified (MDS-U); MDS associated with isolated del(5q) (Swerdlow et al. 2008).

MDS occurring in patients with history of previous cytotoxic chemotherapy or radiation therapy for a prior neoplastic or non-neoplastic disorder are classified as therapy-related Myeloid Neoplasms (t-MN). Borderline disorders presenting with both proliferative and dysplastic findings are classified separately from MDS as Myelodysplastic/Myeloproliferative neoplasms (MDS/MPN).

Karyotype, bone marrow blast percentage, number of peripheral blood cytopenias, WHO morphologic classification and transfusion requirements concur to define score systems, such as the International Prognostic Score System (IPSS) and the WHO classification-based Prognostic Score System (WPSS), which stratify patients in risk classes associated to different survival and risk of AML evolution (Greenberg et al. 1997; Malcovati et al. 2007).

Several factors contribute to set the bone marrow failure in MDS. Despite peripheral cytopenias, bone marrow in MDS patients is often hypercellular with coexistence of increased proliferation and excessive apoptosis, resulting in hematopoietic precursors prematurely eliminated in the marrow without reaching the peripheral blood. Bone marrow microenvironment and a deregulated cytokine network concur to MDS pathology and progression, according to a model of *"seed and soil"* disease. Nevertheless, the clonal hematopoietic stem cell (HSC) plays the main role in MDS pathogenesis, with the acquisition of several genetic and epigenetic changes that finally alter its properties of proliferation, self-renewal, differentiation and interaction with the microenvironment. In particular, MDS hematopoietic cell progenitors are characterized by increased apoptosis, decreased proliferation and survival, and depletion of early hematopoietic cells (Nimer 2008).

Gene expression profiling (GEP) based on the microarray technology represent a powerful tool to study disease biology, allowing the simultaneous quantification of the expression of several thousands of genes, through the hybridization of patient genetic material to oligonucleotides, followed by digital evaluation of the fluorescence intensity of the tagged genetic material. In particular, microarrays consist of a high number of regularly spaced DNA probes which are immobilized on a solid surface. The pool of transcripts in a given patient sample is labeled with a fluorescent dye and hybridized to the microarray. The fluorescent signal bound to the probe serves as an indicator of the expression of the corresponding transcript. The probe on the array may be represented as cDNA, oligonucleotides, or larger genomic fragments. Currently available commercial arrays have now reached whole transcriptome coverage (Bacher et al. 2010).

More than a decade ago, Golub et al. (1999) first reported that GEP is able to predict leukemia subclasses. Subsequently, many GEP studies in onco-hematology have been able to identify well

characterized disease subclasses ("class prediction") and discover novel clinically relevant subgroups of patients ("class discovery"), with significant contribution to diagnosis, classification and prognostic stratification of hematological malignancies (Bacher et al. 2010; Theilgaard-Mönch et al. 2011).

GEP have been analyzed in MDS using different purified or unselected bone marrow hematopoietic cells, microarray chips and patient populations, producing a huge amount of data with wide result variability. Nevertheless, this approach has allowed a better comprehension of MDS biology through the identification of several differentially expressed genes and crucial deregulated cellular pathways, which can distinguish MDS stem cell from their normal counterpart, characterize distinct disease entities, predict prognosis and response to treatment (Bacher et al. 2010; Theilgaard-Mönch et al. 2011).

Compared to other hematopoietic and lymphoid malignancies, the impact of GEP on MDS classification has been hampered by the heterogeneity of the bone marrow population, with blast percentage representing less than 20 % of bone marrow nucleated cells, therefore requiring the purification of selected cell populations. This issue is even more challenging when the aim of GEP studies is to characterize myelodysplastic stem cells.

Leukemia-initiating cells (LICs) or leukemia stem cells (LSCs) are defined by their ability to form tumours after xenotransplantation in immunodeficient mice and represent a small fraction of the neoplastic population (Testa 2011). What is the real myelodysplastic stem cell is still an unsolved question. The application of immunodeficient mouse xenograft models to MDS has been difficult. The *non-obese diabetic/severe combined immunodeficient* (NOD/SCID) mice were not a good model to allow expansion of clonal MDS precursors (Benito et al. 2003), while lineage negative (Lin-) bone marrow cells from MDS patients have been able to repopulate, at least transiently, NOD/SCID-beta2microglobulin−/− mice and produce abnormal differentiation patterns (Thanopoulou et al. 2004).

Normal HSCs able to reconstitute normal bone marrow are derived from CD34+CD38- cells and represent less than 0.1 % of total bone marrow cells. The CD34+CD38-Thy1+ fraction appears to harbour the HSCs in patients with MDS and carries the same cytogenetic abnormalities of the MDS clone (Nilsson et al. 2002). Several immunophenotypic abnormalities of the CD34+CD38-Thy1+ HSCs population have been described in MDS patients, showing frequent coexpression of CD13, CD33, CD117, CD133, and HLA-DR and variable expression of CD2, CD5, CD7, CD44, CD96, and CD123 (Xie et al. 2010).

GEP studies in MDS used different bone marrow or peripheral blood cell populations, with or without immunomagnetic selection. Most commonly, bone marrow AC133+ cells or CD34+ cells have been immunoselected for GEP in MDS. Despite severely limiting sample size with obviously technical amplification problems, these populations still present considerable heterogeneity and the true MDS stem cells represent a even smaller subset within these cell types.

Only few studies analyzed the most primitive CD34+CD38-Thy1+ fraction. It was reported by Nilsson et al. (2007) that myelodysplastic CD34+CD38+Thy1- progenitor cells contain a high number of differentially expressed genes compared to the normal counterpart, while only few distinct differential genes distinguish myelodysplastic and normal CD34+CD38-Thy1+ HSCs.

In this chapter, we will consider only those studies performed on immunoselected bone marrow cells coarsely representative of myelodysplastic stem cells. Data presented in the following paragraph will summarize most of the actual knowledge about GEP in MDS. A collection of the main studies on GEP on immunoselected hematopoietic progenitor cells in MDS are reported in Table 6.1.

## Gene Expression Profiling (GEP) Studies on Purified Hematopoietic Stem Cells (HSCs) in Myelodyplastic Syndromes (MDS)

The first study in MDS (Miyazato et al. 2001) performed GEP on AC133+ bone marrow hematopoietic cells from three patients with

**Table 6.1** Main studies on GEP on immunoselected hematopoietic progenitor cells in MDS

| Reference | Patients | Cell type | Technology | Results |
|---|---|---|---|---|
| Miyazato et al. (2001) | 1 MDS (RAEB)<br>2 MDS associated leukemia<br>3 AML | AC133+ BM cells | Commercial (HO-3) and custom-made array (Mergen) | Highly selective expression of the Dlk gene in MDS |
| Hofmann et al. (2002) | 10 low-risk MDS<br>9 high-risk MDS<br>4 normal BM | CD34+ BM cells | GeneChip HG-U95Av2 (Affymetrix) | Identification of 11 genes able to discriminate between low- and high-risk MDS |
| Ueda et al. (2003) | 11 RA<br>5 RAEB<br>14 MDS-associated leukemia | AC133+ BM cells | Commercial (HO-3) and custom-made array (Mergen) | Identification of stage-specific sets of genes in MDS |
| Chen et al. (2004) | 4 MDS with −7<br>13 MDS with +8<br>8 normal BM | CD34+ BM cells | GeneChip HG-U95Av2 (Affymetrix) | Different GEP associated to recurrent cytogenetic abnormalities in MDS |
| Sternberg et al. (2005) | 14 normal karyotype low-risk MDS<br>9 normal BM<br>5 non-MDS anemia | CD34+ BM cells | GeneChip HG-U133A (Affymetrix) | Down-regulation of B-cell lineage–affiliated genes in normal-karyotype low-risk MDS |
| Pellagatti et al. (2006) | 55 MDS (18 RA, 19 RARS, 18 RAEB)<br>11 healthy control | CD34+ BM cells | GeneChip HG-U133 Plus 2.0 (Affymetrix) | Up-regulation of ISG in most MDS; up-regulation of mitochondrial-related genes in RARS; down-regulation of genes located on 5q in del5q MDS |
| Prall et al. (2009) | 16 MDS<br>6 normal controls | CD34+ BM cells | AtlasHuman 1.2 I arrays (Clonotech) | Distinct GEP between normal and MDS CD34+ cells. Identification of two MDS subgroups with different survival |

| | | | | |
|---|---|---|---|---|
| Vasikova et al. (2009) | 4 early MDS<br>4 advanced MDS | CD34+ BM cells | Human 1A (V2) arrays (Agilent Technologies) | Discrimination between early and advanced MDS according to 286 differently expressed genes |
| Sridhar et al. (2009) | 35 MDS<br>6 healthy controls | CD34+ BM cells | 40 000 gene chip (Stanford Functional Genomics Microarray Facility) | Discrimination between normal and MDS CD34+ cells and between stable and subsequently transformed MDS |
| Pellagatti et al. (2010) | 183 MDS (55 RA, 48 RARS, 37 RAEB1, 43 RAEB2)<br>17 healthy control | CD34+ BM cells | GeneChip HG-U133 Plus 2.0 (Affymetrix) | The largest study identifying distinct GEP and deregulated pathways in early and advanced AML and in MDS with recurrent cytogenetic abnormalities |
| Baratti et al. (2010) | 7 RARS<br>4 healthy controls | CD34+ BM cells | Custom designed 44 k intron-exon oligoarrays (Agilent) | 30% of differentially-expressed genes codify for non-coding transcripts |
| Boultwood et al. (2007) | 10 5q- Syndromes<br>14 RA<br>16 normal controls | CD34+ BM cells | GeneChip HG-U133 Plus 2.0 (Affymetrix) | Most genes down-regulated in 5q- Syndrome belong to the CDR on 5q (gene dosage effect) |
| Nilsson et al. (2007) | 11 5q- syndrome MDS<br>10 healthy controls | CD34+CD38-Thy1+ HSC | Human Genome Oligo Version 2.1 and Human Genome Oligo Set Version 2.1 Upgrade (Operon Biotechnologies) | Fewer differences between normal and 5q-syndrome CD34+CD38-Thy1+ HSC than normal and 5q- syndrome CD34+CD38+Thy1- progenitors |

AML and three with MDS (1 RAEB and 2 MDS associated leukemia). By this approach, a number of genes were shown to be expressed in a disease-specific manner. Among these, the gene encoding the protein Delta-like (Dlk) was found to be selectively overexpressed in MDS samples. Results were confirmed by real time quantitative RT-PCR on a large cohort of MDS, AML and normal samples, suggesting a pathogenetic as well as diagnostic role for Dlk gene in MDS.

Hofmann et al. (2002) compared GEP of CD34+ BM cells in low-risk, high risk MDS and healthy subjects, identifying by class membership prediction 11 genes whose expression was able to accurately discriminate between patients with low-risk, high-risk MDS, and healthy controls. CD34+ cells from patients with low-risk MDS showed significant down-regulation of genes codifying for defensive proteins, such as RAI3, IEX1 and STIP1, that may lead to increased susceptibility to cell damage. These results support the clinical relevance of GEP for risk evaluation in MDS at the time of initial diagnosis. Similarly, GEP performed on AC133+ BM cells of 30 MDS patients identified sets of genes whose expression was specific to either indolent or advanced stages of MDS (Ueda et al. 2003). Among differentially expressed genes, the pro-apoptotic PIASy was markedly overexpressed in blasts from control subjects or RA patients, compared to blasts from RAEB or leukaemia secondary to MDS patients, making it a potential stage-dependent molecular marker in MDS.

Distinct GEP have been associated to the presence of recurrent cytogenetic abnormalities, such as monosomy 7 or trisomy 8 in CD34+ BM cells isolated from MDS patients (Chen et al. 2004). In trisomy 8, up-regulated genes were primarily immune and inflammatory response genes, while down-regulated genes were mainly involved in apoptosis inhibition. CD34+ cells in monosomy 7 MDS showed up-regulation of genes involved in leukemia transformation and down-regulation of genes controlling cell growth and differentiation. Sternberg et al. (2005) reported that expression of B-cell lineage–affiliated genes was decreased in CD34+ bone marrow progenitor cells from normal-karyotype/low-blast-count MDS patients, compared to age-matched controls and patients with "non-MDS" anemia. Consistently, flow cytometry on unfractionated marrow from independent samples also demonstrated reduced B-cell progenitors in MDS patients, compared to healthy controls.

Significant up-regulation of interferon-stimulated genes was found in CD34+ cells isolated from 55 MDS patients compared to normal subjects (Pellagatti et al. 2006). Up-regulated genes included 15 out of 22 genes previously identified in normal BM CD34+ HSCs and stromal cells treated *in vitro* with γ-interferon (Zeng et al. 2006). Among the most significantly up-regulated genes there were IFITM1 and IFIT1. In this study, CD34+ cells from RARS patients were characterized by up-regulation of mitochondrial-related genes and, in particular, of those associated to heme synthesis, such as ALAS2. Moreover, a distinct gene expression profile was observed in CD34+ cells from patients with del5q, characterized by down-regulation of genes assigned to 5q and up-regulation of the histone HIST1 gene cluster at chromosome 6p21 and of genes related to the actin cytoskeleton (Pellagatti et al. 2006).

Analyzing differential GEP of bone marrow CD34+ cells from 16 MDS patients and 6 healthy controls, Prall et al. (2009) showed increased expression of genes involved in proliferation, induction of apoptosis, DNA-repair and detoxification-/stress-response in MDS samples. Moreover MDS CD34+ cells presented lower expression of anti-apoptotic genes and several genes encoding cytokines, inflammation-related proteins and growth factors/-receptors. The unsupervised hierarchical clustering analysis was able to separate controls from MDS patients and identify two MDS subgroups associated to different median survival (8 months versus 48 months, $P=0.0016$). A less significant association was found between GEP MDS subgroups and WHO type ($P=0.032$), but no association was found with other clinical parameters. "Short survival" MDS subgroups showed increased expression of six genes encoding the proteasome subunits, while the most down-regulated gene was the anti-apoptotic gene IEX-1. Vasikova et al. (2009) were able to discriminate between early and

advanced MDS according to the expression levels of 286 differently expressed genes in CD34+ BM cells. Of these, 136 genes were up-regulated and 150 down-regulated in early MDS compared to advanced MDS. Among identified genes, BIRC5 expression decreased in advanced stages of MDS, while higher ADAM8 and MPL expression was observed in advanced MDS.

Sridhar et al. (2009) found 1,175 genes differentially expressed in MDS versus normal CD34+ BM cells, with a minimal classifier requiring 39 genes, 26 of which were overexpressed and 13 underexpressed in MDS. Among these, there was a predominance of genes involved in transcription, cytoskeleton, metabolism, and signaling/transport. Distinctive GEP were found between MDS evolving into AML and stable MDS. In particular, a “poor risk” gene signature identified six overexpressed genes (RPL23, RPS4X, RPS25, RPS19, KLK3 and TPP2) including four genes codifying for ribosomal proteins, which were associated with AML transformation and provided additive prognostic information for IPSS Intermediate-1 patients. In leukemia secondary to MDS compared to normal controls, the gene set enrichment analysis (GSEA) identified several genes involved with ribosomal protein biosynthesis, Myc and Wnt signalling pathways, while increased levels of apoptosis-related genes were present in stable MDS. Comparison of del(5q) with other MDS subtypes identified 1924 differentially expressed genes, with underexpression of 1,014 genes, 11 of which were within the 5q31-32 commonly deleted region.

The largest study so far performed to characterize the gene expression profile of MDS HSCs compared CD34+ BM cells of 183 MDS patients versus 17 healthy controls (Pellagatti et al. 2010). Thirty-five genes resulted up-regulated in more than 50% of MDS patients, whereas 139 genes were down-regulated. The most significant for commonly up-regulated genes was the ‘interferon signaling’ pathway, whereas the most significant for commonly down-regulated genes include the ‘Wnt/β-catenin signaling’ pathway. In early MDS, mainly deregulated pathways were ‘immunodeficiency,’ ‘B-cell receptor,’ ‘IL4’, ‘chemokine’, and ‘apoptosis signaling’. Advanced MDS were characterized by deregulation of cell cycle control and DNA damage response (DDR). In particular most interesting down-regulated genes related to DDR in RAEB-2 were RAD51 and BRCA2, implicated in the Double Strand Break Repair through Homologous Recombination, and FANCA and FANCD2, two genes which are involved in the pathogenesis of Fanconi anemia. Distinct gene expression profiles and deregulated gene pathways were found in patients with del5q, trisomy 8 or monosomy 7/del7q. Patients with trisomy 8 were characterized by deregulation of pathways involved in the immune response, whereas patients with del(5q) showed deregulation of ‘Wnt/β-catenin signaling’, ‘integrin signaling’ and ‘cell cycle regulation’. Multiple pathways that have an important function in cell survival, differentiation, apoptosis and growth were significantly deregulated in monosomy 7/del(7q) MDS, including SAPK/JNK, NF-kB, PI3K/AKT and ceramide signaling pathways. Noteworthy, R-Ras2, a gene with a significant role in tumorigenesis and cell growth regulation, was up-regulated in MDS patients with monosomy 7/del(7q) but not in other MDS cytogenetic groups. R-Ras2 oncogenic signals are mediated by the PI3K/Akt and NF-KB signalling pathways either found deregulated in −7/del(7q) MDS patients,

## Gene Expression Profiling and Myelodyplastic Syndromes Subtypes

Refractory anemia with ringed sideroblasts (RARS) is a low-risk MDS where an excess of iron accumulates in the perinuclear mitochondria of ringed sideroblasts, in the form of mitochondrial ferritin. GEP studies have shown distinct genetic signature associated to RARS, characterized by up-regulation of mitochondrial-related genes and, in particular, of those of heme synthesis, such as ALAS2 (Pellagatti et al. 2006). Among differentially expressed genes emerged the ATP-binding cassette, sub-family B (MDR/TAP), member 7 (ABCB7), which is implicated in the transport of Fe-S clusters from mitochondria,

where they are formed, to the cytosol, where they can be incorporated into proteins, maintaining in this way the mitochondrial iron homeostasis. ABCB7 mutations have been described in X-linked sideroblastic anemia and ataxia. ABCB7 expression was shown to be significantly lower in CD34+ BM cells and in erythroblast cultures from RARS patients compared to other MDS subtypes and healthy controls. No mutations or differences in ABCB7 promoter methylation were found, compared to healthy controls (Boultwood et al. 2008).

Baratti et al. (2010) compared GEP of CD34+ cells from RARS patients to controls using custom-designed combined intron-exon expression oligoarrays, including probes for protein-coding genes and for both sense and antisense strands of non-coding RNAs. Differentially expressed protein-coding genes were related to mitochondrial iron metabolism, heme biosynthesis, transferrin trafficking, B-cell receptor pathway signalling, cell adhesion, apoptosis, ion transport and regulation of transcription. Interestingly 65 out of the 216 differentially expressed genes in RARS codified for non-coding RNAs (32 down-regulated and 33 up-regulated), suggesting that they may play an important role during the development of myelodysplastic syndromes.

The 5q- syndrome is a distinct clinical entity, often diagnosed in elderly women, defined by bone marrow blast counts of less than 5 % , macrocytosis, anaemia, normal or high platelet counts, hypolobulated megakaryocytes and the presence of the del(5q) as the sole karyotypic abnormality (Swerdlow et al. 2008). The immunomodulatory drug lenalidomide, which has been shown to have dramatic therapeutic effects in patients with del(5q) MDS , is now the treatment of choice. Several studies have shown that CD34+ cells from MDS patients with del(5q) present a GEP distinct from other MDS subtypes and healthy controls (Pellagatti et al. 2006, 2010; Sridhar et al. 2009; Boultwood et al. 2007). Most differentially expressed genes in 5q- patients map to the chromosome 5q, with significant down-regulation of most genes mapping to the commonly deleted region (CDR), consistent with a gene dosage effect due to the loss of one allele of chromosome 5q (Boultwood et al. 2007). The gene pathway analysis performed on differentially expressed genes found several pathways deregulated in the 5q- syndrome, including the Wnt/β-catenin signalling, protein ubiquitination and actin cytoskeleton signalling pathways. Among interesting down-regulated genes, there were the tumour suppressor gene SPARC, the component of the 40S ribosomal subunit RPS14 and the RNA-binding protein RBM22.

Nilsson et al. (2007) studied the global gene expression profiling of highly purified 5q-deleted CD34+CD38-Thy1+ cells in 5q- MDSs. The number of differentially expressed genes was considerably less when comparing 5q-CD34+CD38-Thy-1+ cells with normal CD34+CD38-Thy-1+ cells than when comparing with more mature normal or 5q-CD34+CD38+Thy-1 progenitors. These findings support the origin of the 5q- clone from the true HSC. Expression of BMI1, encoding a critical regulator of self-renewal, was up-regulated in 5q- stem cells, while the myeloid transcription factor CEBPA presented a stage-specific expression with increased or normal levels in CD34+CD38-Thy1+ cells in most 5q- patients, and consistent dramatic down-regulation in 5q- CD34+CD38+Thy-1- progenitors.

## Lineage Specific Gene Expression Profiling and Drug-Induced Changes in Gene Expression

Beside the definition of gene expression signature in immunoselected MDS HSCs, GEP approach has been used to characterize alterations within the transcriptional program in MDS derived CD34+ cells during lineage-specific differentiation. Gueller et al. (2010) analyzed gene expression patterns of erythro-, granulo-, and megakaryopoiesis at defined time points during *in vitro* lineage-specific differentiation of bone marrow CD34+ cells derived from three healthy donors and from eight MDS patients (four with high-risk and four with low-risk). Gene expression of cells collected at days 0, 4, 7, and 11 during *in vitro* differentiation were analyzed by oligonucleotide microarrays (HG-U133A).

Compared to the continuous course of gene expression observed in normal hematopoietic differentiation, erythropoietic differentiation in MDS was associated with 33 % up-regulated (7 of 21) and 10 % down-regulated (6 of 58) genes; in myelodysplastic granulopoiesis, there were 9 % up-regulated (8 of 91) and 3 % down-regulated (1 of 37) genes, while myelodysplastic megakaryopoiesis was associated with 19 % (4 of 21) and 13 % (4 of 30) up-regulated and down-regulated genes, respectively. Categorization of differentially expressed genes in all three hematopoietic lineages according to the molecular function, yielded seven functional groups, including genes involved in binding, catalytic, signal transduction, transcriptional regulation, structure, enzymatic regulation, and transport activity. Several genes associated with basic erythropoietic functions were dysregulated in MDS cells, including Ankyrin 1, Tropomodulin, and genes coding for components of signaling pathways, such as RAP1GA1, ARHGAP1 and PP2A. Genes deregulated during myelodysplastic granulopoiesis included genes of the granulocytic membrane and prostaglandin-endoperoxide synthase 2. Although up-regulated in MDS stem cells, Dlk1 gene expression continuously decreased during megakaryopoietic differentiation, in contrast to normal cells.

Effects of new therapeutic strategies have been studied by GEP in myelodysplastic stem cells. Promoter hypermethylation leading to silencing of tumor suppressor genes significantly contributes to establish gene expression signatures and has been identified as the dominant mechanism involved in MDS progression to AML (Jiang et al. 2009). In the last decade, hypomethylating agents have emerged as standard treatment for patients with intermediate-2/high risk MDS, and have been able to reduce transfusion requirements and leukemic evolution as well as increasing survival (D'Alò et al. 2010). To study specific biologic pathway reactivated by hypomethylating treatment in myelodysplastic stem cells, our group studied the GEP of intermediate-2/high risk MDS bone marrow CD34+ hematopoietic cells treated *in vitro* with the hypomethylating agent 5-aza-2′deoxycytidine (decitabine) for 72 h (Giachelia et al. 2011). Despite the huge variability in differential gene expression, a significantly higher number of genes were upregulated by hypomethylating treatment in myelodysplastc cells, especially with abnormal karyotype, compared to the normal counterpart. Seven genes were exclusively induced by decitabine in MDS, but not in normal CD34+ cells and were down-regulated in mock-treated MDS CD34+ cells compared to normal CD34+ cells, including CD9, GPNMB, PLA2G7, TPM1, ANGPT1, FUCA1 and ARHGEF3. A significantly higher number of genes were induced by decitabine in abnormal karyotype MDS (224 induced and 68 suppressed genes), versus normal karyotype MDS (18 induced and 30 suppressed), suggesting a more pronounced effect of hypomethylating treatment in MDS with abnormal karyotype.

Lenalidomide is highly effective in patients the 5q- syndrome, resulting in complete cytogenetic remission in more than 60 % of patients. To gain insight into molecular targets of lenalidomide, Pellagatti et al. (2007) investigated effects of this drug on CD34+ cells isolated for MDS patients with del(5q) and grown *in vitro* in isolated erythroblast cultures. Lenalidomide inhibited growth of differentiating del(5q) erythroblasts, but did not affect cytogenetically normal cells. By GEP analysis, lenalidomide significantly influenced the pattern of gene expression in del(5q) intermediate erythroblasts, with the VSIG4, PPIC, TPBG, activin A, and SPARC genes up-regulated more than twofold in all samples, and many genes involved in erythropoiesis, including HBA2, HBB, SPTA1, GYPA, GYPB, ALAS2, and KLF1, down-regulated in most samples.

## Distinctive MicroRNA Expression Profiles in CD34+ Cells from Myelodyplastic Syndrome Patients

MicroRNAs (miRNAs) are endogenous small non-coding RNAs, made by 18–25 nucleotides, able to negatively regulate the expression of complementary genes by translational inhibition and target mRNA degradation, finally resulting in gene silencing. Since several pathways and

cellular processes are under control of miRNAs (more than 60 % of gene are miRNA target), the evaluation of miRNA expression may be directly correlated with the gene expression changes found in MDS HSCs.

Several studies have addressed the differential miRNA expression in MDS compared to normal bone marrow (Rhyasen and Starczynowski 2011), but only two of them analyzed CD34+ immunoselected bone marrow hematopoietic progenitors (Dostalova Merkerova et al. 2011; Votavova et al. 2011).

Dostalova Merkerova et al. (2011) performed miRNA microarray analysis on purified CD34+ BM cells obtained from 6 healthy donors and 39 patients with MDS and AML evolved from a previous MDS. The expression levels of 1,145 miRNAs were tested and, among these, 371 miRNAs resulted unexpressed, whereas the remaining 774 miRNAs were used for the evaluation of miRNAs expression profiling in CD34+ BM cells. A set of 22 miRNAs differentially expressed between patients and controls was identified: 13 were up-regulated (miR-299-3p, miR-299-5p, miR-323-3p, miR-329, miR-370, miR-409-3p, miR-431, miR-432, miR-494, miR-654-5p, miR-665, HS_40 and HS_206), and 9 down-regulated (miR-196a*, miR-423-5p, miR-525-5p, miR-507, miR-583, miR-940, miR-1284, miR-1305 and HS_122.1) in MDS patients. Interestingly, 9 of 13 up-regulated miRNAs mapped within the 14q32 cluster, which comprises 40 miRNA implicated in cell development and oncogenesis and whose imprinted expression is controlled by a distant differentially methylated region (DMR). Authors hypothesized that the cluster up-regulation might be caused by altered imprinting in DMR, possibly because of uniparental disomy and/or deregulated epigenetic mechanisms in 14q32. Differences in miRNA expression were observed between early and advanced MDS. In particular RAEB-1 was clearly distinguished from RAEB-2, with a miRNA expression profile very similar to early MDS and controls. On the contrary, RAEB-2 miRNA profiles resembled those of secondary AML (Dostalova Merkerova et al. 2011).

The characterization of miRNA expression in 5q- syndrome gave alternative results according to different platform and assays performed. Dostalova Merkerova et al. (2011) reported that 13 miRNAs are elevated and seven reduced in 5q- patients. Among miRNAs located in the commonly deleted region in the 5q- syndrome, only miR-378 and miR-143* (minor variant of miR-143) were down-regulated, whereas no differences were found in the expression of miR-146a and miR145. On the contrary, studying miRNA expression in CD34+ BM cells from seven patients with 5q- syndrome and five healthy controls, Votavova et al. (2011) found down regulation of miR-378, and miR-146a but not miR-143*. Moreover, 21 miRNAs were differentially expressed between 5q- MDS patients and controls (17 over-expressed and 4 under-expressed). Unsupervised hierarchical clustering performed on this set could discriminate between patients and controls. The most up-regulated miRNA was miR-34a, a proapoptotic transcriptional target of p53. Since the expression of miR-34a promotes apoptosis through inhibition of the Bcl2 target genes, high miR-34a expression could concur to the increased apoptosis of bone marrow progenitor cells in 5q- patients. Among up-regulated miRNAs there were miR-10a and miR-10b, a couple of miRNAs localized in the cluster of HOX genes and implicated in haematopoiesis and leukemogenesis. Their expression correlates with HOX gene expression, suggesting their modulation by the same regulators as those of HOX genes (Votavova et al. 2011).

In conclusion, GEP studies are a deductive research approach, which has sometimes lead to long lists of deregulated genes and biologic pathways, where it has been hard to discriminate between important phenomena and background noise. Notwithstanding, GEP has allowed a clear discrimination of the myelodysplastic hematopoietic CD34+ cells (comprehensive of the real myelodysplastic stem cell) from the normal counterpart. Considering that MDS diagnosis relies primarily on the morphologic assessment of the patient's bone marrow and peripheral blood cells (and morphologists do never completely agree), sometimes without any helpful cytogenetic and immunophenotypic markers of dysplasia, the experience derived from GEP could be applied in

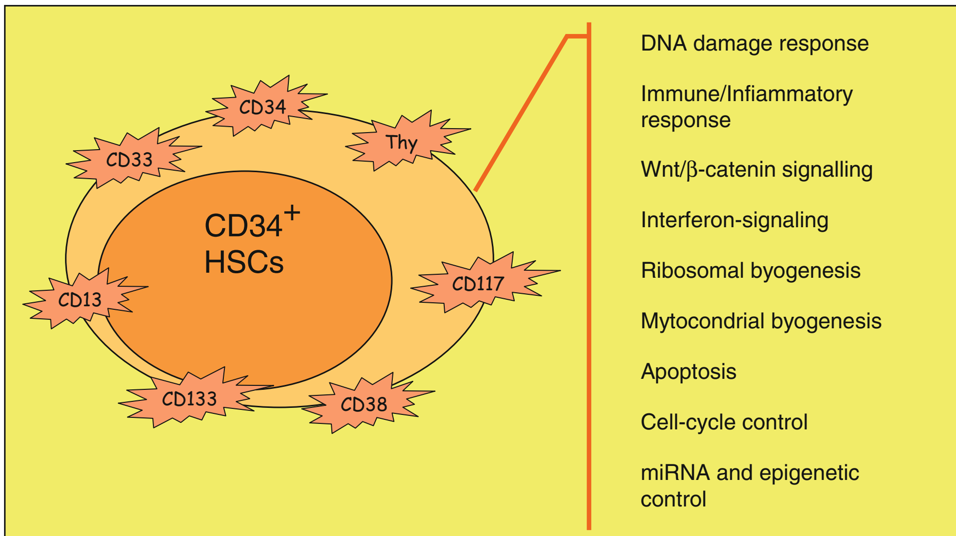


**Fig. 6.1** Principal pathways dysregulated in myelodysplastic CD34+ cells

the next future to refine the diagnostic process. The identification of a limited set of discriminating genes able to distinguish between normal and myelodysplastic hematopoiesis, morphologic and cytogenetic MDS subtypes, early and advanced MDS may lead to patient-tailored MDS management. Moreover, GEP would add further prognostic information besides current clinical and laboratory parameters and could contribute to identify subsets of patients likely to take advantage by a certain therapy.

Noteworthy, in the last decade we have learnt more about MDS biology from GEP studies than from any other approach, by the identification of crucial cellular pathways deregulated in myelodysplastic hematopoiesis. Most data generated by GEP studies are in accordance with what we already knew about MDS pathobiology, and represent a huge step toward the comprehension of MDS development and behavior. Among the most significant deregulated pathways in MDS HSCs emerged those related to interferon-signaling and ribosomal protein biogenesis, as well as those related to immune response, apoptosis, cell-cycle control and DNA damage response (Fig. 6.1). Further studies are required to make of all these data the basis for future development of targeted therapies for MDS.

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# Part III

# Mesenchymal Stem Cells

# Human Mesenchymal Stem Cell Transdifferentiation to Neural Cells: Role of Tumor Necrosis Factor Alpha

7

Christian Ries and Virginia Egea

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**Abstract**

Mesenchymal stem cell (MSC)-based therapy is a promising new approach in regenerative medicine and anti-tumor strategies. The MSCs are easily accessible and can be rapidly expanded *in vitro*. Additionally, MSCs possess the useful characteristics of stem cell plasticity or transdifferentiation, cytokine/chemokine secretion, and tumor tropism. After *in vivo* administration, MSCs induce peripheral tolerance and migrate to sites of injury where they promote tissue repair and regeneration. Moreover, MSCs turned into a “Trojan horse” by introducing specific transgenes have potential as therapeutic vehicles to target aggressive inoperable tumors. Although local transplantation or injection of hMSCs may prove therapeutically useful, the ability to target MSCs to specific tissues with high efficiency will be crucial in developing new treatments. This article introduces MSCs as multipotent and multifunctional cells, addresses especially the properties of MSCs transdifferentiated by tumor necrosis factor (TNF-)α into neural cells, and discusses the potential of MSC-based strategies in the therapy of neurodegenerative diseases and injuries of the central nervous system.

## Introduction

Adult mesenchymal stem cells (MSCs), also referred to as multipotent (or mesenchymal) stromal cells, were first isolated from bone marrow and described as spindle-shaped cells that

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can serve as feeder layers for the culture of hematopoetic stem cells (Friedenstein et al. 1974). Bone marrow-derived stromal cells were later recognized for their ability to differentiate into mesodermal cell types such as adipocytes, chondrocytes, and osteocytes (Pittenger et al. 1999). In recent years, the notion that multipotent stem cells are restricted in their potency to form cell types of the same germ layer has been challenged. Several studies have reported that also MSCs have potential of crossing the germ layer boundary to form cell types of alternative layers – a process termed transdifferentiation – by adopting expression profiles as well as functional phenotypes of both endodermal and ectodermal cells (Phinney and Prockop 2007). Another fundamental property of MSCs is their secretion of a broad range of bioactive molecules regulating the function and behaviour of cells present in the MSC microenvironment (Singer and Caplan 2011). Based on their particular features MSCs have demonstrated significant potential for clinical use in regenerative medicine (Bernardo et al. 2012). The therapeutic applications of MSCs are focused to repair damaged tissues and treat inflammation resulting from cardiovascular disease, myocardial infarction, brain and spinal cord injury, cartilage and bone injury, Crohn's disease, and graft-versus-host disease during bone marrow transplantation (Phinney and Prockop 2007). Moreover, there is especially great interest in the use of MSCs to treat neurodegenerative disorders of the central nervous system (CNS) such as multiple sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease, as well as amyotrophic lateral sclerosis (Sadan et al. 2009). In contrast, therapeutical concepts based on the application of embryonic and neural stem cells to cure neurological diseases are limited by ethical and logistic problems. Thus, the clinical utility of MSCs is supported by several issues: (i) lack of ethical controversy; (ii) easy isolation from bone marrow or fat tissue; (iii) convenient expansion in cell culture; (iv) absence of significant immunogenicity permitting allogenic transplantation without immunosuppressive drugs; and (v) lack of tumorigenicity.

## Mesenchymal Stem Cells in Physiology and Tissue Repair

MSCs are a heterogenous population of cells that proliferate (self-renew) *in vitro* as plastic-adherent cells, have fibroblast-like morphology, and can differentiate into bone, cartilage, and fat cells. Although stromal cells apparently fulfilling the MSC criteria were isolated from almost every type of tissue and organ (da Silva et al. 2006), MSCs have mainly been characterized after isolation from the bone marrow. The adherent nature of MSCs facilitates their rapid enrichment from heterogenous starting cultures. In contrast to hematopoietic stem cells, MSCs can be expanded over several doublings without differentiation. Because, no specific and unique MSC marker exists so far, there is a general consensus to confirm the MSC phenotype by monitoring the positive expression of characteristic molecules including CD105, CD73, CD44, and STRO-1 and the negative expression of cell surface markers such as CD14, CD34, and CD45 which usually are antigens of hematopoietic cells.

The detection of MSCs in the peripheral blood of adult humans and other mammalian species (He et al. 2007) supports the hypothesis that MSCs can migrate from bone marrow or other organs into distant tissue sites. It is well known that damaged and inflamed tissues release increased amounts of inflammatory cytokines and chemokines including transforming growth factor β (TGF-β), interleukin 1β (Il-1β), stromal cell-derived factor 1 (SDF-1), and tumor necrosis factor α (TNF-α). These factors were shown to attract human MSCs (hMSCs) *in vitro* and facilitate their migration by upregulating the biosynthesis and secretion of specific matrix metalloproteinases (MMPs) in these cells (Ries et al. 2007). Thus, MSCs appear to be mobilized in response to inflammation or injury and target specific tissues via active mechanisms.

Two major paradigms describe the contribution of MSCs in the repair of tissues: (i) MSCs engraft and (trans-)differentiate to replace injured

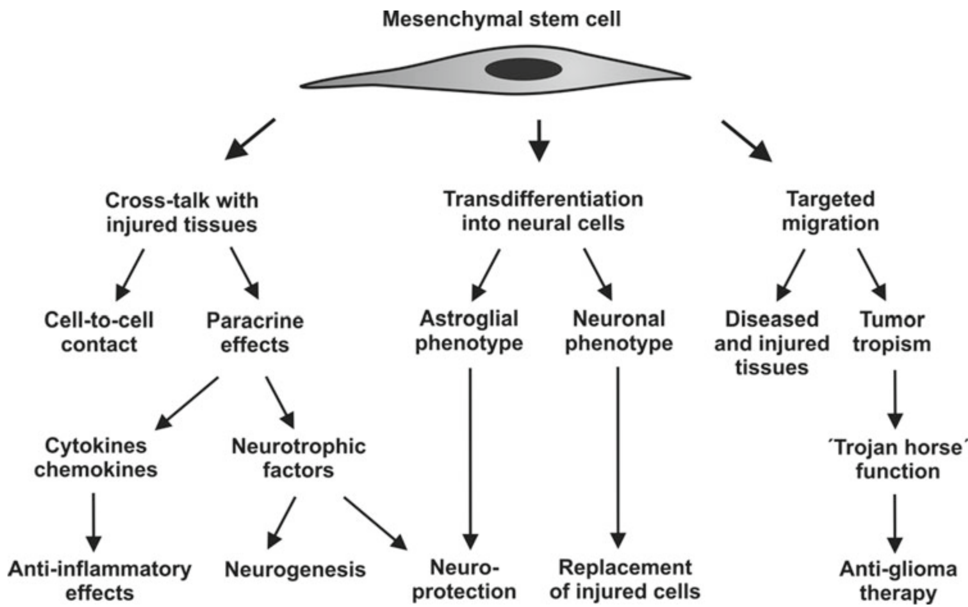


**Fig. 7.1** Possible mechanisms and effects of MSC-based clinical applications in patients with neurological diseases and injuries

cells; and (ii) MSCs engage in cross-talk with injured tissues thereby generating microenvironments that promote regeneration. Although there are still controversies about certain aspects of MSC functionality *in vivo* and the underlying molecular mechanisms, the beneficial effects observed in various animal models clearly indicate MSCs to be powerful therapeutic agents (Prockop 2009). Frequently, MSCs produce functional amelioration in injured and diseased tissues without engraftment and (trans-)differentiation but rather through cell-to-cell contact and release of paracrine factors. In culture, MSCs secrete a large number of cytokines, chemokines, and other factors with (neuro-) trophic, anti-inflammatory, and anti-apoptotic functions (Singer and Caplan 2011) (Fig.7.1). Apparently, these features of MSCs contribute to their broad immunoregulatory activities: MSCs were demonstrated to control gene expression, cell proliferation, and maturation in various immune cells such as dendritic cells, natural killer cells, neutrophil granulocytes, T-cells, and B-cells (Uccelli et al. 2008).

## Transdifferentiation of MSCs to Neural Cells

With respect to stem cell-based approaches in the therapy of neuronal disorders, neural stem cells are of primary interest as cellular resources for regenerative medicine in order to repair damage or disease of the CNS (Miller 2006). Principally, neural stem cells are able to differentiate into all major cell lineages of the brain, such as neurons, astroglia, and oligodendroglia. A major drawback in the use of neural stem cells, however, is their limited access from nervous tissues. In contrast, MSCs can be easily obtained from bone marrow or adipose tissue. During the past years numerous reports have described *in vitro* culture conditions demonstrating putative germ layer crossing and transdifferentiation of MSCs toward astroglial and/or neuronal phenotypes (Fig. 7.1). Some protocols are based on supplementation of differentiation media with simple chemicals or cocktails of small molecules (Woodbury et al. 2000), while others use mixtures of various growth factors or neurotrophic molecules (Hermann et al. 2006),

and apply transgenic expression of particular genes (Dezawa et al. 2004). Another approach is based on cocultures of MSCs with neural cell types in which MSCs are induced as a result of direct cell-cell interaction and/or interplay by soluble factors (Jiang et al. 2003).

Critical evaluation is required for protocols showing short-time transdifferentiation of hMSCs into neural cells by the use of chemical compounds as outlined by Bertani et al. (2005). The doubts predominantly arise from the fact that neural differentiation is a highly complex process that *in vivo* requires the spatial and temporal regulation of a wide array of genes. It is unlikely that MSCs undergo these complex molecular events to form mature, functional neural cells within a matter of hours. For instance, the *in vitro* differentiation of MSCs into their characteristic mesenchymal germ layer cell types of bone, fat, and cartilage occurs over a timescale of several days and weeks, suggesting that similar if not longer periods of time are required for MSC transdifferentiation into neural cells.

For evaluation of MSC development into a new characteristic cellular phenotype such as neural cells, a set of criteria should be fulfilled including the adoption of the morphology and functionality of native cells as well as the expression of cell type-specific markers and the cessation of expression of naive markers (Krabbe et al. 2005).

## Effect of TNF-α on hMSC Morphology and Neural Gene Expression

In physiological and pathological processes regulating the function and development of various cell types including neural stem/progenitor cells, cytokines such as TNF-α seem to play a major role (Bernardino et al. 2008). As shown by us in hMSCs, TNF-α acts as a potent chemoattractant and modulates the expression and activity of certain MMPs associated with the migratory potential of these cells (Ries et al. 2007). Our more recent studies revealed that long-term cultivation of hMSCs for 2–4 weeks in the presence of TNF-α (50 ng/ml) induces transdifferentiation into cells with a neuroglial-like morphology (Egea et al. 2011). After 2 weeks of treatment with TNF-α, hMSCs loose their typical flat spindle-shaped morphology and display a more elongated and contracted cell contour compared to non-treated cells. Upon 4 weeks of TNF-α exposition, the majority of cells develops spherical refractile cell bodies which prevalently show radial and/or branched cellular extensions (Egea et al. 2011). This star-shaped appearance is similar to that of astrocytes, the main type of glial cells in the CNS. Astrocytes are present in brain and spinal cord where they have various supporting functions and act as adult neural stem cells during repair processes following traumatic injuries (Buffo et al. 2010).

In addition to the adoption of glial cell morphology our microarray analysis of hMSCs after 14 days of incubation with TNF-α revealed upregulation of numerous gene transcripts which are characteristically expressed in neural cells and known for their relevance in the development of these cells such as GFAP (glial fibrillary acidic protein), MAP2 (microtubule-associated protein 2), LIF (leukemia inhibitory factor) and its receptor LIFR, BMP2 (bone morphogenetic protein 2), SOX2 (SRY box 2), as well as CXCR4 (CXC motive chemokine receptor 4). In contrast, the transcription level for nestin and vimentin were reduced in TNF-α-treated hMSCs (Egea et al. 2011). These findings confirm an astroglial nature of TNF-α-treated hMSCs because astrocytes are characteristically able to synthesize GFAP, a cytoskeletal filament protein, whereas the production of nestin and vimentin is abrogated in these cells (Laywell et al. 2007). Nestin is an intermediate filament which is essential in neuroepithelial stem cell function and becomes downregulated during maturation of these cells (Wiese et al. 2004). The rapid diminishment of nestin in TNF-α-treated hMSCs may be a consequence of the astroglial differentiation process initiated by this cytokine.

The transcription factor SOX-2 that is typically present in embryonic and neural stem cells being responsible for the maintenance of progenitor characteristics (Pevny and Nicolis 2010), is elevated in TNF-α-treated hMSCs on protein level suggesting an immature progenitor-like status

of these cells. Remarkably, marker proteins characteristically expressed in mature oligodendrocytes and neurons such as galactocerebroside and β-tubulin III or MAP-2, respectively, are not synthesized in hMSCs after incubation with TNF-α, eliminating the possibility of a neuronal phenotype in these cells (Egea et al. 2011).

It has been reported that neuroepithelial progenitor cells can be differentiated toward the astrocytic lineage by the combined application of the cytokines LIF and BMP-2 (Nakashima et al. 1999). Interestingly, TNF-α-treated hMSCs express elevated levels of BMP-2, LIF and LIF receptor (Egea et al. 2011). Thus, it can be hypothesized that the addition of TNF-α to hMSCs triggers the secretion of LIF as well as BMP-2 from the cells and thereby promotes astroglial differentiation by an autocrine stimulatory mechanism.

TNF-α-induced effects in cells can be mediated by different signal transduction pathways including that of the mitogen-activated protein kinases (MAPKs) (Cuschieri and Maier 2005). Our studies in hMSCs revealed that activity of the MAPK ERK1/2 is essential in the TNF-α-modulated expression of the neural genes *LIF, BMP2, SOX2,* and *NES (nestin)* (Egea et al. 2011). This mechanism may be causative for TNF-α-driven differentiation of hMSCs toward the neural lineage. Similar findings obtained in bone marrow stromal cells from other species (Yang et al. 2008) confirm the requirement of ERK activity and signalling in transdifferentiation of MSCs into neural cells.

## Effect of TNF-α on hMSC Migration and Tropism to Gliomas

A prerequisite for endogenous and transplanted stem cells to promote tissue repair and regeneration is their ability for directed migration into the areas of damage, chronic disease, or inflammation (Fig. 7.1). In tissues of the CNS, elevated concentrations of SDF-1 (or CXCL12) are present during development and injury attracting neural stem cells that typically synthesize high levels of the chemokine receptor CXCR4 specific for SDF-1 (Imitola et al. 2004) . This mechanism allows directed mobilization and recruitment of cells migrating along the SDF-1 gradients. Similar to neural stem cells, we could demonstrate that hMSCs express CXCR4 and are attracted by SDF-1 (Ries et al. 2007). Importantly, the pretreatment of hMSCs with TNF-α significantly upregulates the biosynthesis of CXCR4 in these cells resulting in an increased capacity for SDF-1α-directed invasion through barriers of human extracellular matrix (Egea et al. 2011) representing a requirement for tissue targeting by MSCs *in vivo*.

A striking feature of hMSCs is their pronounced tropism for malignant gliomas (Bexell et al. 2010). After intravascular or local delivery, hMSCs specifically integrate into gliomas attracted by the release of cytokines and growth factors from the tumor cells. Glioblastoma multiforme is the most aggressive form of glioma with a median survival time of only 14.6 months for patients after conventional therapy including resection, chemotherapy, and radiation (Stupp et al. 2005). Many high-grade gliomas fail to respond to therapy for a variety of reasons, including extensive seeding of microsatellites of tumor cells into normal brain and the resistance of glioma cells to radiation and chemotherapy. Thus, innovative therapeutic strategies are desperately needed. Accumulating evidence from numerous *in vitro* and *in vivo* studies has indicated the usefulness and therapeutic efficacy of MSCs from different sources including bone marrow, adipose tissue, and umbilical cord blood, applied as cellular vectors for anti-tumor gene delivery to malignant gliomas (Bexell et al. 2010). The genetic manipulation turning hMSCs into a ‘Trojan horse’ that produces for instance immunotherapeutic cytokines such as interferon-ß (Nakamizo et al. 2005), TNF-related apoptosis-inducing ligand (TRAIL) (Sasportas et al. 2009), or interleukin-12 (Ryu et al. 2011) has shown profound anti-tumor effects and significantly increased survival upon specific engraftment into gliomas grown in animal models (Fig. 7.1).

The success of these novel therapeutic strategies critically depends on an effective recruitment of hMSCs to glioma cells and tissues. Accordingly,

we could recently show that TNF-α-pretreatment of hMSCs promotes their local migration to and invasion into spheroids of glioma cells *in vitro* and enhances the *in vivo* tropism of hMSCs toward experimental glioma after their application by intravenous injection in nude mice (Egea et al. 2011). This effect can be explained by at least three major properties observed in TNF-α-treated hMSCs: (i) the adoption of a gene expression profile similar to neural progenitor cells known for their tropism to brain tumors; (ii) the upregulation of CXCR4, the receptor of the chemokine SDF-1 that is highly expressed in glioblastoma tissues and almost absent in normal brain; (iii) the elevated expression and proteolytic activity of MMPs (Ries et al. 2007) shown to facilitate the interpenetration of hMSCs and glioma cells (Egea et al. 2011).

A current strategy for clinical MSC-based cancer therapy is the direct implantation of genetically modified MSCs into inoperable tumors (Bexell et al. 2010). This approach, however, requires operative access to the tumor. Therefore, a less invasive alternative in the treatment of malignant gliomas might be the enhancement of tumor tropism in hMSCs by treatment with TNF-α prior to intravenous administration of these cells in patients.

## MSC-Based Therapy in Neurological Disorders

MSCs are one of the most promising sources for stem cell-based therapy of neurodegenerative diseases which are characterized by dysfunction and loss of neural cells and tissue (Sadan et al. 2009). In this context, MSC-based therapeutic approaches include (i) the replacement of damaged cells by transdifferentiation of MSCs and/or their *in situ* fusion with host neural cells, (ii) the improval of the environment of diseased neural tissues by release of neuroprotective and trophic factors from MSCs, and (iii) the induction or enhancement of neurogenesis (Fig. 7.1).

Parkinson's disease is characterized by loss of specific dopaminergic neurons in the substantia nigra. Studies in a rat model of Parkinson's disease provide evidence, that transplanted hMSCs after neural differentiation display dopaminergic properties and have regenerative effects in the diseased brain (Levy et al. 2008). Moreover, autologous MSC-derived neural progenitor cells after multiple intrathecal injections promote repair and recovery in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (Harris et al. 2012). In addition, MSCs programmed to become neural cells by engineering overexpression of neurogenin 1 exhibit characteristics of neuronal cells, and were demonstrated to integrate into ischemic brain tissue and improve motor function in a rat stroke model (Kim et al. 2008). These and additional studies in animal models strongly suggest a great potential of MSC-based cell therapy to ameliorate the symptoms of various neurological diseases.

As a first step in humans, a clinical trial conducted on patients suffering from amyotrophic lateral sclerosis proved intrathecal transplantation of hMSCs to be safe and tolerable (Mazzini et al. 2010). The results encourage future trials to demonstrate efficacy. Similar to neurodegenerative diseases, major human brain and spinal cord injuries currently do not have effective treatments. Transplantation of MSCs from humans, primates, and rodents has provided functional improvement in various animal models of nervous tissue injuries including ischemic stroke, traumatic brain injury, and traumatic spinal cord injury (Parr et al. 2007). The transplantation of MSCs may repair injured nervous tissues through replacement of damaged cells, neuroprotection, and/or the creation of an environment conductive to regeneration by endogenous cells.

Promising data indicate that grafted MSCs provide a source of growth factors to enhance the axonal elongation across spinal cord lesions (Wright et al. 2011). A recent report demonstrating the positive contribution of astrocytic progenitor-like cells to the healing and regeneration process after brain tissue damage (Buffo et al. 2010) strongly suggests the potential usefulness of hMSC-derived astrocytic cells for reparative strategies in the CNS.

In conclusion, accumulating evidence suggests that the application of MSCs represents a

promising therapeutic route for the treatment of neurodegenerative diseases and CNS injuries. The mechanisms of MSC-mediated neural functional recovery include the capacity of MSCs for directed migration and engraftment into neural tissues, transdifferentiation of MSCs into neural cell types, and/or MSC secretion of paracrine factors (Maltman et al. 2011). The pretreatment of autologous or allogenic MSCs with TNF-α is one possible strategy to enhance the therapeutical potential of these cells *ex vivo* by inducing a neural progenitor cell-like phenotype with an increased efficiency in targeting neural tissues and appropriate secretion of neurotrophic/neuroprotective factors. Remarkably, the neural cells generated by incubation of hMSCs with TNF-α are fully mitotic (Egea et al. 2011) favouring their *in vitro*-expansion before transplantation into patients.

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# 8 Role of Mesenchymal Stem Cells (MSC) in HIV-1 Associated Bone and Lipid Toxicities

Eoin J. Cotter, Robert T. Maughan, and Peter P. Doran

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**Abstract**

In addition to its well-recognised impact on the immune system, HIV-1 infection has long been associated with disorders of other tissue types, and with the advent of long term anti-retroviral therapy specific toxicities of both bone and lipid tissues have emerged. These issues are of significant clinical importance in a population receiving life-time therapy. The mechanisms underpinning these toxicities have yet to be fully understood, and the relative contribution of virus and treatment (especially in the case of bone toxicities) fully elucidated.

Mesenchymal stem cells (MSCs) are pluripotent cells that can differentiate into cells, amongst others, of osteoblast and adipocyte lineage. They are largely resident in the bone marrow – although mesenchymal/stromal precursor cell 'pools' can be found in other tissues including adipose tissue. Increasingly MSCs are considered to play an important role in tissue homeostasis, repair and response to injury. In addition to their roles as bone/fat progenitor cells, they can also express receptors which could allow their infection by HIV-1. This chapter will deal with the prevalence of HIV-1 associated toxicities of lipid and bone, and treat of the existing evidence for a role for MSCs in the pathogenesis of these phenomena.

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## Introduction

From a clinical standpoint, HIV-1 infection has in recent decades evolved from an inherently fatal condition to one which can be managed by appropriate highly active antiretroviral therapy (HAART). The objectives of HAART are to stably reducing plasma viral load below 50 copies/ml and to normalise CD4+ cell counts in the long-term; thus, restoring immune function. HAART is a combination therapy; it usually consists of at least two-drug antiretroviral regimen comprising, (1) HIV-1 protease inhibitor (PI, which prevents the production of the viral capsid) and, (2) HIV-1 reverse-transcriptase inhibitor (nRTI, nnRTI – which prevents the production of viral DNA in initial infection) drugs. Administration of HAART has proved to significantly decrease patient mortality even in those with advanced disease.

However, despite the successes of HAART, significant clinical problems have emerged in the HIV-1 infected population. These include abnormalities of lipid metabolism; including lipoatrophy/lipodystrophy, dyslipidemia, increased cardiovascular risk, and reduced bone mineral density (McComsey et al. 2010; Feeney and Mallon 2011). While these abnormalities have been increasingly well described clinically, their underlying causes remain to be fully understood. In addition to the prevalence of these toxicities is the persistence of so-called 'viral-reservoirs', populations of latently infected cells which allow the HIV-1 virus to 'survive' the onslaught of HAART – meaning that HIV-1 therapy only succeeds in controlling rather than eradicating the virus.

Mesenchymal stem cells (MSCs) are pluripotent cells which have been shown to play a vital role in the regeneration and repair of an ever increasing number of tissue types, as well as possessing important immunomodulatory properties (Bai et al. 2009). They were originally considered to be solely resident in the bone marrow niche, however more recently 'pools' of MSCs and MSC-like cells have been detected in a diverse range of tissues, from the vascular wall to adipose tissue. They are particularly interesting in the context of HIV-1 infection as they are the cells from which both adipocytes(ACs) and osteoblasts(OBs) are derived, and play an important role in bone and lipid homeostasis. In addition, they can express the receptors necessary for HIV-1 fusion and entry (CD4, CCR5 and CXCR4), and there is *in vitro* and *ex vivo* evidence to suggest that they may be infected by HIV-1 (Cotter et al. 2011; Gibellini et al. 2011). In this chapter we will discuss briefly the prevalence and potential etiologies of both HIV-1 associated bone and fat abnormalities, before examining in detail the existing evidence for a role for MSCs in these phenomena.

## Bone and Lipid Toxicities in HIV-1 Infection and Treatment

### Reduced Bone Mineral Density in HIV-1 Infection and Treatment

Osteoporosis is defined as a reduction in bone mass and disruption of the micro architecture of bone which leads to a greatly increased risk of fractures, while osteopenia is a lesser reduction in bone density and strength which may remain asymptomatic, but can precede actual osteoporosis. Osteoporosis/osteopenia may be diagnosed using dual-energy x-ray absorptiometry (DXA). The world health organisation (WHO) definitions specify t-scores between −1 and −2.5 as being indicative of osteopenia, while t-score of less than −2.5 are indicative of osteoporosis (McComsey et al. 2010).

At a cellular level, the drivers of bone homeostasis are osteoblasts(OBs), the cells which deposit extracellular matrix(ECM) and calcium to form bone, and osteoclasts(OCs) the cells responsible for bone resorption. Their actions are closely linked; for example OB produced RANK-L stimulates circulating OC progenitors to become active OCs, while it has been suggested that the acidic conditions of the bone resorption 'pit' activate TGF-β,which in turn can stimulate the differentiation of MSCs into OBs (Tang et al. 2009). Any dysregulation of this balance has obvious implications for BMD.

Reduced bone mineral density has become a recognised consequence of long term HIV-1 infection and treatment; it is estimated that the prevalence of osteoporosis is some three times higher in HIV-1 infected individuals than in the general population (McComsey et al. 2010; Triant et al. 2008). In addition increased fracture rates have been reported in the HIV-1 infected population (interestingly, regardless of treatment status), where HIV-1 infected individuals were reported to be 30–70% more likely to present with a fracture of any kind than uninfected individuals (Triant et al. 2008). In addition, at least one other study suggests poorer healing of fractures in a HIV-1 infected population, suggesting a fundamental impairment of the regenerative capacity of bone in these individuals (Richardson et al. 2008).

The causes of these bone abnormalities are most likely multi-factorial, with HAART, underlying HIV-1 infection, high levels of tobacco and alcohol use, and reduced Vitamin D levels all potentially involved. Some ART drugs have been strongly associated with reduced bone mineral density. A higher incidence of reduced BMD has been clinically associated with both PI and NRTI use. Tebas et al. determined that in HIV-1 patients receiving PIs some 50% of the patients had osteopenia and 21% had osteoporosis. This incidence is significantly increased compared to patients without therapy or normal controls (Tebas et al. 2000). Studies by Moore et al. (2001) confirmed that 71% of HIV-infected patients on PI therapy have reduced BMD. Similarly, Carr et al. reported that 3% of 44 HIV-infected patients receiving NRTIs developed osteoporosis and 22% developed osteopenia, while in a study examining HIV-1 infected men Mallon determined a reduction in BMD beginning at 48 weeks post initiation of treatment (Mallon et al. 2003; Carr et al. 2001). Tsekes et al. (2002) determined BMD and whole body fat by (DEXA) of HIV-infected patients receiving zidovudine and other NRTIs and found significant decreases in both body fat and BMD. In addition, the analysis by Brown and Qaqish also reported 2.5-fold increased odds of reduced BMD in ART-treated patients compared with ART-naive patients (95% CI 1.8, 3.7) (Brown and Qaqish 2006). More recently the NRTI tenofovir has been strongly associated with reduced BMD, which it has been suggested occurs due to the loss of phosphates as a result of the renal tubular toxicities associated with this drug (McComsey et al. 2010).

In addition, it has been noted that HIV-infected patients have increased risk for osteonecrosis of the hip. Keruly et al. (2001) reported 15 cases of avascular hip necrosis in HIV-infected patients and suggested that the incidence of osteonecrosis in HIV-infected patients was higher than the general HIV-negative population. It is not known whether this phenomenon is attributable to HIV-1 infection itself, HAART, or other HIV-associated complications.

The mechanisms by which either HIV-1 or its treatment induce reduced BMD are as yet unclear, and several researchers have suggested that reduced Vitamin D levels observed in HIV-1 infected patients, and particular the reduced levels of the biologically-active metabolite $1{,}25(OH)_2D$ (which is the natural ligand for the Vit. D receptor, VDR), may contribute to reduced BMD. Studies have demonstrated that the level of $1{,}25(OH)_2D$ in HIV-1 infected patients are between 5 and 50 % lower in infected patients. In addition, studies have indicated that patients receiving treatment are more likely to have greater reductions in $1{,}25(OH)_2D$, with at least one study suggesting that NNRTI treatment may increase the risk of Vit D deficiency (Cotter et al. 2009b).

There is also *in vitro* evidence to suggest a viral mediated impact on bone mineral density, with at least two studies linking viral proteins (including gp120 and p55-gag) with increased OC activity, reduced OB activity, and induction of apoptosis in OB cells. Studies by our group also showed that gp120 treatment decreased the activity of the RUNX-2 transcription factor (which drives OB differentiation and maintenance of an osteoblastic phenotype), and increased the activity of the pro-adipogenic PPARγ (interestingly, other studies have demonstrated that induction of PPARγ in OBs induces apoptosis). Taken in the light of clinical studies, these findings add weight to the idea that HIV-1 may have a direct effect on the cells responsible for the maintenance of BMD (Cotter et al. 2008; Gibellini et al. 2008;

Cotter et al. 2007). HIV-1 associated bone disorders are an increasingly well-defined clinical issue. However, the molecular events underpinning these clinical disorders remains to be fully explained.

## HIV-1 Associated Lipid Disorders

Patients living with HIV-1 and taking ART are at risk of developing a number of disorders related to dysfunctions in lipid metabolism. These disorders of fat metabolism are generally referred to as HIV associated lipodystrophy (HIVLD) and are estimated to effect up to half of all patients receiving ART. The most common manifestations of HIV associated Lipodystrophy are the abnormal progressive redistribution of body fat namely lipohypertrophy/lipoatrophy and alterations in serum cholesterol and lipid profiles namely dyslipidemia.

The fat redistributions associated with HIVLD can vary widely, however patients most commonly present with a central gain of visceral adipose tissue (lipohypertrophy) and/or a peripheral loss of subcutaneous adipose tissue (lipoatrophy). A central gain of body fat frequently presents as abdominal visceral obesity, hypertrophy of the breast in women and the enlargement of the dorsocervical fat pad (commonly called the 'buffalo hump'). Peripheral loss of body fat is characterised by the atrophy of peripheral subcutaneous adipose tissue which is particularly visible in the limbs, buttocks and face (Troll 2011). These HIV associated fat redistributions have in many cases a significant impact upon the health and quality of life of the affected individual particularly in terms of cosmetic appearance and an increased need for healthcare services. This can often lead to suboptimal adherence to therapy by the patient as well as placing an increased burden upon the healthcare services in terms of cost and utilisation.

In addition to the complications of lipohypertrophy/lipoatrophy, patients with HIV-associated lipodystrophy commonly have significantly altered serum cholesterol and lipid profiles similar to those observed in Metabolic Syndrome in the general population. The common pattern of dyslipidemia in HIV patients receiving antiretroviral therapy includes an increase in LDL cholesterol (hypercholesterolemia) and circulating triglyceride levels (hypertriglyceridemia) as well as a decrease in HDL cholesterol levels (Feeney and Mallon 2011). Profiles of dyslipidemia such as these are associated with atherosclerosis which is indicative of increased cardiovascular and cerebrovascular risk. Thus, it is likely that the aforementioned dyslipidemias play a large role in the reports of an increased incidence of myocardial infarction in the HIV infected population receiving ART. However, other common cardiovascular risk factors are also frequently present in HIV positive individuals such as high smoking rates, diabetes and insulin resistance, endothelial dysfunction, inflammation and hypertension (Feeney and Mallon 2011).

Existing preventative strategies for HIVLD include diet and lifestyle changes, careful ART regimen selection and the administration of pharmacotherapies to correct dyslipidemia. However, in many cases these have little or no effect and the administration of pharmacotherapies in tandem with HAART is undesirable as this can potentially lead to adverse drug-drug interactions. Therefore, a great deal of research has focused on the etiology of HIVLD syndromes and how to prevent them.

In further understanding these disorders of adipose tissue and lipid metabolism, a closer look must be taken at adipose tissue (AT) itself. AT is a complex, well vascularised tissue with multiple and often functionally distinct depots throughout the body. The functions of adipose tissue were once assumed to be restricted to lipid storage and release, local tissue architecture, thermal insulation and organ cushioning. It is now understood that in addition to being essential to energy metabolism adipose tissue is also a key endocrine organ and has been referred to as an integrator of a wide array of homeostatic processes (Caron-Debarle et al. 2010). As such adipose tissue expresses and secretes a variety of bioactive peptides, called adipokines, which act both locally (autocrine/paracrine) and systemically (endocrine). As an endocrine organ, adipose tissue plays a role in a number of important systems such as Blood Pressure Control (via the Renin Angiotensin System), insulin sensitivity

(via adiponectin and others), and the metabolism of the gonadal steroids. The importance of AT as an endocrine organ is emphasized by the adverse metabolic consequences associated with clinical conditions of both adipose tissue excess and deficiency. For example obesity is frequently associated with insulin resistance, hyperglycemia, dyslipidemia, hypertension, and proinflammatory states. Such consequences are presumably due to variations in adipokine secretion and failure to store triglycerides in fat, which can lead to dysfunction and lipotoxicity in adipocytes and other cells. Similar metabolic sequelae are also frequently present in HIVLD syndromes to varying degrees, as previously discussed (Avram et al. 2007).

Disorders of AT excess and in some cases adipose tissue deficiency are facilitated by the remarkable dynamic capability of AT to achieve significant changes in its tissue mass primarily in response to caloric storage requirements. AT mass increase is achieved by an increase in adipocyte volume (hypertrophy) and a subsequent recruitment and differentiation of adipocyte precursors into mature adipocytes (hyperplasia) (Avram et al. 2007). New adipose cells can be acquired throughout life via the differentiation of resident preadipocytes cells into mature fat cells in a process called Adipogenesis. Adipogenesis is a carefully orchestrated process requiring the appropriate hormonal, neuronal and nutritional stimuli as well as the activity of a selected set of transcription factors (Avram et al. 2007). The dysregulation of this complex process by ART and/or the effects of HIV infection is apparent in HIVLD syndromes. In the case of weight loss, studies show that the corresponding reduction in adipose tissue mass is attained via a reduction in both adipocyte number and volume (Avram et al. 2007). This clearly implies that adipocyte cell loss must occur *in vivo* in order to allow the dynamic control of tissue mass and adipocyte number. However, the mechanisms for such a cell loss in the absence of pathology are not well understood at present. In the case of HIV associated lipoatrophy adipose tissue loss is generally attributed to adipocyte apoptosis and due to toxicity caused by certain types of ART as will be discussed.

HIVLD was recognised as a significant issue in the HIV population rapidly after the widespread introduction of HAART in the mid-1990s. Since then exposure to several drug components of HAART combination therapy have been clearly linked to the lipodystrophic phenotype. In particular considerable evidence links HAART regimens containing certain drugs of the classes Nuceloside Reverse Transcriptase Inhibitors (nRTIs) and Protease inhibitors (PIs) to the development of HIVLD.

More specifically, ART with drugs of the class Thymidine Analogue nRTIs (t-nRTIs), such as Stavudine and Zidovudine, are known to contribute to the development of subcutaneous peripheral lipoatrophy (Caron-Debarle et al. 2010). Lipoatrophy due to t-nRTIs is primarily associated with their induction of mitochondrial toxicity in adipose tissue sites. *In vitro* and *ex vivo* studies of adipocytes have shown that t-nRTIs and their metabolites accumulate in adipocytes and are capable of inducing severe mitochondrial dysfunction as demonstrated by increased oxidative stress and reductions in mtDNA content as well as mtRNA expression. Severe mitochondrial dysfunction such as this could ultimately lead the apoptosis of adipocytes and subsequent lipoatrophy. t-nRTIs are also reported to downregulate the expression of the transcription factor PPARγ in adipose tissue, PPARγ is an important regulator of adipocyte function which is involved in Adipogenesis. ART mediated downregulation of PPARγ and consequential inhibition of preadipocyte differentiation may explain the characteristic Lipoatrophy seen in HIVLD (Feeney and Mallon 2011). Due to overwhelming evidence implicating tNRTIs in HIVLD, drugs such as Zidouvodine and Stavudine are no longer used as front-line antiretroviral therapies in the western world.

Protease Inhibitors (PIs) have long been strongly implicated in HIV associated lipid toxicities and insulin resistance. PI use is particularly associated with dyslipidemias and insulin resistance. It is known that PIs are capable of directly impairing the important metabolic functions of adipose tissue via a number of mechanisms. PIs can directly inhibit lipolysis in adipocytes by inhibiting the action of lipoprotein lipase, this

can lead to an impaired uptake of trigycerides resulting in increased levels of circulating TGs and a decrease in adipocyte volume (Feeney and Mallon 2011). In addition certain PIs have been shown to inhibit the activity of the insulin-responsive glucose transporter isoform GLUT-4 thus contributing to the insulin resistance often observed in HIVLD. The dyslipidemias associated with PI exposure can also be explained by the effects the drugs have on other tissues that are important for lipid homeostasis such as the liver. For instance, the exposure of mice to therapeutic doses of Ritonavir has been shown to result in excess fatty acid synthesis and hepatic, steatosis. PIs are also known to inhibit adipogenic differentiation. This inhibition may be due to the reported inactivation and downregulation of key transcription factors involved in the tightly regulated process of adipogenic differentiation such as SREBP-1 and PPARγ (Feeney and Mallon 2011).

These toxicities can frequently be severe in certain patients, in these cases switching patients to protease inhibitor sparing regimens is often successful in preventing the progression of the syndrome. The most severe forms of lipodystrophy are associated with first generation PIs and tNRTIs the use of which has greatly declined. Thankfully, more recently developed drugs have much improved lipid toxicity profiles. These drugs have a reduced overall prevalence of lipid toxicities and in the cases where toxicities do develop: they appear after a much longer duration of treatment (Caron-Debarle et al. 2010). Nonetheless even with careful selection of ARTs these syndromes cannot always be avoided and because of the considerable variation in their prevalence and presentation, clinicians have little choice but to deal with each patient's condition on a case by case basis.

Although the weight of clinical and scientific evidence suggests HIV-1/ART associated lipid abnormalities occur largely as a result of treatment, it is likely that HIV infection itself also contributes to the overall HIVLD phenotype. Indeed, prior to the widespread introduction of HAART, it was recognised that HIV infection itself could cause dyslipidemia and lipoatrophy in advanced disease (Feeney and Mallon 2011). Furthermore, several studies report that the severity of HIV infection, as measured by HIV viremia and/or immunodeficiency, correlates with an increased prevalence of lipodystrophy (Caron-Debarle et al. 2010). The exact mechanisms for the involvement of HIV infection itself in HIVLD are as of yet not clear. However, one emerging hypothesis suggests that HIV-infected macrophages persist in adipose tissue and that this leads to the dysregulation of AT function. This local inflammation would lead to the release of pro-inflammatory cytokines such as IL-6 and TNF-α from infected and activated macrophages. Exposure to TNF-α is known to dysregulate adipocyte function and adipocyte precursor differentiation, partly via suppression of PPARγ. The presence of extracellular HIV proteins secreted from nearby infected cells may also impact negatively upon AT. In particular HIV proteins Vpr and Nef have also been shown suppress the transcriptional activity of PPARγ resulting in similar effects as those described above (Caron-Debarle et al. 2010).

HIV infection may also contribute to the HIVLD syndromes by impacting upon multipotent adult stem cell populations such as MSCs. MSCs are present in the adipose tissue and are thought to serve as a reservoir of adipocyte precursors for adipogenesis. Work by our group and others suggest that HIV itself and its proteins are capable of dysregulating the differentiation and normal function of these cells (Cotter et al. 2009a). Such effects may explain in part, the contribution of HIV infection to lipodystrophic syndromes.

In summary HIVLD syndromes are broad and complex issues which have a multifactorial etiology. In recent years intense research in this field has yielded significant improvements in both our understanding of these syndromes and how to prevent them. For instance the prevalence of peripheral lipoatrophy syndromes has greatly declined due to improved ART and the reduced use of Thymidine nRTIs (Carr 2007). However, lipohypertrophy syndromes and the metabolic conditions which accompany them remain a concern and are poorly understood.

## Mesenchymal Stem Cells in Sickness and Health

The past two decades has seen an explosion in our understanding of mesenchymal stem cells (MSCs), driven by their potential applications in a host of regenerative therapeutics, auto-immune disorders, and as a therapy for acute conditions such as acute kidney injury (AKI) and myocardial infarction (MI). MSCs now are understood to have roles in a wide variety of both normal physiological processes and responses to disease and injury. Of particular interest to this review is their role in the formation of bone and adipose tissue.

MSCs are a population of self-renewing non-hematopoietic multipotent precursor cells initially isolated from the bone marrow but MSC 'pools' are known to be present in many tissues, including adipose tissue and the vascular wall (Caplan 1991; Bai et al. 2009). Circulating MSCs have also been detected. These cells are usually isolated and identified by their expression of the STRO$^{1+}$ CD34$^{-}$ antigens as well as their characteristic of plastic adherence in *in vitro* culture. They may also express CD105, CD44 and CXCR4 (the latter two playing important roles in MSC migration and 'homing') (Gibellini et al. 2011). MSCs can differentiate into a number of cell types including ACs, OBs, chondrocytes, skeletal myocytes. Their ability to differentiate into a wide range of cell types and their capability of migrate to sites of tissue injury allows them to play an important role in tissue repair and regeneration. These attributes have made them attractive candidates for clinical cell therapy for the treatment of a variety of disorders (Caplan 2007; Cotter et al. 2009a). In the bone marrow compartment MSCs are considered to have important hematopoietic supportive functions-possibly due to their ability to secrete a vast panel of cytokines, growth factors and chemokines with angiogenic, immunosuppressive, anti-apoptotic, and proliferative properties.

Regardless of MSC 'pool' or functional end-point, the MSC must be capable of performing a number of important steps; it firstly must retain an ability to renew itself by cell division; secondly must be able to migrate to its end niche by responding to appropriate chemoattractant signals (e.g., SDF1α) or by interacting with the extracellular matrix; and finally it should be able to perform it's end function, be it either through differentiating to form new tissue, or by performing an immunomodulatory role (Caplan and Dennis 2006). All of these stages are governed by complex interactions with a host of cellular signals, and any impairment or disregulation of these processes may have profound consequence for tissue homeostasis and repair. For example, impaired MSC renewal (premature senescence) has been suggested as an underlying cause in metabolic syndrome, and age related osteoporosis has been associated with increased bone marrow adiposity (disregulated differentiation) (Verma et al. 2002; Mansilla et al. 2011). From a therapeutic standpoint it has been demonstrated in models that administration of exogenous MSCs engineered to over-express CXCR4 (therefore increasing their ability to migrate toward an SDF1α gradient) improves their utility in an animal model of MI (Cheng et al. 2008).

MSCs are of particular interest in the case of HIV-1 bone and lipid toxicities as they are the cell type from which Osteoblasts (OB) and Adipocytes (AC) are derived. A HIV/ART mediated interference in the function of these cells could contribute to the observed HIV associated bone and fat toxicities. In addition, their reported expression of CXCR4, CD4 and CCR5 raises the possibility that they potentially could be infected by both R5 and X4 trophic HIV-1 (Gibellini et al. 2011).

It is considered that the processes of osteogenic and adipogenic differentiation of MSCs are antagonistic events, i.e. that AC formation is at the expense of OB formation and vice versa (Gimble et al. 2006). The activities of the transcription factors Peroxisome Proliferator-activated Receptors Gamma (PPARγ) and Runt-related Transcription Factor-2 (RUNX-2) in the MSC are known to be vital in this balancing process. The activity of RUNX-2 is essential for driving osteogenic differentiation and maintenance of the OB phenotype while PPARγ is similarly important for adipogenic differentiation. The balance between the effects of PPARγ and RUNX-2 is

thought to be a critical factor in driving MSCs toward an adipogenic or osteogenic lineage. The interrelated nature of bone and fat formation has been demonstrated in studies which have shown that mice deficient in PPARγ display a lipodystrophic phenotype with increased bone mass, while PPARγ ligands of the thiazolidinedine (TDZ) class of drugs reduced bone mineral density, and could reduce RUNX-2 activity (Gimble et al. 2006).

## Evidence for Viral Interaction with Mesenchymal Stem Cells

A relatively limited number of studies have examined the impact of HIV-1 exposure on MSCs over the past decade. Though recent studies show a consensus emerging on both the mechanism and effect of HIV-1 interaction with MSCs, the overall impact of these effects on the bone and lipid toxicities observed with HIV-1 remain to be determined.

Cells of the bone marrow compartment in general, and MSCs in particular, have been long been considered as potential target for HIV-1 infection. Indeed, infection of long term bone marrow stromal cell cultures has been demonstrated, and this is considered to contribute to the loss of their hematopoietic supportive functions. As mentioned previously, MSCs have been shown to express the extracellular receptors necessary to allow HIV-1 infection – and some of their differentiated progeny (OBs for examples) have been shown to permissive to infection. However, to our knowledge, no study has ever comprehensively determined *in vivo* infection of human MSC (derived from any 'pool') by HIV-1. Studies carried out in a fetal primate model reported that although only a small proportion of MSC express CD4 and CCR5 *in vivo,* simian immunodeficiency virus (SIV) can readily infect these cells *in vitro* (Lee et al. 2004) .

Despite the lack of *in vivo* evidence for MSC interaction with HIV-1 there have been a number of *in vitro* studies which have examined the impact of HIV-1 exposure on MSCs. In 2002 Wang and co-workers published data which demonstrated *in vitro* infection of bone marrow stromal derived cells with HIV-1. The productive phase of this infection (as measured by HIV-1 p24 titer in the supernatant) was short-lived (approx. 4 weeks), but the presence of HIV-1 specific nucleic acids and Tat protein were also determined. Interestingly, infection with HIV-1 resulted in a marked reduction of the potential of these cells to differentiate into pure colonies of fibroblasts or mixed colonies of ACs and fibroblasts. This phenomena was replicated by treatment of uninfected cells with exogenously added Tat protein, an effect which was potentiated by TNF-α. Although this study examined a mixed population of bone marrow derived cells (containing MSCs and blastoid cells), and did not confirm infection of MSCs, this was the first study to suggest that the differentiation and proliferative capacity of MSCs may be impaired by exposure to HIV-1(Wang et al. 2002).

More recently our group has examined the impact of HIV-1 exposure on the differentiation of bone marrow derived MSC using *in-vitro* and *ex-vivo* models, namely exposure of cultured cells to viral proteins, and exposure of MSCs to serum collected from HIV-1 infected donors. We demonstrated that the HIV-1 proteins REV and p55 can alter the degree of osteoblast formation from MSCs. Further analysis of this phenomenon revealed that in MSCs undergoing differentiation exposure to HIV proteins caused significant changes in the timing and magnitude of important osteogenic events and signals. Treatment with REV increased the overall degree of mineralization, and induced earlier increases in CTGF levels, RUNX-2 activity and BMP-2 secretion, than those observed in the normal course of differentiation. In contrast, p55-gag reduced the overall level of osteogenesis, and reduced bone morphogenic protein (BMP)-2 secretion, RUNX-2 activity, connective tissue growth factor (CTGF) levels and alkaline phosphatase (ALP) activity at many of the timepoints examined. Also, in cells cultured in basal conditions, treatment with HIV proteins did not in and of itself induce a significant degree of differentiation over the time period examined – we hypothesised that these in vitro findings provide evidence for a

generalised dysregulation of MSC differentiation by HIV-1 (Cotter et al. 2008).

Following these investigations an *ex vivo* model of MSC/HIV-1 exposure was developed. This involved treatment of MSC with serum collected from treatment naive HIV-1 infected individuals. Sera were divided into three groups; HIV-negative, HIV-positive low viral load [LVL] (VL range 120–4,000 copies per ml) or high viral load [HVL] (VL range 100,000–500,000 copies per ml). It was found that exposure of MSCs to serum from HIV-1 individuals during the course of adipogenesis drove a significant, viral load-dependent, increase in adipogenesis (although surprisingly a concomitant decrease in osteogenesis was not observed). Exposure to HIV-1 infected sera in basal (ie non- differentiating conditions) also increased expression of PPARγ mRNA and lipoprotein lipase (LPL) levels, while reducing RUNX-2 mRNA and alkaline phosphatase (ALP) activity – suggesting the induction of a 'pro-adipogenic' phenotype. Interestingly, many of these effects could be replicated by treatment with exogenously added gp120 and Tat proteins. Blockade of the CD4 receptor with pyridoxal 5 phosphate (p5p) attenuated the pro-adipogenic effects of HIV-1 infected sera (again, indicating a role for gp120) – as did the nucleoside reverse transcriptase inhibitor (NRTI) zidovudine (AZT), suggesting that these events were not only HIV-1 dependent, but involved direct infection of the MSCs by HIV-1. While no evidence for productive infection of MSCs was determined (as measured by production of p24), we putatively identified integrated viral DNA using a PCR assay, and cellular expression of the Rev protein using immunocytochemistry. We also identified expression of the CD4, CCR5 and CXCR4 receptors using immunocytochemistry – indicating that the MSCs were theoretically permissive to HIV-1 infection. In short, our conclusions were that HIV-1 infection of, and interaction, with MSCs may dysregulate normal MSC differentiational capacity and drive a pro-adipocytic phenotype (Cotter et al. 2011).

These conclusions have been validated by recently published work by Gibellini et al. In this study MSCs extracted from the vascular wall were shown to express the CD4/CCR5/CXCR4 receptors and to be permissive to infection by laboratory strains of HIV-1 (HIV-1 IIIB; as identified using a nested PCR based assay for HIV-1 DNA integration) – again this infection appeared to be non-productive, as production of p24 was not determined. Exposure to HIV-1 was shown to increase both induced – adipogenesis of these MSCs, and to increase MSC apoptosis. An increase in PPARγ activity was also observed. Treatment with heat inactivated HIV-1 did not produce these pro-adipogenic effects. As demonstrated previously, inclusion of the CD4 antagonist p5p attenuated these effects – while treatment with the recombinant gp120 protein alone was sufficient to drive increased adipogenesis and PPARγ activity, as well as increased MSC apoptosis (Gibellini et al. 2011).

Taken together the results of these recent investigations raise intriguing questions about the role of MSCs in HIV-1 bone and fat toxicities. Functional impairment of MSC differentiation has potential consequences for a variety of tissues, but the distinct 'pro-fat' phenotype observed in these studies could explain either reduced bone mass and altered visceral and subcutaneous fat levels observed in HIV-1, as MSC are predisposed to form adipose rather than bone tissue. Likewise – the presence of 'pro-adipogenic' MSCs in the vasculature is of interest not only in the context of altered lipid profiles of HIV-1 infected individuals, but may be of particular clinical consequence in a population considered to be at increased risk of cardiovascular events.

## Evidence for Treatment Mediated Disregulation of Mesenchymal Stem Cell Function

As discussed previously, our understanding of HIV-1 bone and lipid toxicities increasingly points to their being numerous causal factors – most importantly HIV-1 infection and therapy. While no studies have examined the effects of HAART on MSCs *in vivo*, a limited number of studies have examined the impact of HAART drugs on MSCs.

Jain and Lenhard (2002) published investigations examining the effects of a variety of protease inhibitor (PI) drugs on a range of cell types involved in bone and fat homeostasis, including bone marrow derived MSCs. Using relatively high concentrations of the PI drugs (5–20 μM), they demonstrated that the drugs nelfinavir (NFV) and lopinavir (LPV) could significantly decrease the production of OBs from MSCs, as measured by diminished calcium deposition and osteoprotegrin expression, while other PI drugs did not. In addition they found that induced adipogenesis was strongly inhibited by squinavir (SQV) and NFV, while ritonavir (RTV) and LPV induced a more moderate reduction in adipogenesis. In addition the expression of diacylglycerol transferase, a marker of adipocyte differentiation was also decreased by NFV. They hypothesised that this data indicated that some PIs could impair the differentiation capacity of MSCs, resulting in a reduced ability to produce cells of an OB and AC lineage (Jain and Lenhard 2002).

More recently, a series of elegant experiments by Lagathu et al. (2010) have shed light on a process which may have significant implications for our understanding of HIV-1 associated lipid abnormalities. Their investigations used the PIs atazanavir (ATZ) and LPV, which are currently prescribed, and are generally 'boosted' by RTV (RTV increases the drugs intercellular concentrations by inhibiting their metabolism by cellular enzymes). They found that exposure to these drugs in both their boosted (10 μM ATZ/LPV with 2 μM RTV) or unboosted forms significantly reduced MSCs ability to differentiate into both OBs (as measured by calcium deposition, and RUNX-2 and OSX expression). This occurred parallel to an increase in cellular ROS production, while LPV/r also reduced the expression of several markers involved in extracellular matrix (ECM) production.

They also examined markers of MSC cell senescence in response to long term exposure (10–40 days) with PIs. They found that long term PI treated cells had impaired proliferative capacity compare with untreated controls; while LPV/r and ATZ/r both increased levels of β-galactosidase activity (a marker of cell senescence), levels of the cell cycle inhibitors p21/WAF and p16/INK4A, and accumulation of prelamin A – a marker of cell aging. Most interestingly MSCs exposed to PIs for 30 days displayed altered capacity to differentiate into either OBs or ACs, with LPV and LPV/r reducing differentiation into both cell types, while ATZ treatment reduced osteogenesis but increased adipogenesis (Lagathu et al. 2010).

These data suggests that what has been called 'MSC exhaustion'; i.e. premature senescence, reduced proliferative capacity and altered differentiation potential, may contribute to the lipid abnormalities associated with HAART. Indeed, it has been suggested that metabolic syndrome (which shares many of the hallmarks of HAART/HIV-1 associated lipid toxicities), may be driven in part by impairment of MSCs (Mansilla et al. 2011).

In conclusion, in the past decades we have begun to understand the complex and important role of MSCs in the human body. These cells, it seems, play a part not just in the maintenance of tissue homeostasis and repair, but in acute response to injury. In recent years their putative role in the bone and fat disorders associated with HIV-1 infection and treatment has begun to be deciphered. However, despite some compelling *in vitro* and *ex vivo* evidence, we are far from a comprehensive picture of their role in these toxicities. It is clear that further studies are required to tease out their role *in vivo*.

However given the existing evidence, it is possible to speculate that HIV-1 and its treatment may induce a generalised impairment of MSC function. Taking into consideration both the general physiological importance of MSCs, and that the HIV-1 infected population are subject to disorders not only of bone and fat, it may be that loss of the regenerative and ameliorative functions of MSCs contribute to a wide array of HIV-1/HAART associated toxicities.

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# Mesenchymal Stem Cell Therapy: Immunomodulation and Homing Mechanisms

9

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**Abstract**

Mesenchymal stem cells (MSC) have been explored as a new clinically relevant cell type to repair injured tissue. A growing corpus of studies have highlighted two important aspects of MSC therapy: (1) MSCs can modulate T-cell mediated immunological responses, and (2) systemically administered MSCs home to sites of ischemia or injury. In this chapter, we describe the known mechanisms of immunomodulation and homing of MSCs. First, we examine their niche *in vivo*, with several new peripheral locations identified. Next, we discuss the paracrine and direct interactions between MSCs and innate (dendritic cells (DC)) and adaptive immune cells (T lymphocytes) with a focus on the roles of interferon γ (IFN-γ), prostaglandin

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E2 (PGE2), and indoeamine 2,3-dioxygenase (IDO). We transition to outline the steps of activation, rolling/adhesion, and transmigration of MSCs into target tissues during inflammatory or ischemic conditions. These aspects of MSC grafts – immunomodulation and homing – are contextualized to provide insight into a reported side-effect of MSC therapy – cancer development. Finally we discuss how MSCs home to malignant tissue sites, which may facilitate future therapeutic application of MSCs in the treatment of malignant tumors.

## Introduction

Mesenchymal Stem Cells (MSC) belong to a category of clinically relevant cell types that may be utilized for potential cell-based therapies, as complicated culturing or handling techniques are not required to yield clinically practical quantities. MSCs stably express large quantities of immunologically active molecules such as growth factors and chemokines, and maintain their multipotency for many passages (Caplan 1991). Recent clinical studies have demonstrated that MSCs have therapeutic potential in Graft Versus Host Disease (GVHD), Crohn's disease, chronic obstructive pulmonary disease (COPD) and Type 1 diabetes. Although ample experimental evidence exists supporting the therapeutic potential of MSCs, the mechanism of MSC homing and recruitment to specific active locations is still not well understood; as such therapeutic site-specific and targeted immunomodulatory protocols cannot yet be designed and adequately optimized without risking clinical complications. This chapter reviews recent findings related to the mechanisms of MSC-induced immunomodulation as it relates to inflammation and tumors. MSC recruitment to sites of inflammation and their potential role in malignant tissue progression will also be discussed.

## Mesenchymal Stem Cells: Their Niche *In Vivo*

Although previous studies revealed that the primary MSC stem cell niche is in bone marrow, there are several reports that also identify additional peripheral locations, e.g., adipose tissue, salivary glands, tendon, periodontal ligament, skin, muscle, lung and most recently intestinal lamina propria (Powell et al. 2011). A report by da Silva Meirelles et al. (2008) posits that one of the important MSC stem cell niches is the perivascular region, which, as a residual aspect of embryogenesis, might explain the presence of MSCs in many different tissue types. Others have suggested that MSCs might originate in the bone-marrow and subsequently be recruited distally to specific sites of tissue injury (Stappenbeck and Miyoshi 2009). Nonetheless, there appear to be two origins of MSC populations. One population present in peripheral locations where they interact with perivascular cells (an embryonic remnant), and the second population, originates in the bone marrow, where MSCs form their primary stem cell niche and respond appropriately following tissue injury. MSCs secrete various families of active molecules including cytokines, chemokines, and growth factors, which regulate the local bone marrow environment, as well as modulate the systemic immune response to inflammatory events. Although there have been numerous reports demonstrating that MSCs can repair tissue by directly differentiating toward mesenchymal lineages, recent work has established that, instead of, or perhaps in addition to differentiation, MSCs can enhance the differentiation of other progenitor cells into functional somatic cells. In addition, they may contribute to other aspects of local tissue repair via paracrine mechanisms.

Mesenchymal stem cells can function as immune suppressive and anti-inflammatory agents as well as stimulators of tissue repair and regeneration. However, the difference between MSCs that are recruited from the bone marrow versus peripherally located MSCs, in executing these distinct roles, in unclear. Recently, Brandau et al. (2010) compared the differentiation potentials of local resident and bone marrow-derived MSCs, and suggested that the two populations were not identical.

## Mechanism of Immune Modulation in Local and Systemic Inflammation

### The Role of IFN-γ in the Immune Modulation of MSC

*In vivo* inflammatory sites are rich in pro-inflammatory cytokines, in particular TNF-α and IFN-γ, secreted by activated T and NK cells. MSCs can inhibit allogeneic immune responses by suppressing T- or NK-cell proliferation, thereby diminishing IFN-γ production. This suppressive activity is not contact-dependent and requires the presence of IFN- γ, to stimulate MSC indoleamine 2 3-dioxygenase (IDO) activity, which in turn inhibits the proliferation of activated T or NK cells. High level expression of PD-L1, IDO and PGE-2 by MSC are differentially regulated by IFN-γ and TNF-α, leading to further regulation of T-cell, NK-cell, as well as macrophage function, and contributing essential, non-redundant roles in modulating alloantigen-driven inflammatory cell proliferation. The beneficial effect in GVHD, of graft/MSC co-infusion, may be due to the activation of the immunomodulatory properties of MSCs by T cell- derived IFN-γ as described above. In addition, the surface expression of PD-L1 on MSCs induced by IFN-γ, may have an important role in modulating the antigen presentation capability of MSCs. Thus, MSC expression of immunomodulatory factors dramatically changes in a pro-inflammatory environment, and IFN- γ in particular has an important role in regulating MSC immunomodulatory factor expression (English et al. 2007) (Fig. 9.1).

### The Contribution of MSC Soluble Factors and Cell-Cell Interaction

Several studies have shown that MSCs actively inhibit the function of several immune system cell lineages through secreted cytokines, growth factors and enzymatic action. For example, the immunosuppressive function of lung resident-MSCs was noted in the absence of direct cell contact. Collectively, secreted molecules from MSCs delivered by a bolus injection of concentrated conditioned medium or by MSC extracorporeal bioreactor treatment, can reverse a rat model of multi-organ dysfunction syndrome. The effectiveness of the MSC secretome in attenuating inflammation is also supported by studies using alginate encapsulated MSC, where the cells are physically separated from the inflammatory environment. In vitro, a dramatic reduction in LPS-activated macrophage TNF-α secretion was detected following co-culture with both encapsulated MSC/macrophages and within a transwell membrane co-culture system. Parallel results were demonstrated *in vivo*, where macrophage activation was attenuated following implantation of encapsulated MSC post-spinal cord injury in a rat model (Barminko et al. 2011).

In contrast to those studies that support a central role for MSC soluble factors, others suggest that cell-cell contact is more important. Tse et al. (2003) demonstrated that close proximity to MSCs was important in suppressing T cell responsiveness and suggested that direct contact between lymphocytes and MSCs was more important than soluble mediators in the inducing immunosuppressive MSC function. Krampera et al. (2003) stated that inhibition requires both the presence of MSCs in culture and direct MSC-T-cell contact. Recently, several reports suggested the importance of combined soluble factors and cell-cell contact in MSC-mediated immunosuppression. In order for MSCs to provide a pleiotropic immunomodulatory effect that is responsive to different stimulants such as cytokines and chemokines and targets different effecter cells such as T-cells, NK-cell and DCs, it seems reasonable for MSCs to employ both direct and soluble mediators to coordinate a multi-pronged therapeutic effect.

### Modulation of Immune Responsive Cells by Soluble Factors and Cell-Cell Interaction

Natural Killer (NK)-cell proliferation and NK-cell mediated cytotoxicity: In effect, MSCs modulate

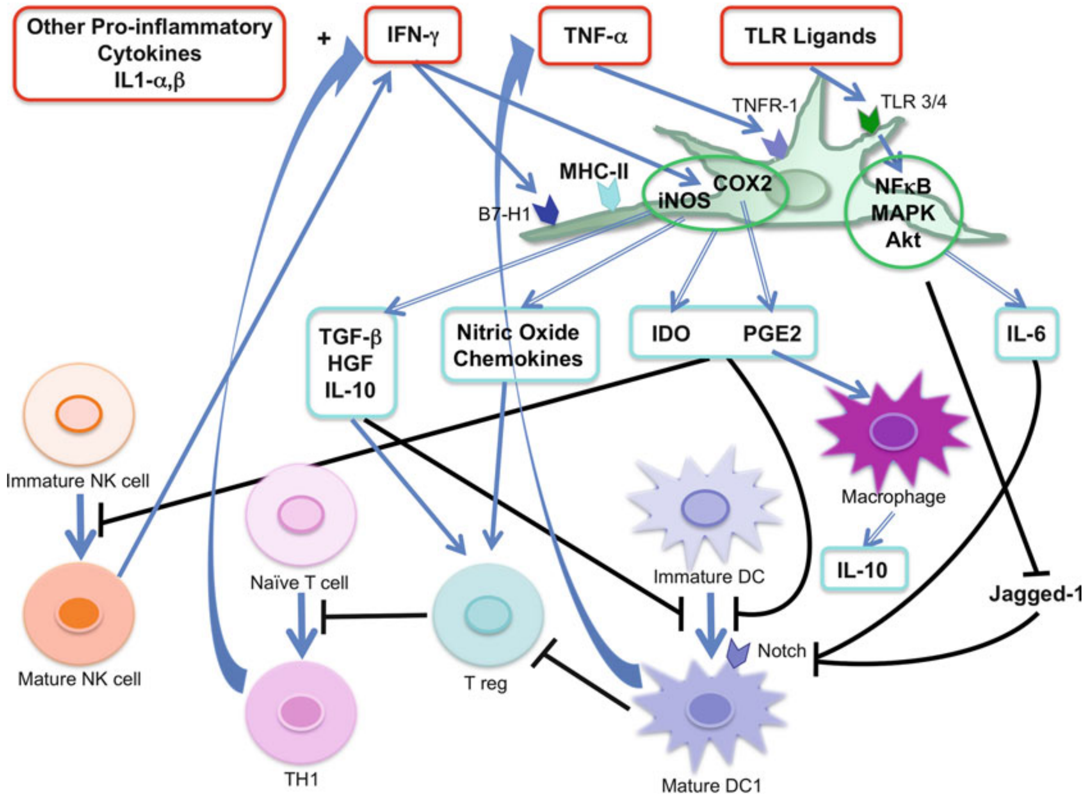


**Fig. 9.1 Mechanism of immune modulation in local and systemic inflammation**. MSCs can inhibit allogeneic immune responses by suppressing T- or NK-cell proliferation, thereby diminishing IFN-γ production. This suppressive activity is not contact-dependent and requires the presence of IFN- γ, to stimulate MSC IDO activity, which in turn inhibits the proliferation of activated T or NK cells. High level expression of PD-L1, IDO and PGE-2 by MSC are differentially regulated by IFN-γ and TNF-α, leading to further regulation of T-cell, NK-cell, as well as macrophage function, and contributing essential, non-redundant roles in modulating alloantigen-driven inflammatory cell proliferation. *IL* Interleukin, *IFN* Interferon, *TNF(R)* Tumor Necrosis Factor (Receptor), *TLR* Toll Like Receptor, *TGF-β* Transforming Growth Factor-β, *HGF* Hepatocyte Growth Factor, *PGE* Prostaglandin, *IDO* Indoleamine 2,3-Dioxygenase, *COX2* Cyclooxygenase-2, *iNOS* Inducible Nitric Oxide Synthase, *NFκB* Nuclear Factor Kappa B, *MAPK* Mitogen-Activated Protein Kinase, *NK cell* Natural Killer cell, *DC* Dendritic Cell, *T reg* Regulatory T cell, *TH1* Type 1 Helper T cell1

different components of the immune response including for example, inhibition of DC differentiation, skewing CD4+ T helper population phenotypes, and modulating CD8+ cytotoxic T lymphocyte and NK cell functions. NK cells are major effector cells of innate immunity and are generally thought to play a fundamental role in antiviral responses. MSCs and NK cells have been shown to interact in vitro. The outcome of this interaction may depend on the state of NK-cell activation and/or the cytokines present in the milieu. Previous studies have indicated that cytokine-induced proliferation of NK cells was highly susceptible to MSC-mediated inhibition. In addition, the surface expression levels of activating NK receptors were positively correlated with NK-cell function. There is growing evidence that IDO, PGE2, and TGF-1 may control MSC-mediated inhibition of NK-cell function. Spaggiari et al. (2006) demonstrated a strong inhibition of NK-cell proliferation when NK cells were cultured in the presence of MSCs. Remarkably, the simultaneous blocking of IDO and PGE2 could almost completely restore NK-cell proliferation. They also showed that the ability of NK-cells to kill target cells was markedly

reduced when cultured with MSCs. These data further confirm that IDO and PGE2 may exert a synergistic effect in the immunosuppressive activity mediated by MSCs.

Dendritic Cell (DC) Maturation: Dendritic Cell (DC) plays a key role in the initiation of primary immune responses and in tolerance, depending on the activation and maturation stage of DCs. Immature DCs behave as sentinels in peripheral tissues, with highly efficient antigen uptake and processing, and low ability to stimulate T cells. Locally produced inflammatory cytokines or microbial components promote the maturation of DCs from a processing to a presenting stage, characterized by up-regulation of MHC class II and costimulatory molecules (CD80 and CD86), production of IL-12, and migration to lymphoid tissue. Dendritic cell maturation is a prerequisite to induce immunogenic T cell responses, whereas tolerance is observed when antigens are presented by immature or semi-mature DCs. Therefore, DC maturation plays a key role in initiating T cell responses. Recent evidence demonstrates that MSCs disrupt the three major functions that characterize the transition of DC from immature to mature stages, namely the up-regulation of antigen presentation/costimulatory molecule expression, the ability to present defined antigens, and the capacity to migrate in response to CCL19. Furthermore, reduction of IL-12 secretion was also observed after MSC co-culture.

In addition, MSCs produce several cytokines, including interleukin (IL)-6, and monocyte-colony stimulating factor (M-CSF), which have been shown to have an important role in DC differentiation. It is now clear that modulation of DC maturation by MSC requires IL-6 and a contact-dependent signal. Djouad et al. (2007) observed that MSCs secrete higher levels of IL-6, which was involved in the reversion of the maturation of DC to a less mature phenotype and in the partial inhibition of bone marrow DC progenitors. PGE2 has also been observed to contribute to the modulation of DC maturation. Chen et al. (2007) demonstrated that blockage of PGE2 synthesis in MSCs could revert most of the inhibitory effects on DCs differentiation and function. Taken together, these findings suggest that PGE2 and IL-6 can mediate MSC control of DC maturation, which ultimately leads to T-cell suppression.

Mesenchymal stem cells induce T-Cell Suppression through NFκB Pathway: Toll-like receptors (TLRs) are a conserved family of receptors that recognize pathogen-associated molecular patterns and promote the activation of immune cells. Among their functions, TLRs mediate stress responses of bone marrow-derived progenitor cells. A recent study described the importance of TLRs in migration and immune regulation of MSCs. A common signaling feature among TLRs is the activation of the transcription factor nuclear factor κB (NFκB), which has been implicated in controlling the expression of inflammatory cytokines and cell maturation molecules. Pevsner-Fischer et al. (2007) demonstrated that cultured MSCs express TLR molecules 1–8. Activation of MSCs by TLR ligands induced IL-6 secretion and NFκB nuclear translocation. Liotta et al. (2008) demonstrated that LPS was able to induce NFκB activation in MSCs, as well as production of cytokines and chemokines including IL-6, IL-8, and CXCL10. However, the addition of LPS in culture could significantly reduce the suppressive activity of MSCs on T-cell proliferation. They showed that ligation of TLR3 or TLR4 on MSCs inhibited their suppressive effect on T-cell proliferation by hampering Jagged-1 expression, which impaired signaling to its cognate Notch receptor expressed on T cells. TLRs were critical players in the immunoregulation observed when MSCs were transplanted *in vivo*. The intersection of IFN-γ regulated pathways and their combined effect on MSC immunomodulatory function, is mediated by the upregulation of COX-2 and PGE-2 and downstream NFκB activation. In addition, TNF-α has been shown to be a critical modulator of NFκB, and there is evidence that MSCs can express TNF receptors (TNFR) (Yagi et al. 2010). Recently, Nemeth et al. (2009) reported that MSCs, when activated by LPS or TNF-α, could release PGE2, which acts on EP2 and EP4 macrophage receptors, thereby reprogramming them for the treatment of sepsis. These findings suggest that NFκB activation, via both TLR and

TNFR expression on MSCs, can modulate the immune response by suppressing T-cell proliferation, macrophage activation and DC maturation.

Recently Waterman et al. proposed a new MSC paradigm based upon downstream TLR signaling, which classifies MSC into two homogenously acting phenotypes, MSC1 and MSC2 (Waterman et al. 2010). MSC primed via TLR4 developed a "pro-inflammatory" phenotype (MSC1), whereas TLR3-primed MSC developed an "anti-inflammatory/immunosuppressive" phenotype (MSC2). Though not conclusive, that study did demonstrate the potential to generate distinct populations of MSC depending on the activating agent and specific TLR that is targeted.

Mesenchymal stem cells increase regulatory T cell population: Recent reports demonstrated that MSCs can effectively suppress inflammatory allogeneic responses, through induction and activation of CD4(+)CD25(+)FoxP3(+) regulatory T cells, which can significantly (1) inhibit the differentiation of autoreactive/inflammatory T-helper1 and (2) generate IL-10–secreting T cells, which leads to the down-regulation of both Th1-driven autoimmune and inflammatory responses. Taken together, MSCs may act as tolerogenic cells directly generating anergic T cells with regulatory functions. Thus, MSCs emerge as key regulators of immune tolerance and as attractive candidates for a cell-based therapy for solid organ allo-transplantation, allergic conditions, GVHD, autoimmune diseases, asthma and Crohn's disease. Crohn's disease is a chronic inflammatory disease characterized by severe Th1 cell-driven inflammation of the colon, partially caused by a loss of immune tolerance against mucosal antigens.

Mesenchymal stem cells enhance immune suppressive capacity of macrophages: A recent study suggested that macrophages are positive regulators of the MSC niche and are required to maintain the expression of various hematopoietic stem cell retention factors, including CXCL12 (Ehninger and Trumpp 2011). The close interaction between MSC and macrophages might be mediated by proteins including IGF-1, IL-1, TNF, and IL-10. Indeed, large amounts of IL-10 are known to be produced by subsets of T cells, monocytes and macrophages. Interleukin (IL)-10 was up-regulated when MSCs were co-cultured with macrophages exposed to inflammatory conditions. Moreover, IL-10 serum levels were increased in a septic mouse model following treatment with MSCs (Nemeth et al. 2009). The beneficial effect of MSCs during sepsis was eliminated by macrophage depletion or pretreatment with antibodies specific for IL-10 or IL-10 receptor. In addition, macrophages could be reprogrammed by MSCs, via the release of PGE2, which subsequently acts on prostaglandin EP2 and EP4 macrophage receptors. This effect was eliminated if the MSCs lacked the genes encoding TLR4, myeloid differentiation primary response gene-88, TNFR-1α or COX2.

An additional MSC role in attenuating macrophage mediated inflammation was recently described by Barminko et al. (2011) Anti-inflammatory function was assessed by co-culturing free or encapsulated MSC with LPS-activated macrophages. Relative to the free MSC, encapsulated MSC increased the conversion of macrophages into an anti-inflammatory M2 phenotype with increased CD206 expression and decreased TNF secretion. These studies support a dual anti-inflammatory role, as MSC may both attenuate M1 inflammatory macrophages and convert macrophage populations into anti-inflammatory M2 cells.

## Immune Reactive Soluble Factors Secreting from MSC

Mesenchymal stem cell-derived lipid intermediates suppresses T-cell proliferation: Prostaglandin E2 (PGE2) is a lipid intermediate that has been implicated as one of the potential candidates responsible for T cell inhibition by MSCs. Like all prostanoids, PGE2 is synthesized from arachidonic acid via the actions of either the constitutive cyclooxygenase-1 (COX-1) or the inducible COX-2 enzyme. Murine and human MSC constitutively express COX-2. Prostaglandin E2 production is upregulated after co-culture of human

MSC with peripheral blood mononuclear cells (PBMCs), suggesting communication between MSC and lymphocytes, that leads to the increased production of immunosuppressive factors such as PGE-2. Both COX-2 and PGE-2 expression by MSC were up-regulated by IFN-γ and TNF-α, suggesting a level of control based on the inflammatory microenvironment. Aggarwal and Pittenger (2005) demonstrated that human MSCs produced elevated PGE2 in co-culture with T cells, and inhibitors of PGE2 production mitigated MSC-mediated immunomodulation in vitro. Also, Jarvinen et al. (2008) reported PGE2 synthetic capacity of lung allograft-derived MSCs isolated from bronchioalveolar lavage samples from lung transplant recipients. Lung resident (LR)-MSCs significantly inhibited proliferation of third party, HLA-mismatched T cells. This effect was abrogated by COX inhibitors. These findings suggest that PGE2 is one of the key molecules involved in the immunomodulatory effect of MSCs.

Mesenchymal stem cell Metabolism of Tryptophan suppresses T-cell proliferation: Recent data has suggested a role for indoleamine 2,3 deoxygenase (IDO) in human MSC mediated immune suppression. Indoleamine 2,3 deoxygenase (IDO) is the rate-limiting enzyme involved in the catabolism of the essential amino acid tryptophan into its breakdown product kynurenine. Indoleamine 2,3 deoxygenase (IDO) is involved in the inhibition of T cell proliferation by dendritic cells. Mesenchymal stem cells do not constitutively express IDO, however IDO levels increase following stimulation with IFN-γ, but not TNF-α MSCs can be induced to express IDO and previous reports suggesting a role for this enzyme in human MSC suppressive effects. Ryan et al. (2005) demonstrated the contribution of IDO to IFN-γ induced immunomodulation by MSC via an antagonist of IDO, 1-methyl-L-tryptophan. Moreover, the tryptophan catabolite kynurenine was examined in mixed lymphocyte reactions (MLR), and was found to block alloproliferation. These findings support a model where IDO exerts its effect through the local accumulation of tryptophan metabolites rather than through tryptophan depletion. However, Tse et al. (2003) did not find a significant restoration of proliferation by IDO. This finding indicates that, IDO is not an exclusive mechanism for MSC immunomodulation in basal states, but may be essential for MSC suppression in the presence of IFN-γ.

Nitric Oxide and Chemokines from MSCs as a stress mediator: Nitric oxide (NO) has been recognized as one of the most versatile players in the immune system. It is involved in the pathogenesis and control of infectious diseases, tumors, autoimmune processes and chronic degenerative diseases. Sato et al. (2007) demonstrated that Stat5 phosphorylation in T cells is suppressed in the presence of MSCs and that NO is involved in the suppression of Stat5 phosphorylation and T-cell proliferation. In addition, they showed that NO was readily detected in the medium in the presence of MSCs and MSCs from iNOS$^{-/-}$ mice had markedly reduced abilities to suppress T-cell proliferation. The expression of high levels of inducible NO synthase (iNOS) by MSCs is regulated by cytokine combinations including IFN-γ with TNF-α, IL-1α or IL-1β. These proinflammatory cytokines are required to induce immunosuppression by MSCs through the concerted action of NO, suggesting that NO produced by MSCs is one of the major mediators of T-cell suppression by MSCs.

The role of TGF-β, HGF and other soluble factors in MSC immunotherapy: Previous studies demonstrated that exposure of MSC to IFN-γ could induce expression of HGF and TGF-β1 at concentrations that suppressed allo-responsiveness. Another report showed that quantitative real time PCR confirmed significant HGF mRNA up-regulation by IFN-γ and TNF-α. Di Nicola et al. (2002) suggested that HGF worked in synergy with TGF-β1 to resist T cell recognition by simultaneous neutralization of HGF and TGF-β1, in the latter study restoring T cell proliferation by MSC.

## Mechanism of Homing and Migration Toward Inflamed/Injured Tissues

To assess the mechanism of homing of MSC toward inflammatory sites, it is important to sequentially examine the whole process, beginning with

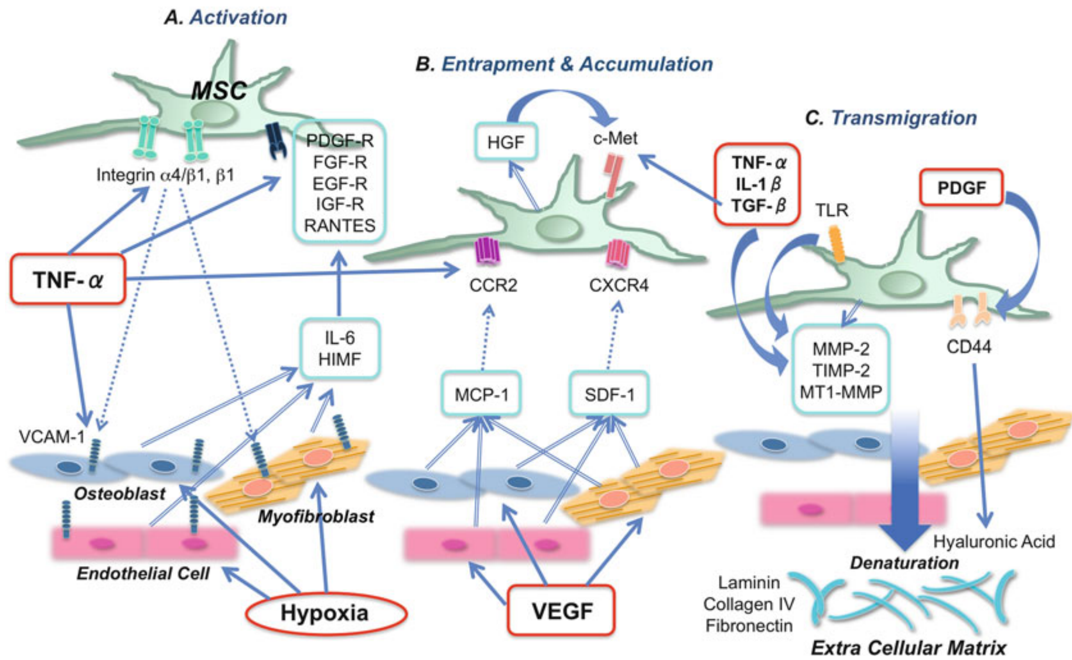


**Fig. 9.2 Mechanism of homing and migration toward inflamed/injured tissues.** Expression of VEGF can enhance the up-regulation of chemokines such SDF-1 or MCP-1, secreted from local immune regulatory cells including Osteoblast, Myofibroblast or Endothelial Cells. MSCs are known to express receptors for several chemokines, including CXCR4 or CCR2, which are activated in response to TNF-α, a pro-inflammatory cytokine also secreted from inflamed/injured tissues. This process is probably the most important and widely studied mechanism to understand the homing and migration capacity of MSCs. Finally, to produce an immune modulatory effect on local tissues, MSCs have to transmigrate into the perivascular or parenchyma through the tight junctions of microvascular endothelial barriers. Pro-inflammatory cytokines secreted from target tissues including PDGF, TNF-α, IL-1b or TGF-β lead to increased expression of MSC cell surface receptors. CD44, MMP-2, TMP-2 or MT1-MMP, which denature extracellular matrices components, such laminin, collagen IV and fibronectin and thereby strongly enhance cell migration toward the parenchyma. *PDGF(-R)* Platelet Derived Growth Factor (-Receptor), *FGF-R* Fibroblast Growth Factor-Receptor, *EGF-R* Epidermal Growth Factor-Receptor, *IGF-R* Insuline-like Growth Factor-Receptor, *TNF* Tumor Necrosis Factor, *IL* Interleukin, *HIMF* Hypoxia-Induced Mitogenic Factor, *VEGF* Vascular Endothelial Growth Factor, *HGF* Hepatocyte Growth Factor, *c-Met* Hepatocyte Growth Factor Receptor, *CCR2* Chemokine (C-C motif) Receptor, *CXCR4* Chemokine (C-X-C motif) Receptor, *MCP-1* Monocyte Chemotactic Protein-1, *SDF-1* Stromal cell Derived Factor-1, *TGF-β* Transforming Growth Factor-β, *MMP-1* Matrix Metalloproteinase-1, *TiMP-2* Tissue Inhibitor of Metalloproteinase-2, *MT1-MMP* Membrane Type-1 Matrix Metalloproteinase

MSC activation, through transmigration into parenchyma of inflamed or injured tissue, and to understand the mechanisms that govern MSC regulation via different cellular properties in each step along the way (Fig. 9.2).

## Activation of MSC and Local Immune Responsive Cell

Systemic up-regulation of cytokines such TNF-α, IL-6 or HIMF, due to inflammation/injury or tissue hypoxia, is a first important stimulatory step for MSC recruitment. Those soluble factors activate the secretion of multiple immune modulators from MSCs, including PDGF-AB, IGF-1, HGF, EGF, RANTES, MDC, and SDF-1, which can modulate systemic and local immune responses. In addition, specific up-regulation of integrin α4/β1 or β1 on the cell surface of MSCs strongly enhance their attachment to osteoblast, myofibroblast or endothelial cells. This group of cells collectively regulate local cell migration for systemically circulating cells, such as

hematopoietic stem cells as well as bone barrow stromal cells. Upon activation, those cells also express increased levels of cell adhesion molecules such VCAM-1, which interact with integrins expressed on MSC and may thus regulate the first step of cell activation and initiate the cell homing process.

### Entrapment and Accumulation of MSC Toward Local Immune Responsive Cells

After MSCs arrive at the target tissue, direct cell-cell contact is required before transmigration into the parenchyma of the target lesion. This entrapment and accumulation process is partly regulated by VEGF from inflamed/injured tissues. Expression of VEGF can enhance the up-regulation of chemokines such SDF-1 or MCP-1, secreted from local immune regulatory cells including Osteoblast, Myofibroblast or Endothelial Cells. MSCs are known to express receptors for several chemokines, including CXCR4 or CCR2, which are activated in response to TNF-α, a pro-inflammatory cytokine also secreted from inflamed/injured tissues. This process is probably the most important and widely studied mechanism to understand the homing and migration capacity of MSCs. SDF-1/CXCR4 interaction between MSC and local immune responsive cells mainly contributes to this process, which may also similarly regulate the migration process of MSC into tumors. Additionally, MSC auto- regulation is controlled by the ligand, secreted HGF and its receptor, c-Met, both regulated by TGF-β (Neuss et al. 2004).

### Transmigration of MSC into Parenchyma

Finally, to produce an immune modulatory effect on local tissues, MSCs have to transmigrate into the perivascular or parenchyma through the tight junctions of microvascular endothelial barriers. Pro-inflammatory cytokines secreted from target tissues including PDGF, TNF-α, IL-1b or TGF-β lead to increased expression of MSC cell surface receptors. One of the expressed MSC receptors, CD44, can denature hyaluronic acid, an extracellular matrix component. In addition, inflammatory molecules can stimulate TLR on MSC, leading to the secretion of proteinases from MSCs. These include MMP-2, TMP-2 or MT1-MMP, which denature other important extracellular matrices components, such laminin, collagen IV and fibronectin and thereby strongly enhance cell migration toward the parenchyma (Sackstein et al. 2008).

## Role of Mesenchymal Stem Cell in Cancer Formation

The role of MSC in cancer development is still controversial. There is evidence that MSC promotes tumor progression by immune modulation (Karnoub et al. 2007). In contrast, some reports describe the tumor suppressive effect of MSC via modification of Akt signaling (Khakoo et al. 2006). The discrepancy between these results may come from the different tissue sources, individual donor variability, and injection timing. The expression of critical receptors such as TLR is variable in each time point of the treatment, which may also determine the effect of MSC on tumor progression. Whether MSC supports or suppresses tumor progression, it is clear that systemically administered MSC can be recruited and migrated toward tumors. These findings are important as they can be used as a basis for initiating studies to explore the incorporation of engineered MSCs as novel anti-tumor carriers, for the development of tumor targeted therapies.

## Mechanism of Homing and Migration to Malignant Tissue Sites

The migration capacity of MSC into multiple inflamed or injured tissues has been studied in various models, as described. Recently interest into understanding MSC homing and migration into tumors has also grown, and several investigators have begun to compare the two processes.

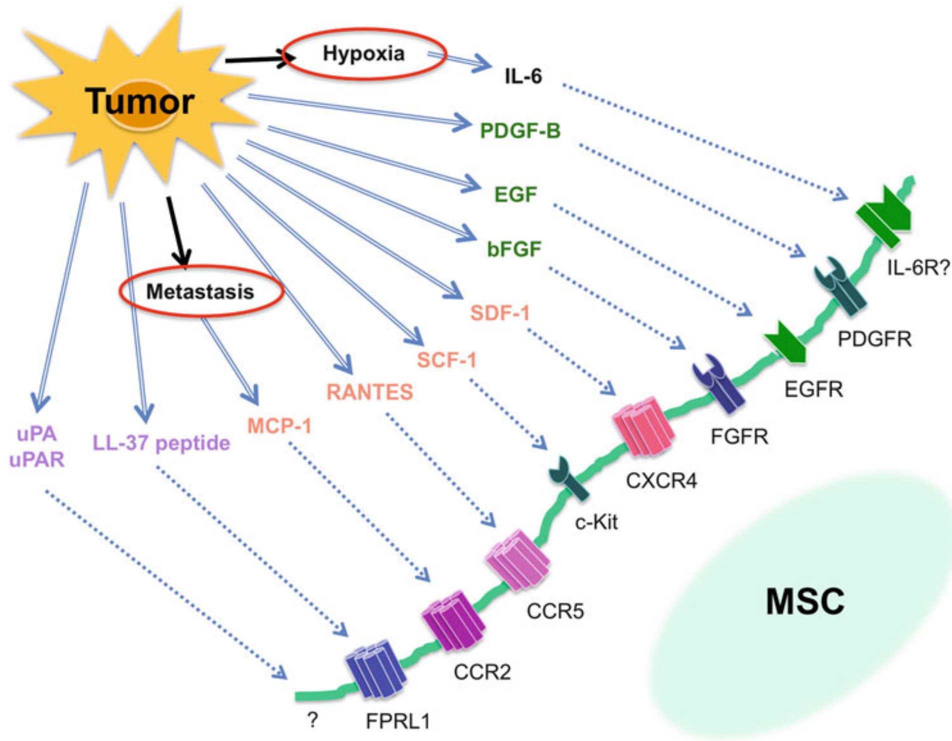


**Fig. 9.3 Regulating factors of homing and migration to malignant tissue sites.** The figure represents the reported factors that can regulate homing and migration of MSC into malignant tissue sites. Cytokines, growth factors, chemokines and other molecules released from tumor interacting to each receptor expressed on the surface of MSC. The release of these factors can be enhanced by hypoxic condition or metastasis of the tumor. *IL-6(R)* Interleukin-6(Recepator), *PDGF(-R)* Platelet Derived Growth Factor(-Receptor), *EGF(R)* Epidermal Growth Factor (Receptor), *bFGF(R)* basic Fibroblast Growth Factor (Receptor), *SDF-1* Stromal cell Derived Factor-1, *CXCR4* Chemokine (C-X-C motif) Receptor, *SCF-1* Stem Cell Factor-1, *CCR* Chemokine (C-C motif) Receptor, *MCP-1* Monocyte Chemotactic Protein-1, *LL-37* leucine, leucine-37, *FPRL1* Formyl-Peptide Receptor-Like 1

Since the process of tumor progression is highly related to inflammation, recent studies have revealed the importance of epithelial mesenchymal transitions in cancer development (Kalluri and Weinberg 2009), and the role of MSC in carcinogenesis is a new attractive concept in cancer therapy. In this section, we review recent reports demonstrating the homing mechanism of MSC. Several different mediators have been reported to be involved in this process. Some of these molecules such as growth factors, chemokines or cytokines have already been described as mediators which can regulate cell migration toward inflammatory sites, e.g. SDF-1 and SCF-1, CCL5/CCR5, CCR2, TNF-α or some other peptides (Fig. 9.3).

## Growth Factors

Vascular endothelial growth factor (VEGF) seems to be one of the important factors which enhance and direct stem cell motility. Indeed MSC demonstrated intensive migratory and invasive behavior in the presence of gliomas, which express high levels of VEGF. Ritter et al. (2008) demonstrated that VEGF, as well as bFGF secreted by breast cancer cells, induced migration of MSCs. They also demonstrated that receptors for these molecules were expressed on MSCs and that depletion of these growth factors by antibodies reduced their migration capacity. MSCs are known to express EGF and PDGF receptors on their surface, and the antibodies which block

PDGF-B or EGF were also able to attenuate the migration of MSCs. Those reports demonstrated that some malignant tumors such as glioma or breast cancer, which have highly specialized vasculature and stroma, could provide permissive environments for the selective engraftment of MSCs. Taken together, the tropism of MSCs for tumors may be mediated, at least in part, by specific growth factors and receptors expressed by MSCs, thereby using a recruitment mechanism similar to inflamed or injured tissue.

## Chemokines and Cytokines

Effect of Stromal cell derived factor (SDF) 1a / Chemokine (C-X-C motif) receptor 4 (CXCR4) in tumors: Stromal cell derived factor (SDF) 1α, which is a well-established chemo-attractant for leukocytes, acts directly on cancer cells by stimulating proliferation through the SDF-1α receptor CXCR4 expressed on the cancer cells. However, SDF-1α secretion also leads to recruitment of endothelial progenitor cells to the growing tumor, thereby promoting angiogenesis. Some reports demonstrated that SDF-1α secreted from cancer cells enhanced MSC tropism and the antibody that blocks SDF-1α could attenuate the migration of MSCs. An infection-related cancer development process, such as helicobacter infection in gastric cancer, can give rise to an environment conducive to the recruitment of MSCs and this can be regulated by SDF-1 and SCF-1. Houghton et al. (2004) demonstrated an interesting model, reporting that in response to chronic Helicobacter infection, bone marrow–derived cells could home to and repopulate the gastric mucosa and contribute over time to metaplasia, dysplasia, and cancer.

Effect of Chemokine (C-Cmotif) ligand 5 (CCL5 or RANTES)/Chemokine (C-Cmotif) receptor 5 (CCR5) in tumors: Chemokine (C-Cmotif) ligand 5 (CCL5) is one of the chemokines that can enhance stem cell migration during inflammation. Karnoub et al. (2007) demonstrated that the actions of CCL5 were responsible for MSC-induced metastasis by breast cancer cells. The breast cancer cells stimulated de novo secretion of the chemokine CCL5 (also called RANTES) from mesenchymal stem cells, which then acted in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis. This result indicated the critical importance of the CCL5–CCR5 paracrine interactions in enabling MSC to induce cancer metastasis.

Response to irradiation through the secretion of cytokines: The role of inflammatory-related cytokines and chemokines in radiation-enhanced MSC migration was reported by Klopp et al. (2007). They identified the cytokines and chemokines implicated in this chemotaxis towards irradiated tumor microenvironments. The low-dose irradiation of murine tumors enhanced the tropism for and engraftment of MSC in irradiated tumor environments. They demonstrated that tumor cells were able to increase the production and secretion of cytokines e.g., VEGF, PDGF-BB, or TGF-β, following irradiation, that enhanced the migratory properties of MSC. In addition, the chemokine receptor CCR2 was found to be up-regulated in MSC exposed to irradiated tumor cells as it was undetectable or expressed at low levels on untreated cells but could be up-regulated by inflammatory cytokines such as TNF-α. In addition inhibition of CCR2 led to a marked decrease of MSC migration in vitro. Taken together, these experiments suggested that radiation can increase the expression of inflammatory mediators that can secondarily enhance the recruitment of MSC into the tumor microenvironment.

Hypoxic condition in tumors and the up-regulation of IL-6: Hypoxia plays a role in tumor progression, metastasis and poor clinical outcomes. However, the role of hypoxia in MSC recruitment to the tumor microenvironment has not been described well. Rattigan et al. (2010) demonstrated that hypoxic breast cancer cells enhance their production of IL-6, which promotes the recruitment of MSC. Secreted IL-6 acts in a paracrine fashion on MSC, stimulating the activation of both Stat3 and MAPK signaling pathways to enhance migratory potential and cell survival. Specifically, increased migration is dependent on IL-6 signaling through the IL-6 receptor. Generally, IL-6 is a multifunctional cytokine that

plays a role in apoptosis, cell proliferation and survival. It binds to its cognate receptor leading to activation of the JAK/STAT signal transduction pathway. A similar pathway may contribute to the migratory capacity of MSC toward hypoxic tumors.

### Other Exogenous Molecules

Leucine, leucine-37 (LL-37) peptide from tumors: Previous studies have shown that LL-37 (leucine, leucine-37), the C-terminal peptide of human cationic antimicrobial protein 18, stimulates the migration of various cell types and has similar expression patterns in tumors, damaged tissue, and inflammation, where MSCs are prominent. These peptides also have the ability to stimulate chemotaxis of various cell types. Coffelt et al. (2008) demonstrated that LL-37 peptide promotes ovarian tumor progression through recruitment and engraftment of MSC into tumors, where these cells provide pro-angiogenic and immunomodulatory factors to support tumor growth and progression. Indeed, neutralization of LL-37 *in vivo* significantly reduces the engraftment of MSC into ovarian tumor xenografts, resulting in inhibition of tumor growth as well as disruption of the fibrovascular network. The LL-37-mediated migration of MSC to tumors likely occurs through formyl peptide receptor like-1. These data indicate that LL-37 facilitates tumor progression through recruitment of progenitor cell populations to serve as pro-angiogenic factor-expressing tumor stromal cells. Expressed factors include IL-1 receptor antagonist, IL-6, IL-10, CCL5, VEGF, and matrix metalloproteinase-2 (MMP-2). The overall consequence of LL-37's actions, through its recruitment of MSCs, is advancement of tumor progression.

Urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor (uPAR): Urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor (uPAR) are up-regulated in tumors of various origins, where they play critical roles in the development of invasive and chemo-resistant cancer phenotypes. The activation of uPA and uPAR in malignant solid tumors (brain, lung, prostate, and breast) augments MSC tropism. Gutova et al. (2008) revealed that chemo-attraction of MSCs to cancer cells strongly correlated with uPAR expression levels on tumor cells, which may be important for development of optimal stem cell-based therapies.

## Development of MSCs as a Stem Cell Therapy

Since there are some reports that describe sarcoma development following MSC transplantion into animals (Tolar et al. 2007), determination of MSC therapeutic efficacy and safety is now required for its clinical application. From a practical perspective, MSCs seems to be one of the most promising cell sources to use in stem cell therapy for tissue impairment, given that MSC can home to inflamed or injured tissues, as well as tumors, probably without differentiating into somatic cells. It is important to identify the utility of MSC in the clinical setting while understanding their complicated mechanisms as immune and inflammatory regulators. As shown in this chapter, the most promising clinical aspects of MSCs might be the immune-modulatory and anti-inflammatory effects. However a major challenge still remains in understanding both the actual benefits and side effects in human disease.

This chapter provided a review regarding the importance of IFN-γ as one of the key factors in inflammation as well as SDF-1, which plays a critical role in both inflammation and tumor related cell homing. Thus control of the level of these key factors in target tissues may be able to increase the specificity of MSC application to the tissues, which may also lead to a reduction in the total cell number needed for the therapy and in concert, reduce the potential side effects, such as malignant transformation. The receptors for the reviewed key factors expressed on MSC, including TLR or CXCR4, can also be potentially modified genetically via transfection, which may augment the efficacy of MSC in clinical settings and decrease the migration of MSC to non-targeted sites.

On the other hand, the clinical application of MSC for cancer treatment is still challenging, since the role of MSC in tumor development is not fully understood, particularly in the field of epithelial mesenchymal transition (EMT), which is a key mechanism of cancer invasion and might be partly affected by localized MSC. This chapter reviewed the migratory potential of MSC to malignant tissues, which is largely similar to MSC migration into inflammatory tissue. However, the factors released from the malignant cells, as well as surrounding tissues and vascular systems, are still regarded as a "black box"; thus, it is yet difficult to provide a specific role for MSC in cancer development after they migrate and home into different tissues. Although some reports demonstrated a tumor suppressive MSC effect, others described a tumor supportive potential. But in any case, these reports encourage the notion that MSC may play a critical role in cancer development and may be useful as a novel therapeutic delivery system that can target malignant tumors, potentially superior to existing therapeutic molecular therapies. While MSC can react to surrounding microenvironments, molecular therapies cannot. Thus it is imperative that scientists continue to investigate the role and mechanism of MSCs in tumor progression in order to harness MSC therapeutic potential to regulate both inflammatory and metastatic diseases.

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# Mesenchymal Stem Cells: The Role of Endothelial Cells and the Vasculature

10

Sally James, Julia Marshall, Kelly Langford, and Paul Genever

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**Abstract**

Mesenchymal stem cells/multipotent stromal cells (MSCs) are a population of stem cells that have been isolated from virtually every adult tissue. MSCs are able to differentiate into skeletal lineages, such as bone, cartilage and fat, and as such represent valuable research tools for unravelling the complex mechanisms that control fate determination and developmental processes. Their intrinsic tissue-forming capacity has also elevated MSCs to the forefront of regenerative medicine applications and tissue engineering strategies, particularly for orthopaedic interventions. However, the *in vivo* MSC remains poorly understood, with a paucity of selective markers and a relatively ill-defined niche. Recent work suggests that MSCs are associated with cells of the vascular system, which would permit a broad tissue distribution and may provide a form of supportive niche microenvironment. This review will provide an introduction to MSC biology, an overview of potential *in vivo* interactions and how this knowledge may advance MSC research and clinical goals.

## Introduction

MSCs are currently receiving a high level of interest in the fields of stem cell biology and regenerative medicine. The MSCs' multipotent differentiation capacity coupled with the relative ease of isolation and *ex vivo* amplification has

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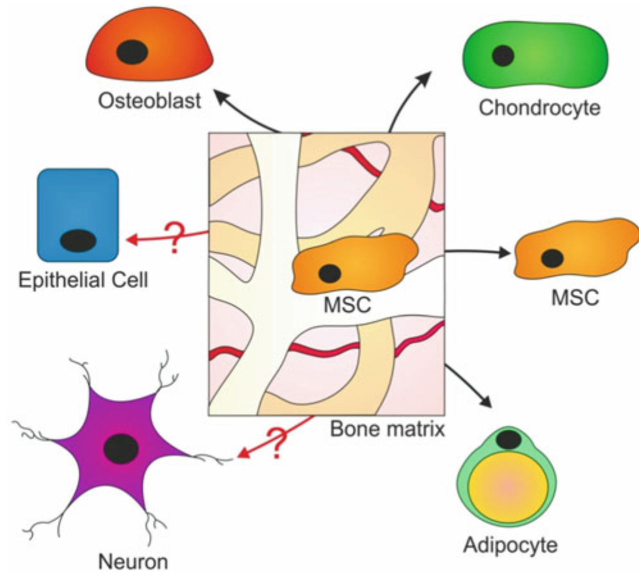


**Fig. 10.1** Multilineage differentiation of MSCs into skeletal cell types including osteoblasts, chondrocytes and adipocytes. There is some evidence to suggest that MSCs can partially differentiate into non-skeletal lineages, such as epithelial cells and neurons

made these cells attractive candidates for a broad range of cell-based therapies but despite this appeal, challenges remain. One issue relates to the *in vivo* location of MSCs and how coresident tissue cells may influence their behaviour. A proposed perivascular MSC niche has focused attention on the relationship between MSCs, pericytes and endothelial cells and how a deeper insight into these heterotypic cell-cell interactions can be translated into further research and clinical advances.

## Mesenchymal Stem Cells

It was in the 1970s that Friedenstein first isolated a mesenchymal stem cell-like population from bone marrow. Friedenstein characterised these cells as adherent multipotent cells with a fibroblast-like morphology with the ability to form colonies (Friedenstein et al. 1976). Today, different cell populations isolated from various adult tissues and defined as "MSCs" (mesenchymal stem cells or multipotent stromal cells) are characterised by their plastic-adherence and tri-lineage differentiation potential, into osteogenic, adipogenic and chondrogenic cell types (Fig. 10.1). There is currently no selective cell surface marker for MSCs, therefore a non-specific panel is frequently used. MSCs are described as being positive for CD166, CD105, CD90, CD73, CD44 and CD29 and negative for CD235a, CD144, CD79a, CD45, CD34, CD19, CD14, CD11a and HLA-DR (Dvorakova et al. 2008; Karp and Leng Teo 2009), though other markers have been described, including Stro-1, CD146 and CD271 (Shi and Gronthos 2003).

*Ex vivo* MSCs are useful models to study skeletal differentiation in simplified in vitro conditions, by exposing the cells to a cocktail of inductive stimuli. Osteogenic differentiation is induced by supplementing the culture medium with dexamethasone, β-glycerophosphate and ascorbic acid; adipogenic differentiation through the addition of insulin, isobutylmethylxathine, indomethacin and dexamethasone and chondrogenic differentiation requires dexamethasone, ascorbic acid, insulin transferring selenium, transforming growth factor-β3 and proline. Whilst the ability of MSCs to differentiate into skeletal (mesoderm-derived) lineages is a defining characteristic, some evidence suggests that MSCs can also differentiate into cell and tissue types from the endoderm and ectoderm. For example, there is evidence that MSCs can be induced into a neuron-like lineage using β-mercaptoethanol, retinoic acid, forskolin, platelet

derived growth factor, basic fibroblast growth factor and glial growth factor (Mahay et al. 2008). Such a broad, even pluripotent, differentiation potential for adult stem cells would have pervasive research and clinical benefits, but this area of research remains controversial.

## MSC-Based Therapies

The field of regenerative medicine has been gaining momentum over the past decade or so with MSCs emerging leading players of this diverse area. MSCs are known to be immunomodulatory, to release large numbers of growth factors, and to undergo tri-lineage differentiation. The therapeutic application of MSCs has also gained attention as it potentially bypasses the ethical debate regarding use of fertilized embryos to generate stem cells (Chanda et al. 2010), a fact that has made their use appealing for many companies developing regenerative medicine-type products.

There are a number of ways in which MSCs could be used therapeutically. There are already a number of clinical trials underway, focusing on the use of MSCs for immunological disorders such as GVHD and Crohn's disease (Sensebe et al. 2010). These trials make use of the inherent immunomodulatory effects demonstrated by MSCs both *in vitro* and *in vivo* (Roberts et al. 2011). In addition, the immunoprivileged status of MSCs provides the potential for an allogeneic, off-the-shelf therapy to be developed which would greatly improve the cost, availability and possibly the efficacy of any MSC therapy commercially.

There is a wealth of evidence suggesting that MSCs are good candidates for skeletal repair. Within bone, MSCs contribute to the normal remodelling and repair processes by providing a pool of osteoblasts necessary to form the mineralized matrix of bone (Arthur et al. 2008). However, in many types of diseases and conditions such as non-union, this ability to remodel skeletal tissues is impaired (Arthur et al. 2008). Therapeutic applications of MSCs in bone disease could aid in the supply of increased numbers of 'healthy' MSCs and aid acceleration of healing and/or alleviate symptoms associated with defective MSC populations. There are already a number of *in vivo* and clinical studies which show promise for the therapeutic application of MSC for diseases such as osteogenesis imperfecta, infantile hypophosphatasia and osteoporosis (Chanda et al. 2010).

A key disease area for MSCs is in the treatment of osteoarthritis, a disorder which is estimated to affect 8.5 million people in the UK alone (Roberts et al. 2011). Within a goat model of osteoarthritis, MSCs have been shown to promote regeneration of meniscal tissue following intra-articular injection (Murphy et al. 2003). This regeneration has been shown to occur with very little integration of administered MSCs to the newly formed tissue, a phenomenon that is often repeated within the literature. Disappointingly, this meniscal regenerative capacity was not seen clinically, although an improvement within patients in terms of pain and reduction of degenerative bone changes was noted, the primary endpoint of meniscal regeneration was not seen (Osiris 2011). This leads to intriguing questions in terms of the translation of preclinical work into clinical therapies, the types of patients that may be applicable for these types of treatments, and the time elapsed between injury and treatment. Interestingly it has been proposed that osteoarthritis, a disease typically considered as occurring due to damage to the cartilage/subchondral bone layers, also has an inflammatory element (Goldring and Otero 2011). Potentially MSCs delivered to diseased joints may be altering this inflammation in some way and contributing to repair of damaged tissues. Whilst administration of stem cells alone appears to provide a benefit for haematopoietic, neuronal and cardiac regeneration, for skeletal repair, structural and mechanical support is often required, usually provided in the form of a scaffold (Arthur et al. 2008). There is a wealth of research on the use of scaffold technologies for stem cell delivery. These range from natural and synthetic biomaterials (Seo and Kun 2011), and nano-textured scaffolds (Seo and Kun 2011) through to complex self-assembling peptide gels (Miller et al. 2010). A deeper knowledge of the *in vivo* niche, and its structure and organisation in different tissue types, will also contribute to new strategies for regulating the proliferation and differentiation status of MSCs in a 3D context.

## MSC Tissue Sources

Historically, MSCs isolated from adult bone marrow have received most attention, however wider MSC tissue sources have been discovered. For example, MSCs have been sourced from adipose tissue, lung, liver, spleen, dental pulp and umbilical cord blood. The tissue sources MSCs have been derived from all have a close association with the vascular system; therefore, MSCs might reside within a perivascular origin (Crisan et al. 2008). MSCs located from different sources have shown the same general cell surface markers, morphology and function. However, the anatomical location of MSCs may affect some specific characteristics; for example, Sakaguchi and co-workers (Sakaguchi et al. 2005) showed that MSCs derived from synovium underwent chondrogenesis more readily compared to the bone-marrow derived cells. This implies that MSCs from a specific tissue are partially programmed in a way that may augment local repair. This concept is further supported by evidence that adipose-derived MSCs achieve adipogenesis more effectively than bone marrow-derived MSCs.

Due to the wide variety of tissues in which MSCs have been discovered, it has been hypothesised that MSCs have the ability to enter the circulatory system from various tissues and travel to a site of injury. However, several studies have failed to detect MSCs in peripheral blood, which argues against this theory. The broad tissue distribution also questions the nature of the specialised microenvironment, or niche, in which MSCs are located. It may be that the specific organisation and composition of the MSC niche varies with tissue-type dependency. Another possibility is that MSCs are associated with the vasculature, in contact or close association with endothelial cells and pericytes.

## The Stem Cell Niche

The concept of a stem cell niche was first introduced by Schofield (1978), who presented the hypothesis that a stem cell must associate with "other cells which determine its behaviour", and wherein "its maturation is prevented and as a result, its continued proliferation as a stem cell is assured". The concept of a niche has since evolved and is now used to refer to the defined anatomical compartment in which stem cells are located. This incorporates not only specific cell types but also the microenvironment in which these cells are located. Multiple niche effectors have been found to instruct stem cell fate, maintenance, proliferation and differentiation. These include biochemical signalling cues from neighbouring cells, soluble factors or the extracellular matrix (ECM), or physical factors such as matrix elasticity, sheer force and oxygen tension (Wang and Wagers 2011).

The MSC niche is perhaps less well defined than for other adult stem cells. Initial studies of the adult stem cell niche focused on localised tissues such as the hair follicle, where clear spatial constraints made identification of niche components more straightforward. However, one of the most well characterised stem cell niches is that of haematopoietic stem cells (HSCs). HSCs are known to reside within the bone marrow, the primary site of adult haematopoiesis and where MSCs co-exist. Whilst HSCs are a rare cell population in bone marrow, comprising generally less than 1% of the mononuclear cell fraction, they are considerably more abundant than the even rarer MSCs, and have a clearly defined surface marker profile. The ability to purify HSCs based on this profile, modify them and reintroduce them to the *in vivo* setting, combined with the use of new and elegant techniques, have provided key insights into the origins, physiological regulation and dynamics of the HSC niche. Like the MSC niche, the haematopoietic niche may not be anatomically restricted, and the signals provided to HSCs may be spatially as well as temporally regulated. A critical role for biomechanical forces in haematopoietic development was recently revealed, (Adamo et al. 2009) both *in vitro* and *in vivo* by demonstrating that fluid sheer stress, to which foetal HSCs are subject, increases expression of the transcription factor RUNX1, a master regulator of haematopoietic development. Indeed, the quality of the mechanical support provided by

the extracellular matrix has been reported to affect the differentiation of many stem cell subsets and several studies have shown that exposure of MSCs to sheer stress in vitro may influence their proliferation and differentiation (Keung et al. 2010).

It has been hypothesised that the presence of low oxygen tension in stem cell niches offers a selective advantage that is well suited to their particular biological roles. Embryogenesis, for example, is heavily influenced by oxygen gradients, and it has long been postulated that HSCs reside in a hypoxic niche. Upregulation of hypoxia inducible factor 1α (HIF1α) in HSCs in response to hypoxia has been reported to cause a shift towards glycolysis rather than mitochondrial oxidative phosphorylation, which promotes HSC survival in a hypoxic environment. Deletion of HIF1α was shown to promote HSC proliferation, resulting in an exhaustion of their repopulation capacity. However, over-stabilisation of HIF1α also leads to HSC loss, suggesting that there is a narrow window of hypoxia, optimal for HSC maintenance (Wang and Wagers 2011). Tracer studies of HSCs in bone marrow cavities, and the creative use of Hoechst dye as a marker for hypoxia, have revealed that HSCs exist in poorly perfused niches and most likely reside coexisting with osteoblasts, where MSCs were located in closer proximity to the vasculature. However, the different tissues where MSCs are found may themselves exhibit low oxygen tension. MSCs derived from bone marrow or adipose tissue, when cultured in hypoxic conditions, show decreased differentiation into adipogenic or osteogenic cells, promoting the notion that low oxygen levels may promote “stemness”. However, hypoxia can also induce MSC apoptosis in a caspase-dependent manner (Mohyeldin et al. 2010), and promote MSC migration.

Cellular interactions are also of vital importance in the niche environment. MSCs in culture are known to require cell-cell contact for efficient differentiation to occur, an effect that can be mimicked through the use of N-cadherin on culture plates (Gobaa et al. 2011). In the case of HSCs, interaction with osteoblasts at the interface between bone and the bone marrow space, in what is termed an endosteal niche, is known to be vital for stem cell function. Osteoblast ablation results in the loss of HSCs, where genetic manipulation to increase osteoblast numbers promotes a concomitant increase in the number of HSCs. The mechanisms underlying this regulation are still unclear, although osteoblasts produce the cytokines GM-CSF, G-CSF and IL-6 which support both HSC survival and differentiation, and other factors that can regulate HSC numbers, such as angiopoietin, N-cadherin, osteopontin, Wnt and Notch. *In vitro* co-culture experiments have further demonstrated the ability of osteoblastic cells, or cells with osteoblastic potential, to maintain HSC growth and function. Culture of HSCs with endosteal cells is sufficient to maintain the long term reconstituting capacity of HSCs. Furthermore, this “endosteal” population is heterogeneous and capable of differentiation into adipocytes, osteoblasts and chrondrocytes, with each subtype having a differential ability to support HSCs (Wang and Wagers 2011). Ablation of nestin-positive MSCs results in a considerable reduction in the number of long term HSCs in the bone marrow. Thus it is proposed that a dual niche may exist within the bone marrow, where both HSCs and MSCs reside and simultaneously respond to, and interact with, multiple different niche cell inputs (Mendez-Ferrer et al. 2010).

## MSCs and the Vascular Niche

The lack of definitive, selective markers of MSCs combined with the wide range of tissues in which they may be found mean the exact identity and nature of the MSC niche has remained elusive (Fig. 10.2). MSC niches may be housed in specific tissues but capable of migration through the circulation to additional sites of requirement. However, multiple studies have failed to detect MSCs circulating in normal peripheral blood. There is growing evidence that pericytes may be the native form of *ex vivo* MSCs. Pericytes, also known as mural or Rouget cells, mesangial cells in the kidneys, Ito cells in the liver, or advential reticular cells in the bone marrow, are branched cells located on the abluminal side of small

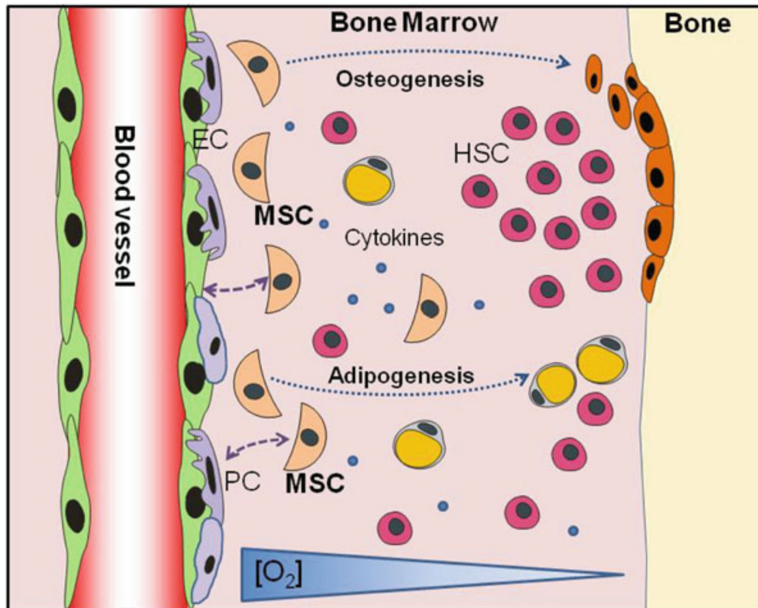


**Fig. 10.2** Schematic representation of the proposed interactions between MSCs and microenvronmental niche components in proximity to the vasculature. *EC* endothelial cell, *PC* pericyte, *HSC* haematopoietic stem cell

blood vessels, in close association with the vessel's endothelial cells. They function in vessel stabilisation, synthesis of matrix proteins and immunological defence, and have macrophage-like properties and, possibly, mesenchymal potentiality. Pericytes isolated from a variety of tissue sources have been shown to exhibit an *in vitro* phenotype similar to that of MSCs; they express MSC markers such as CD44, CD73, CD90 and CD105, and are capable of in vitro differentiation into adipocytes, osteocytes or chondrocytes (Saleh and Genever 2011; Augello et al. 2010). Conversely, Stro1-positive bone marrow stromal stem cells were found to express the pericyte markers α-smooth muscle actin and CD146. Large similarities in the global expression profiles of primary MSC and pericyte cultures have been observed (Crisan et al. 2008). Canfield's research group have shown that, under defined conditions, isolated pericytes, housed in diffusion chambers implanted into athymic mice, were capable of differentiating into bone, cartilage and adipose tissue *in vivo* (Doherty et al. 1998; Farrington-Rock 2004). However, prospective cell tracking experiments that could confirm whether pericytes can directly contribute to tissue repair or regeneration by differentiation into mature mesenchymal phenotypes *in vivo* are yet to be performed.

Their proximity to blood vessels would allow MSCs/pericytes to quickly enter the bloodstream and migrate to sites of injury or physiological turnover and replace lost tissues. So, are all MSCs pericytes? There does seem to be a distinct overlap in gene expression profile, surface marker phenotypes and functional properties between mesenchymal cell types in the bone marrow. However, given that both large and small vessels are surrounded by perivascular cells with a wide range of functions quite separate from that of adipo-, osteo- or chondrogenic activity, it can be said that not all pericytes are MSCs. MSCs can also be isolated from avascular tissues such as articular cartilage, implying that MSCs may not be exclusively located in a pervivascular niche. MSCs isolated from different tissues display subtly different properties *in vitro*, and it is likely that MSC/pericytes from developing tissues will behave differently to those from adult tissues involved in homeostasis. The question remains as to whether MSCs are a unique population of

cells, distinct from pericytes, existing in multiple niche environments, or whether there are multiple subsets of MSCs and progenitor cells with subtly different functions.

## MSC Models and Niche-Based Therapies

As highlighted within this review, the niche is a significant and important factor in both the maintenance and mobilisation of MSCs *in vivo*. Once removed from the niche, in situations such as standard tissue culture, MSC are no longer provided with the spatial and biochemical cues that the niche contributes. We do not yet fully understand the impact this has on the MSC itself and the effects this might have on MSC expansion or differentiation potential. Research is now moving towards studying the MSC niche to better understand the cells themselves. One aim of this type of research is the development a simplified *in vitro* MSC niche model to better maintain their biology and aspects of 'stemness'. Research into the co-culture of endothelial cells alongside MSCs in 3D spheroid-type structures and investigation of the effects of endothelial cell-conditioned media on MSC could start to provide some of these answers. Within these studies endothelial cell cues have been shown to increase the CFU-F and osteogenic capacity of MSC *in vitro* (Saleh et al. 2011a). 3D MSC-endothelial cell spheroid co-cultures also self-organise and develop primitive capillary-like networks (Saleh et al. 2011a, b). The development of niche-like models could pave the way for clinical niche-based therapies, with greater efficacy compared to single cell-type suspensions. These have the potential to broaden the use of MSCs clinically, perhaps enabling in situ reprogramming of diseased or aged MSCs or increasing MSC potency resulting in lower therapeutic cell numbers being required.

In conclusion, it is clear that a better understanding of the *in vivo* localisation of MSCs would benefit *ex vivo* MSC applications. Increasing numbers of reports that MSCs are associated with a perivascular niche would explain their apparent location in virtually all tissues and it may be that the MSC niche is not uniformly defined across different tissue types. The isolation of tri-potent MSCs with broad mesenchymal differentiation potential from mesodermal, endodermal and ectodermal sources would appear to contradict known developmental programmes and MSCs may experience imprinting from their tissue of origin that determines how they behave *ex vivo*. These biological phenomena should be considered during MSC extraction, growth and in vitro manipulation to maximise the potential of these intriguing cell types.

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# Use of Cancer/Testis Antigens in Immunotherapy: Potential Effect on Mesenchymal Stem Cells

11

Felipe Saldanha-Araujo and Rodrigo Haddad

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**Abstract**

Currently, the identification of appropriate target antigens is the most critical step for the development of antigen-specific cancer immunotherapy. The cancer testis antigens (CTAs) have emerged as potential targets for cancer therapies due to their expression in a range of malignant cells with low or highly restrict expression in normal tissues. Several clinical trials involving CTAs are now being conducted with some promising results. Conversely, the identification of CTAs expression in normal stem cells, such as mesenchymal stem cells (MSCs), should be considerate in these studies. The possible side effects of CTA-based immunotherapy in MSCs cannot be ignored or overlooked, taking into account their biological properties.

## Introduction

The development of strategies for cancer therapy has been one of the major challenges of the last decades. Conventional treatments for cancer, such as chemotherapy and radiotherapy, are able to shrink the tumors, but fail to destroy the cells that maintain the tumoral growth and metastasis, known as cancer stem cells (CSCs) (Soltanian and Matin 2011). The discovery of CTAs expression in cancer cells and immunologically privileged tissues, such as germ cells of the testis, and new evidences that CSCs may express these markers (Costa et al. 2007) permitted that CTAs

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were considered as potential targets for cancer treatment. In contrast, CSCs and normal stem cells (especially mesenchymal stem cells – MSCs) shared some similar characteristics including asymmetric division, induction of angiogenesis, and cell migration (Soltanian and Matin 2011). In addition, MSCs from different sites also express CTAs (Cronwright et al. 2005; Saldanha-Araujo et al. 2010), and thus these cells could be destroyed by therapy based on CTAs. Considering the biological properties of MSCs, could cancer vaccine targeting CTAs lead to non-desired effects because the off-target effects on MSCs? Based on this question, this chapter will discuss some aspects about CTAs immunotherapy, MSCs biological properties and possible implications on MSCs in CTA-based immunotherapy.

## Cancer Testis Antigens

Over the last decades, the search for human tumor antigens or tumor-associated antigens (TAAs) has been attractive in order to explore their potential as immunotherapeutic targets for antibody-based therapies and cancer vaccines. Transfection-based assays led to the discovery of the first TAA, the human cancer antigen termed melanoma antigen-1 (MAGE-1, later renamed MAGE-A1). It was first recognized as targets for CD8 T cell recognition of autologous human melanoma cells (van der Bruggen et al. 1991). Subsequently, the same researchers identified another member of MAGE-A family, termed MAGE-3, and two additional families of TAA (GAGE and BAGE). Further studies showed that these TAAs were expressed in a large number of cancer cells and testis normal cells, but not in any other normal somatic tissues or cells (Boon et al. 1997).

Soon afterward, the development of SEREX (serological analysis of recombinant cDNA expression library) methodology led to the identification of several tumor antigens. After these immunological methods, many methodologies based on comparison of mRNA expression between tumor cells and normal cells or testis cells and other tissues cells were used to identify genes with expression restricted to tumor cells, testis and placenta (and in the ovary occasionally). To segregate the genes that share this pattern, the term Cancer/Testis Antigens (CTAs) was coined in 1997 (Caballero and Chen 2009). With the expansion of number of CTAs genes, it became necessary the establishment of a database. Currently, at least 70 families of CTAs have been indentified and registered in a database established by the Ludwig Institute for Cancer Research (http://www.cta.Incc.br) (Almeida et al. 2009).

The most of CTAs genes are encoded by X chromosome clusters, particularly at a telomeric end between Xq24 to Xq28 and centromeric region from Xp11.2 to Xp11.4. On the other hand, there are some CTAs that are not encoded by X chromosome clusters, but by single-copy genes distributed throughout the genome. In addition, no additional chromosomal clustering is found for these genes. The differences in the localization reflect on their expression pattern and biological function, and lead to the classification of these genes in X-CTA genes (encoded by X chromosome) and non-X-CTA (Simpson et al. 2005).

Based on experimental evidences, the expression of some CTAs genes is regulated by epigenetic mechanisms. The most widely characterized epigenetic events controlling the expression of CTAs genes are DNA methylation and histone post-translational modification (Fratta et al. 2011). Experiments demonstrated that the reactivation of CTA expression in cancerous cells occurs when methylated regions of chromatin are demethylated. Moreover, the acetylation of histones, usually deacetylated, may trigger the CTA expression in cancer cells. So far, it is not known whether this reactivation is an anomaly of deregulated expression or is related with proliferative and metastatic potential of cancer cells (Cronwright et al. 2005).

Recent studies on cancer cells showed that some CTAs are likely involved in cell cycle progression and regulation, cell survivor, apoptosis and transcriptional control. In addition, increased evidences indicate that CTAs are possible associated

to carcinogenesis and spermatogenesis processes, but the biological function of the most of CTAs are poorly understood. Furthermore, both negative and positive effects of CTAs expression were related with the prognosis of cancer patients, supporting a potential implication of these proteins in tumor cells (Fratta et al. 2011).

## Cancer Testis Antigens as Targets for Immunotherapy

The CTAs have some particular characteristics. First, the immunogenicity of some CTAs was demonstrated by cytotoxity T-cell cloning (e.g. MAGE-A1 and MAGE-A3). The tumor cells expressing these CTAs may be recognized by CD8+ T lymphocytes, which are restricted by HLA class I antigens and mediate rejection responses (Boon et al. 1997). Second, the CTAs have the capacity to stimulate the humoral immune response, as initially determined by SEREX method (e.g., NY-ESO-1 and SSX). Anti-CTA antibodies have been detected in serum of patients with many cancer types, including lung cancer, breast cancer, cutaneous T-cell lymphoma, and melanoma, etc. (Caballero and Chen 2009). Third, the expression of CTAs in a wide variety of malignant tumors and their restricted expression in immunologically privileged tissues, such as germ cells of the testis. In fact, the lack of HLA class I expression in the germ cells and the blood-testis barrier (also known as "Sertoli cell barrier") avoid the recognition of CTAs as non-self structures by immune system (Fratta et al. 2011). Taken together, these features are considered essential to make the CTAs as excellent targets for vaccine development. Furthermore, some of them are biomarkers of different types of tumors, such as ovarian cancer, breast cancer, lung cancer, testicular cancer and others (Caballero and Chen 2009).

Multiple clinical trials at different phases involving CTAs as therapeutic agents are now being conducted with some promising results. On the other hand, recently completed clinical trials showed that even though the immunotherapies against some CTAs shrink the tumors, the survival rate of the treated patients group versus placebo group was not increased (Fratta et al. 2011; Kawada et al. 2012). Classic examples of CTAs that elicit both humoral and cytotoxity T-cell responses are the NY-ESO-1 and MAGE-A family, and both were and are currently being evaluated in phase I and II clinical trials. For instance, a series of trials with MAGE-A3 recombinant protein (combined or not with adjuvant) showed, for some patients, the specific T-cell response and increased titers of circulating anti-MAGE-A3 antibodies. Also, immunological responses, including high levels of antibodies and T-cell recognition were observed for NY-ESO-1 clinical trials. In addition, the administration of anti-CTLA-4 monoclonal antibody in melanoma patients enhanced NY-ESO-1-specific T-cell response and titers of circulating anti-NY-ESO-1 antibodies. Thus, the combination of non-specific immunotherapy approaches, to NY-ESO-1 cancer vaccines, seems to be a promising strategy. Despite of abundance of studies involving MAGE-A3 and NY-ESO-1, studies for other CTAs have been very limited to date (Fratta et al. 2011; Caballero and Chen 2009), thus encouraging further studies.

## Biological Properties of Mesenchymal Stem Cells

The most well characterized adult stem cells are in the bone marrow. Besides the hematopoietic stem cells that give rise to blood cells, this tissue contains another population of cells called mesenchymal stromal cells or mesenchymal stem cells (MSCs). These non-hematopoietic stem cells were first recognized by Friedenstein, more than 40 years ago. In this classic study, a population of plastic-adherent fibroblastic cell that could differentiate *in vitro* into bone, cartilage, muscle and fat was described (Friedenstein et al. 1968). Although the cells were initially identified in bone marrow, it was later demonstrated that these expandable spindle-like plastic-adherent cells could be obtained from virtually all adult and fetal human tissues (Covas et al. 2008). Currently,

MSCs are identified by their plastic adherence, by phenotypic analysis and by their capacity of differentiation *in vitro* into bone, cartilage and fat.

## Immunophenotype

MSCs obtained from bone marrow, the principal source to research, do not express the hematopoietic markers CD11, CD14, CD34 and CD45, neither costimulatory molecules (CD40, CD80 and CD86) or adhesion molecules, such as CD18, CD31 and CD56. On the other hand, more than 95% of these cells, when measured by flow cytometry, must express CD105, CD73 and CD90 (Horwitz et al. 2005). Some factors, such as source and methods used for isolation and culture, can alter MSCs phenotype and make difficult the interpretation and comparison of results obtained by different groups. In addition, none of markers listed above are specific to MSCs. Therefore, the search for a specific marker for these cells is essential to allow more advances in MSCs research.

## Differentiation

Besides the phenotypic analysis, MSCs can be identified by their capacity to differentiate into bone, cartilage and fat. The osteogenic differentiation *in vitro* is performed by the treatment of a monolayer of MSCs cultured in medium supplemented with dexamethasone, ascorbic acid and β-glycerophosphate. After 14–21 days, osteocytes can be detected by von Kossa stain. Cultivated in the presence of 1-methyl-3-isobutylxanthine, dexamethasone, insulin and indomethacin, the MSCs differentiate into adipocytes. These cells express peroxisome proliferation-activated receptor g2 (PPARg2), lipoprotein lipase (LPL) and the fatty acid– binding protein aP2, and their lipid vacuoles can be detected with oil red O staining. The chrondrogenic differentiation from MSCs is performed culturing these cells in the presence of transforming growth factor-β3 (TGF-β3). The cells produce type II collagen, that is typical of articular cartilage and are positive for chondrocytic markers (Pittenger et al. 1999).

## Immunological Properties of MSCs

Recently, MSCs have attracted the attention of researchers by their capacity of modulate T-cells response. As noted above, these cells do not express costimulatory molecules and, moreover, MSCs lack expression of MHC class II (Tse et al. 2003). However, INF-γ can induce MSCs to express MHC class II and even under this condition, these cells are not capable of inducing immune responses (Le Blanc et al. 2003; Gotherstrom et al. 2004). MSCs play a role on suppressive T lymphocytes by three main mechanisms: cell-cell contact, production of soluble factors and by inducing regulatory T-cells (Tregs). Both intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) have an important role in T-cell immunomodulation by MSCs. In addition, these cells are able to produce several suppressive molecules such as adenosine and IDO, and can modulate immune response by induction of classical Tregs and other subtypes of suppressive T-cells (Saldanha-Araujo et al. 2011, 2012; Maccario et al. 2005; Ren et al. 2010; Meisel et al. 2004). The suppressive property of MSCs encouraged many researchers to use this cell clinically, especially for the prevention and treatment of GVHD. Although many results are promising, there is not a comprehensive view of the behavior of MSCs *in vivo*.

## Off-Target Effect of CTA-Based Immunotherapy on MSCs

It is well-established that CTAs are expressed in a large number of tumors and in a restrict set of normal tissues, including germ cells of the testis, fetal ovary and placental tissues. Furthermore, some immature cells (e.g., spermatogonia and oogonia cells) and non-gametogenic tissues (e.g., pancreas, liver and spleen) may also display CTAs expression, although at low levels (Scanlan

et al. 2004). Numerous studies have shown that CTAs are expressed at different types of stem cells, such as MSCs from different adult and fetal tissues, CD34+ hematopoietic stem cells and embryonic stem cell lines (Lifantseva et al. 2011; Saldanha-Araujo et al. 2010; Steinbach et al. 2002). However, little is known about the specific function of CTAs in these cells. For stem cells, some authors suggest that CTAs play a role at earlier stages during embryonic development and stem cell self-renewal (Costa et al. 2007). For germ cells, reports showed that MAGE-A and GAGE families are expressed specifically in the human germ line during the development of male and female reproductive systems. Also, MAGE-A family are expressed in somatic cells, particularly in the central nervous system and peripheral nerves development, and in the myotome myoblast at the early stages. Moreover, GAGE proteins were expressed in the early ectodermal and neuroectodermal cells (Gjerstorff et al. 2008). Taken together, the function of CTAs appears to be related to differentiation of stem cells, germ cells and somatic cells in the early stages of development.

The expression of CTAs in normal stem cells may be explained by consistent links between these cells and cancer stem cells (CSCs). The traditional model of cancer development suggests that a sequential mutation in normal cells, resulting from genetic instability and/or environmental factors, promotes the tumor formation. However, the mature cells of many tissues in which the tumors arise (e.g., blood and skin) have limited lifespan. Thus, the accumulation of mutations necessary for tumor development is a major hurdle for this model. On the other hand, a second model has been proposed based on CSCs. In which, the tumors originate after the deregulation of self-renewal process of normal stem cells or progenitor cells, leading to CSCs (Soltanian and Matin 2011).

Some authors suggest that a restricted cell population in the tumor mass retain stem cell properties (possible the CSCs), promoting the tumor maintenance, proliferation and metastasis (Costa et al. 2007). These CSCs could harbor drug resistance and low radiosensivity, resulting in tumour recurrence after various treatments (Yawata et al. 2010). In addition, the CTAs expression in tumor tissues could be restricted to these cells (Costa et al. 2007). These findings suggest that the eradication of CSCs by targeting CTAs could lead to a complete cure of cancer. In contrast, some CTAs are expressed on adult stem cells and the implication of CTA-based immunotherapy on MSCs cannot be disregarded.

Recently, the epithelial mesenchymal transition (EMT), an important concept to embryology, has been studied in oncology, especially to process of cancer progression and metastasis (Thiery et al. 2009). Interestingly, mammary epithelial cells induced to EMT generate a cell population enriched for CSCs (Gupta et al. 2009). Although the exact origin of CSCs is not known yet, it is possible that these cells are originated from adult stem cells. In this sense, CSCs posses similarities when compared with MSCs, such as asymmetric division, identical expression of integrins (CD134, CD133 and CD49e), induction of angiogenesis, resistance to apoptosis and cell migration (Jorgensen 2009; Soltanian and Matin 2011). In addition, as well as CSCs, MSCs also express CTAs (Cronwright et al. 2005; Saldanha-Araujo et al. 2010; Costa et al. 2007).

As previously discussed, MSCs are capable to induce immunoregulatory cells generation. Interestingly, this MSCs property is similar to that observed at the tumor stromal cells because many immunomodulatory factors (such as IDO, PGE2 and TGF-β) are similarly secreted by the tumor stromal microenvironment and by MSCs (Kim et al. 2006; Shi et al. 2011). Although MSCs present in the tumor niche may provide an immune escape mechanism influencing cancer growth and spread (Direkze and Alison 2006), MSCs located in the wall of the vasculature throughout the body (Covas et al. 2008) could contribute to the peripheral homeostasis of the immune system. Considering the CTAs expression by MSCs, the cancer therapy involving CTAs could kill the MSCs that favor the tumor growth. Otherwise, MSCs located in vasculature throughout the body could also be affected, leading to a deregulation of homeostasis in the immune system. Studies have shown that MSCs express

NY-ESO-1, a CTA investigated as target for cancer immunotherapy trials. In these trials, the possible side effects on MSCs were not evaluated. The choice of targets that is not expressed on normal stem cells could be a good strategy to avoid non-desired effects.

In summary, the expression of CTAs on stem cells population, especially MSCs, should be evaluated taking into account the risk of the off targets effect. The cancer vaccine targeting CTAs could cause a range of deleterious effects by targeting the MSCs due to their features, including the differentiation and immunomodulatory properties. On the other hand, despite of the expression of CTAs on MSCs has been demonstrated, it is unclear whether this expression occurs *in vivo* or is activated during their cultivation *in vitro*. Furthermore, the fact that MSCs have immunoevasive properties, including CTL-recognition escape, encourages the use of CTAs as targets for cancer therapy. In conclusion, additional studies evaluating CTAs expression in normal stem cells, including MSCs, need to be performed in order to demonstrate the roles of these proteins. Whether the cancer vaccine targeting CTAs will have off-targets effect on MSCs is still unknown and require additional investigation.

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# Part IV

# Reprogramming and Differentiation of Stem Cells

# 12 Regulation of Self-Renewal and Pluripotency of Embryonic Stem Cells: Role of Natriuretic Peptide Receptor A

Essam M. Abdelalim and Ikuo Tooyama

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**Abstract**

Embryonic stem (ES) cells have two defining properties: self-renewal and pluripotency, and these make them a promising source for cell transplantation therapies. Oct4, Nanog, and Sox2 are the main transcription factors in regulating ES cell pluripotency. These key factors have also been identified that form an intrinsic core-regulatory circuit that maintains ES cells in the pluripotent state *in vitro*. The precise mechanism of how these processes are regulated remains largely unknown. Thus investigation of the molecular and cellular mechanisms of stem cell self-renewal and pluripotency provide the necessary tools to harness the regenerative potential of ES cells for therapeutic purposes. Recently, we have showed that natriuretic peptide receptor A (NPR-A), a specific receptor for atrial and brain natriuretic peptide (ANP and BNP), is expressed in preimplantation embryos and in ES cells, and is functional in ES cells. In this chapter, we will provide an overview on the importance of identifying the expression and function of NPR-A in maintaining ES cell characteristics.

## Introduction

Embryonic stem (ES) cells are derived from inner cell mass (ICM) of the blastocysts (Evans and Kaufman 1981). They can undergo unlimited self-renewal and characterized by their potential

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to differentiate into any cell types in the body. Therefore, ES cells are thought to hold great promise for regenerative medicine. A comprehensive understanding of the molecular mechanisms underlying the special properties of ES cells is required to achieve the goal of clinical applications.

ES cell pluripotency is characterized by transcriptional profiles that promote self-renewal and suppress differentiation specific gene expression. Key pluripotency factors (Oct4, Nanog, Sox2, and Klf4) are required to maintain ES cell pluripotency and when Introduced in combination can reprogram differentiated cells back to a pluripotent state (Loh et al. 2006; Liu et al. 2007; Okita et al. 2007). However, the precise mechanisms involved in regulating ES cells pluripotency are largely unknown.

Natriuretic peptide receptor A (NPR-A) is the receptor for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). ANP and BNP, members of the natriuretic peptide family, have been extensively studied for their functions in regulating blood pressure (Levin et al. 1998; Pandey 2005). In addition to this cardiovascular profile, there is evidence that ANP can also act as an autocrine/paracrine factor (Morishita et al. 1994). Previous studies have reported that ANP has apoptotic (Wu et al. 1997), anti-apoptotic, anti-hypertrophic (Kato et al. 2005), anti-mitogenic (Cao and Gardner 1995), mitogenic effects (You and Laychock 2009), and plays a role in tumor cell growth (Kong et al. 2008). ANP and BNP signal through NPR-A or natriuretic peptide receptor B (NPR-B) by increasing cGMP and activating cGMP-dependent protein kinase (PKG), which in turn can activate different downstream effectors involved in cell growth, apoptosis, proliferation and inflammation (Levin et al. 1998; Silberbach and Roberts 2001). ANP does not bind to NPR-B, but some mitogenic effects of BNP are mediated through its binding to NPR-B (Yasoda et al. 1998). Furthermore, it has been found that exogenous BNP can enhance clonal propagation in murine ES cells (Ogawa et al. 2004). Our recent studies reported that natriuretic peptides and their receptors are expressed in undifferentiated ES cells and play a role in maintaining ES cell functions (Abdelalim and Tooyama 2009, 2011a). In this chapter, we focus on the role of NPR-A in self-renewal and pluripotency of ES cells. We also discuss the mechanisms by which NPR-A affects ES cells.

## Expression of NPR-A in Pluripotent ES Cells and Pre-implantation Embryos

There are several studies focused on the functions of NPR-A in adult (Oliver et al. 1997; Kuhn et al. 2002; Ellermers et al. 2002). Although these studies focused on adult animal only, a recent study reported that NPR-A is critically required for early development (Scott et al. 2009), suggesting that this receptor plays a role during early development.

NPR-A mRNA and protein are expressed in undifferentiated murine ES cells (cultured in the presence of LIF) and levels are reduced after induction of differentiation (without LIF). NPR-A is expressed in Oct-4-positive cells in undifferentiated ES cells (Abdelalim and Tooyama 2009). Under the conditions that promote differentiation (without LIF), the mouse ES cells lost their pluripotent stem cell markers, such as Oct4 and Nanog.

The inner cell mass (ICM) of the blastocyst is the *in vivo* compartment from which ES cells originate. Immunofluorescence analysis showed that NPR-A is expressed in 3.5-day-old murine blastocysts. The expression of NPR-A is co-localized to those of Oct4 (Abdelalim and Tooyama 2009, 2011a). During development, Oct-4 expression is required to maintain the pluripotent cell population of the inner cell mass (ICM) and epiblast. In addition, Oct4 is a crucial regulator of ES-cell pluripotency, and acts as a gatekeeper to prevent ES-cell differentiation (Nichols et al. 1998). Taken together, these findings indicate that loss of NPR-A may affect development *in vivo*. These results are in agreement with a recent study in mice in which ablation of the NPR-A gene led to reduced viability of the embryo (Scott et al. 2009).

The expression of NPR-A in the blastocysts and undifferentiated ES cells suggests that signaling through this receptor may play a role in maintaining ES cell functions. Furthermore, ANP

and BNP, the ligands for the NPR-A receptor, are specifically expressed in undifferentiated murine ES cells (Abdelalim and Tooyama 2009, 2011a, b), suggesting their involvement in mediating NPR-A function in ES cells.

## Role of NPR-A in ES Cell Proliferation

ES cells can proliferate without apparent limit and can be readily propagated if they are cultured under conditions that prevent their differentiation (in the presence of LIF). Rapid division of ES cells is associated with an unusual cell cycle structure, characterized by a short G1 phase and a high proportion of cells in the S phase (Burdon et al. 2002), which is associated with a unique mechanism of cell cycle regulation.

Our previous study reported reduced cell growth and increased apoptosis when BNP expression is down-regulated in ES cells. BNP knockdown suppresses ES cell proliferation by reducing the percentage of cells in S phase and accumulation of cells in G1 and G2/M phases (Abdelalim and Tooyama 2009). In agreement with this finding, NPR-A has been reported to be involved in ES cell self-renewal. NPR-A is a specific receptor for ANP and BNP (Levin et al. 1998). Silencing of NPR-A in ES cells using RNA interference (siRNA) causes a significant reduction in ES cell number and colony size. The ability of single ES cells to form colonies after NPR-A knockdown were investigated by seeding ES cells at low density in the presence of LIF for 6 days to form secondary ES cell colonies. The number of formed colonies of NPR-A siRNA-treated ES cells is significantly reduced in comparison to those of control siRNA-treated cells (Abdelalim and Tooyama 2011a). Also, it has been found that NPR-A knockdown inhibits ES cell proliferation by blocking cell cycle progression in G0/G1 and by preventing G1-S transition (Fig. 12.1) (Abdelalim and Tooyama 2011a). Recently, it has been reported that lengthening of G1 phase appears to be a cause, rather than a consequence of ES cell differentiation (Lange and Calegari 2010).

Analysis of the genes involved in the G1-S transition showed that NPR-A knockdown leads to a down-regulation of cyclin D1 and an up-regulation of p21. Cell cycle progression is controlled by cyclin-dependent kinases (Harper et al. 1993). p21 has a crucial role in the regulation of the G1-S transition, in which it works as an inhibitor of the G1 cyclin-dependent kinase-cyclin complexes (Harper et al. 1993). Recent study showed that down-regulation of Oct4 in ES cells leads growth arrest by up-regulating p21 and blocking the cell cycle progression in G0/G1 (Lee et al. 2010).

ES cells express low level of cyclin D1, which is regulated by the Phosphoinositide-3-kinase (PI3K)-dependent pathway. PI3K/Akt signaling

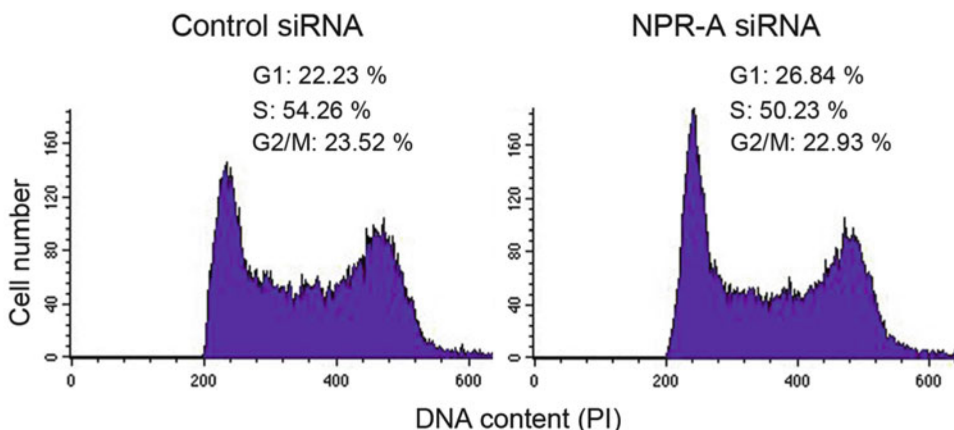


**Fig. 12.1** Effect of NPR-A knockdown on ES cell cycle. Cell cycle profile analysis of ES cells 48 h after transfection with control siRNA or NPR-A siRNA

pathway plays a critical role in the G1-S transition and maintenance of ES cell pluripotency (Jirmanova et al. 2002; Paling et al. 2004; Storm et al. 2007). NPR-A knockdown down-regulates phosphorylated Akt. In contrast, ES cells treated with exogenous ANP showed an up-regulation of phosphorylated Akt (Abdelalim and Tooyama 2011a). These findings indicate that NPR-A is essential for ES cell proliferation and cell cycle progression.

## Role of NPR-A in ES Cell Pluripotency

The pluripotent identity of ES cells is controlled by a group of transcription factors. The transcription factors Oct4, Nanog and Sox2 contribute to the hallmark characteristics of ES cells by activating target genes that encode pluripotency and self-renewal mechanisms and by repressing signaling pathways, which promotes differentiation (Burdon et al. 2002; Loh et al. 2006). Also, ES cell self-renewal and pluripotency require inputs from extrinsic factors and their downstream effectors (Chambers and Smith 2004). Self-renewal of murine ES cells under conventional culture conditions depends on the leukemia inhibitory factor (LIF). Withdrawal of LIF induces differentiation (Burdon et al. 2002).

Silencing of NPR-A gene using siRNA technique induced changes in the morphology of ES cells indicating their differentiation (Fig. 12.2). Also, the alkaline phosphatase activity (AP), which is a marker of the pluripotency, is markedly reduced in ES cells treated with NPR-A siRNA in comparison to those treated with control siRNA. The pluripotency factors Oct4 and Nanog are down-regulated at gene and protein levels after NPR-A knockdown in ES cells. Oct4 and Nanog are the master regulators of ES cell self-renewal and pluripotency (Abdelalim and Tooyama 2011a).

Furthermore, down-regulation of NPR-A in ES cells induces the expression of early differentiation marker genes such as GATA-4 and GATA-6 (extra-embryonic endoderm), AFP (visceral endoderm), Brachyury (mesoderm), nestin (ectoderm) and Cdx2, Hand1 and Eomes (trophectoderm) (Abdelalim and Tooyama 2011a). This increase in multi-lineage specific genes indicates that cells were differentiated along several different pathways.

Oct4 is an essential regulator of ES cell pluripotency, and acts as a gatekeeper to prevent ES cell differentiation (Nichols et al. 1998). It has been shown that reduction of Oct4 expression below 50% of normal levels induces differentiation of ES cells into trophectoderm by up-regulating the transcription factors Cdx2 (Niwa et al. 2000; Chen et al. 2009). Knockdown of NPR-A reduces Oct4 expression and induces the expression of the trophectodermal markers, Cdx2, Hand1 and Eomes (Abdelalim and Tooyama 2011a). It has been reported that Cdx2 and Oct4 antagonize each other at the transcription level (Niwa et al. 2005). These findings suggest that the ANP/NPR-A system has

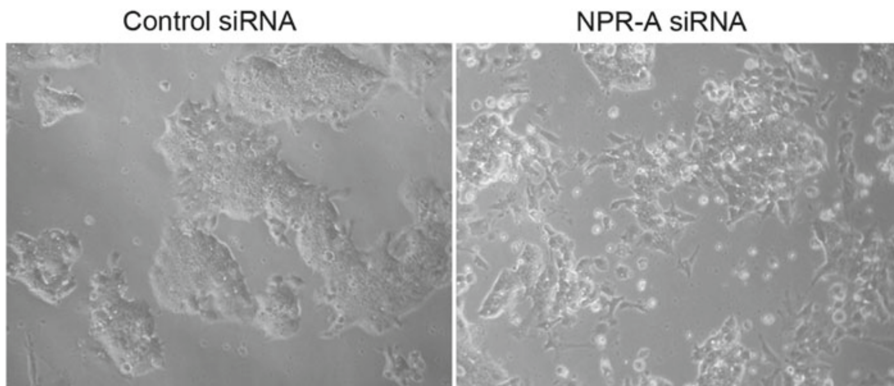


**Fig. 12.2** NPR-A knockdown induces ES cell differentiation. Morphologies of murine ES cells 72 h after transfection with the control siRNA or BNP siRNA transfection in the presence of LIF

a role in preventing the differentiation of ES cells into trophectoderm by regulating Oct4 expression.

Nanog has been shown to block the differentiation of murine ES cells into extra-embryonic endoderm cells (Mitsui et al. 2003; Chambers et al. 2003). Moreover, deficiency of Nanog disruption in ES cells results in differentiation to endoderm lineages, and over-expression of Nanog in murine ES cells can maintain them in a pluripotent state in the absence of LIF (Chambers et al. 2003; Mitsui et al. 2003). Knockdown of NPR-A markedly reduces Nanog expression at mRNA and protein levels, and induces the expression of extra-embryonic endoderm and trophectodermal markers. In addition, treatment of ES cells with ANP induces Nanog expression via an NPR-A-dependent pathway and ANP is able to maintain Nanog expression in the presence of PI3K inhibitor (Abdelalim and Tooyama 2011a). Taken together, these findings suggest that ANP/NPR-A in ES cells contributes to maintaining the undifferentiated state and supports ES cell self-renewal by regulating the expression of Oct4 and Nanog.

Furthermore, seeding of NPR-A-deficient ES cells at low density in the presence of LIF showed that the majority of colonies formed by the NPR-A deficient cells appears differentiated. Also, the quantification of the AP-positive colonies showed a significant decrease in the proportion of undifferentiated colonies after NPR-A knockdown (Abdelalim and Tooyama 2011a). These findings indicate that NPR-A is required for ES cell pluripotency.

Pharmacological inhibition of NPR-A in ES cells using NPR-A antagonist (anantin) results in reduction in the protein levels of Oct4 and Nanog. In addition, inhibition of cGMP-dependent protein kinase (PKG) using KT5823 (PKG inhibitor) leads to down-regulation of Oct4 and Nanog (Abdelalim and Tooyama 2011a). These findings indicate the involvement of NPR-A/cGMP pathway in ES cell pluripotency.

## NPR-A Ligands Enhance ES Cell Self-Renewal

The physiological effects of ANP and BNP are initiated by binding to NPR-A (Levin et al. 1998). The abilities of natriuretic peptides to modulate cell growth and cell proliferation have received attention (Silberbach and Roberts 2001). Cell-based studies have shown that ANP and BNP exhibit important autocrine and paracrine functions such as modulating myocyte growth, apoptosis and proliferation in smooth muscle cells (Abell et al. 1989). Furthermore, signaling through NPR-A has been found to play a crucial role in tumor growth (Kong et al. 2008).

Our previous report showed that ANP and BNP are specifically expressed in self-renewing ES cells (Abdelalim and Tooyama 2009, 2011a), suggesting their involvement in mediating NPR-A function in ES cells. Treatment of ES cells with exogenous ANP promotes ES cell pluripotency by inducing the expression of Oct4, Nanog, and phosphorylated Akt. The positive effect of ANP on the expression of pluripotency factors was mainly dependent on ANP binding to its specific receptor, NPR-A, involving the cGMP/PKG pathway, because pre-treatment with a specific NPR-A antagonist (anantin) or PKG inhibitor (KT5823) abrogated the ANP-induced upregulation of Oct4 and Nanog (Abdelalim and Tooyama 2011a). These two transcription factors, Oct4 and Nanog, work in concert to maintain the undifferentiated state of ES cells (Niwa et al. 2000; Mitsui et al. 2003; Chambers et al. 2003). Also, knockdown of ANP in ES cells exhibits a change in morphology with the presence of differentiating cells. The AP-staining, is markedly reduced in ES cells treated with ANP siRNA in comparison to those treated with control siRNA. Consistent with this reduction in the number of AP positive colonies, knockdown of ANP also led to reduction in the expression of markers of pluripotency, including Nanog and Oct4. The effect of ANP knockdown on ES cell pluripotency are lower than those observed in NPR-A-deficient cells due to incomplete inhibition of ANP and the presence of endogenous NPR-A (Abdelalim and Tooyama 2011a).

BNP knockdown suppresses ES proliferation without affecting on ES cell pluripotency (Abdelalim and Tooyama 2009). In addition, it has been found that exogenous BNP can enhance clonal propagation in murine ES cells (Ogawa et al. 2004), suggesting the presence of functional natriuretic peptide receptors in ES cells. Furthermore, BNP knockdown in ES cells leads to a marked decrease

in intracellular cGMP level and a significant reduction in mRNA level of NPR-B, suggesting that the reduction in the cGMP level in ES cells is a reflection of the reduced NPR-B mRNA level. This hypothesis represents the signaling pathway through NPR-B as a possible regulator for ES cell proliferation (Abdelalim and Tooyama 2009, 2011b). These findings suggest that BNP may exert its proliferative effects through either NPR-A, its preferred receptor or through NPR-B, for which it has reasonable affinity.

Overall, these findings indicate that ANP and BNP have different functions in ES cells. It has been found that BNP has a low affinity for NPR-A with a potency approximately tenfold lower than that of ANP, which has led to speculation that may be an additional BNP-specific receptor might play a role (Goy et al. 2001). In agreement with this, Chusho et al. (2000) suggested that it is possible that there is an as yet unidentified receptor with high affinity to BNP. Also, it has been shown that in the absence of NPR-A, testis and adrenal glands retain significant high-affinity response to BNP that can only be accounted for the presence of a novel receptor in these tissues that prefer BNP over ANP. Although the physiological significance and the biochemical component of this receptor remains to be established, its existence does reinforce the notion that ANP and BNP are likely to carry out at least some independent action.

## PI3K is Involved in NPR-A Functions in ES Cells

The phosphoinsitide 3-kinase (PI3K) signaling pathway has been shown to be important to maintain pluripotency in mouse ES cells, and inhibition of PI3K signaling by a PI3K inhibitor LY294002 leads to differentiation of murine ES cells in the presence of LIF (Paling et al. 2004; Storm et al. 2007). Knockdown of NPR-A in ES cells leads to down-regulation of Akt phosphorylation, the downstream target of PI3K. Furthermore, treatment of ES cells with ANP induced Akt phosphorylation (Abdelalim and Tooyama 2011a). The PI3K signaling pathway has been linked to ANP- and NPR-A-mediated cell proliferation in endothelial cells (Kook et al. 2003). These results indicate that the involvement of the PI3K/Akt pathway in the effect of the ANP/NPR-A signaling on the self-renewal and pluripotency of ES cells.

The relationship between ANP/NPR-A and PI3K signaling pathway has been investigated using LY294002, a specific inhibitor of PI3K pathway, to inhibit PI3K signaling (Storm et al. 2007). ES cells cultured in presence of ANP and LY294002 together showed less differentiation than those of cells treated with LY294002 only. Also, Treatment of ES cells with ANP and LY294002 maintains Nanog expression at a higher level of those cultured in the presence of LY294002 alone (Abdelalim and Tooyama 2011a). It is known that PI3K inhibitor (LY294002) induces ES cell differentiation by suppressing Nanog expression in ES cells (Storm et al. 2007). These results suggest that the ANP/NPR-A system is able to partially maintain ES cell pluripotency in the presence of PI3K inhibitor by maintaining Nanog expression.

## Differentiation of NPR-A-Deficient ES Cells

ES cells are commonly differentiated *in vitro* by spontaneously self-assembling in hanging drop and suspension culture methods into 3-dimensional cell aggregates called embryoid bodies (EBs), which model many of the hallmarks of early embryonic development. The NPR-A–deficient ES cells induced to differentiate in the presence of retinoic acid (RA), showed that NPR-A siRNA-derived EBs are much smaller in size compared to EBs derived from control siRNA-treated cells. Also, EBs derived from NPR-A-deficient cells expressed greater levels of endoderm (GATA4, GATA6 and AFP), ectoderm (nestin), and trophectoderm (Hand1 and Eomes) markers. However, Brachyury, a marker of mesoderm, is slightly reduced by NPR-A knockdown, indicating that NPR-A knockdown predisposes ES cells to endodermal, ectodermal and trophectodermal differentiation. Pluripotency markers (Oct4 and Nanog)

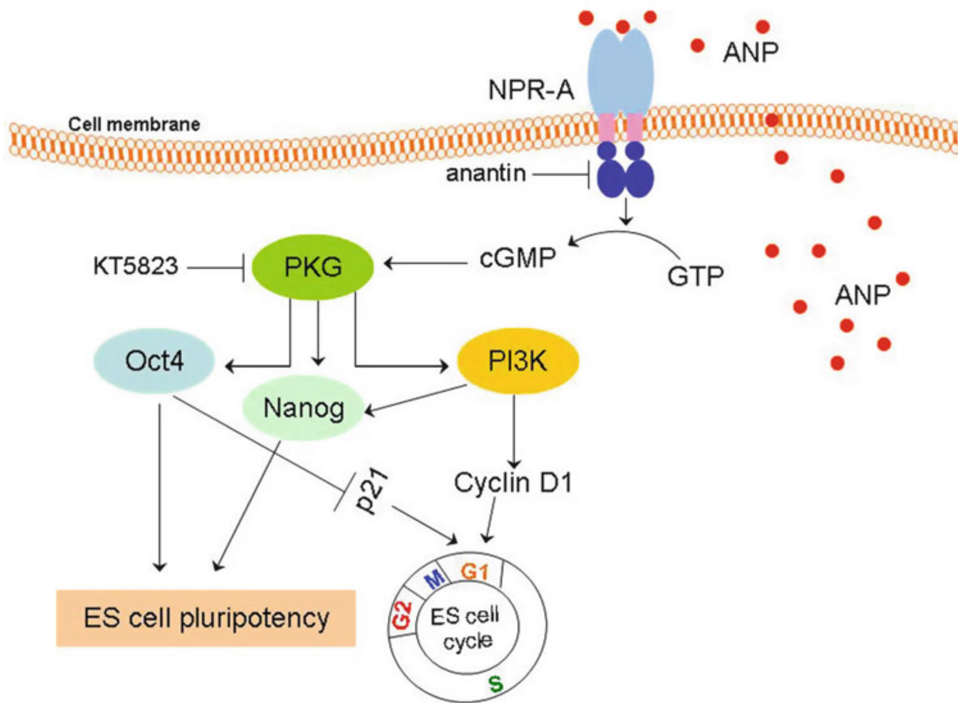


**Fig. 12.3** Simplified schematic presentation of the role of NPR-A in murine ES cells. NPR-A and ANP are expressed by undifferentiated ES cells. Activation of NPR-A by ANP stimulates cGMP-PKG signaling pathway, leading to increased expressions of Oct4 and Nanog, and reduced expression of p21, which in turn suppresses ES cell differentiation and maintains ES cell pluripotency. In another pathway, binding of ANP to NPR-A activates the phosphoinositide 3-kinase (*PI3K*) signaling pathway enhancing G1-to-S progression through induction of cyclin D1, and maintains pluripotency via activation of Nanog expression (This figure is reproduced from Abdelalim and Tooyama 2011a)

are detected at higher levels in NPR-A siRNA-derived EBs than those of control siRNA-derived EBs, most likely as a result of the incomplete differentiation of a subset of cells (Abdelalim and Tooyama 2011a).

In conclusion, the results summarized in this chapter suggest that NPR-A receptor is required for the self-renewal and pluripotency of ES cells (Fig. 12.3). Knockdown of NPR-A suppresses the expression of genes encoding the pluripotency factors (Oct4, Nanog and Sox2), which subsequently leads to the differentiation of ES cells. In addition, NPR-A ligands, ANP and BNP are expressed in undifferentiated murine ES cells, and they play a role in maintaining the self-renewal of ES cells. Exogenous ANP positively regulates ES cell pluripotency, involving its receptor NPR-A in a PKG-dependent manner (Fig. 12.3). However, BNP has been reported to promote ES cell proliferation without affecting on ES cell pluripotency, suggesting the involvement of other receptor in mediating its function. Thus, ANP/NPR-A system is functional in undifferentiated ES cells and it is crucial for maintaining the ES cell pluripotency. The findings reported here provide novel molecular mechanisms involved in the self-renewal and pluripotency of ES cells, which is the fundamental requirement for applying ES cells for the regenerative therapy. Further studies are required to address the molecular mechanism of how NPR-A signaling pathways interact to regulate ES cell self-renewal.

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# Reprogramming and Pluripotency of Epiblast Stem Cells

# 13

Anne-Clemence Veillard, Julien Maruotti, and Alice Jouneau

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**Abstract**

Pluripotent stem cells established *in vitro* from mammalian embryos are essential tools to understand pluripotency. From the mouse embryo, two kinds of stem cells can be derived, both being pluripotent: embryonic stem cells (ESCs) are derived from the pluripotent cells in the inner cell mass of the blastocyst, whereas epiblast stem cells (EpiSCs) are derived from the post-implantation late epiblast, at the time of gastrulation. These two types of stem cells share some common properties but each of them also display specific features that clearly define two states of pluripotency, now referred as naive for ESCs and primed for EpiSCs. Although being derived from the inner cell mass at the blastocyst stage, human ESCs are in fact distinct from mouse ESCs and closer to the primed state. In this chapter we describe how EpiSCs are obtained in the mouse and what their molecular and functional characteristics are in comparison with mouse ESCs. We present the two states of pluripotency and their *in vivo* equivalence. In other species where pluripotent stem cells derived from the embryo are available, we show that these cells are indeed in the primed state. Then, we present a state of art of experiments exploring pathways

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allowing conversion of EpiSCs into ESCs or vice-versa and means to stabilize cells in one or the other state. From the current knowledge, it seems that the primed state not only represents a more advanced epiblast developmental stage, but may also be a more easily stabilized state, compared to the naive one. At last we expose reprogramming experiments in the mouse, where somatic cells are reprogrammed into the primed state, through induction using exogenous factors or by nuclear transfer into enucleated oocytes.

## Introduction

Pluripotency is defined as the capability of a cell to give rise to all cell types of an organism. In the embryo, pluripotent cells are present only transiently, from preimplantation (morula) to pre-gastrulation stage. Pluripotency can be captured *ex vivo*: pluripotent stem cells (PSCs) serving as a paradigm for understanding pluripotency. In the 1980s, a major breakthrough was the discovery by Gail Martin and Martin Evans that cells harvested from the inner cell mass of the mouse blastocyst could be isolated and maintained *in vitro* (Smith 2001). These mouse embryonic stem cells (mESCs) soon became a gold standard, as they have been shown to succeed in pluripotency tests of increasing stringency: *in vitro* differentiation, *in vivo* formed teratomas, extensive colonization of chimeras after blastocyst injection and germline transmission, and last but not least, tetraploid complementation. In this last test, the host tetraploid cells will only contribute to the extra-embryonic component, while mESCs support the development of entire ESC-derived animals. Notably, not all mESC lines can pass this test and the molecular characteristics conferring this ability are still poorly understood.

Mouse Embryonic Stem Cells can be derived from the inner cell mass of the early blastocyst and also earlier at the morula stage (Tesar 2005). More recently stem cells have been derived from later, post-implantation stages, from the epiblast just before the onset of gastrulation. These cells showed different features that distinguish them from mESCs and were thus called epiblast stem cells (EpiSCs) (Brons et al. 2007; Tesar et al. 2007). Epiblast Stem Cells are now considered as a new state of pluripotency. In humans, pluripotent stem cells have been established from early blastocysts and were thus called ESCs (hESCs). However, it has become clear that they are in fact different from mESCs while showing more similarities with mouse EpiSCs. In this chapter we will describe the characteristics and properties of EpiSCs, in comparison with both mESCs and hESCs. We will also review different studies exploring the nature of the two states of pluripotency represented by ESC and EpiSC and their relationships. At last we will present some data about reprogramming of somatic cells into EpiSCs, through nuclear transfer and induction with transcription factors.

## Derivation of EpiSCs in the Mouse

Mouse Embryonic Stem Cells are derived directly from the inner cell mass (ICM) of the blastocyst between E3.5 and E4.5 and are thus considered as an *in vitro* equivalent of the early epiblast. *In vivo,* cells of the ICM will progressively separate in two lineages, the pluripotent epiblast and the primitive endoderm. In the mouse, the embryo will then implant in the uterus before starting to gastrulate around E6.5. Epiblast Stem Cells have been derived from post-implantation mouse embryos from E5.5 to E7.5. At this stage the epiblast is harvested from the surrounding extra-embryonic tissues (visceral endoderm and trophoblast) and plated *in vitro* to form an outgrowth. A couple of pioneering studies in this area have resulted in two preferred cell culture strategies (Brons et al. 2007; Tesar et al. 2007): either on a mouse feeder layer in the presence of FGF2 and knockout serum replacement (KSR), or on fibronectin-coated plates in a chemically defined medium supplemented with Activin A and FGF2. Incidentally, both media are also used for growing hESCs. During the derivation process, undifferentiated colonies identified by their pluripotent morphology are manually picked and replated every 2–3 days. When most colonies display homogenous

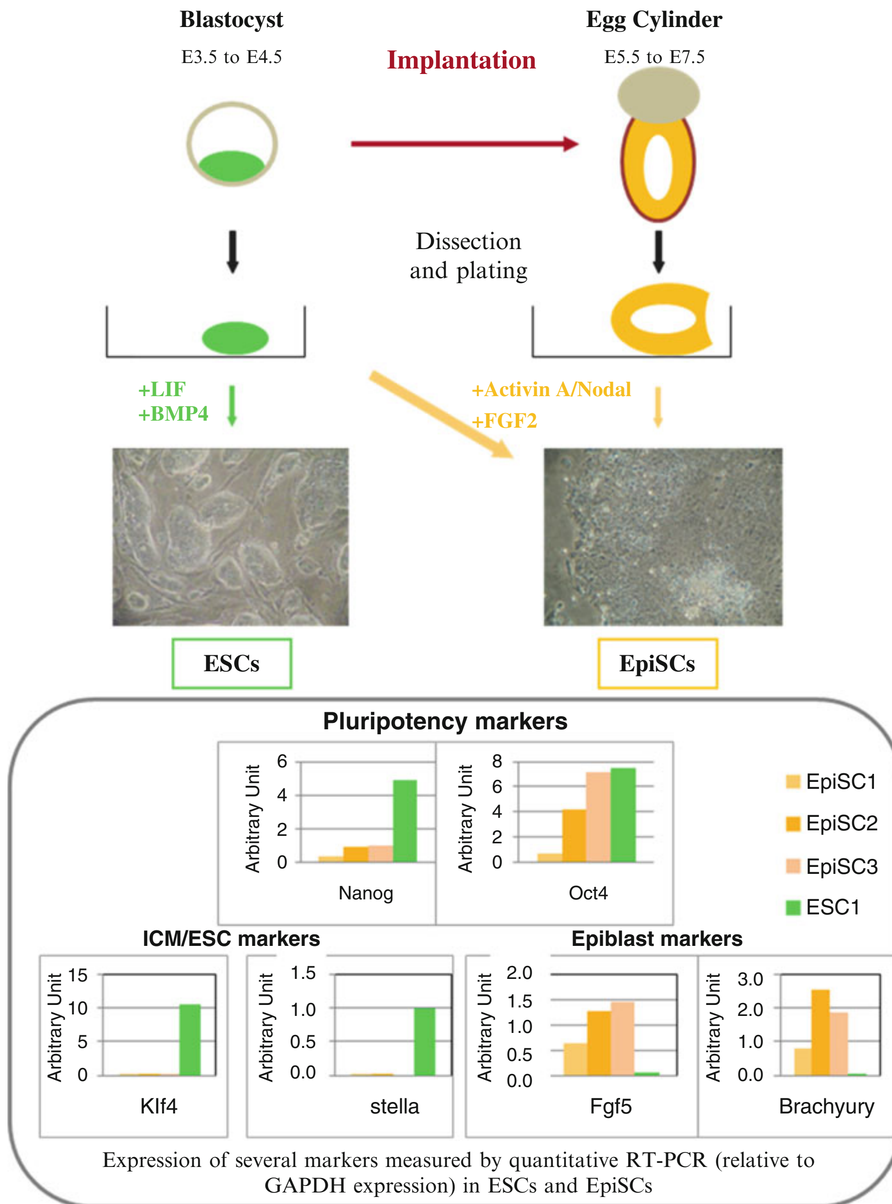


**Fig. 13.1** Characteristics of EpiSCs compared to ESCs in mouse

pluripotent morphology, they can be passaged as small clumps enzymatically with collagenase. In contrast to mouse ESCs and similar to human ESCs, EpiSCs cannot be replated at clonal density. In addition, EpiSCs grow as a monolayer, forming large flat colonies different from the small domed structures characteristic of ESC colonies (Fig. 13.1).

A stable cell line takes approximately 2–3 weeks to establish. Although EpiSCs have been obtained from different mouse strains, we have noticed that the pace and efficiency of derivation are dependent on the genetic background of the host species. We compared the derivation of EpiSCs from embryos of three strains and observed important differences. Whereas 28% of the epiblasts

give rise to EpiSC lines using B6D2 F1 mice, the rate drops to only 8% using 129B6 F1 strain (Maruotti et al. 2010 and our unpublished data). Interestingly, as previously observed for ESC derivation (Batlle-Morera et al. 2008), the best derivation efficiency (51%) in our experiments was reached using pure 129 embryos. This may be explained by a different behavior during the derivation process: in B6D2 F1 or 129B6 F1 explanted epiblasts, most cells rapidly loose the pluripotent morphology (Maruotti et al. 2010). Re-appearance of pluripotent colonies then occurs after 7–10 days. By contrast, most cells of the explanted epiblasts from 129 embryos maintain their pluripotent character after plating and therefore expand faster than the other strains. Despite these different derivation characteristics, up to now EpiSC have been derived from all the strain tested, even those considered as "non-permissive" for ESC derivation, such as non-obese diabetic (NOD) embryos (Brons et al. 2007).

Recently it has been shown that EpiSCs can also be derived from E3.5 mouse pre-implantation embryos (Najm et al. 2011). The authors plated such blastocysts on feeders using a modified medium without adding exogenous LIF or FGF2, so as to be potentially permissive to both ESCs and EpiSCs derivation (feeders themselves produces LIF and Activin). After 6 days, cells were dissociated and replated in the same culture conditions. Once pluripotent colonies showed morphological characteristics of either ESCs (domed colonies) or EpiSCs (flat colonies) the cells were cultured under conditions adapted to each cell type (Feeders and LIF for ESC, feeders and FGF2 for EpiSCs). Using this protocol the authors succeeded in obtaining stable EpiSC lines in addition to ESC lines. These new EpiSC lines presented the same molecular profile as those derived from post-implantation embryos. Overall the two types of pluripotent cells were obtained from strain 129 blastocysts with a similar (and high, 25%) efficiency. Noteworthy at least one embryo was able to give rise to both types of pluripotent cell lines. This could indicate a stochastic behavior of inner cell mass cells during the derivation process in these permissive conditions, some cells progressing towards a late epiblast-like state *in vitro* and becoming EpiSCs while others are caught at an earlier developmental stage and giving rise to ESCs.

## Signaling Pathways Controlling EpiSC Self-Renewal

Epiblast Stem Cells use different signaling pathways than mESCs. For mESCs, the main pathways are LIF/Stat3, BMP4 and Wnt3 (Berge et al. 2011). The presence of LIF and BMP4 is inhibitory to EpiSC derivation (Brons et al. 2007). Epiblast Stem Cells derived in the presence of JAK/STAT inhibitors in fact display a higher plating efficiency highlighting the negative role of these factors on EpiSC derivation (Tesar et al. 2007). Similarly, Noggin, an inhibitor of BMP signaling, does not preclude EpiSC derivation (Brons et al. 2007). By contrast, Epiblast Stem Cells are cultured in the presence of both Activin and FGF2 (Fig. 13.2).

Epiblast Stem Cells have a strict dependence on Activin/Nodal signaling pathways for both their derivation and maintenance. This has been highlighted by experiments exhibiting decreased EpiSC derivation efficiencies in the presence of the Activin receptor inhibitor SB431542 as well as a rapid down-regulation of pluripotency markers and induction of neuroectodermal genes in EpiSCs treated with this inhibitor (Brons et al. 2007; Tesar et al. 2007). Activin/Nodal binds to heterodimeric receptors Acvr1b/Acvr2b in EpiSCs leading to the phosphorylation of the downstream effectors Smad2/3 (Fig. 13.2). Nodal is expressed by EpiSCs and it has been suggested that at high density this autocrine/paracrine signaling may be sufficient to maintain the undifferentiated state (Greber et al. 2010). Activin/Nodal signaling maintains pluripotency in EpiSCs by controlling *Nanog* expression, which in turn prevents differentiation (Vallier et al. 2009a). Phosphorylated Smad2/3 directly binds to Nanog protein and this complex activates *Nanog* promoter. Activin/Nodal signaling has a dual effect on EpiSCs as it is also an inducer of mesendoderm differentiation, both *in vivo* and *in vitro* (Shen 2007; Vallier et al. 2009b). In this regard, the role of Nanog may also be to fine-tune the Activin signaling,

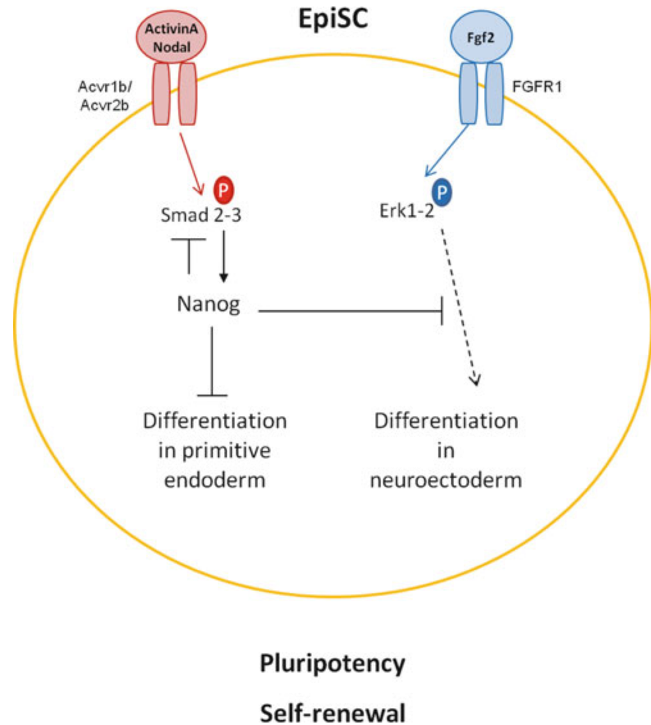


**Fig. 13.2** Signalling pathways controlling EpiSCs self-renewal. According to the expression data in EpiSCs, the main receptor for Activin/Nodal is Acvrlb/ Acvr2b, and for FGF2 it is Fgfrl (our unpublished data)

depending on the partners it interacts with (Teo et al. 2011).

Fibroblast Growth Factor signaling helps to expand EpiSCs although it is not required for derivation. It does not stimulate cell proliferation; however, it improves cell survival after plating (Greber et al. 2010). Its downstream signaling effector is the MAP kinase Erk1/2, which is phosphorylated following activation of FGF receptors (Fig. 13.2). The mechanisms mediating the effect of FGF signaling in EpiSCs is not well known. As for Activin, it is also involved in directing differentiation, although there have been contradictory studies on its role in neuroectoderm differentiation (inhibition: Greber et al. 2010; stimulation: Vallier et al. 2009a).

## Molecular and Epigenetic Characteristics of EpiSCs

Epiblast Stem Cells present typical features differentiating them from other pluripotent stem cells such as ESCs. The pluripotency factors *Oct4* (*Pou5f1*), *Nanog* and *Sox2* are expressed in EpiSCs like in ESCs, although *Nanog* is expressed at a lower level (Fig. 13.1). As described above, pluripotency is not controlled by the same signaling pathways in the two populations of stem cells. For example, the expression of *Oct4* is regulated by two different enhancers. Whereas the distal enhancer is active in ESCs, in EpiSCs it is the proximal one. The global transcriptome profile shows that EpiSCs are close to the post-implantation epiblast, whereas ESCs share more characteristics with ICM (Brons et al. 2007). For example, Embryonic Stem Cells express some ICM markers like *Gbx2*, *Klf4*, *Zfp42* (*Rex1*) or *Pecam1*, which are expressed only at low levels, if at all, in EpiSCs (Brons et al. 2007; Tesar et al. 2007). Conversely, EpiSCs express late epiblast markers such as *Nodal*, *Fgf5* or *Brachyury* (*T*), which are almost absent in ESCs (Fig. 13.1). Moreover germline markers such as *Dppa3* (*Stella*), *Dazl* or *Stra8* are expressed by ESCs whereas EpiSCs show no or less expression of them (Tesar et al. 2007). Another significant trait is that EpiSCs have no alkaline phosphatase (AP) activity, unlike ESCs for which AP is routinely used as a marker.

We have recently established the profile of miRNAs in EpiSCs and compared it to that of ESCs (Jouneau et al. 2012). The main clusters of miRNAs associated with and controlling pluripotency, including miR290-295, miR302-367 and miR17-92, are expressed in both types of cells. Notably, the relative importance of the first and the second cluster is reversed in EpiSCs compared to ESCs. In addition, many miRNAs that are expressed in somatic cells are also present in EpiSCs.

For several genes, the differences at the transcriptional level have been correlated with the epigenetic status. For instance, the *Dppa3* locus is enriched in histone H3 with trimethylated lysine 4 (H3K4me3) in ESCs, whereas in EpiSCs this mark is removed and the promoter region is methylated (Hayashi and Surani 2009; Tesar et al. 2007). Two other genes, *Zfp42* and *Fbxo15,* are also hypermethylated in EpiSCs (Hayashi and Surani 2009). Moreover, *Dnmt3b,* which is responsible for *de novo* DNA methylation, is more highly expressed in EpiSCs than in ESCs (Hayashi and Surani 2009). Another important epigenetic difference between ESCs and EpiSCs is the X inactivation state. Both X chromosomes are active in female mESCs. By contrast, in female EpiSCs the random inactivation of one X chromosome has already occurred as shown by accumulation of the repressive histone mark H3K27me3 and the random expression of a GFP reporter carried by one X-chromosome (Bao et al. 2009; Guo et al. 2009).

## Differentiation Properties

The essential property of pluripotent stem cells is the capacity for each single cell to differentiate along the three primordial lineages (ectoderm, endoderm and mesoderm). To assess this capacity, EpiSCs have been subjected to different tests of pluripotency, *in vitro* differentiation, teratoma and chimera formation. The *in vitro* differentiation potential of EpiSCs has been tested by growing them as floating embryoid bodies (EB) followed by growth as an adherent monolayer. Epiblast Stem Cells grown in this fashion can differentiate into mesodermal, endodermal and ectodermal derivatives, as demonstrated by the expression of different markers assessed by RT-PCR or immunostaining (Brons et al. 2007; Tesar et al. 2007). Importantly these results were obtained using both a population of EpiSCs and clonal sublines. Differentiation experiments were also driven *in vitro* using hESCs protocols, showing that the signaling pathways controlling differentiation of hESCs are similar for mouse EpiSCs while distinct for mESCs (Vallier et al. 2009b).

Epiblast Stem Cells apparently express trophoblast-associated genes like *Cdx2*, *Hand1, Eomes, H19* in response to BMP4 treatment (Brons et al. 2007). As the lineages are already well established and separated at the post-implantation stages, it seems paradoxical that cells derived from the late epiblast could differentiate into trophoblast. Recently, the identity of the cells differentiating from BMP4-treated EpiSCs was questioned (Bernardo et al. 2011). This study has demonstrated that *Brachyury* drives a gene regulatory network which is necessary for the downstream *Cdx2* activation. It concludes that BMP4 induces mouse EpiSCs and hESCs to form several mesodermal derivatives (including extra-embryonic mesoderm) but not trophoblast.

Epiblast Stem Cells can also differentiate properly into germ cells *in vitro* (Hayashi and Surani 2009). *In vivo*, mouse primordial germ cells (PGCs) originate from the post-implantation proximal epiblast and are precursors of the germ cell lineage. At E6.25 they start to express *Prdm1* and then at E7.25 *Dppa3* is upregulated. Hayashi and colleagues have shown that in the presence of KSR (serum replacement, known to exhibit a BMP4 activity) 10–50 % of EpiSCs are expressing *Prdm1* (Hayashi and Surani 2009), a small proportion of them being also *Dppa3* positive. These double positive cells were shown to share several transcriptional and epigenetic characteristics with early germ cells. In conclusion, the *in vitro* differentiation ability of EpiSCs is characteristic of a true pluripotent cell.

Epiblast Stem Cells injected into immunodeficient mice form teratomas *in vivo* in which a wide range of cell types such as liver, neuronal or epithelial cells have been identified (Brons et al. 2007; Tesar et al. 2007). The formation of chimeras is another, more stringent pluripotency test.

Injection of EpiSCs into a blastocyst at E3.5 gives no (Tesar et al. 2007) or very few (2/385 in Brons et al. 2007) chimeras. To rule out the possibility that this was due to the mortality of EpiSCs in single cell conditions, aggregation experiments at 8-cell or morula stages were performed using clumps of EpiSCs. However, the cells remained as a cluster without mixing with host blastomeres. Moreover chimera experiments using EpiSCs expressing GFP under the control of *Oct4* promoter have shown a rapid loss of *Oct4* expression which indicates either differentiation or cell death (Guo et al. 2009). These experiments suggest a more restricted pluripotency in EpiSCs than in ESCs that more readily participate in chimera formation with germ line transmission. However the explanation for this very low rate of chimeras using EpiSCs could be a temporal incompatibility. Indeed EpiSCs are derived from post-implantation epiblast and keep a lot of characteristics of this developmental stage (see below). We hypothesize that a pre-implantation embryo is, therefore, a non-permissive host for those cells. For example, *Nodal* is faintly expressed in ICM and activation of Nodal pathway is necessary for EpiSCs self-renewal (Fig. 13.2). So EpiSCs introduced in this environment could possibly differentiate. Another explanation could reside in a role of cadherins which are glycoproteins involved in cell-cell adhesion. Embryonic Stem Cells and EpiSCs both express *Cdh1* (*E-Cadherin*) and *Cdh2* (*N-Cadherin*), however, the relative level of each may be different in the two types of cell (unpublished data). This may contribute to the absence of intermingling of EpiSCs with ICM cells after their injection into a blastocyst. In conclusion, the very low chimera formation rate does not allow to readily test the possibility of germline transmission.

## The Two States of Pluripotency: Primed State in Contrast to Naive State. Correlation with *In Vivo* Development

From all the data presented above, it is clear that ESCs and EpiSCs present features that allow defining two different states of pluripotency, that differ in terms of molecular profiles, X-chromosome status and *in vivo* differentiation properties (Fig. 13.3). They are now commonly referred as naive pluripotency for ESCs and primed pluripotency for EpiSCs (Nichols and Smith 2009). Mouse Embryonic Stem cells are considered as being the *in vitro* equivalent of the very early naive epiblast within the blastocyst before implantation. By contrast EpiSCs are derived from the epiblast at post-implantation stages. In mouse, when the embryo is implanted, the late epiblast is surrounded by extra-embryonic tissues, the visceral endoderm and the trophoblast that supply it with inductive signals that will subsequently lead to the orderly appearance of the three primary lineages and the patterning of the embryo. As a consequence it is primed for differentiation while retaining pluripotency, exhibiting at the same time expression of Oct4, Sox2 and Nanog and different markers of the primary lineages such as Brachyury and Gsc, even before their morphological appearance (Perea-Gomez et al. 2004). Similarly, Epiblast Stem Cells express different markers of these primary lineages. The abundance of somatic miRNAs is also in agreement with the primed state (Jouneau et al. 2012). Moreover, we have seen above that the repression of expression of some ICM/ESC specific genes is associated with the methylation of these loci, such as *Dppa3*, *Zfp42* or *Fbxo15*. Indeed, the passage from an ICM to an epiblast is accompanied *in vivo* by the *de novo* methylation of the genome. Another manifestation of this extensive epigenetic remodeling is the X-chromosome inactivation in female embryos. The presence of an inactivated (at least partially) X chromosome in female EpiSCs is another landmark of their primed state, separated from the naive one by an epigenetic barrier. The difference in the X-chromosome inactivation status in the two states of pluripotency opens an avenue to understand the role of gene-dosage compensation in the stabilization of pluripotent cells in one or the other state. Cells from the late epiblast were shown to be unable to incorporate into the blastocyst, in contrast to the early epiblast from implanting embryos (Tesar et al. 2007). This highlights again the "late epiblast" origin of EpiSCs, which are also unable to form chimeras (see above). The signaling pathways involved in EpiSC maintenance,

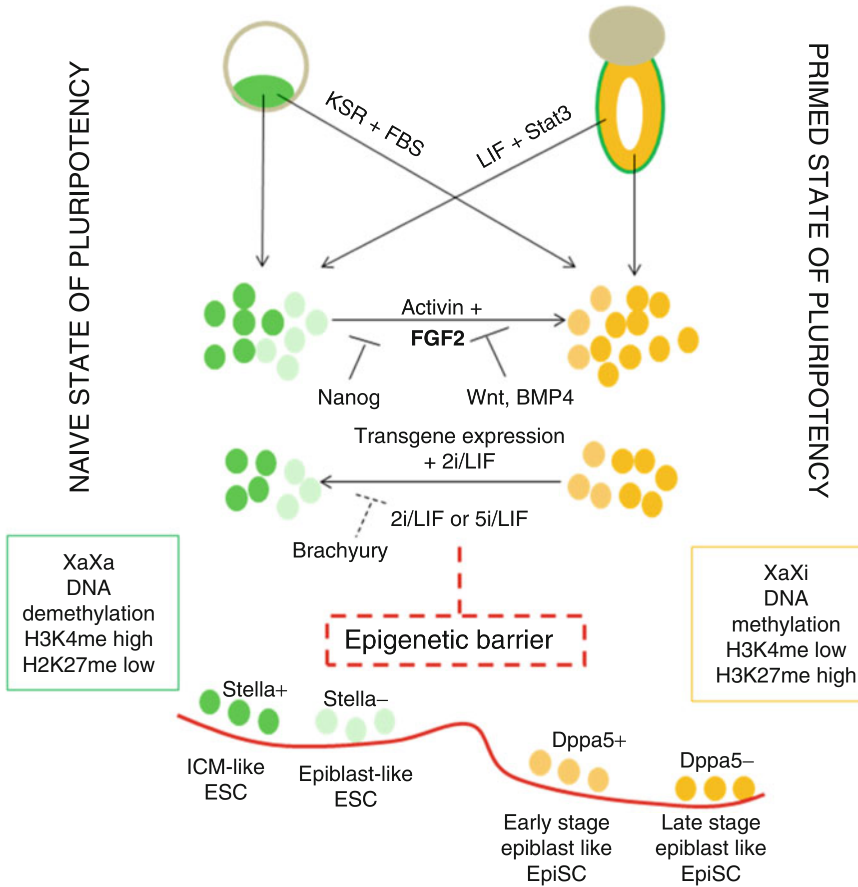


**Fig. 13.3** The two states of pluripotency and the relationships between ESCs and EpiScs

Nodal/Smad2/3 and FGF/Erk1/2, are of utmost importance *in vivo*, but they have been more viewed as patterning molecules than for pluripotency maintenance. However, in the absence of *Nodal* the expression of *Oct4* is greatly reduced and the epiblast adopts an early neural fate (Shen 2007). Erk activity probably triggers the transition from the naïve to the primed state *in vivo*, as it does in mESCs, preparing the epiblast to respond to the lineage inductive cues (Nichols et al. 2009; Osorno and Chambers 2011).

## Derivation of EpiSCs in Non-murine Species

Epiblast Stem Cells have been derived in few other species. Rat Epiblast Stem Cells have been obtained from two strains, Sprague–Dawley and Wistar (Brons et al. 2007). Recently, pig Epiblast Stem Cells have been obtained using isolated epiblast from elongating blastocysts (Alberio et al. 2010). These cells were shown to be dependent on FGF2 and Activin and were able to differentiate *in vitro* into the three lineages. Noteworthy, no ESCs have ever been successfully derived from pig blastocysts. Only when ICM cells were transduced with two factors, *Oct4* and *Klf4*, could LIF-dependent pluripotent cells be generated in pig (Telugu et al. 2011). In the search of rabbit ESCs, two groups have established pluripotent cells from blastocysts but they subsequently demonstrated that these cells were in fact LIF-independent in contrast to mESCs and instead required Activin and FGF, like mouse EpiSCs (Honda et al. 2009). Human Embryonic Stem Cells have been derived for the first time in 1998. It became soon clear that they were different

from the canonical mESCs, in terms of culture conditions, morphology and even molecular profile. The first description of mouse EpiSCs revealed that they were indeed quite similar to human ESCs: morphology, dependence on FGF2 and Activin signaling, X-chromosome inactivation, absence of alkaline phosphatase activity (Tesar et al. 2007). Later on, Vallier and colleagues highlighted that the role of Activin and Nanog in the control of self-renewal and the signaling pathways controlling differentiation were very similar in mouse EpiSCs and human ESCs (Vallier et al. 2009a, b).

Therefore, in species such as rabbit and human, despite starting from the same tissue at an equivalent embryonic stage (the ICM of the blastocyst), the pluripotent cells are in fact similar to cells derived from a later stage. Even in mouse, some strains such as non-obese diabetic (NOD) behave like rabbit and human, with explanted ICM giving rise only to EpiSC-like cells, unless a combination of exogenous factors stabilizes the ESC-state (Hanna et al. 2009; Najm et al. 2011). Conversely, mouse strains that easily generate ESC such as 129, can also efficiently give rise to EpiSCs, from both ICM and late epiblast stage (Najm et al. 2011). From these data we can speculate that *in vitro* the primed state is the common state of pluripotency and the naive one the exception, only reached in some mouse strains. Derivation of EpiSC and/or ESC lines in other mammalian species may help to support this hypothesis.

There are clearly some strain-specific endogenous modulators that prevent or restrict access to the naive state, while being permissive for the primed state (Hanna et al. 2009). Susceptibility of the epiblast cells towards Erk signaling pathways probably plays a role, as suggested by the improvement of mESC derivation in recalcitrant strains when MEK inhibitors are added continuously (Batlle-Morera et al. 2008). Thanks to the simultaneous existence of both ESC and EpiSC, the mouse is the model of choice for a comprehensive study of the molecular clues controlling the stabilization of cells in one or the other state. The state of art of the relationships between ESC and EpiSC will be examined in the next part.

## Relationships Between EpiSC and ESCs: Clues from Reversion/Conversion Studies

Mouse Embryonic Stem Cells and EpiSCs represent two distinct and stable pluripotency states: naive and primed, respectively. In each of these cell populations, when gene expression is studied at the level of the individual cells, a growing list of genes has been found to be heterogeneously expressed between cells (Han et al. 2010; Osorno and Chambers 2011). Heterogeneous expression has been shown in some cases to correlate with differences at the epigenetic level and in developmental potential as it was demonstrated for *Dppa3* and *Zfp42* in ESCs, or *Dppa5* in EpiSCs (Han et al. 2010; Hayashi et al. 2008; Osorno and Chambers 2011): thus, Dppa3 or Zfp42 negative mESCs are closer to the primed state, whereas Dppa5 positive EpiSCs are closer to the naive state. Importantly, Dppa3+/Dppa3- or Zfp42+/Zfp42- mESC populations and Dppa5+/Dppa5- EpiSCs are interconvertible, as indicated by the fact that, once selected, each population restores the original proportion of each type of positive and negative cells (Han et al. 2010; Hayashi et al. 2008).

When considered at the cell level, mESCs and EpiSCs may therefore individually adopt different epigenetic states that bring them closer or further apart, in a dynamic and metastable equilibrium (Fig. 13.3). This raises the question of whether the balance that maintains mESCs and EpiSCs within their respective epigenetic states could eventually be tipped. In other words, what does it require to cross the epigenetic barrier that keeps naive and primed pluripotent cells distinct? In this part, we will review the current literature concerning EpiSC to mESC reprogramming (or reversion), and conversion of mESCs into EpiSCs.

## EpiSC to ESC Conversion: Overcoming the Epigenetic Barrier

### Reversion to the Naive State

In order to achieve reversion of EpiSCs into ES-like cells, different transcription factors have

been overexpressed in EpiSCs, such as Klf4, Nanog, c-Myc, the orphan nuclear receptor Nr5a and a JAK/Stat3 activating receptor (Guo et al. 2009; Hanna et al. 2009; Osorno and Chambers 2011; Silva et al. 2009; Yang et al. 2010).

After virus transduction (Hanna et al. 2009) or lipofection with piggyBac vectors (Guo et al. 2009; Silva et al. 2009; Yang et al. 2010), EpiSC over-expressing defined factors are transferred to media containing MEK and GSK3 inhibitors (2i) in addition to LIF: such media (2i/LIF) have proven to support ground state ESC self-renewal (Nichols and Smith 2009). Following transfer to 2i/LIF, colonies with typical ESC morphology (tighly packed and domed aggregates) emerge and can be further expanded into stable cell lines (Guo et al. 2009; Hanna et al. 2009; Silva et al. 2009). Notably, established cell lines remain stable even after Cre-mediated transgene excision (Guo et al. 2009; Yang et al. 2010).

Concomitant with over-expression of the aforementioned factors, EpiSCs have also been successfully reprogrammed into ESC looking cells by simple switch onto mouse feeders with ESC medium (Bao et al. 2009), into 2i/LIF containing medium (Greber et al. 2010; Hanna et al. 2009), or in some case with medium containing a combination of five inhibitors targeting respectively the histone demethylase LSD1, ALK4/5/7, MEK, FGFR and GSK3 (Zhou et al. 2010).

Reprogramming of Epiblast Stem Cells into ES-like cells is usually an inefficient process, with 0.1–2% of the cells reverting, while the others differentiate or die. It indicates that the primed state of EpiSCs is stable. However there are a few notable improvements:

– *Nanog* overexpression, or transient *Nr5a* expression together with stable *Klf4* expression, increased the rate of reversion up to 10% (Silva et al. 2009). Forced expression of *Nanog* even allowed EpiSCs to be reprogrammed in LIF/BMP4 alone without any inhibitor, albeit with a lower efficiency (Silva et al. 2009).
– Combination of five inhibitors was reported to reprogram EpiSCs into ESC-like cells with about 15% efficiency (Zhou et al. 2010). In this case the reversion was facilitated by the use of an epigenetic modulator together with chemicals that inhibit Activin and FGF signaling pathways while stimulating the Wnt pathway.
– The Dppa5+ fraction of EpiSCs reverted to ES-like cells with 15% efficiency, compared to 1 % for the Dppa5- cells (Han et al. 2010).

Intriguingly, while some teams could successfully reprogram EpiSCs to ES-like cells by simply switching culture conditions, others were unable to do so and had to over-express exogenous factors in their EpiSC lines. It has been proposed that the ability to revert could be correlated to distinct initial developmental states of EpiSCs (Bernemann et al. 2011; Han et al. 2010).

## Molecular and Functional Evidence for Reversion to the Naive State

In most reports, reprogramming of EpiSCs into ESCs is first evidenced by changes in morphology, from flat colonies to three-dimensional aggregates. A more refined approach involves the use of an Oct4-ΔPE-GFP reporter with only the distal enhancer for Oct4: this reporter shows preferential expression in ESCs but not in EpiSCs and is therefore activated in the subset of reprogrammed EpiSCs (Bao et al. 2009; Greber et al. 2010; Hanna et al. 2009).

Epiblast Stem Cells reverted into ESCs (subsequently called rESC) can be passaged by clonal propagation to form established cell lines (Bao et al. 2009; Guo et al. 2009). They have upregulated expression of ESC specific markers such as *Dppa3*, *Zfp42* and *Fbx15*, whereas EpiSC specific markers such as *Fgf5*, *Brachyury* and *Lefty* are down-regulated (Bao et al. 2009; Greber et al. 2010; Guo et al. 2009; Silva et al. 2009). Further whole genome expression analysis in different studies tends to support the view that rESCs and genuine ESCs are transcriptionally indistinguishable (Greber et al. 2010; Hanna et al. 2009; Zhou et al. 2010).

Results from *in vitro* differentiation assays also suggest that rESCs are closely related to ESCs, while different from EpiSCs: rESCs were not affected by treatment with a SMAD 2/3 inhibitor, contrary to EpiSCs; conversely, LIF inhibition induced rESCs and ESCs differentiation, whereas

EpiSCs were unaffected (Greber et al. 2010). After BMP4 treatment, rESCs and ESCs were unable to generate extra-embryonic derivatives, contrary to EpiSCs (Zhou et al. 2010). Further directed differentiation of rESCs yielded beating cardiomyocytes as efficiently as with ESCs, while very little were produced with EpiSCs (Zhou et al. 2010).

Despite these resemblances, and because pluripotency is defined by the ability to give rise to any cell belonging to the three germ layers including the germ line, it was of importance to assess how rESCs compared to ESCs in terms of *in vivo* developmental potential. When rESCs were injected into diploid host embryos, high-contribution chimeras were obtained, with germline transmission (Bao et al. 2009; Guo et al. 2009; Hanna et al. 2009; Yang et al. 2010; Zhou et al. 2010). Therefore, rESCs show the same *in vivo* developmental potential as ESCs.

Epigenetic mechanisms play a key role in ESCs for correct gene expression and developmental potential. Due to their relevance for biomedical applications, iPSCs and ESCs have been systematically scrutinized at a whole genome scale for epigenetic differences. Concerning rESCs and ESCs, however, epigenetic comparisons have mainly been performed at the single gene level so far: *Dppa3*, *Zfp42* and *FGF4* loci appeared demethylated in rESCs and ESCs, while hypermethylated in EpiSCs (Bao et al. 2009; Han et al. 2010; Zhou et al. 2010). Moreover, H3K4 and H3K27 methylation patterns of *Dppa3* in rESC were similar to that observed in ESCs, but distinct from that in EpiSCs (Zhou et al. 2010). Confirming epigenetic reprogramming from a primed to a naive state, loss of H3K27me3 nuclear body was evidenced in female rESCs, which indicates X chromosome re-activation in these cells, whereas one X chromosome remains inactive in EpiSC (Bao et al. 2009; Guo et al. 2009; Silva et al. 2009; Yang et al. 2010).

Reversion of Epiblast Stem Cells, from their primed pluripotent state, to a more naive state is therefore accompanied by epigenetic remodeling, which leads to rESCs that are similar to genuine ESCs on the molecular and developmental level. Because an epigenetic barrier has to be overcome when EpiSCs are reverted into rESCs (Bao et al. 2009; Hayashi et al. 2008), stringent culture conditions with inhibitors, or defined factor overexpression, must generally be used. In contrast, conversion of ESCs into EpiSCs, since it emulates *in vitro* the *in vivo* transition from a naive ICM to a primed epiblast, should be less demanding in terms of culture conditions.

## ESC to EpiSC: Going Down Waddington's Canal

### Conversion to the Primed State

Conversion of Embryonic Stem Cells into EpiSC-like cells is achieved by simply changing culture conditions, to media supplemented with Activin A and FGF2 (Berge et al. 2011; Guo et al. 2009; Hanna et al. 2009). Embryonic Stem Cells adopt a flattened morphology, and stable EpiSC-like cell lines are passaged as small clumps with collagenase (Berge et al. 2011). While FGF2 is thought to facilitate the transition from ESCs to EpiSC-like (Zhang et al. 2010), addition of BMP4 or WNT to the culture medium inhibit conversion into EpiSC-like cells (Berge et al. 2011; Zhang et al. 2010). Of interest, overexpression of Nanog in ESCs has the same effect (Osorno and Chambers 2011). Conversion efficiencies have not been reported precisely in the studies above, however, our own observations indicate that most ESCs can be converted: after an initial phase of limited cell death upon transition to Activin A and FGF2 medium, most ESC colonies survive and display an EpiSC-like morphology.

### Molecular and Functional Evidences for Conversion to the Primed State

Besides their flattened morphology, ESCs converted into EpiSC-like cells (thereafter named cEpiSCs) also stain negatively for alkaline phosphatase (Berge et al. 2011) and acquire marker-gene expression profile similar to that of EpiSCs. While *Oct4* and *Nanog* are maintained in Epi-ESCs (albeit at lower level for the latter), the ESC markers *Essrb*, *Dppa3*, *Zfp42* and *Klf4* are downregulated (Berge et al. 2011; Osorno and Chambers 2011). In contrast,

EpiSC markers such as *Nodal*, *Otx2* and *Claudin6* are upregulated (Berge et al. 2011; Zhang et al. 2010).Genome-wide profiling in one study suggests that cEpiSCs are closely related to EpiSCs, but distinct from their parental ESC lines (Zhang et al. 2010).

On the functional level, cEpiSCs become dependent on Activin signaling through the ALK (AcvR) receptors, because differentiation follows treatment by ALK inhibitors (Berge et al. 2011). They are also able to differentiate *in vitro* and *in vivo* -through teratoma- into derivatives of the three lineages (Zhang et al. 2010). cEpiSCs therefore display the same developmental potential as genuine EpiSCs.

On the epigenetic level, increased expression of the *de novo* DNA methyl transferase Dnmt3b in cEpiSCs denoted the induction of the DNA methylation machinery, in accordance with the establishment of the epigenetic barrier (Berge et al. 2011). In addition, in female cEpiSCs, H3K37me3 foci were observed, indicating the presence of an inactive X chromosome (Berge et al. 2011; Guo et al. 2009).

From the studies described above, cEpiSCs have recapitulated so far the hallmarks of EpiSCs, regarding marker gene expression as well as key epigenetic features. Because in conversion from a naive state to a primed state, the epigenetic barrier is established according to a path that mimic the epigenetic changes happening during *in vivo* development it is somewhat easier than the reverse process and doesn't require specific conditions besides a simple medium switch.

In conclusion, in the transition of embryo-derived stem cells across the epigenetic barrier, between naive and primed states of pluripotency, one key element lies in the cell environment: media switches are necessary for the transition between ESCs and EpiSCs to occur. Even overexpression of an exogenous factor, such as *Nanog* or *Klf4*, cannot convert EpiSCs into rESCs without concomitant changes in culture conditions (Guo et al. 2009; Silva et al. 2009). However, in this case, our knowledge is very limited on how the signaling pathways interact with the induced factor and what precisely the hierarchy of the events is. The signaling pathways switches may not only be necessary to sustain either the naive or the primed state of pluripotency but may also play a more direct role in the transition. Another point which remains to be highlighted is the precise role of the X-chromosome inactivation/reactivation during the conversion or reversion process: it is not yet known whether it is a passive role (as a landmark underlying global epigenetic changes) or a more active one in the gene dosage compensation that may be essential for cell survival in the primed state. Furthering our understanding of the events underlying this medium-mediated epigenetic reprogramming could potentially lead to new strategies for the resetting of more differentiated cells back to the pluripotent state.

## Reprogramming of Somatic Cells into EpiSCs

### Induced Reprogramming

A few years ago it has been demonstrated that the forced expression of four transcription factors was sufficient to reprogram somatic cells into pluripotent ones, called induced pluripotent stem cells (iPSCs). In mouse the iPSCs are similar to ESCs, in terms of culture requirements, transcriptome and epigenetic profiles. Mouse and human iPSCs behave like the corresponding species-specific ESCs, so they are in the naive and primed state, respectively. As in the mouse both states of pluripotency can be obtained, it was of interest to test whether it was possible to generate mouse iPSCs in the primed state. In the two studies addressing this question, FGF and Activin dependant iPSCs were obtained, although the pace of formation of the reprogrammed cells was slower than for ESC-like iPSCs (Di Stefano et al. 2010; Han et al. 2011). Interestingly, the status of these cells was different in the two papers. The induced Pluripotent Stem Cells described in Di Stefano and colleagues' paper were in fact in the naive state, as indicated by molecular profiling, X-chromosome reactivation and chimera formation. However, they are also clearly Activin and FGF dependant, as demonstrated by the rapid differentiation or death when cultured in the

presence of inhibitors of Activin or FGF signaling, respectively. By contrast, Han and colleagues obtained cells in the primed state, very similar to EpiSCs, in terms of Oct4 enhancer utilization, epiblast marker expression, methylation at the Stella locus and absence of chimera formation. They showed that these EpiSC-like cells were directly derived from fibroblasts without transiting through an ESC like state. Obtaining EpiSCs-like iPSCs was made possible by the use of culture conditions compatible only with the maintenance of primed state (defined medium containing only Activin and FGF and no feeders, known to produce LIF) that selected only EpiSC-like colonies. From these studies, it seems that reprogramming towards the primed state is a more difficult and longer process. From a developmental point of view, this may appear as paradoxical and awaits further investigation to better understand the transition from somatic towards either the naive or primed state of pluripotency. It also suggests that we may not have the optimum conditions to reprogram somatic cells in the primed state. As in humans the iPSCs that are generated are also in the primed state as do human ESCs, the mouse model appears the model of choice for such investigations..

## Reprogramming by Nuclear Transfer

Before the breakthrough of induced reprogramming, reprogramming of somatic cells to pluripotency has been achieved using nuclear transfer (NT) in enucleated oocytes. From blastocysts generated by NT, ESCs, called NT-ESCs, were derived and they were shown to be faithfully reprogrammed, and thus undistinguishable from their fertilize counterparts. Recently, we derived EpiSCs from post-implantation NT embryos. The efficiency of derivation was similar to that of fertilized embryos and the EpiSC lines display the characteristic markers of pluripotency and could differentiate *in vitro* into the three germ lineages (Maruotti et al. 2010). However, among the three NT-EpiSC lines that were analyzed, two of them exhibited some abnormalities in their transcriptome, indicating that they were probably not correctly reprogrammed (Maruotti et al. 2010). Interestingly, some of the genes showing a down-regulated expression were hypermethylated. Our current hypothesis is that epigenetic abnormalities may have arisen during the transition from ICM to late epiblast stage, when the genome of epiblast cells is subjected to *de novo* methylation. Analysis of several other NT-EpiSC lines is necessary before reaching a definitive conclusion.

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# Epigenetic Modifiers and Stem Cell Differentiation

14

Ahmed Taher El-Serafi

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**Abstract**

During stem cell differentiation, genes related to their unique characters should be silenced and genes related to the target lineage must be activated. Epigenetics is well known to control gene expression and changes of the epigenetic status during differentiation were reported. Epigenetic modifiers are chemical agents that can remove the epigenetic silencing markings and consequently activate the affected genes. The role of the epigenetic modifiers is well established in cancer treatment. Reports about enhancing stem cells differentiation (both murine and human) with treatment of the modifiers are accumulating. The modifiers have no differentiation ability by themselves but they enhance the differentiation upon culturing the cells in the corresponding conditions. This effect has been shown in monolayer culture as well as three-dimensional pellets. Epigenetic modifiers could be a valuable additive to the classical differentiation protocols, although various questions have yet to be answered including the safety of the agents.

## Introduction

Epigenetics and stem cells are two independent growing fields of interest for many scientists. As epigenetics influence the gene expression and stem cells differentiation requires switching on and off different sets of genes, the two fields could be related. With this background, modifying the

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epigenetic status of the stem cells could enhance their differentiation, which is the target for most of the researchers working in the field. This chapter discusses the basics of epigenetics, epigenetic modifiers and how they have been used to enhance stem cell differentiation.

## Components of Epigenetics

Epigenetics is classically defined as 'the study of heritable changes in gene expression that occur without a change in DNA sequence' (Wolffe and Matzke 1999). The term 'epigenome' has emerged to describe the epigenetic modifications all over the genome in both normal cellular processes and abnormal events. The branch of science that called 'epigenetics' has three main components: DNA methylation, histone modifications and small RNA control. The first component 'DNA methylation' refers to the condition where a methyl group is covalently added to the carbon number five in the cytosine nitrogenous base attached to the deoxyribonucleotide skeleton of the DNA strand, provided that this cytosine base is adjacent to guanine (Szyf 2008; Vaissiere et al. 2008). DNA methylation is related to gene silencing and is extensively studied as an important mechanism of epigenetic control, including the process of X chromosome inactivation in females. Methylation can suppress gene transcription through one of two mechanisms. The first mechanism (direct approach) is through physical interference with the binding of specific transcription factors to their recognition sites in the gene promoter area, allowing improper interaction between the transcription machinery and the targeted gene (Jones and Takai 2001). The second mechanism (indirect approach) depends on the binding of methylated DNA to specific transcriptional repressors, which in turn suppress the gene expression (Rountree et al. 2000).

The second component of epigenetics is histone modifications. Histones are five basic nuclear proteins that form the core of the nucleosome, around which the DNA double strand is wrapped. Histones function was thought to be the packing of DNA to fit inside the nucleus. This concept proved to be inaccurate. Histone tails that protrude through the DNA gyri into the space surrounding the nucleosomes are subjected to post-translational covalent modifications and those modifications affect gene transcription. Those modifications include histone methylation, acetylation, ubiquitination, sumoylation, phosphorylation or ribosylation. Of those, the role of histone acetylation was extensively studied and clearly established. Histone acetylation enhances gene transcription. The additional acetyl group to the histone protein neutralizes the positive charge of the histone tails, decreasing their affinity for DNA, opening the chromatin and allowing the transcriptional machinery to reach its target and consequently gene activation (Kornberg and Lorch 1999).

The last component of epigenetics is Small RNA. This population of RNA includes the micro RNA (miRNA) and the small interference RNA (siRNA). Both are 21–25 nucleotides in length. Small RNA binds their complementary mRNA and thus dsRNA is formed, recognized as foreign RNA and thus cleaved and degraded. When the matching between the small RNAs and mRNA is not perfect, while it may not be fully recognized but at least the incomplete binding blocks the translation (Mattick and Makunin 2005). Besides this post-transcriptional epigenetic control, siRNA guides homologous DNA methylation and thus inactivates the corresponding gene (Matzke et al. 2001).

## Epigenetics in Health and Disease

The interaction between any organism and the environment is of significant importance for adaptation during life. This interaction takes place through modulation of the epigenetic status of the key genes in response to the external as well as internal signals induced by such effectors. One of the most interesting examples in mammals is the effect of diet on epigenetic markings. Feeding pregnant mice of two strains with different doses of methyl supplement diets (which would function as methyl donors and thus facilitate DNA methylation) will result in offspring with different phenotypes, i.e. fur colour in the case of Agouti

mice. The mice colour ranged from yellow to brown according to the dose of the methyl donor supplements (Cooney et al. 2002). In human, maternal life style and diet constituents, even before pregnancy, will affect the health of the offspring, from early childhood, adulthood and old age in an environmental compatible manner (Gluckman et al. 2009).

Epigenetics is thought to be involved in a number of diseases, including cancer. Methylation silencing of tumour suppressor genes, aberrant expression of DNA methyl transferase or demethylation of oncogenes can lead to the conversion of a normal cell to a malignant cell. In addition, chromosomal instability and inactivation of the DNA repair system has both the genetic and epigenetic backgrounds (Esteller and Herman 2002). There is also a stringent relationship between the histone modifications and tumour formation. In addition, the recurrence of certain cancers is predicted by the presence of certain histone modifications (Kurdistani 2007). Furthermore, the prognosis of certain malignancies can be affected by the epigenetic status (Sakuma et al. 2007). Osteoarthritis is another example for the relationship between epigenetics and disease. Progressive destruction of the articular cartilage was induced by inflammatory cytokines that cause abnormal demethylation of the promoter of different metalloproteinases within the chondrocytes, causing degradation of the cartilage matrix and is associated with disease progression (Hashimoto et al. 2007).

## Epigenetic Modifiers

Epigenetic modifiers are those chemicals that are able to change the characteristics of the cells in correspondence to alteration of their epigenetic status. Alteration of DNA methylation and histone acetylation causes a shift between active/silent chromatin and consequently gene expression. Epigenetic modifiers were initially used as differentiating agents in cell culture and with time, the modifiers were substituted by more specific agents. In the last few years, several modifiers were approved as line of management for malignant tumors (reviewed in Piekarz and Bates 2009). These agents change the epigenetic status of malignant cells and thus activate silent tumour suppressor genes, which renders the modifiers themselves to be used as treatment or adjuvant to the standard regimen (Daskalakis et al. 2002; Shang et al. 2007).

### DNA Methylation Modifiers

Modification of the DNA methylation status is through introducing a cytosine analogue into the newly formed DNA, provided that this base could not be methylated. Reports about 5-Aza-deoxycytidine (5-Aza-dC) could be found in literature as early as the sixties of the last century. During the S phase of the cell cycle, 5-Aza-dC is converted to a deoxynucleotide triphosphate and incorporated into newly synthesized DNA, replacing cytosine. This fraudulent base bonds covalently to the DNA methyl transferase and traps the enzyme. Thus 5-Aza-dC would inhibit the DNA methylation in the dividing cells. This epigenetic change is inherited to the next generation of cell as the replication fork will proceed without the methylase (Szyf 2008). 5-aza-cytidine (5-Aza-C) is another nucleoside-based DNA methylation inhibitor, which is the corresponding analogue to cytidine. It was initially synthesized as an antimetabolite that is specific for acute myelogenous leukemia. 5-Aza-C affects DNA, RNA and protein, while 5-Aza-dC affects DNA only. Both 5-Aza-dC and 5-Aza-C are cytotoxic and can lead to chromosomal breakages. The DNA demethylation effect could be seen on optimal dose, which differs among cell types (reviewed in Christman 2002).

### Histone Acetylation Modifiers

Histone deacetylases (HDACs) are those enzymes catalyse the removal of the acetyl group from the histones. HDACs are 18 enzymes in mammalian cells that are divided into two families: (a) zinc metalloenzymes that catalyse the hydrolysis of acetylated specific residues on the histone tails

and includes class I, II and IV HDACs and (b) NAD-dependant Sir2 deacetylases which are considered as class III HDACs (Glaser 2007).

There is a long list of histone actylation modifiers, called histone deacetylase inhibitors (HDI). Those chemicals inhibit histone deacetylases non-competitively and reversibly. HDI have a zinc binding domain and serve as substrates to chelate the zinc ion contained in the deacetylases. The consequences of deacetylation include: (a) preservation of already acetylated histones in their active state; (b) a change in the balance between the histone acetylating and deacetylating enzymes resulting in increased histone acetylation (Szyf 2008). Trichostatin A (TSA) is the most widely known HDI. TSA was isolated in the 1970s from *Streptomyces hygroscopicus*, as an antifungal antibiotics, which is active against Trichophyton. *In vitro*, TSA increases histone acetylation and arrests the cell cycle in both $G_1$ and $G_2$ phases (reviewed in Yoshida et al. 1995). As a consequence, hyperacetylated histones that are attached to the promoter regions should activate corresponding genes transcription.

## Epigenetics Elements and Modifiers Interaction

The three components of epigenetic control do not work separately, but they interact together in a complicated and as yet not fully understood sense. It has been reported that methylated DNA is associated with deacetylated histones in the inactive areas of DNA but the order of silencing and what follows is still a question to be resolved (Vaissiere et al. 2008). It has been shown that DNA methylation can enhance histone modifi cation. In addition, inactive transcription *per se* is insufficient to change the acetylation status of histone but DNA methylation is required for such change to occur, methylated DNA will attract methylated DNA binding domain proteins such as MeCP2. MeCP2 in turn will recruit histone deacetylases, compiling two components of epigenetic silencing. While the carboxyl terminal (catalytic domain) of a DNA methyl transferase add the methyl groups to the DNA; the N-terminal interacts with HDAC1 and 2 mediating histone deacetylation at the same time, resulting in gene silencing (Rountree et al. 2000).

Thus, the two ends of MeCP2 may connect the action of the epigenetic modifiers. As 5-Aza-dC inhibits DNA methylation and TSA inhibits histone deacetylases, MeCP2 can link between TSA and DNA methylation as well as 5-Aza-dC and histone deacetylation. Thus, indirect effects of each of the modifiers on the alternate epigenetic element could be expected. In mammalian cells as well as in Neurospora, TSA does not induce global demethylation. Certain genes are signifi cantly activated in a selective manner that was comparable to the effect of the DNA demethylating agents. The proposed genes are those silenced by DNA methylation then MeCP2 bound to the methyl groups followed by the recruitment of HDACs. Heavily silenced genes such as those on the inactive X chromosome cannot be activated by TSA alone. Thus DNA methylation could be considered to be in dynamic balance between the activity of demethylases and the degree of histone deacetylation (Kawamoto et al. 2007).

## Stem Cells and Epigenetic Modifiers

Bone marrow stromal cells are those cells forming the stroma supporting the hematopoietic forming cells and are known to be the richest source of stem cells. There are always problems encountering the differentiation of those cells into more specialized cells. Stem cell differentiation is particularly less efficient when the target is cells belong to another lineage. Alexanian has shown that the differentiation of murine BMSCs into neural-like cells was enhanced when the cells were treated with 5-Aza-dC and/or TSA and cultured in neuronal induction media (Alexanian 2007). Thus, the epigenetic modifiers enhanced the differentiation of neural cells, originally derived from the ectoderm, from BMSCs that have a mesodermal origin. The author suggested a potential role for epigenetics in governing stemness, commitment and differentiation, as well as maintenance of these states. In the Alexanian study, 5-Aza-dC and TSA had similar effects.

In addition, there was a synergistic effect of the combined treatment, which was in agreement with a study performed on the Oct-4 gene in which the authors showed activation of the Oct-4 gene in primary murine trophoplastic cells by one or both agents. In the murine embryonic fibroblast cell line (NIH/3T3), which is known not to express Oct-4, only the combination of treatments could up-regulate the gene (Hattori et al. 2004).

Epigenetic markings are accumulating with time, which was shown by comparative studies of DNA methylation between 3 and 50 year old twins (Fraga et al. 2005). The methylation map was very comparable at the age of 3 years but apparently different at 50 years old. The individual exposure to various environmental challenges would be reflected on the individual's epigenetic map. Accumulating epigenetic markings may need to be erased before the multipotential cells are directed to a specialized cell type. This erasure is documented in the early developmental stages. After fertilization, demethylation takes place in both the male and female pro-nuclei, with the exception of imprinted genes. Demethylation of the paternal pro-nuclei takes place faster than the maternal counterpart; which is assumed to be an active process in the paternal pronuclei but passive in the maternal pronuclei. Following which, reprogramming takes place in a tissue specific manner (Niemitz and Feinberg 2004).

The role of epigenetic modifiers in human derived cells is very comparable. Differentiation of human-derived BMSCs into specialized cell lineages could be enhanced with the modifiers, provided that the cells were cultured in corresponding conditions (El-Serafi et al. 2011a). The main difference was the differential effects of 5-Aza-dC and TSA. 5-Aza-dC was shown to enhance the osteogenic differentiation of BMSCs when cultured in ostegenic conditions in monolayer as well as three dimensional pellet culture for 3 weeks, while TSA enhanced chondrogenic differentiation when the cells were cultured in corresponding conditions in comparison to controls. The effects of 5-Aza-dC and TSA were not reciprocal; i.e. cells treated with 5-Aza-dC did not show chondrogenic differentiation in long term pellet cultures in chondrogenic media and vice versa. In addition, 5-Aza-dC and TSA did not show osteogenic or chondrogenic effects alone. Osteogenic and chondrogenic induction was not found when pellets were cultured in basal conditions. The corresponding media was essential to show differentiation (El-Serafi et al. 2011a). Thus the modifiers did not have osteogenic or chondrogenic potential on their own. Similar effects were shown for neural, oligodendrocytes and beta cells differentiation (Alexanian 2007; Liu et al. 2010; Tayaramma et al. 2006). One assumption for explaining these findings is that epigenetic modifiers 'sensitize' the cells for the action of the media supplement, which in turn guides the differentiation of the stem cells. This assumption based on 'generalized' demethylation of DNA or acetylation of histones with consequence removal of epigenetic silencing. The chromatin in this active status can respond more effectively to the culture conditions. Supporting evidence was that the differentiation of fetal derived skeletal cells into the osteogeinc and chondrogenic lineages were more efficient than BMSCs (El-Serafi et al. 2011b) and epigenetic modifiers did not prove enhancement in the fetal cell differentiation (unpublished data). According to the studies on twins (Fraga et al. 2005), fetal derived cells could be expected to have less epigenetic silencing markings and thus can respond to the culture conditions more efficiently.

Global DNA demethylation or histone acetylation could not be expected as an effect of the epigenetic modifiers. Such assumption would interfere with the stability of the chromosomes and consequently the cell survival. For unknown reasons, epigenetic modifiers may have tendency to affect some genes and not the others. Surprisingly, TSA may also decrease the transcription and the translation of some genes; Wilms tumour gene 1 is an obvious example in this context. For yet undetermined reasons, TSA enhances the gene silencer, which consequently decreases gene expression. In addition, TSA enhances the transcription of a certain proteosome, which digests the Wilms tumour protein. The net result is marked decrease in the Wilms tumour protein (Makki et al. 2008). This harmonized action may raise the issue of selectivity. The inhibition

of Wilms tumour at both the transcription and translation levels may be caused by one or more factors that selectively antagonise this protein and co-operate with the TSA or expose their target genes to the TSA. This factor could be an epigenetic modulation, a transcription factor or other regulatory molecule. In another study, only 8 genes of 340 examined genes have enhanced transcription profile in response to TSA treatment (VanLint et al. 1996) while in a microarray study 0.44% of the analysed genes were affected by TSA treatment for 6 h. Of these, 46 genes (representing 53%) were up-regulated including transcription factors, while 41 genes (47%) were down regulated (Crabb et al. 2008).

Menendez et al. (2007) have studied the genes responsive to 5-Aza-dC treatment in an ovarian cancer line using a microarray approach. From 47,000 transcripts, only 465 (1%) were up-regulated and 366 (0.8%) were down-regulated (Menendez et al. 2007). Similar results were found with BMSCs using PCR-array method (unpublished data). These authors anticipated that the downregulation may have resulted from access of gene repressors or negative regulatory factors to the hypomethylated promoter.

The most important question is what are the genes susceptible to epigenetic modifiers effects? Epigenetic silencing varies among genes. For example, genes carried on the second X chromosomes in females are subjected to cascades of DNA demethylation and histone deacetylation. Such genes are not expected to respond to the modifiers action. In different cells, epigenetic silencing map is different; for example, insulin gene is active only in beta cells of the pancreas but silenced in other cells. Similar effect could be expected for genes responsible of differentiation. Those genes were switched off at early stages of life, with special emphasis on genes responsible for the other lineages. Stem cells derived from mesenchymal origin and settled down in a niche of the same origin for years, such as BMSCs, could have epigenetic silence markers on the ectodermal lineage gene. That is why differentiation of stem cells derived from the desired lineage is more efficient than across lineages, i.e. transdifferentiation. The effect of 5-Aza-dC has also been proved in hematopoietic stem cells (Hu et al. 2010). 5-Aza-dC helped in keeping the stem specific genes, self renewal as well as their ability to respond to the differentiation triggers.

Thus, the sequence of events could be expected as following; (a) Some genes are more susceptible to the action of epigenetic modifiers than others. Those genes are different based on many factors such as the cell type, age, environment and any other factor that can affect the epigenome. (b) Upon treatment with the modifiers, those genes are activated and can respond to the triggers of differentiation, such as growth factors, steroids, vitamins or different physical challenges. (c) Being active and occupied with the transcription factors, responded genes will sustain its epigenetically active status. Other genes would acquire the default epigenetic silencing. (d) Cells differentiate into the activated lineage.

## Safety of Epigenetic Modifiers

The safety of the modifiers has yet to be defined. Several questions have to be answered before applying the utilization of epigenetic modifiers in standard stem cell differentiation protocols. Which genes that will be affected by each modifier in every cell type? What are the unwanted genes that could be affected? Activation of an oncogene for example, would be a serious adverse effect. In breast cancer cell line, 5-Aza-dC has been shown to induce the metastasis related genes in addition to the tumor suppressor genes (Ateeq et al. 2008). In spite, 5-Aza-dC was approved by the Food and Drug Adminstration in USA (FDA) for treatment of myelodysplastic syndrome. Two different histone deacetylase inhibitors (Vorinostat and Romidepsin) were approved by FDA for treatment of cutaneous T cell lymphoma.

Another important question is what will be the effect of ex-vivo treatment of stem cells on the human body upon reimplantation? What is the most suitable time frame to reimplant the cells? Short term in vitro phase is expected to have active genes that will differentiate into the corresponding cell type when implanted in the proper niche *in vivo*. Although, long term *in vitro* phase

will help in silencing unwanted genes prior to implantation.

In conclusion, Epigenetic modifiers are those chemicals that can activate genes through changing their epigenetic status, rendering stem cells more responsive to the differentiation triggers in classical differentiation protocols. The modifiers are in their early stages of studies in this field although being in clinical use for treating certain diseases. The safety issues on the long term cultures as well as upon transplanted in vitro has yet to be determined.

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# Enrichment and Selection of Stem Cell-Derived Tissue-Specific Precursors

15

Harold S. Bernstein and William C. Hyun

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**Abstract**

Human embryonic stem cells have the capacity for self-renewal and pluripotency, making them a primary candidate for tissue engineering and regenerative therapies. They also provide an opportunity to study the development of human tissues ex vivo. To date, numerous hESC lines have been developed and characterized. In this chapter, we will review the strategies used to direct tissue-specific differentiation of embryonic stem cells. We also will discuss how these strategies have produced new sources of tissue-specific progenitor cells. Finally, we will describe the next generation of methods being developed to identify and select stem cell-derived tissue precursors for experimental study and clinical use.

## Introduction

Stem cells have the ability to maintain long-term proliferation and self-renewal. Under specific conditions, stem cells can differentiate into a diverse population of mature and functionally specialized cell types. There are two main types of human stem cells classified according to their source and developmental potential: embryonic and adult, or tissue-specific, stem cells. Human embryonic stem cells (hESCs) are pluripotent cells that can differentiate into all types of somatic and in some cases, extraembryonic tissues. Human adult stem cells are derived from non-embryonic tissues and are capable of generating specific

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cells from their organ or tissue of origin. Because of the unrestricted potential of hESCs, these cells have become a highly desirable experimental tool for understanding human development, and are especially attractive for therapeutic applications. In addition, methods for inducing a pluripotent state in human somatic cells has created the opportunity to study and utilize patient-specific stem cells, or induced pluripotent stem cells (iPSCs), and their derivatives. For these reasons, methods for directing the differentiation of pluripotent stem cells and selecting these for analysis and clinical use has become an area of intense investigation.

## Enrichment Strategies

Pluripotent stem cells are defined in part by their capacity to differentiate, which can be tested using *in vivo* and *in vitro* methods. A test of pluripotency *in vitro* involves determining the ability of hESCs and iPSCs to form human embryoid bodies (hEBs) when cultured in a non-adherent cell suspension in the absence of feeder cell layers. hEBs are spherical colonies of differentiating stem cells that contain cell types representative of all three embryonic germ layers (King et al. 2009). The most commonly used *in vivo* method to test pluripotency involves the transplantation of undifferentiated stem cells into immunodeficient mice to induce the formation of teratomas (King et al. 2009, 2011; Ritner and Bernstein 2010). Teratomas are benign tumors comprised of disorganized tissue structures characteristic of the three embryonic germ layers. Analysis of embryonic tissues found in teratomas from engrafted stem cells can be used to test their differentiation potential (Fig. 15.1).

The ability of hESCs and iPSCs to mimic *in vitro* and *in vivo* the events occurring during human development makes them valuable tools for understanding the mechanisms involved in developmental processes, and steppingstones toward the generation of desired cell types suitable for cell therapies. Recent studies have shown it is possible to generate lineage-restricted progenitors that are capable of differentiating into specialized post-mitotic cell types such as cardiomyocytes, pancreatic islet cells, chondrocytes, hematopoietic cells, endothelial cells, and neurons. Furthermore, the ability of pluripotent stem cells to divide indefinitely makes these a potential large-scale source of specific progenitors. In the following sections, we will provide examples of how stem cell differentiation can be directed towards specific cell/tissue types.

## Recapitulating Development

Some of the most effective strategies for directing the differentiation of pluripotent stem cells into specific cell types have taken advantage of our understanding of human development (Table 15.1). Endodermal derivatives include cells that populate the lung, liver, and pancreas. Directing the differentiation of hESCs and iPSCs toward definitive endoderm would help generate specific cell types, such as islet cells or hepatocytes, which could be used towards treatment of diseases such as diabetes or liver disease, respectively. D'Amour et al. (2005) showed that selective induction of endoderm could be achieved through the addition of high concentrations of Activin A, under low serum conditions, and in a stage-specific manner. Activin A mimics the action of Nodal, a ligand that activates TGFβ signaling, which in turn leads to the induction of endoderm differentiation. The effect of Activin A in inducing definitive endoderm is enhanced when additional factors such as Wnt3a and Noggin are present, or when coupled with the suppression of the phosphoinositide 3-kinase pathway.

Induction of definitive endoderm can lead to the generation of specific progenitor populations following the addition of other factors. Among the most successful examples to date is the generation of pancreatic islet progenitors devised by Kroon et al. (2008), accomplished through the sequential exposure of hESCs to Activin A and Wnt3A, followed by the addition of keratinocyte growth factor or fibroblast growth factor-7 to induce the formation of the primitive gut tube. Subsequently, retinoic acid, cyclopamine, and

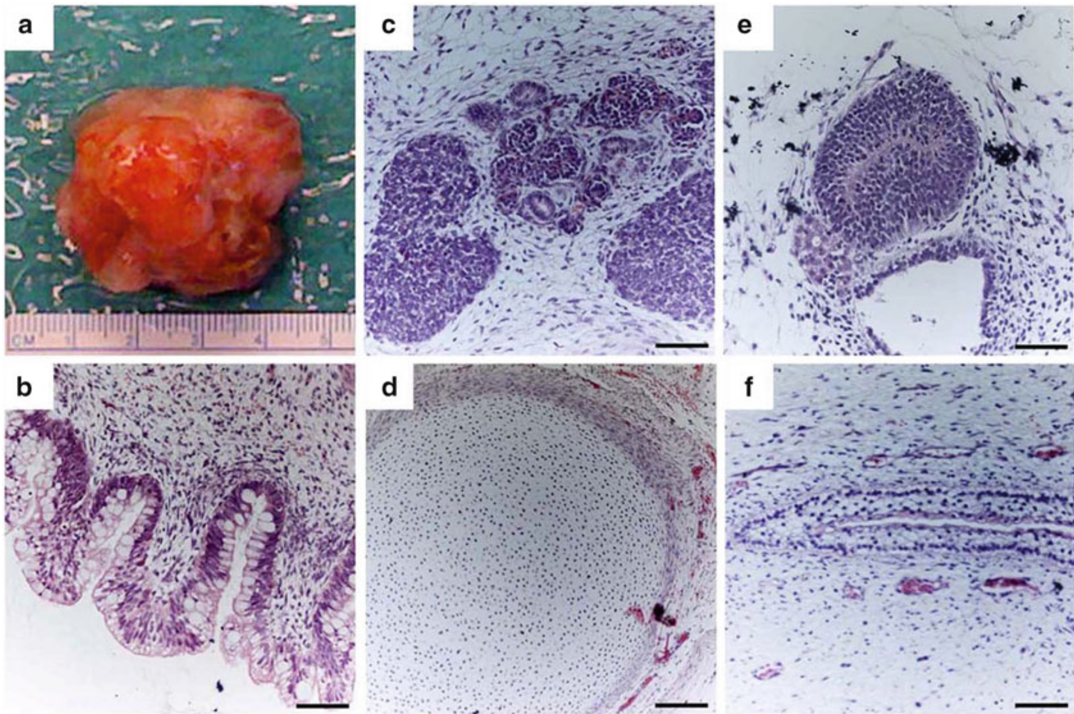


**Fig. 15.1** ***In vivo*** **differentiation of hESCs by teratoma formation**. Proliferating cultures of hESCs were used to form teratomas by renal capsule grafting using established methods (Ritner and Bernstein 2010). (**a**) An explanted teratoma is shown. (**b–f**) Teratomas were sectioned and stained with hematoxylin and eosin to identify embryonic tissues. Representative tissues from all three embryonic germ layers can be seen, including endoderm (**b**), mesoderm (**c, d**) and ectoderm (**e, f**). (**b**) Glandular intestinal structure. (**c**) Nascent renal tubules and glomeruli within bed of primitive renal epithelium. (**d**) Cartilage surrounded by capsule of condensed mesenchyme. (**e**) Nascent neural tube. (**f**) Primitive squamous epithelium. Bar, 100 μm

Noggin are added to inhibit hedgehog and TGFβ signaling, and thus induce the differentiation of posterior foregut cells, the source of pancreatic cell progenitors. These are cultured further to generate pancreatic endoderm cells. When these cells are engrafted in immunodeficient mice, they display the histological and structural characteristics of pancreatic islet cells, and are able to sustain insulin production for at least 100 days.

In a similar manner, hepatocytes can be obtained after differentiation of hESCs into definitive endoderm (Agarwal et al. 2008). A robust population of functional hepatocytes was generated with the sequential addition of low serum medium, collagen I matrix, and hepatic differentiation factors that include FGF, BMP4, hepatocyte growth factor, oncostatin M, and dexamethasone. These cells expressed known markers of mature hepatic cells, exhibited appropriate function, and were able to integrate and differentiate into mature liver cells when injected into mice with liver injury.

Directing the differentiation of stem cells into mesoderm requires activation of the TGFβ signaling pathway and can be accomplished through the stepwise and dosage-dependent addition of Activin A, BMP4, and growth factors, VEGF and bFGF (Dravid and Crooks 2011). Mesodermal derivatives have also been successfully obtained by spontaneous differentiation of hESCs through hEB formation without first directing them toward mesoderm. Robust differentiation of hESCs into hematopoietic lineage cells, which give rise to all blood cell types and components of the immune system, has been achieved under serum-free conditions through spin hEB formation.

**Table 15.1** Examples of directed differentiation of hESCs into specific cell types

| | Differentiation factors and/or culture conditions | Example of differentiated cells |
|---|---|---|
| Endoderm | FGF, BMP4, hepatocyte growth factor, oncostatin M, dexamethasone | Hepatocytes |
| | Activin A, Wnt3A, keratinocyte growth factor/FGF7, retinoic acid, cyclopamine, noggin | Pancreatic islet progenitors |
| Mesoderm | Serum-free conditions; BMP4 | Dendritic cells |
| | Micromass of dissociated embryoid bodies; BMP2 | Chondrocytes |
| | High density culture of dissociated embryoid bodies; Ascorbic acid, dexamethasone | Chondrocytes |
| | Co-culture with primary chondrocytes; poly-D, L-lactide scaffold | Chondrocytes |
| | Serum-free conditions | Blood cells |
| | Dense monolayer of hESCs; Activin A, BMP4 | Cardiomyocytes |
| | BMP4, BMP4/bFGF/Activin A,VEGF/DKK1, VEGF/DKK1/bFGF | Cardiomyocytes |
| Ectoderm | FGF8, SHH | Dopaminergic neurons |
| | ciliary neutrotrophic factor, neuregulin 1β, dbcAMP | Schwann cells |
| | Retinoic acid, SHH | Motor neurons |
| | Withdrawal of FGF2, BDNF; Addition of GDNF, NGF, dibutyryl cyclic AMP | Peripheral sympathetic and sensory neurons |
| | Serum-free conditions; Activin A, nicotinamide | Retinal pigment epithelium |
| | B27, thyroid hormone, retinoic acid, FGF2, EGF, insulin | Oligodendrocytes |
| | BMP4, ascorbic acid | Basal keratinocytes |

*BMP* bone morphogenetic protein, *DKK1* Dickkopf-related protein, *EGF* epidermal growth factor, *FGF* fibroblast growth factor, *GDNF* glial cell-derived neurotrophic factor, *NGF* nerve growth factor, *SHH* sonic hedgehog, *VEGF* vascular endothelial growth factor

Specific hematopoietic cells, such as functional dendritic cells, have been successfully differentiated from hESCs through spontaneous hEB formation under serum-free conditions with the addition of BMP4 at specific time points. Hematopoietic progenitor cells that give rise to functional T and natural killer cells capable of targeting human tumor cells both *in vitro* and *in vivo* have also been derived from hESCs co-cultured with stromal cells. Thus, the ability to differentiate hESCs into hematopoietic lineage cells promises to be useful in improving existing therapies that require blood cell transplantation, and in immune therapies that require induction of the immune response in an antigen-specific manner (Dravid and Crooks 2011).

Cardiomyocytes, which represent another therapeutically important derivative of mesoderm, have been successfully generated from pluripotent stem cells using several methods (reviewed in Wong and Bernstein 2010). Spontaneous differentiation of stem cells under appropriate culture conditions can produce cardiomyocytes that exhibit morphological, molecular, and electrophysiological properties similar to adult cardiomyocytes, and display quantifiable responses to physiological stimuli reminiscent of atrial, ventricular, and pacemaker/conduction tissue. Cardiomyocytes have also been generated by directed differentiation with Activin A and BMP4 on a dense monolayer of stem cells; these cells successfully form specific cardiac lineages when transplanted *in vivo*. Another study used additional medium supplements that included VEGF, and the Wnt inhibitor, DKK1, followed by the addition of bFGF to promote cardiomyocyte differentiation in culture. Success of these studies was measured by the expression of proteins specific for mature cardiac cells such as cardiac troponin T, atrial myosin light chain 2, and the cardiac transcription factors, Tbx5 and Tbx20.

The dominant differentiation pathway in hESC cultures leads to the formation of ectoderm, which makes up cells of the nervous system and the epidermis (Gaspard and Vanderhaeghen 2010). hESC-derived neural progenitor cells are characterized by rosette-like neural structures that form in the presence of growth factors, FGF2 or EGF, through either spontaneous differentiation from an overgrowth of hESCs or after hEBs are plated onto adherent substrates. These neural rosettes have become the signature of hESC-derived neural progenitors, capable of differentiation into a broad range of neural cells in response to appropriate developmental signals. Thus, many studies are exploring ways to enhance the formation of neural rosettes in order to generate an enriched population of specific neural cell types. One example is the use of specific stromal cell lines. With this method, stromal cells provide ectodermal signaling factors required for neural induction, as determined in animal model studies, and therefore promote the formation of neural rosettes.

The withdrawal of FGF2 and EGF, and the addition of specific compounds can lead to the differentiation of neural rosettes into specific neural subtypes (reviewed in Gaspard and Vanderhaeghen 2010). For example, hESC-derived neural progenitors treated with FGF8 and sonic hedgehog give rise to dopaminergic neurons, while treatment with sonic hedgehog and retinoic acid induce motor neuron differentiation. Neural crest stem cells derived from neural rosettes can differentiate into peripheral sympathetic and sensory neurons by withdrawing FGF2/EGF and adding BDNF, GDNF, NGF and dbcAMP, or into Schwann cells in the presence of CNTF, neuregulin 1β and dbcAMP. Neuroglial cells, such as oligodendrocytes, are generated with B27, thyroid hormone, retinoic acid, FGF2, epidermal growth factor, and insulin.

## Chemical Factors

In some cases, the manipulation of key pathways during germ layer development have allowed for significant enrichment of specific cell types, without the complete recapitulation of embryonic development. Treatment with 5-azacytidine at days 6–8 of hESC differentiation significantly increases cardiac αMHC expression and enhances cardiomyocyte differentiation, suggesting that DNA demethylation is a key factor in directing tissue-specific differentiation (Xu et al. 2002). Similarly, exposure to SB203580, a small molecule inhibitor of $p38^{MAPK}$, has been shown to significantly improve cardiomyocyte differentiation of hESCs grown in medium conditioned by mouse END2 cells, supporting a role for $p38^{MAPK}$ signaling in regulating human cardiomyocyte differentiation (Graichen et al. 2008). SB203580-treated hEBs display an increase in expression of both early mesoderm markers (Brachyury T, Tbx6, Mesp1) and cardiac αMHC, as well as increased cardiomyocyte numbers. Gaur et al. subsequently showed that $p38^{MAPK}$ inhibition occurs in a dose and stage-dependent manner, that it also causes the accelerated differentiation of hESC-derived cardiomyocytes using the standard hEB formation method, and that it appears to act at the ectoderm/mesoendoderm branch point during hESC differentiation (Gaur et al. 2010).

In the original study with SB203580, cells were subjected to an adapted differentiation system in which hESCs were differentiated in a suspension culture using serum-free medium conditioned by the mouse END2 cell line. Medium conditioned by END2 cells exhibits cardiomyocyte-inducing activity during hESC differentiation (Graichen et al. 2008), and biochemical as well as microarray analysis of END2 conditioned medium and END2 cells, respectively, identified prostaglandin I2 (PGI2), a product of prostaglandin synthase enzymes, as an inducing factor in hESC cardiac differentiation (Xu et al. 2008). Two key enzymes involved in PGI2 synthesis are upregulated in END2 cells compared to control MES1 cells (Xu et al. 2008), which lack cardiogenic activity. PGI2 levels are between six- and ten-fold higher in END2 conditioned medium compared to control conditioned medium from MES1 cells. Moreover, insulin, a common supplement in media formulations, was discovered to be an inhibitor of hESC cardiac differentiation. END2 conditioned medium supplemented

with increasing concentrations of insulin results in a dramatic decrease in hESC cardiomyocyte differentiation. Thus, addition of PGI2 in combination with insulin-free, unconditioned medium yields effective cardiac induction similar to that produced by END2 conditioned medium. Cardiac differentiation is further augmented in the presence of SB203580. Taken together, these three components provide a basic, synthetic recipe for directing cardiomyocyte differentiation of hESCs.

## Mechanical Factors

Since cardiac muscle is one of the few tissues that develops under the effects of dynamic force, it is not surprising that conditions generated by the force of fluids in motion can enhance cardiomyocyte differentiation as well. Supplying a constant rotary orbital motion for 7 days to suspension cultures of differentiating mouse EBs results in a significantly increased number of beating mEBs compared to mEBs cultured in static suspension (Sargent et al. 2010). Analysis of gene expression shows higher levels of mesodermal and cardiac proteins (Brachyury, GATA4, Nkx2-5, MEF2c, αMHC, MLC2v) in rotary mEBs than in static mEBs. In addition, a greater proportion of rotary mEBs stain positive for α-sarcomeric actin compared to static EBs. The enhanced cardiomyocyte differentiation is independent of rotary speed ranging from 25 to 55 rpm as determined by the expression of cardiomyogenic genes (Sargent et al. 2010).

Domian et al. have examined the effects of surface tension on cardiomyogenic differentiation of murine cardiac progenitors (Domian et al. 2009). Embryonic- and mESC-derived progenitors are cultured on either fibronectin-coated slides or micropatterns of fibronectin alternating with a surfactant that blocks cell adhesion. When grown on these micropatterned surfaces, a population of cells form longitudinally aligned myocardial fibers. In addition, culturing this population on micropatterned surfaces results in a statistically significant increase in the proportion of cardiomyocytes, supporting a role for microenvironmental forces in cardiac muscle differentiation.

Studies of substrate stiffness and elasticity during stem cell differentiation have also demonstrated effects on skeletal muscle and bone development. Myosin/actin striations only occurred when myoblasts were cultured on gels with stiffness typical of normal muscle, and muscle stem cells cultured on hydrogel substrates that mimic the elasticity of muscle self-renew and fuse to existing myofibers with greater efficiency than cells grown on rigid plastic (Gilbert et al. 2010). Substrate stiffness has also been shown to favor osteogenic differentiation from ESCs.

## Epigenetic Manipulation

MicroRNAs (miRNAs) are small, noncoding RNAs thought to regulate the expression of 30% of protein-coding genes. Their biological importance in stem cell biology is underscored by recent studies demonstrating that mESCs lacking the miRNA processing enzyme Dicer display differentiation and proliferation defects. MiR-1 and miR-133 specifically are expressed in the mouse heart, and targeted deletion or knockdown of these miRNAs results in dysregulation of cardiac morphogenesis, electrical conduction, cell-cycle, and cardiac hypertrophy. Recently, Ivey et al. showed that miR-1 and miR-133 regulate the differentiation of mESCs and hESCs into the cardiac lineage (Ivey et al. 2008). Both miRNAs are enriched in mESC-derived CMs. Lentiviral introduction of either miR-1 or miR-133 into mESCs enhances early mesoderm differentiation as evidenced by increased expression of Brachyury. miR-1 and miR-133 also reinforce mesoderm lineage decisions by repressing endoderm and neuroectoderm differentiation. When stimulated to differentiate into either endoderm or neuroectoderm lineages, mEBs expressing either miR-1 or miR-133 express lower levels of endodermal and neural markers compared to control mEBs. However, further differentiation revealed opposing roles of miR-1 and miR-133. miR-1 promotes differentiation of mesoderm into the cardiac and

skeletal muscle lineages as determined by enhanced Nkx2-5 and myogenin expression, respectively, whereas miR-133 blocks induction of both markers. Importantly, the differentiation of hESCs in the presence of miR-1 behaves comparably to that of mESC differentiation. Overexpression of miR-1 in hESCs increases Nkx2-5 expression and yields more than a three-fold higher number of beating hEBs compared to wild type controls.

While miRNAs direct cell lineage determination by controlling protein dosage, epigenetic regulation through chromatin remodeling has been shown to control cell fate as well. Takeuchi et al. have identified a minimal set of factors necessary to execute the cardiac transcriptional program (Takeuchi and Bruneau 2009). Baf60c, a cardiac-enriched subunit of the Swi/Snf-like BAF chromatin remodeling complex, in combination with cardiac transcription factors GATA4 and Tbx5, is able to induce cardiac differentiation in mouse embryos when ectopically expressed. With this combination, 90% of the transfected embryos display expression of the early cardiac marker, Actc1, and 50% of the transfected embryos exhibit beating tissue. GATA4 together with Baf60c is essential in initiating the cardiac gene program as assessed by expression of Actc1. None of the other transcription factors tested alone (Tbx5, Nkx2-5) or in concert with Baf60c are able to induce Actc1 expression. GATA4/Baf60c, however, is not sufficient for generating beating embryos: Tbx5 is required to achieve contracting CMs.

## Selection Strategies

As discussed earlier in this chapter, enrichment of specific cell types can be achieved using molecules introduced at specific time points during culture. However, many of these methods yield only moderate enrichment that is not yet scalable for clinical application. In addition, it may be desirable to enrich first for partially differentiated, proliferative stem cell intermediates with specific fates. These could then be expanded before further differentiation into cells for therapy. In the following sections, we will discuss the variety of methods utilized to select tissue-specific precursors and their derivatives.

### Cell Surface Markers

Even before the tools of genetic engineering were employed to manipulate cells, proteins expressed on the cell surface were used to identify distinct populations of stem cells and their ontogeny. Perhaps the best known application of this approach is to the intermediates of hematopoiesis (Weissman and Shizuru 2008). Over the past 10 years, similar attempts to create lineage maps of other tissues have focused on the identification of lineage-specific cell surface markers (Table 15.2). For example, pluripotent stem cells can be identified by the expression of Stage-specific embryonic antigens (SSEA)-3 and 4, and the embryonal carcinoma marker, Tra-1-60 on their surface (King et al. 2009). The expression of the cell surface antigen, CD133, on proliferating hESCs identifies cells predestined toward a neuroectodermal fate (King et al. 2009). Hemangioblasts that ultimately give rise to hematopoietic stem cells, smooth muscle progenitors, and endothelial progenitors can be selected based on the surface expression of CD143, E-cadherin, VE-cadherin, platelet endothelial cell adhesion molecule-1 (PECAM-1), and vascular endothelial growth factor receptor-2 (VEGFR2) (Ogawa et al. 2001). Mesenchymal stem/stromal cells that differentiate into muscle, fat, cartilage, and bone cells can be identified by the expression of the cell surface proteins, CD29, CD44, CD51, CD73, Thy-1, and mast/stem cell growth factor receptor (SCFR) (Keating 2006). Recently, cardiomyocytes that have until now eluded detection by specific surface protein expression have been shown to express signal-regulatory protein $\alpha$ (SIRPA) (Dubois et al. 2011).

Two main approaches have been used to identify lineage-specific surface markers. One employs labeled cell fate mapping to assess the tissue-specific fate of subpopulations of pluripotent

**Table 15.2** Examples of surface markers expressed on stem, progenitor, and differentiated cells

| | Stem cell | Progenitor | Differentiated cell |
|---|---|---|---|
| Pluripotent Stem Cell | **SSEA-3/4, TRA-1-60** | | |
| Ectoderm | Neural stem cell<br>**CD133, ABCG2** | Neuron-restricted progenitor<br>**NCAM** | Neuronal cell<br>**PSD-95, Synaptophysin** |
| | | Glial-restricted progenitor | Oligodendrocyte<br>**MOG, CD140a** |
| | | **FGFR** | *Type 2 Astrocyte* |
| | | | Type 1 Astrocyte<br>**FGFR3** |
| | | *Motor neuron progenitor* | *Motor neuron* |
| | *Surface ectoderm* | Epidermal stem cell<br>**α6-integrin$^{high}$, CD71$^{low}$** | Epithelial cell<br>**CEACAM-1, EpCAM** |
| Mesoderm | Hemangioblast<br>**CD143, E-cadherin, VE-cadherin, PECAM-1, VEGFR2** | Hematopoietic stem cell<br>**CD34, CD44, CD150, VEGFR2, Sca-1** | Myeloid/lymphoid lineages |
| | | Smooth muscle progenitor<br>**VE-cadherin** | Smooth muscle cell<br>**VE-cadherin** |
| | | Endothelial progenitor<br>**CD31, CD105, CD144, CD146, VEGFR2, VWF** | Endothelial cell<br>**CD31** |
| | Mesenchymal stem cell<br>**CD29, CD44, CD51, CD73, Thy-1, SCFR** | Myogenic precursor<br>**α7-integrin, M-cadherin** | Cardiac muscle<br>**SIRPA** |
| | | | Skeletal muscle<br>**α7β1-integrin** |
| | | *Pre-adipocyte* | Adipocyte<br>**Glut4** |
| | | *Chondrocyte precursor* | Chondrocyte<br>**CD44** |
| | | *Osteoblast* | *Osteocyte* |
| Endoderm | *Primitive endoderm* | | |
| | *Definitive endoderm* | Hepatic Progenitor<br>**c-Met, CD49f** | Hepatocyte<br>**Il-6** |
| | | *Pancreatic progenitor* | *α cell* |
| | | | β cell<br>**Glut2** |
| | | | *PPγ cell* |
| | | | *δ cell* |
| | | | *ε cell* |

*ABCG2* ATP-binding cassette sub-family G member 2, *CEACAM* carcinoembryonic antigen-related cell adhesion molecule, *EpCAM* epithelial cell adhesion molecule, *FGFR* fibroblast growth factor receptor, *Glut* glucose transporter, *MOG* myelin oligodendrocyte glycoprotein, *NCAM* neural Cell Adhesion Molecule, *PECAM* platelet endothelial cell adhesion molecule, *PSD* postsynaptic density protein, *SCFR* mast/stem cell growth factor receptor, *SIRPA* signal-regulatory protein alpha, *SSEA* stage-specific embryonic antigen, *VEGFR* vascular endothelial growth factor receptor, *VWF* Von Willebrand factor

stem cells expressing specific surface proteins (King et al. 2009), while the other uses a high-throughput flow cytometry screen of available antibodies against stem cell derivatives (Dubois et al. 2011). Although there are still large gaps in our ability to identify and select derivatives of all three germ layers based on surface marker expression, progress continues to be made.

## Biochemical Characteristics

Although detection of lineage-specific genes underlies most methods for selecting specific cell types from differentiating stem cell cultures, a few cell types, such as cardiac muscle cells, bear unique biochemical properties that aid in their isolation. For example, Percoll gradient centrifugation has been described to purify hESC-derived cardiomyocytes (Yeghiazarians et al. 2012), based on their buoyancy characteristics. Differentiating hESCs are applied to a discontinuous Percoll gradient consisting of 40.5% Percoll layered over 58.5% Percoll. Following centrifugation, the majority of cardiomyocytes reside within the 58.5% Percoll layer and express cardiac troponin I, sarcomeric myosin heavy chain (MHC), αMHC, βMHC, and N-cadherin. hESC-derived cardiomyocytes of ≤70% purity have been obtained using this approach.

A second purification strategy is based on the observation that cardiomyocytes have high mitochondrial content compared to that of non-myocytes (Hattori et al. 2010). Using the fluorescent dye, tetramethylrhodamine methyl ester (TMRM), which freely diffuses into the mitochondrial matrix, Hattori *et al.* found that TMRM fluorescence in embryonic rat hearts increases with developmental stage suggesting that mitochondrial biogenesis is linked to myocardiogenesis. In whole rat embryos, TMRM fluorescence in the heart is more robust than in other tissues, and when analyzed by flow cytometry, flow-sorted populations with the highest TMRM fluorescence are observed to express cardiac α-actinin. TMRM-labeled cardiomyocytes derived from mESCs are positive for both Nkx2-5 and α-actinin. The cardiomyocyte content in cultured cells sorted from day 12 to day 25 mEBs is >99% as determined by Nkx2-5 and α-actinin expression. Most notably, >99% cardiomyocyte purity is also obtained in cultured cells sorted from differentiating hEBs.

## Transgenic Reporter Cell Lines

The use of fluorescent reporters of gene expression offers an approach to selecting tissue-specific cells by high throughput, fluorescence-activated cell sorting. Alternatively, the use of reporters that activate expression of a selectable marker can drive a population of cells to near-homogeneity by restricting survival to a specific cell type. The paucity of specific cell surface markers with which to select cardiac cells until recently (Dubois et al. 2011) has led to the development of a variety of reporter lines with which to select stem cell-derived cardiomyocytes and cardiac progenitors. Huber et al. used lentiviral vectors to produce stable hESC lines in which enhanced GFP is expressed under control of the cardiac-specific human ventricular myosin light chain-2 promoter (Huber et al. 2007). Kita-Matsuo et al. designed a set of lentiviral vectors to generate multiple stable hESC lines with eGFP and mCherry reporters or with puromycin resistance downstream of the mouse αMHC promoter (Kita-Matsuo et al. 2009). Most recently, Ritner et al. generated a cardiac-specific hESC reporter line using a lentiviral construct consisting of a fragment of the mouse αMHC promoter upstream of eGFP. The specific promoter fragment allowed for the identification and analysis of early cardiac progenitors expressing Nkx2-5, but before the onset of cTnT or chamber-specific MLC expression (Ritner et al. 2011).

Whereas isolation of hESC-derived cardiomyocytes from these reporter lines are based on positive selection, Anderson *et al.* implemented a negative selection strategy to deplete undifferentiated, proliferating hESCs from cultures of hESC-derived cardiomyocytes (Anderson et al. 2007). Their hESC line utilized a Herpes thymidine kinase (HTK)/gancyclovir (GCV) suicide gene system under the control of a constitutive phosphoglycerate kinase promoter. Following administration of the antiviral drug GCV, cells expressing HTK phosphorylated GCV, which then incorporated into nascent DNA chains of proliferating cells causing chain termination and cell death. The increased number of α-actinin positive cells after GCV treatment lead to an almost sevenfold enrichment of cardiomyocytes. An important caveat of this approach, however, is that other non-proliferating cell types would remain in the culture while proliferating cardiomyocytes would be depleted. The culture would

still need to undergo a cardiac purification step, and the excluded proliferating cardiomyocytes and cardiac progenitors may be of greater benefit for transplantation than fully differentiated, nonproliferating cells (Wong and Bernstein 2010).

Reporter lines have similarly been derived for the isolation of other tissue-specific cells of biological and clinical interest, including motor neurons, germ cells, hepatocytes, and pancreatic progenitors. However, the effects of random genomic integration on disrupting endogenous gene expression, as well as unregulated transgene expression, are potential limitations to this approach. Reporter lines must be rigorously evaluated to confirm authentic representation of gene expression without altering cell behavior (Ritner et al. 2011).

## Homologous Recombination

To address the limitations of virally-mediated introduction of reporter transgenes (e.g., constitutive transgene expression, effects of random integration), many investigators have approached reporter development using homologous recombination to 'knockin' a fluorescent protein into a specific genetic locus (Leavitt and Hamlett 2011). Initially, gene targeting of ESCs by homologous recombination was slowed by the difficulties of single-cell cloning, transfection efficiency using conventional methods, and karyotypic instability with dissociation of ESCs. However, these obstacles have been overcome to some degree, with the result of several successfully targeted ESC lines.

The hESC reporter lines currently in use represent a variety of targeting strategies (reviewed in Leavitt and Hamlett 2011). An H1.1-derived OCT4-eGFP line targeting exons 3-5 of the octamer-binding transcription factor 4 gene by positive selection alone has been used to sort pluripotent hESCs. Similarly, a MIXL1-GFP line targeting exon 1 of the Mix1 homeobox-like 1 gene in HES3 cells by positive selection has been used to identify blood precursors. Another group used the FEZ family zinc finger 2 promoter to direct eYFP expression in HUES9 cells as a means to isolating neuronal precursors using cre-mediated recombination with both positive and negative selection steps, while a similar approach using the oligodendrocyte lineage transcription factor 2 promoter driving eGFP expression in BG01 hESCs identified a subset of glial-restricted progenitors with oligodendrocyte fate.

Cre-mediated homologous recombination has also been used to generate two cardiac reporter lines, one using the second heart field-specific Islet-1 promoter directing dsRed expression in H9 hESCs (Bu et al. 2009) and another recently described pair of hESC lines in which the promoter of the early cardiac transcription factor, NKX2-5, was used to drive eGFP expression in MEL1 (male) and HES3 (female) cells (Elliott et al. 2011). While these accomplishments demonstrate that the various unrelated ESC lines currently available are all amenable to homologous recombination, optimal targeting vector designs and selection strategies are still being explored.

## Molecular Beacons

Although homologous recombination technology avoids some of the concerns with transgenic reporter cell lines generated by random integration, the resulting cells have still undergone genomic modification. As an alternative to genetically modified stem cell lines for tracking and isolating tissue-specific progenitors, King et al. adapted dual-fluorescence resonance energy transfer (FRET) "molecular beacon" technology for transient, real-time detection of gene expression during ESC differentiation (King et al. 2011). Molecular beacons are single-stranded oligonucleotide probes that have been employed to assay gene expression *in vitro*, as in real-time polymerase chain reaction assays, and *in vivo* using microscopy. These consist of short sequences capable of forming stem-loop structures bearing a fluorescent reporter group at one end and a fluorescent quencher at the opposite end. In the absence of a target sequence, the oligonucleotide self-anneals, forming a stem that brings the reporter and quencher in close proximity, thereby quenching fluorescence (Fig. 15.2).

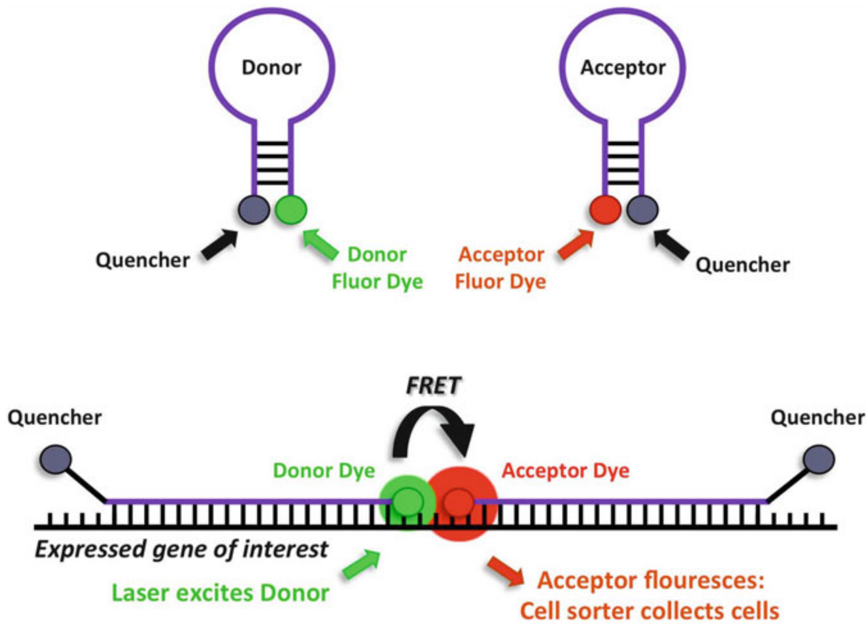


**Fig. 15.2 Dual-FRET molecular beacon system.** In solution, molecular beacons assume a stem-loop structure that juxtapose the fluorescent dye and quencher, inhibiting fluorescence (*top*). With transcription of a gene of interest, molecular beacons preferentially undergo sequence-specific base pairing with the gene transcript (*bottom*). This removes the fluorescent dyes from quencher proximity, and allows for laser excitation of the donor dye, fluorescence-resonance energy transfer (*FRET*) that excites the acceptor dye, and detection/collection of cells based on acceptor dye emission

In the presence of a target sequence, the oligonucleotide anneals to the target, separating the reporter and quencher, thereby allowing fluorescence. These investigators showed that appropriately designed, dual-FRET molecular beacon pairs can identify the expression of specific mRNAs by microscopy and flow cytometry, and facilitate the collection of specific stem cell populations by cell sorting, while leaving the stem cell genome intact (King et al. 2011).

## Spectral Flow Cytometry

Conventional flow cytometers and cell sorters use a series of dichroic mirrors and filters matched to the fluorescence emission profile of reporter fluorochromes to identify and quantify specific biomarkers using photomultiplier tubes to detect each parameter. Historically, this technology has relied on dichroic mirrors that reflect light of specific wavelengths and bandpass filters that allow incident light within a specific wavelength range to get to the detector. The detector, or photomultiplier tube (PMT), detects photons and turns them into electrons or current. The fluorescent photon hits a photocathode and turns into an electron on a one-to-one basis; the electron then passes through a series of dynodes that amplify the electrons based on the voltage generated across the dynode. One electron is multiplied and becomes two or more electrons, creating a wave of electrons based on the single initial photon. At the end of the dynode chain, an anode collects the electron wave into an electronic signal; the more photons delivered to the PMT, the bigger the electronic signal.

A key criterion for distinguishing specific biomarkers over background is the optical signal-to-noise ratio of the detector. For cytometric analysis of ESCs and iPSCs in altered differentiation states and treatment conditions, variable cellular

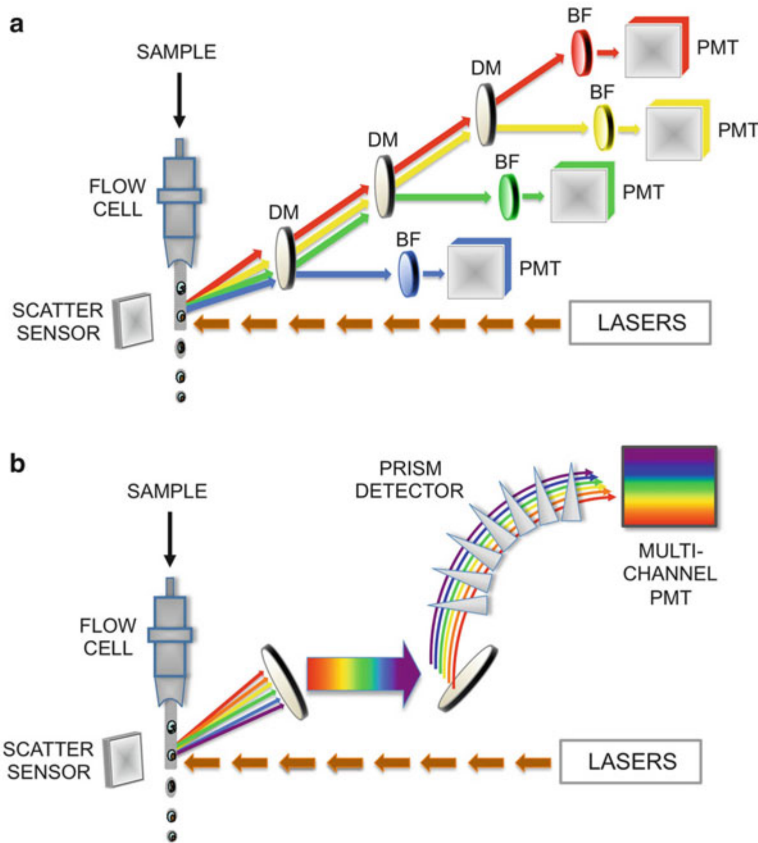


**Fig. 15.3 Spectral flow cytometry.** (**a**) With a conventional flow cytometer, lasers excite cell-associated fluorochromes, and emitted light is filtered by a combination of dichroic mirrors (*DM*) and bandpass filters (*BF*) that reflect and filter light of specific wavelengths, respectively. Light within narrow selected wavelength ranges arrive at a photomultiplier tube (*PMT*), which converts light as photons to an electronic signal. (**b**) In spectral flow cytometry, laser diodes similarly provide initial excitation of reporter fluorochromes, however, emitted fluorescence passes through a prism array into a spectral PMT. Component fluorescence including autofluorescence is linearly unmixed using spectral lookup tables. In contrast to conventional cytometry, almost all light signals are analyzed, and signal-to-noise resolution is dramatically improved

autofluorescence and fluorescence crossover between multiple fluorochromes contribute to the background or "noise." Increased noise decreases the resolving power for dim signals. A new cytometer developed by Sony Corporation replaces filter-based optics with a spectral detection system based on a multi-anode spectral PMT. In the spectral cytometer, the light is directed based on wavelength into discrete channels corresponding to different regions of the color spectrum using a series of prisms. Over 30 channels are distributed across the 500–800 nm color spectrum, which allows almost all photons to be processed, whereas ≥20 % of photons may be lost in the dichroic mirror/bandpass filter system.

The spectral cytometer is able to measure and subtract varying autofluorescence, permitting increased signal-to-noise ratios and improving the resolution of dim signals (Fig. 15.3). Multiple fluorochromes are mathematically unmixed using component analysis. Fluorochromes with overlapping emissions like fluorescein isothiocyante and Alexa 532 can now be used simultaneously where their spectral overlap would prevent their

use in a conventional filter-based cytometer. Unique spectral "fingerprints" can be created to distinguish tissue precursors transfected with combinations of molecular beacons described above, without the need for genome integration of reporter constructs or surface antibody staining that might cause unintended pleiotropic cellular responses. This technology holds great promise for its ability to exploit functional biosensors such as dual-FRET molecular beacons (King et al. 2011) to identify specific stem cell differentiation states of therapeutic value.

## Conclusion

Research on pluripotent stem cells has progressed significantly since the first derivation of hESCs from discarded blastocysts in 1998 (Thomson et al. 1998), and the subsequent discovery that somatic cells can be reprogrammed to a pluripotent stem-like state in 2006 (Takahashi and Yamanaka 2006). The international scientific community has discovered the enormous potential of pluripotent stem cells as newly-derived lines continue to be developed and differentiation methods into various types of cells are optimized. The ability to select and isolate stem cell-derived precursors and differentiated cells with tissue-specific properties holds the key to fully exploiting these cell lines and differentiation methods for scientific investigation and clinical use.

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# Differentiation of Pluripotent Stem Cells into Steroidogenic Cells: Role of SF-1 Regulator

16

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**Abstract**

Replacement of deficient steroid hormones by hormone replacement therapy is necessary for patients with adrenal and gonadal failure. However, patients undergoing these therapies have associated risks during their entire life. Stem cells represent an innovative tool for tissue regeneration and gene therapy, and the possibility of solving these problems. Among various stem cell types, mesenchymal stem cells can be differentiated into steroidogenic cells by stable expression of steroidogenic factor-1 (SF-1) and cAMP treatment. The same approach cannot be applied to other undifferentiated cells such as embryonic stem (ES) cells because of poor survival following expression of SF-1. However, ES cells can also be differentiated into steroidogenic cells with SF-1 *via* the mesenchymal cell lineage. These approaches will provide a source of cells for future regenerative medicine and gene therapy of diseases caused by steroidogenesis deficiencies.

## Introduction

In mammals, steroid hormones are produced from cholesterol mainly in adrenal glands and gonads. Steroidogenesis begins with conversion of cholesterol into pregnenolone in mitochondria by the P450 side chain cleavage enzyme (P450scc/CYP11A1/Cyp11a1), a rate-limiting enzyme for synthesis of all steroid hormones

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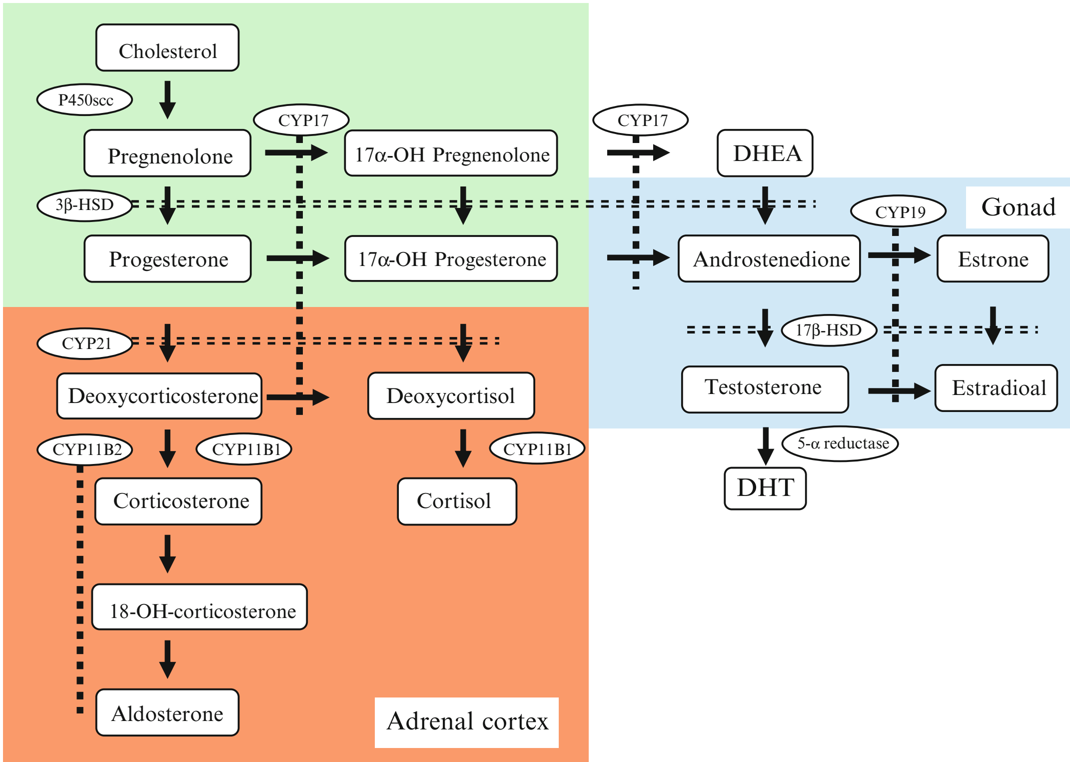


**Fig. 16.1** Steroidogenic pathway in adrenal cortex and gonads

(Fig. 16.1). Thereafter, various hormones are synthesized by tissue-specific P450 hydroxylases and hydroxysteroid dehydrogenases (Miller and Auchus 1988). Steroid hormones are essential for glucose metabolism, the stress response, fluid and electrolyte balance, sex differentiation and reproduction *via* binding to cognate receptors in target tissues. Therefore, steroidogenic abnormality may be life threatening. Congenital adrenal hyperplasia (CAH) is one of the most common disorders caused by deficiency of any enzyme involved in steroidogenesis in adrenal (Claahsen-van der Grinten et al. 2011). Impaired cortisol and aldosterone production increases adrenocorticotropic hormone (ACTH) secretion from the pituitary gland and leads to adrenal hyperplasia and accumulation of adrenal androgens. Female patients are prenatally virilized due to excess androgen, and neonates of both sexes suffer from life-threatening Addisonian crisis. Hormone replacement therapy has been well established for the treatment of such patients, although they require hormone replacement for their entire lifetime. In addition, these patients continue to suffer various side effects and risks from replacement therapy over a long term. It is also very hard to cure patients with overproduction of steroid hormones by endocrine tumors. Stem cells represent an innovative tool for tissue regeneration and gene therapy, and the possibility of solving these problems. In this chapter, we first describe the roles of steroidogenic factor-1 (SF-1) in the development of steroidogenic organs and steroidogenesis. We also provide an overview of recent progress in differentiation and regeneration of steroidogenic cells using stem cells, including our own studies using pluripotent embryonic stem (ES) cells.

## SF-1 and Its Roles in the Development of Steroidogenic Organs and Steroidogenesis

In vertebrates, steroid hormones are primary synthesized in the adrenal cortex (inter-renal tissues in fish and amphibia) and gonadal somatic cells. Although they produce various steroid hormones in adult life, both organs have a common developmental origin, a so-called adrenogonadal primordium (AGP) that mainly originates from intermediate mesoderm and localized on the coelomic epithelia of the developing urogenital ridge (Hatano et al. 1996). As development proceeds, AGP separates into two distinct populations, adrenocortical and gonadal primordia that are characterized by the existence of chromaffin cell precursors and primordial germ cells, respectively, which originate and migrate from other germ layers. During differentiation, adrenal glands and gonads synthesize tissue-specific steroid hormones by specific expression patterns of steroidogenic genes.

SF-1 (also known as Ad4BP) is one of the earliest makers of the appearance of AGP (Hatano et al. 1996). Because SF-1-knockout mice fail to develop gonads and adrenal, SF-1 represents a master regulator for the development of these organs. SF-1 is also essential for steroidogenesis by regulating the transcription of steroidogenesis-related genes. SF-1/Ad4BP was originally discovered by Keith Parker and Ken Morohashi as a transcription factor that binds to the Ad4 sequence (a variation of the AGGTCA motif) in promoter regions of cytochrome P450 steroid hydroxylase genes for transactivation (Lala et al. 1992; Morohashi et al. 1992). They concluded from selective expression of SF-1 in steroidogenic cells and its regulation of all steroid hydroxylase genes, that SF-1 is a determinant factor in cell-specific expression of steroidogenic enzymes. In addition to steroidogenic enzymes, diverse groups of SF-1 target genes, such as other steroidogenic genes, pituitary hormones and cognate receptors and sex differentiation-related genes, have been reported thus far (Schimmer and White 2010). SF-1 belongs to the nuclear hormone receptor superfamily and contains a characteristic zinc finger DNA-binding domain, an intervening hinge region and a carboxyl-terminal putative ligand-binding domain (Fig. 16.2a). It is very similar to liver receptor homolog-1 (LRH-1) and fushi tarazu factor 1 (FTZ-F1). LRH-1 was originally identified in the liver and is known to function in cholesterol and bile acid homeostasis (Fayerd et al. 2004). It is also expressed in gonads and involved in steroidogenesis, as will be discussed later. FTZ-F1 is a Drosophila orphan nuclear receptor, which interacts with homeodomain protein FTZ to define alternate parasegmental regions in embryos. These factors constitute one of the nuclear receptor subfamilies and are designated as NR5A proteins (SF-1 is NR5A1, LRH-1 is NR5A2, and FTZ-F1 is NR5A3).

Consistent with its role in steroidogenesis, SF-1 expression is detected in adults in three layers of the adrenal cortex (zona reticularis, zona fasciculata and zona glomerulosa), testicular Leydig and Sertoli cells, ovarian theca, granulosa cells and to a lesser extent in the corpus lutea (Ikeda et al. 1993). In the corpus lutea, LRH-1, rather than SF-1, is highly expressed and important for progesterone production (Duggavathi et al. 2008; Yazawa et al. 2010). SF-1 is also expressed in pituitary gonadotrophs and the ventral medial hypothalamus, and its roles in these organs are discussed in other reviews (Parker and Schimmer 1997; Schimmer and White 2010). SF-1-knockout mice die shortly after birth due to adrenal insufficiency and exhibit male-to-female sex reversal in external genitalia (Luo et al. 1994). These phenotypes are caused by the complete loss of adrenal and gonads. Although the initial stages of adrenal and gonadal development occur in the absence of SF-1, they regress and disappear during the following developmental stage. Because gonads disappear prior to male sexual differentiation, the internal and external urogenital tracts of SF-1-knockout mice are female types, irrespective of genetic sex. Heterozygous SF-1-knockout mice show decreased adrenal volume associated with impaired corticosterone production in response to stress (Bland et al. 2004), whereas transgenic overexpression of SF-1 increases adrenal size and ectopic adrenal tissue in the thorax

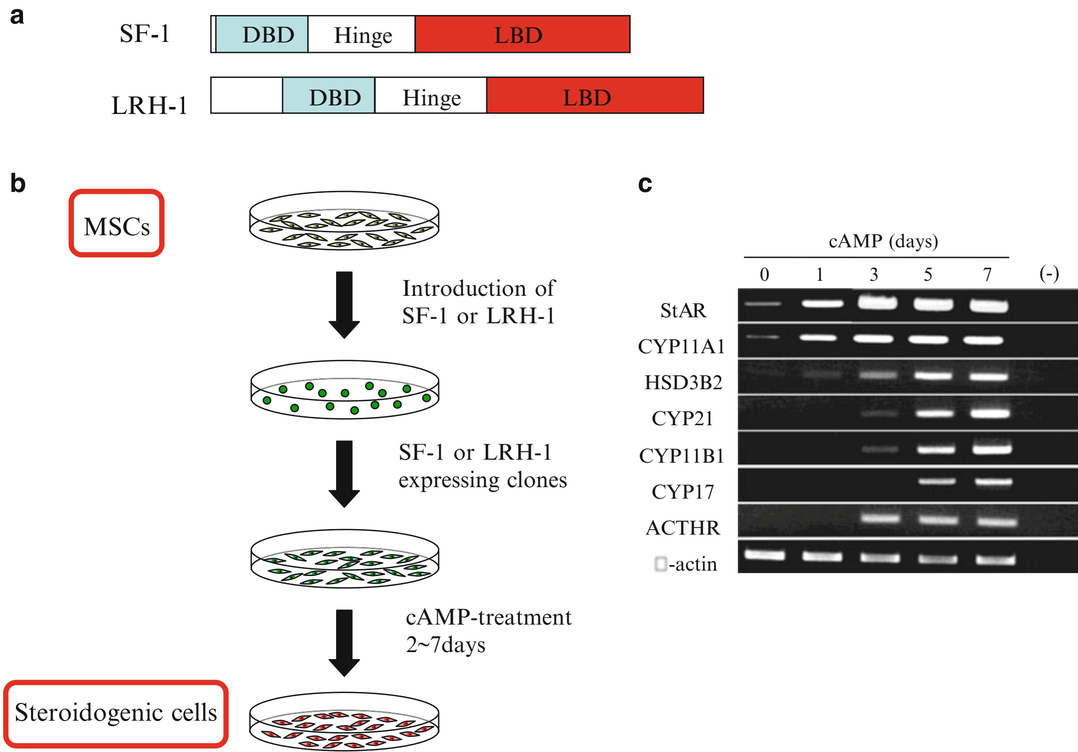


**Fig. 16.2** (**a**) Schematic structure of SF-1 and LRH-1 proteins. (**b**) Protocols for inducing steroidogenic cells from MSCs. (**c**) Time-dependent induction of steroidogenic genes by cAMP in SF-1-expressing human MSCs (RT-PCR)

(Zubair et al. 2009). Total SF-1 disruption in mice demonstrated that SF-1 is crucial for the determination of steroidogenic cell fate *in vivo*. It has also been shown in Leydig cell- and granulosa cell-specific knockout (LCKO and GCKO, respectively) models that SF-1 plays important roles in steroidogenesis following organogenesis (Jeyasuria et al. 2006). In LCKO mice, testicular StAR and Cyp11a1 expression are impaired, indicating a defect in androgen biosynthesis. In support of this inference, the testes fail to descend (an androgen-dependent developmental process) and are structurally abnormal and hypoplastic. In GCKO mice, ovaries are hypoplastic, adults are sterile and ovaries show reduced numbers of oocytes and lack corpora lutea. Gonadotropin-induced steroid hormone production and ovarian Cyp19a1 expression are also markedly reduced in this model (Pelusi et al. 2008).

## Differentiation of Steroidogenic Cells from Mesenchymal Stem Cells

In an early study (Crawford et al. 1997), ectopic expression of SF-1 was shown to direct differentiation of ES cells toward the steroidogenic lineage, and then Cyp11a1 mRNA was expressed after the addition of cAMP and retinoic acid (RA). However, the steroidogenic capacity of these cells is limited and they do not undergo *de novo* synthesis because a membrane-permeable substrate, 20α-hydroxycholesterol, is necessary to produce progesterone, which is the only steroid produced by these cells. In addition, major differences between these differentiated cells and natural steroidogenic cells were shown in cholesterol delivery and the steroidogenic pathway, including deficiencies of steroidogenic acute regulatory protein (StAR; cholesterol delivery

protein from the outer to inner mitochondrial membrane in steroidogenic cells) and steroidogenic enzymes except for Cyp11a1 (Crawford et al. 1997; Yazawa et al. 2006). It is also very difficult to isolate clones expressing SF-1 from ES cells (Yazawa et al. 2006, 2011), because SF-1 overexpression affects the survival and undifferentiated state of ES cells, as will be discussed below. These studies clearly indicate that SF-1 initiates the fate-determination program of the steroidogenic lineage in stem cells, although it is not completed in ES cells.

Based on the above studies, several groups focused on mesenchymal stem cells (MSCs) that have a mesodermal origin, the same as steroidogenic cells (Yazawa et al. 2006; Yanase et al. 2006). MSCs are multipotent adult stem cells and have been shown to differentiate into adipocytes, chondrocytes, osteoblasts and hematopoietic-supporting stroma both *in vivo* and *ex vivo*. Furthermore, MSCs are able to generate cells of all three germ layers. Although MSCs were originally isolated from bone marrow (BM-MSCs), they have also been derived from fat, placenta, umbilical cord blood and other tissues. In addition to their multipotency, MSCs have attracted considerable interest for use in cell and gene therapies because MSCs can be obtained from adult tissues. Indeed, their therapeutic applicability has been assessed in some cases, and particularly for bone tissue engineering.

To investigate the potential of MSCs to differentiate into steroidogenic cells, BM-MSCs from GFP-transgenic rats were transplanted into prepubertal testes. In the prepubertal testis, adult-type Leydig cells, which originate from mesenchymal precursor cells that are present in the testicular interstitium, appear to induce puberty (Dong et al. 2007). After 3 weeks, transplanted GFP-positive cells were located in the interstitium and expressed various steroidogenic enzymes for androgen production (P450scc/Cyp11a1, 3β-HSD I and Cyp17). These results indicate that MSCs have a capacity to differentiate into steroidogenic Leydig cells *in vivo*.

In addition, purified murine BM-MSC lines spontaneously differentiate into steroidogenic cells *in vitro* (Yazawa et al. 2006). A human CYP11A1 promoter-driven GFP reporter, which consisted of a 2.3 kb fragment that drives reporter gene expression selectively in adrenal and gonadal steroidogenic cells, has been transfected into BM-MSCs to detect a cell population committed to the steroidogenic lineage. In some transfected cell lines, GFP fluorescence was detected in very small populations that were also positive for Cyp11a1. Further analysis showed that these cells express several Leydig cell markers including 3β-HSD types I and VI, and LH receptor. These observations further support the *in vivo* findings that MSCs have the capacity to differentiate into steroidogenic cells. Interestingly, SF-1 expression was also detected in GFP-positive cells. Therefore, it is strongly indicated that SF-1 can effectively direct differentiation of MSCs into the steroidogenic lineage.

Indeed, MSCs have been completely differentiated into steroidogenic cells and show their phenotypes after stable expression of SF-1 (using plasmids or retroviruses) and cAMP-treatment (Fig. 16.2b, Yazawa et al. 2006, 2009, 2010, 2011). SF-1 solely induces morphological changes in MSCs, such as the accumulation of numerous lipid droplets, although these cells hardly express steroidogenic enzyme genes or produce steroid hormones at detectable levels. However, SF-1-expressing cells strongly become positive for CYP11A1/Cyp11a1 after cAMP treatment. These cells express many other steroidogenesis-related genes (SR-BI, StAR, 3β-HSD and other P450 steroid hydroxylases) and autonomously produce steroid hormones including androgen, estrogen, progestin, glucocorticoid and aldosterone. Notably, this approach differentiates human BM-MSCs into high cortisol-producing cells in response to ACTH, which are very similar to fasciculata cells in the adrenal cortex (Fig. 16.2c). Adenovirus-mediated transient expression of SF-1 also differentiates MSCs into steroidogenic cells with the capacity for *de novo* synthesis of various steroid hormones (Yanase et al. 2006). In addition to BM-MSCs, various MSC types have been differentiated into steroidogenic cells by SF-1 and cAMP, although their steroidogenic properties are markedly varying and dependent on the derivation tissues and species (Yazawa et al. 2006, 2010; Gondo et al. 2008).

However, as mentioned above, these methods are not applicable to ES cells and terminally differentiated cells such as fibroblasts and adipocytes (Yazawa et al. 2006, 2011; Yanase et al. 2006). In ES cells, this is probably caused by the function of NR5A proteins (endogenously, LRH-1). SF-1 and LRH-1 regulate the expression of similar genes by binding to the same response sequences. This phenomenon also occurs in steroidogenic gene promoters and therefore, LRH-1 induces the differentiation of MSCs into steroidogenic cells, as is the case with SF-1 (Yazawa et al. 2009). In ES cells, LRH-1 is abundantly expressed and necessary for Oct-3/4 expression (an essential gene for maintenance of the inner cell mass and pluripotency of ES cells) at the epiblast stage (Mullen et al. 2007). In addition, Oct-3/4 can be replaced by LRH-1 for the reprogramming of murine somatic cells into induced pluripotent stem (iPS) cells (Heng et al. 2010). As the potential to direct differentiation of MSCs into steroidogenic cells, SF-1 and LRH-1 play similar roles in the regulation of Oct-3/4 expression (Mullen et al. 2007), and therefore the same potential for induction of somatic cells into iPS cells (Heng et al. 2010). Niwa et al. (2000) demonstrated that differential expression of Oct-3/4 defines the fate of ES cells. A less than twofold increase in Oct-3/4 expression causes differentiation of ES cells into primitive endoderm and mesoderm, whereas repression of Oct-3/4 expression induces loss of pluripotency and causes dedifferentiation of cells into the trophectoderm. It has been shown that DAX-1, a common transcriptional inhibitor of Oct-3/4, SF-1 and LRH-1 are also important for the pluripotency and survival of ES cells (Lalli and Alonso 2010). DAX-1 expression is detected in ES cells and its expression is reduced upon differentiation of the cells into each germ layer. DAX-1-knockdown induces a loss of pluripotency even under culture conditions for maintaining the undifferentiated state, whereas complete deletion of DAX-1 by gene targeting results in cell death (Lalli and Alonso 2010). Over-expression of the NR5A family is also cytotoxic to ES cells. These facts strongly suggest that regulated and coordinated expression of NR5A genes is essential for the pluripotency and survival of ES cells. These properties of the NR5A family are likely to cause difficulties in the differentiation of steroidogenic cells by NR5A members directly from ES cells.

## Differentiation of Steroidogenic Cells from Embryonic Stem Cells

The previous section describes studies strongly indicating that MSCs are suitable stem cells for the differentiation of steroidogenic cells. MSC-derived steroidogenic cells may improve symptoms after transplantation into animal models of steroid hormone deficiencies. However, adult stem cells from patients with congenital genetic deficiencies are not useful because autologous cells will contain the same genetic defects. The limited proliferative potential of adult stem cells is also a major issue for the use of adult stem cells in regenerative therapy. Pluripotent stem cells, such as ES and iPS cells, are able to self-renew, while maintaining the ability to differentiate into all cell types of the entire body. Thus, these properties offer the possibility to produce steroidogenic cells for regenerative and gene therapies by correction of defective alleles by gene-specific targeting. However, clinical approaches using MSCs are not applicable to pluripotent stem cells.

To circumvent these problems, the murine ES cell line EBRTcH3, carrying a tetracycline (Tc)-repressible transgene at the *ROSA26* locus (Masui et al. 2005), has been used for regulated expression of SF-1 and induction of differentiation. SF-1 cDNA, together with a gene encoding the fluorescent reporter protein Venus, was integrated into the *ROSA-TET* locus by a knock-in method, and then drug-resistant clones were selected. Withdrawal of Tc from culture medium resulted in Venus fluorescence in virtually all cells within 48 h, whereas no fluorescence was detected in cells cultured with Tc. Although SF-1 mRNA and protein were also expressed in ES cells without Tc, ES cells maintained an undifferentiated state and did not express steroidogenic marker genes including Cyp11a1. However, ES cells ceased to proliferate and died several days after LIF removal from the culture medium.

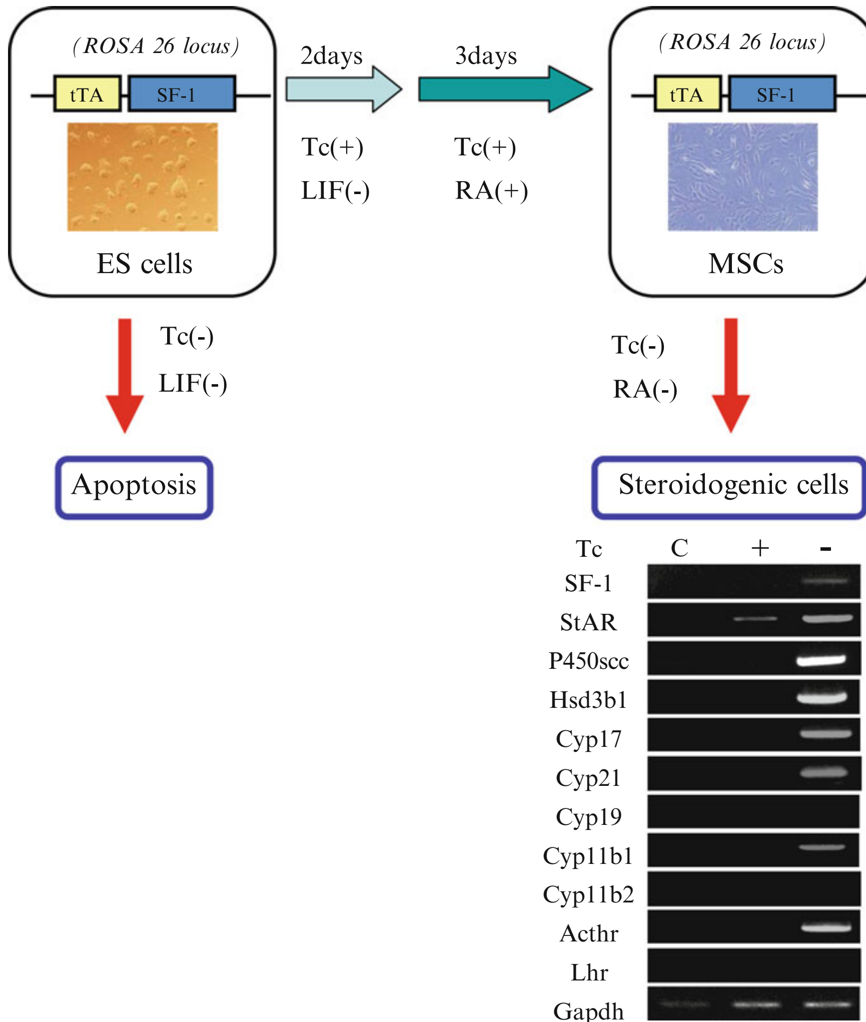


**Fig. 16.3** Induction of steroidogenic cells from ES cells *via* the differentiation of ES cells into MSCs. They differentiated into adrenocortical-like cells

These results are in agreement with the results discussed above, in which steroidogenic cells cannot be directly differentiated from ES cells (Yazawa et al. 2006).

SF-1 expression has been induced after differentiation of ES cells into MSCs. For differentiation of ES cells into MSCs, ES cells were cultured on collagen IV-coated dishes and treated with pulse exposures to RA, as described by Nishikawa and colleagues (Takashima et al. 2007) (Fig. 16.3). Consistent with their reports, various molecular markers of the mesenchymal lineage were induced by RA treatment, indicating that the ES cells were successfully differentiated into mesenchymal cells including MSCs. These cells were cultured further without RA and Tc for 3 days. In contrast to undifferentiated ES cells, the differentiated cells were able to survive following SF-1 expression in the absence of LIF. In these cells, SF-1 expression resulted in the expression of various steroidogenesis-related genes such as StAR, Cyp11a1, Hsd3b1, Cyp17, Cyp21 Cyp11b1 and Acthr. The gene expression pattern was quite similar to that in adrenocortical cells, and particularly fasciculata cells. Consistent with the gene expression profile, corticosterone

was the most secreted steroid hormone from these cells. Cortisol was also secreted from these cells, although it was significantly less than that of corticosterone. These results indicate that ES cells can be differentiated into steroidogenic cells by SF-1 *via* the mesenchymal lineage. It has also been reported that SF-1-transfected ES cells differentiate into steroidogenic cells under specific culture conditions, although these cells show characteristics of the gonadal lineage (Jadhav and Jameson 2011).

In conclusion, it is clear that SF-1 represents a master regulator, not only for the development of steroidogenic organs, but also for steroidogenesis following organogenesis. In addition, SF-1 directs differentiation of non-steroidogenic stem cells into steroidogenic cells. Among various stem cell types, MSCs are suitable stem cells for the differentiation of steroidogenic cells. After pre-differentiation into MSCs, pluripotent stem cells can also be subsequently differentiated into steroidogenic cells using SF-1. These cells may provide a source for regenerative and gene therapies, although various problems should be resolved in future studies. For example, the steroidogenic properties of cells differentiated from MSCs and ES cells are markedly varying dependent on the derivation tissues and species. It is essential to delineate conditions that allow the directed differentiation of specific steroidogenic lineages with the characteristics of testicular Leydig cells, ovarian granulosa and theca cells, as well as various types of adrenocortical cells (reticularis, fasciculata and glomerulosa). In addition, it is necessary for regenerative therapy to establish methods to induce SF-1 expression in stem cells without gene transfer. Further studies will be required for the realization of regenerative and gene therapies of steroidogenic tissues.

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# 17 Human Embryonic Stem Cell Differentiation: Role of Glycosphingolipid Structure

Chien-Huang Liao and John Yu

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**Abstract**

Glycosphingolipids (GSLs) are ubiquitous components of cell membranes that can act as mediators of cell adhesion and signal transduction and possibly be used as cell type-specific markers. A systematic survey of expression profiles of GSLs in human embryonic stem cell (hESC) lines and various differentiated derivatives was carried out using immunofluorescence, flow cytometry, and MALDI-MS and MS/MS analyses. In the undifferentiated hESCs, in addition to the well-known hESC-specific markers, SSEA-3 and SSEA-4, we identified several globosides and lacto-series GSLs, previously unrevealed in hESCs, including Gb4Cer, Lc4Cer, fucosyl Lc4Cer, Globo H, and disialyl Gb5Cer. During differentiation of hESC into embryoid body (EB) outgrowth cells, MS analyses revealed a clear-cut switch in the core structures of GSLs from globo- and lacto- to ganglio-series. To further clarify alterations is correlated with lineage-specific differentiation, we analyzed changes in GSL compositions as hESCs differentiated into neural progenitors or endodermal cells. During differentiation into neural progenitor cells, we found that the core structures of GSLs switched to mostly ganglio-series dominated by GD3. On the other hand, when hESCs differentiated into endodermal cells, patterns of GSLs were totally different from those observed in EB outgrowth or neural progenitors. The most prominent GSL identified by MALDI-MS and MS/SM analysis

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was Gb4Cer, without any appreciable amount of SSEA-3 and 4 antigens, or GD3, in endodermal cells. We also demonstrated that such a switch in GSL profiling was attributable to altered expression of key glycosyltransferases (GTs) in the biosynthetic pathways, suggesting a close association of GSLs with lineage specificity and differentiation of hESCs. Therefore, these results provide new insights into the unique stage-specific transition and mechanism for alterations of GSL core structures during hESC differentiation.

## Introduction

Human embryonic stem cells (hESCs) are pluripotent stem cells derived from the inner cell mass of blastocysts; they possess the capacity for self-renewal and differentiation to form three germ layers. These features allow hESCs to be employed as useful tools for regenerative medicine and research. Moreover, specific surface markers are valuable in the regenerative medicine for monitoring the culture and behavior of stem cells and their status of differentiation. However, many stem cell markers currently in use were adopted from phenotypic characteristics of unrelated-cell types and could only offer limited insight into their functional roles in the particular cell types of interest. Thus, it is important not only to identify *new* markers that can define stem cells distinctly, but also to gain insight into the functional roles of stem cell markers and to understand their downstream regulators in the maintenance of the self-renewal capability and differentiation potentials.

Stage-specific embryonic antigen (SSEA)-3 and SSEA-4 are considered to be important markers of hESCs, whereas O4, O1 antigen, A2B5 antigens, and GD3 are markers of neural lineage cells. Although the epitopes defined by the monoclonal antibodies (mAbs), MC631 (anti-SSEA-3) and MC813-70 (anti-SSEA-4), were delineated (Kannagi et al. 1983a, b), the identities and roles of these well-known surface markers in hESCs are mostly not understood. In addition, these antibodies were shown to cross-react to different degrees with various glycosphingolipids (GSLs) (Kannagi et al. 1983a, b). MC631 recognizes the GalNAcβ1-3Galα1-4Gal epitope which exists in $Gb_5Cer$ of SSEA-3 and sialyl $Gb_5Cer$ of SSEA-4; it also reacts, to a lesser extent, with $Gb_4Cer$ and the Forssman antigen (GalNAcα1-3$Gb_4Cer$), and weakly with Globo H (Kannagi et al. 1983a, b). On the other hand, the MC813-70 epitope is mostly represented by sialyl $Gb_5Cer$ in SSEA-4. This mAb also cross-reacts to different extents with GM1b and GD1a, and a common structure of the core 1 O-glycan glycoprotein, carrying the NeuAcα2-3Galβ1-3GalNAc epitope (Kannagi et al. 1983a, b). Therefore, positive immunostaining with mAbs alone might not necessarily reflect a particular entity of GSLs on hESCs. Instead, a detailed mass spectrometric (MS) analysis coupled with immunostaining is essential to decipher the precise profile of GSLs in hESCs.

## Cell Surface GSLs

GSLs are lipids containing at least one monosaccharide residue and either a sphingoid or a ceramide. They are ubiquitous components of cell membranes and are particularly abundant on surfaces of animal cells. The GSLs in vertebrate animal tissues can be divided generally into three major groups: (a) the ganglio- and isoganglio-, (b) the lacto- and neolacto-, and (c) the globo- and isoglobo-series (Hakomori 2008; Hakomori and Ishizuka 2006). It was suggested that these molecules have important functions as mediators of cell adhesion and signal transduction, and as cell type-specific markers. In vertebrate cells, GSL-enriched surface microdomains organized with various signal transducer molecules seem to be important for modulating cell adhesion and signal transduction (Hakomori 2008, 2010). In addition, the expressions of GSLs are frequently and drastically changed during development and differentiation; therefore, GSLs are useful as lineage-specific differentiation markers.

Numerous studies confirmed that GSLs are key molecules required for the regulation of cellular processes (Hakomori 2008, 2010) and their expressions are developmentally controlled and

cell type-specific (Yu et al. 1988; Yu 1994). For example, SSEA-1, -3, and −4 were identified at defined stages of embryonic development (Kannagi et al. 1983a, b; Solter and Knowles 1978). They were identified as GSLs or as glycan epitopes with uncharacterized carriers. They are highly expressed in mouse and human ESCs and are considered to be specific cell markers (Shevinsky et al. 1982). On the other hand, certain cells possess defined GSL antigens and can be detected by specific antibodies. For example, $Le^{X}$ (CD15) which was found on neutrophils is considered to mediate phagocytosis and chemotaxis (Kerr and Stocks 1992). Human natural killer cell antigen-1 (HNK-1) (sulfo-3GlcAβ13Galβ–14GlcNAcCer), expressed at the cell surface, is uniquely enriched in neural cells and natural killer cells, and is thought to play important roles in cell-cell interactions (Jungalwala 1994).

Recently, functional studies of some GSLs revealed that specific GSLs form clusters at the cell surface and interact with membrane proteins such as caveolin-1, integrins, growth factor receptors, and tetraspanins. Such interactions thus contribute to modulating cell adhesion, growth, and motility (Prinetti et al. 2009; Sonnino and Prinetti 2009). On the other hand, GSLs were also found to be involved in the process of the epithelial mesenchymal transition (EMT) (Guan et al. 2009, 2010). The EMT process is generally recognized as an important phenomenon that occurs during embryonic development and is also implicated in the progression of primary tumors towards metastasis. All these findings suggest that specific GSL patterns or their clustered microdomains at the cell surface can serve as cell-specific markers, and also functionally as complexes to initiate intracellular signaling through interactions with other functional membrane components (Fig. 17.1).

In the following studies, we used matrix-assisted laser desorption ionization- mass spectrometry (MALDI-MS) and tandem mass spectrometry (MS/MS) analyses in addition to immunostaining and flow cytometry to systematically delineate changes in expression profiles of GSLs in undifferentiated hESC and 16-day differentiated EB outgrowth cells (Liang et al. 2010). In addition, we had further investigated the GSL profile of hESCs differentiated toward neural progenitor cells and definitive endodermal lineages, and studied the role of specific glycosyltransferases during the lineage-specific differentiation of hESCs (Liang et al. 2010). We found that there was a striking switch in the core structures of GSLs from globo- and lacto- to ganglio-series during hESC differentiation (Liang et al. 2010, 2011). The results not only uncovered several previously unreported glycans in hESCs but also highlighted the value of such combined strategies to overcome the inherent tribulations associated with cross-reactivities of antibodies (Liang et al. 2010, 2011). In addition, these studies further suggested that specific hESC markers may be expressed in cancers, and specific biomarkers for cancer may also be potential candidates for hESC markers.

## MALDI-MS and MS/MS Analysis of GSLs in Undifferentiated hESCs

SSEA3 and SSEA4 are well known markers of hESC; but previous studies reported various cross-reactivities of mAbs to various GSLs (Kannagi et al. 1983a, b), making it difficult to ascertain the presence of particular entities of GSLs on hESCs using mAbs alone. Due to these cross-reactivities with GSLs containing the same epitopes, positive immunostaining with mAbs alone would not necessarily validate the presence of particular entities of GSLs on hESCs (see below). To decipher a precise profile of GSLs in hESCs, the expressions of GSLs, we used MALDI-MS profiling and MS/MS sequencing, as well as immunofluorescence and flow cytometric analyses, to systematically analyze GSLs in the undifferentiated hESCs and 16-day differentiated embryoid body (EB) outgrowth cells (Liang et al. 2010).

As illustrated in Table 17.1 and Fig. 17.2, GSLs from undifferentiated hESCs were found to comprise $Gb_5Cer$ and its sialylated version, respectively corresponding to the overlapping SSEA-3 and SSEA-4 epitopes; in addition to SSEA-3 and SSEA-4, we demonstrated the

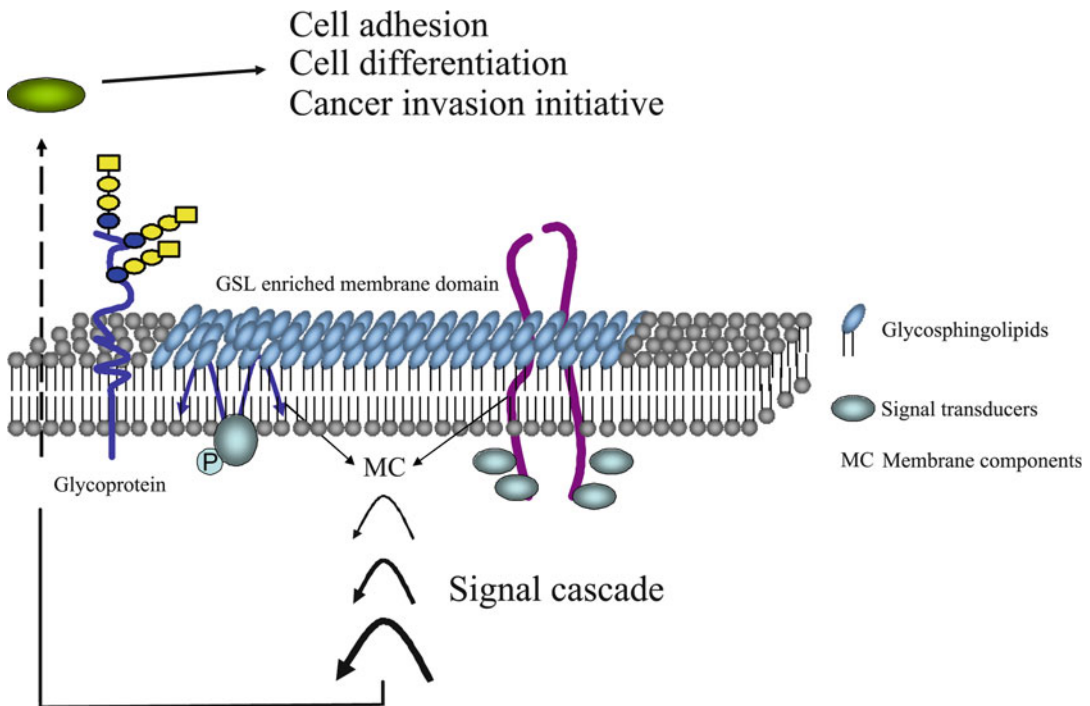


**Fig. 17.1** Changes in GSL enriched membrane domain leading to intracellular signaling

presence of other globosides and lacto-series GSLs, including $Gb_4$Cer, $Lc_4$Cer, fucosyl $Lc_4$Cer, Globo H, and disialyl $Gb_5$Cer in hESCs, that were not previously known to be expressed by hESCs in the undifferentiated hESCs (Liang et al. 2010). SSEA-4 was the sialylated SSEA-3 ($Gb_5$Cer; Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer), whereas disialyl $Gb_5$Cer was the further sialylated SSEA-4. In turn, SSEA-3 was derived from its precursor, $Gb_4$Cer (Table 17.1). On the other hand, Globo H, a hexasaccharide which was overexpressed in epithelial cell tumors such as colon, ovarian, gastric, pancreatic, endometrial, lung, prostate and breast cancers, was the fucosylated SSEA-3 (Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer; Table 17.1). Therefore, the globo series of GSLs such as $Gb_4$Cer, SSEA-3, SSEA-4, Globo H, and disialyl $Gb_5$Cer contained the common core structure as Galα1-4Galβ1-4Glcβ1-1 Cer (Table 17.1).

In our recent collaborations with Drs. C L Liao and M Y Ho at Academia Sinica, we are developing new technology to obtain the information on the linkage patterns of specific GSLs. For example, the molecular ion at m/z 1460 ($Gb_4$Cer) (Liang et al. 2010) has been further confirmed by ESI-MS3 and MS4 analysis. Our preliminary results indicated that this GSL has the Galα1-4Gal linkage and displayed the structure as GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer ($Gb_4$Cer, Table 17.1). In addition, there are several isomer candidates for "disialyl $Gb_5$Cer": i.e. V3NeuAcαIV6NeuAcα-$Gb_5$Cer, V3(NeuAcα) 2-$Gb_5$Cer, etc. (Table 17.1). In fact, we had performed MS/MS analysis for this so-called "disialyl $Gb_5$Cer" in MALDI-MS; so far our analysis suggested that the most likely is V3NeuAcαIV6NeuAcα-$Gb_5$Cer. However, we have not yet completely excluded the existence of the other two possibilities (Table 17.1). We will continue to solve more structural information for these and other newly found glycans on hESCs.

In addition, in the undifferentiated hESCs, $Lc_4$Cer and fucosyl $Lc_4$Cer belonging to GSLs of the lacto series was detected, and the core

**Table 17.1** Structures of glycosphingolipids in hESC and its differentiated EB outgrowth (with IUPAC-style abbreviations)

| | |
|---|---|
| **Globo-series** | Galα1-4Galβ1-4Glcβ1-1 Cer |
| Gb$_3$Cer | Galα1-4Galβ1-4Glcβ1-1 Cer |
| Gb$_4$Cer | GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer |
| Gb$_5$Cer (SSEA-3) | Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer |
| sialyl-Gb$_5$Cer (SSEA-4) | NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer |
| Globo H | Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer |
| disialyl-Gb$_5$Cer | |
| V3NeuAcαIV6NeuAcα-Gb$_5$Cer | NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1’Cer |
| V3(NeuAcα)2-Gb$_5$Cer | NeuAcα2-6(NeuAcα2-3)Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1’Cer |
| No formal nomenclature | NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1’Cer |
| **lacto-series** | Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer |
| Lc$_4$Cer | Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer |
| Fucosyl-Lc$_4$Cer (H type 1) | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer |
| **Ganglio-series** | Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1 Cer |
| GM3 | NeuAcα2-3Galβ1-4Glcβ1-1 Cer |
| GD3 | NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1 Cer |
| GM2 | GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1 Cer |
| GM1a | Galβ1-3GalNAcβ1-3(NeuAcα2-3)Galβ1-4Glcβ1-1 Cer |
| GM1b | NeuAcα2-3Galβ1-3GalNAcβ1-3Galβ1-4Glcβ1-1 Cer |
| GD1a | NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1 Cer |
| GD1b | Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1 Cer |
| GD1c | NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1 Cer |
| GT1a | NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1 Cer |
| GT1b | NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1 Cer |
| GT1c | Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1 Cer |

structure had been determined as Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer (Table 17.1). In contrast, the neo-lacto series have the core as Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer. It is noted that nLc$_4$Cer was not detected in undifferentiated hESCs (unpublished data). Furthermore, fucosylnLc$_4$Cer which had the structure either as IV2FucnLc$_4$Cer or as III3FucnLc$_4$Cer was also not detected in hESCs. The latter had the sequence Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer, (which specified the glycan epitope recognized by SSEA-1 antibody). On the other hand, it is noted that mAbs against H type 1 epitope, Fucα1–2Galβ1–3GlcNAc, clearly stained the undifferentiated hESCs but not the differentiated EB outgrowth cells, confirming not only the abundance of lacto-series GSLs in hESCs, but also indicative of carrying a terminal Fuc-Gal (H antigen) on the Lc$_4$Cer GSL (Table 17.1). The collision-induced dissociation (CID) MS/MS supported the H type 1 epitope, Fucα1–2Galβ1–3GlcNAc (Liang et al. 2010). Base on CID

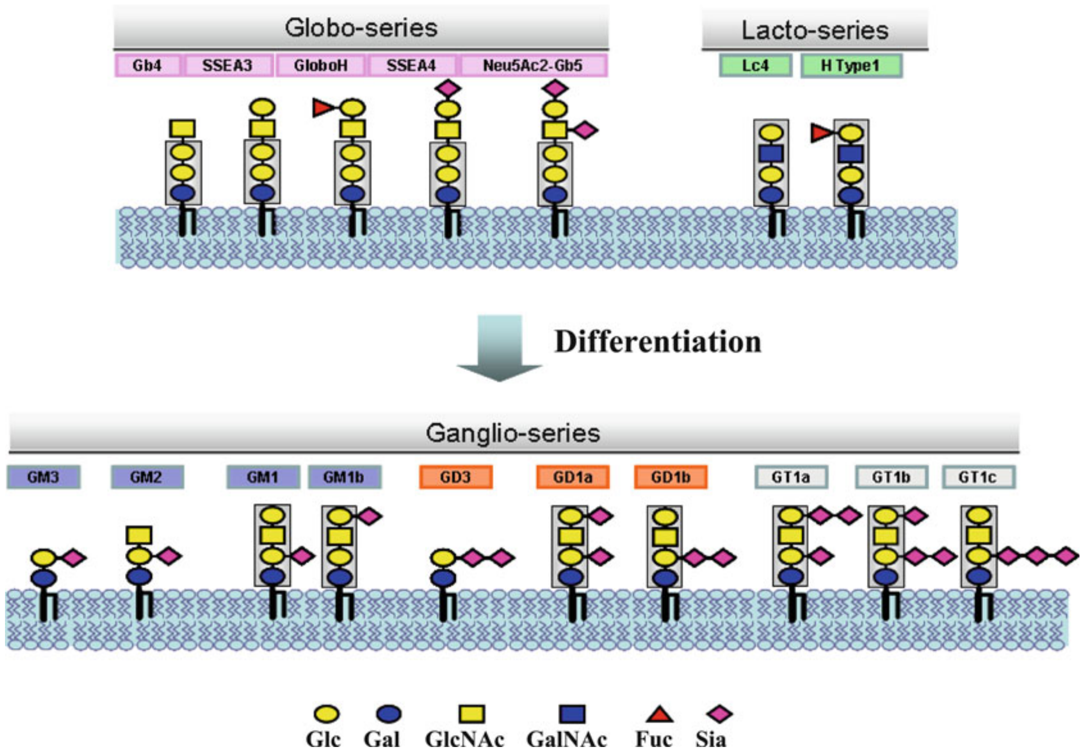


**Fig. 17.2** Switching of the Core Structures of Glycosphingolipids upon Human ESC Differentiation

MALDI-MS/MS analyses of these and a series of other GSLs in Table 17.1, structure of glycosphingolipids found in hESC and its differentiated EB outgrowth are listed.

## Switching of the Core Structures of GSLs from Globo- and Lacto- to Ganglio-Series upon Differentiation

More importantly, there was an almost complete "makeover" of the GSLs when the original embryonic stem cells differentiated to the more developed EB outgrowth cells. It was found that the core structure of GSLs of hESCs made a switch from globo- and lacto-series into a ganglio-series structure upon differentiation (Fig. 17.2). In other word, the globo- and lacto-series of GSLs became rare and replaced by ganglio- series upon differentiation of hESCs.

Based on mass spectrometry analysis, all of the identified $Gb_4Cer$- and $Gb_5Cer$-related GSLs and the $Lc_4Cer$ and Fuc-$Lc_4Cer$ were clearly downregulated upon differentiation to the extent that none was detected at any significant level in the sample from EB cells (Liang et al. 2010). Instead, the differentiated EB cells were dominated by GSLs of the ganglio-series, especially GM3 and GD3 (Liang et al. 2010). At first glance, GD3 was only highly expressed in EB cells and not in hESCs, whereas GM3, along with GD1a/GD1b were commonly expressed in both. Other less-abundant ganglio species including GM2, GM1, and GT1a/GT1b/GT1c, were also more obvious in EB cells, although, in general, it is not possible to categorically rule out the presence of any of the gangliosides at low amounts in hESCs, which may be suppressed or masked by overlapping, more-abundant signals attributed to the globo-series. Additional MS/MS analyses

revealed that isomers of GM1a/GM1b and GD1a/GD1b were present in EB differentiated cells. In table 17.1, gangliosides had the backbone core structure as Galβ1- 3GalNAcβ1-4Galβ1-4Glcβ1-1 Cer with sialic acids. However, the complexities of ganaliosides could be attributed to the complex mixture of EB cells representing individually three different germ cells after gastrulation.

In addition, the results of MALDI-MS indicated that hESC lines, HES5 and H9, exhibited similar expression patterns of GSLs; moreover, similar patterns of GSL expressions were demonstrated after differentiation of these different hESC lines.

Moreover, there was heterogeneity in the ceramide moiety of GM3 and the non-sialylated globe and lacto series in hESCs (Liang et al. 2010). In contrast, the ceramide moieties of gangliosides in EB cells were clearly much more heterogeneous. In addition, the relative amounts of species with different fatty acyl contents distinctly differed in both hESCs and EBs (Liang et al. 2010). These are issues await the future investigation.

Our finding of a switch in the core structures of GSLs from globo- (Galα1-4Galβ1-4Glcβ1-1 Cer) and lacto- (Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer) to ganglio-series (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1 Cer) during hESC differentiation (Table 17.1; Liang et al. 2010 ) is consistent with the observed changes in expression patterns of GSLs during embryonic development of mice (Hakomori 2008). It was reported that globo-series SSEA3, SSEA4, and Globo H are expressed at high levels in four cell stages, and then later decline during mouse embryogenesis (Hakomori 2008). The SSEA1 [III3Fuc-nLc$_4$Cer] is not expressed until the morula stage of mouse embryos (Hakomori 2008). In addition, ganglio-series GM3, GD3, GT3, GM2, and GD2 are expressed in later stages upon neural crest formation in mice (Hakomori 2000; Yu et al. 1988) and GD3, GD1a and GT1b were also expressed after somite formation (Ngamukote et al. 2007; Yu et al. 1988). In addition, based on thin-layer chromatography, the GM1 ganglioside was shown to be present in mouse ESCs (Kwak et al. 2006); furthermore, GM3, GM2, and GD3 were demonstrated using a similar approach (Lee et al. 2007).

In contrast, our studies using hESCs showed that GM3, GM1, and GD1a/GD1b were detected in undifferentiated ESC cells; after differentiation, GM3 and GD1a/GD1b and other gangliosides such as GM2, GD3, and GT1a/GT1b/GT1c all increased.

## Changes in GSL Compositions During Lineage-Specific Differentiation

In addition to EB outgrowth, we had also examined the changes of GSL compositions in hESCs after lineage-specific directed differentiation. We had induced hESCs to differentiate specifically into either neural progenitor cells (97% Nestin$^+$, 99% Pax6$^+$, Sox1$^+$cells) or definitive endoderm cells (SOX17$^+$,FOXA2$^+$). Then MALDI-MS and MS/MS sequencing were used to analyze GSLs in these cells systematically (Liang et al. 2011).

We found that when these hESC-derived differentiated cells became different cell lineages, the profiles of GSLs on MALDI-MS analysis differed from each other. After the annotations of GSL profiles were assigned based on m/z values of each of the major molecular ion signals, the values of m/z for the respective GSLs were fitted to the expected core structures of the three major GSL series (globo-, lacto-, and ganglio-series) along with the usual ranges of the most common permutation of sphingosine and fatty acyl chains, as previously described (Liang et al. 2010). The MS/MS analysis confirmed the presence of SSEA-3, SSEA-4, Globo H, Lc$_4$Cer, and H-type 1 (fucosyl Lc$_4$Cer) in undifferentiated hESCs (Liang et al. 2011). When hESCs differentiated into neural progenitor cells, the GSL expression pattern changed and became dominated by ganglio-series GSLs. GD3 and GM3 were the most abundant GSLs, but other ganglio-series GSLs including GM1, and GD1, were also detected in these neural progenitor cells (Liang et al. 2011). Thus the GSL profile of neural progenitor cells was similar to that for EB outgrowth (Liang et al. 2010), because ectoderm cells are known to be the most dominant cell type among EB outgrowth cells.

However, when hESCs differentiated into definitive endodermal lineages, GSL patterns were quite distinct from those observed in neural progenitor cells (Liang et al. 2011). The most prominent GSL signal for endodermal lineage was found at m/z 1460 corresponding to a mixture of $Lc_4$Cer and $Gb_4$Cer (see below). Minor signals, which could be assigned as GM3, GM1, GD1, Globo H, and fucosyl $Lc_4$Cer/$Gb_4$Cer, were also detected (Liang et al. 2011). Interestingly, it was further demonstrated that the relative amount of $Lc_4$Cer and $Gb_4$Cer that share the same molecular weight and hence giving a common molecular ion at m/z 1460 by MS analysis was shown by further MS/MS analysis to differ significantly in undifferentiated hESCs and definitive endoderm. These MS/MS data strongly indicated that the $Gb_4$Cer structure was more abundant in definitive endoderm, whereas the $Lc_4$Cer structure was the more abundant in hESC (Liang et al. 2011). Therefore, according to our MS/MS analysis, there were significant differences in the compositions between undifferentiated hESCs, neural progenitors and definitive endoderm (Liang et al. 2011).

## Alterations in the Expressions of GSL-Related Glycosyl Transferases (GTs) During Differentiation

Yamashita et al. reported that knockdown of the *Ugcg* gene, a key GT for converting ceramide to glucosylceramide in the initial step of GSL biosynthesis pathway, led to defects in embryonic differentiation after gastrulation, suggesting a vital role of GSL synthesis in development (Yamashita et al. 1999). Recently, Jung et al. reported that in *Ugcg*-suppressed mouse ESCs, there were significant defects in neural differentiation, especially neural maturation related to GFAP and MAP-2 expressions (Jung et al. 2009).

We showed that hESC differentiation was accompanied with a drastic switch of the profiling of GSL on cell surface, which occurred as a result of concerted changes in the expression levels of key GTs involved in the manufacture of GSLs (Liang et al. 2010). For example, several GTs for the globo- of lacto-GSLs on cell surface were downregulated; in contrast, enzymes responsible for the synthesis of GM2, GD3, and GT1b (i.e., B4GALNT1, ST8SIA1, and ST3GAL1, respectively) were upregulated during hESC differentiation. On the other hand, we found that in addition to globo-series GSLs, the undifferentiated hESCs also expressed lacto-series GSLs including $Lc_4$Cer and Fuc-$Lc_4$Cer. And associated with such expression, Lc3 synthase (B3GNT5) and Lc4 synthase (B3GALT5) were expressed by undifferentiated hESCs, but downregulated after differentiation.

When hESCs differentiated into different cell lineages, there were also substantial differences in the GSL profiling and in the expression of key GTs. We found that when hESCs differentiated into neural progenitor cells, the expression of GTs responsible for the biosyntheses of globo-series GSLs, such as FUT1, FUT2 and B3GALT5, were downregulated, as compared to the undifferentiated hESCs (Liang et al. 2011). In addition, the expression of GTs for ganglio-series GSLs, such as ST3GAL1, ST3GAL5 and ST8SIA1, increased by two to tenfolds (Liang et al. 2011). On the other hand, when hESCs differentiated into definitive endoderm cells, the expression of GTs were all downregulated, especially $Gb_5$ synthase was downregulated, causing the accumulation of $Gb_4$Cer (Liang et al. 2011).

## Cross-Reactivities of Antibodies with GSLs Containing the Epitopes

Current studies in stem cell research often relied on the use of mAbs which recognize glycan epitopes; but these epitopes were now shown to be present in various glycoconjugates (Kannagi et al. 1983a). The epitopes of the specific mAbs MC631 and MC813-70 for SSEA-3 and SSEA-4 had been delineated as GalNAcβ1-3Galα1-4Gal and NeuAcα2-3Galβ1-3GalNAc, respectively (Kannagi et al. 1983a, b). However, it is still unknown whether these two stage-specific antigens appeared simply as glycolipids or as glycans conjugated with proteins (i.e. glycoproteins) in hESCs; in other words, the identities

and roles of SSEA-3 or SSEA-4 antigens have not yet been elucidated.

For example, the MC813-70 epitope is mostly represented by sialyl $Gb_5Cer$. This mAb also cross reacts to different extent with GM1b and GD1a, and a common structure of the core 1 O-glycan glycoprotein, carrying the NeuAcα2-3Galβ1-3GalNAc epitope (Kannagi et al. 1983a, b). It was found that SSEA-4 (Neu5Ac-$Gb_5Cer$) was highly expressed in hESCs in immunostaining with mAb MC813-70; but immuno-positive cells decreased to 86% of cells after differentiation into neural progenitor cells. In addition, approximately 96% of definitive endoderm cells also seemed to show positive immunostaining with this mAb. However, the mass spectrometry analysis failed to detect the presence of SSEA-4 (Neu5Ac-$Gb_5Cer$) in either neural progenitor or endodermal cells (Liang et al. 2011). Instead, GD1a and GM1b which cross-reacted with SSEA-4 mAb were detected in both neural progenitor and definitive endoderm cells. In other word, mass spectrometry analysis did not detect SSEA-4 in cells committed toward ectoderm or endoderm lineages, while specific mAb seemed to reveal the presence of SSEA-4$^+$ cells after lineage differentiation in immunostaining. This discrepancy, however, can be reconciled in view of cross-reactivities of mAbs used with various glycoconjugates in the cells containing the specific glycan epitopes. While it is possible that the MS profile may be biased against the SSEA-4 epitope, the lack of immunostaining with mAb MC631 (against SSEA-3) in the neural progenitor cells, which was known to recognize SSEA-4 (Neu5Ac-$Gb_5Cer$) equally well (Kannagi et al. 1983a, b), attested to the absence of SSEA-4 in neural ectoderm cells. Therefore, the SSEA-4$^+$ cells in immunostaining of neural progenitor cells could be attributed to cross reactivities of mAb MC813-70 with GD1a, GM1b or even the extended core 1 o-glycan glycoproteins (Van Dyken et al. 2007).

MC631 recognizes the GalNAcβ1-3Galα1-4Gal epitope which exists in $Gb_5Cer$ of SSEA-3 and sialyl $Gb_5Cer$ of SSEA-4; it also reacts, to a lesser extent, with $Gb_4Cer$ and Forssman antigen (GalNAcα1-3$Gb_4Cer$), and weakly with Globo H (Kannagi et al. 1983a, b). In fact, anti-SSEA-3 MC631 was reactive with essentially all undifferentiated hESCs and became mostly undetectable in neural progenitor cells. These results were confirmed by the absence of MALDI-MS signal at m/z 1664 assigned to $Gb_5Cer$ (SSEA-3) (Liang et al. 2011). In definitive endoderm cells, however, the appearance of apparent SSEA-3$^+$ cells detected with mAb was also decreased, but still present in as much as 62% of cells in immunostaining (Liang et al. 2011). But, MALDI-MS analysis indicated little, if any, amount of *m/z* 1664.2 in the spectrum for this lineage of cells (Liang et al. 2011). Therefore, immunostained MC631$^+$ cells of definitive endoderm cells could be attributed to cross reactivities with mAb against glycoconjugates which carried the MC631 epitope. Indeed our mass spectrometric analysis of definitive endoderm cells confirmed the presence of signal corresponding to $Gb_4Cer$ GSL at *m/z* 1460 (Liang et al. 2011). But, the possibility of cross reaction with other entities uncharacterized could not be excluded.

In addition, Globo H was shown to be highly expressed uniquely in hESCs (Liang et al. 2010) and its polyclonal antibodies reacted with both Globo H and SSEA-3 (Chang et al. 2008). Furthermore, the cholera toxin B subunit, which we used to detect GM1, was reported to bind GM1 as well as fucosyl GM1, asialo GM1, GD1a, GD1b, GT1b, GM2, and GM3 (Cai et al. 2007; Cho et al. 2008; Pankratz et al. 2007).

It was also noted that lactose-series of GSLs, such as $Lc_4Cer$ and fucosyl $Lc_4Cer$ were found both in hESCs and definitive endoderm cells (albeit in smaller amount), and their expressions apparently disappeared in neural ectoderm cells (Liang et al. 2011) in immunostaining and mass spectrometric analysis. Further MS/MS analysis indicated that the signal attributed to $Gb_4$/$Lc_4Cer$ at *m/z* 1460 in hESCs was contributed more by $Lc_4Cer$; but on the other hand, in definitive endoderm, $Gb_4Cer$ was the more abundant component instead (Liang et al. 2011). Therefore, our results showed that the relative abundance of $Lc_4Cer$ versus $Gb_4Cer$ differed in undifferentiated hESCs and definitive endoderm. Epitopes for mAbs against $Lc_4Cer$ and fucosyl $Lc_4Cer$ had been

shown, separately, to be $Le^c$ and H type 1 (Hakomori and Ishizuka 2006), which represent two members of blood group antigens; these glycan determinants were also known to be present on both glycolipids and glycoproteins (Hakomori 1999). In the definitive endoderm, MALDI-MS analysis indeed confirmed the presence of $Lc_4Cer$ and fucosyl $Lc_4Cer$, which were not found in neural progenitor cells (Liang et al. 2011). On the other hand, GD3 was not detectable in hESCs and definitive endoderm cells; but were highly expressed in neural progenitor cells, thus qualifying it as a distinctive marker (Liang et al. 2011), whereas the expression of other ganglio series GSLs such as the GM3, GM1, and GD1 were more universal.

Therefore, various cross-reactivities of Abs to GSLs made it difficult to ascertain the presence of particular entities of GSLs on hESCs using Abs alone. Positive immunostaining with mAbs alone may not reflect a particular entity of GSLs on hESCs. Instead, a detailed mass spectrometric analyses coupled with immunostaining will be essential to decipher a precise profile of GSLs in hESCs. In Table 17.1, we only provide a list of glycolipids that are not necessarily referred to all entities of antigens themselves.

## Cancer and the Reprograming Cells

We hypothesized that stem cell markers are crucial for a variety of cellular functions, such as cell migration, adhesion or signal recognition, and differentiation (Fig. 17.1). Launching from our newly found GSL markers of human ESCs, we had keen interest in the relationship of these newly found, stage-specific glycans present in hESCs and the expression of onco-fetoproteins in cancers or cancer stem cells, because they may serve as candidates for cancer detection and glycan-targeted therapy of cancers (Liang et al. 2010). It was suggested that cancer cells often possess traits reminiscent of those ascribed to normal early embryonic cells, and on the other hand, cancer cells also express many onco-fetoproteins that are found in hESCs. Thus, these newly found stage-specific glycan entities in hESCs may serve as markers for cancer detection or as targets of cancer therapy.

We previously reported that SSEA-3, a marker for hESCs, is highly expressed in 77.5% of breast cancer patients in breast cancer cells and 62.5% in breast cancer stem cells (Chang et al. 2008). On the other hand, we demonstrated that Globo H, a fucosyl $Gb_5Cer$ that was overexpressed in many epithelial cell cancers, was highly expressed by undifferentiated hESCs. It was also demonstrated that immunization of mice with Globo H induced antibodies which are reactive with Globo H and SSEA3, suggesting that Globo H-based immunotherapy would target not only non-breast cancer stem cells, but also breast cancer stem cells (Chang et al. 2008). Recently, we found that the level of epithelial cell adhesion molecule (EpCAM) expression that was correlated with dedifferentiation and malignant proliferation of epithelial cells was expressed in undifferentiated hESCs and was associated with maintenance of the undifferentiated phenotype of hESCs (Lu et al. 2010). Furthermore, microarray studies of gene expressions suggested that histologically poorly differentiated tumors show preferential overexpression of genes normally expressed by ESCs (Ben-Porath et al. 2008). Therefore, these findings are consistent with the notion that specific biomarkers for cancer may be potential candidates as unique hESC markers, and specific hESCs markers may likely be expressed in cancers, which can serve as markers for cancer diagnosis and detection.

We also examined the expression of these unique surface GSLs during reprogramming of human fibroblasts into induced pluripotent stem cells (iPSCs) by transduction with the four reprogramming transcription factors Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006). As shown in Fig. 17.3, the starting population of fibroblasts expressed almost no Globo H, $Lc_4Cer$ and H type 1 antigens, but fully reprogrammed iPSCs that had reactivated an endogenous Oct4 gene displayed levels of these GSL markers comparable to those in ESCs. In contrast, after 30 day differentiation of iPSCs, the expression levels of these markers declined significantly to levels typical of fibroblasts before reprogramming.

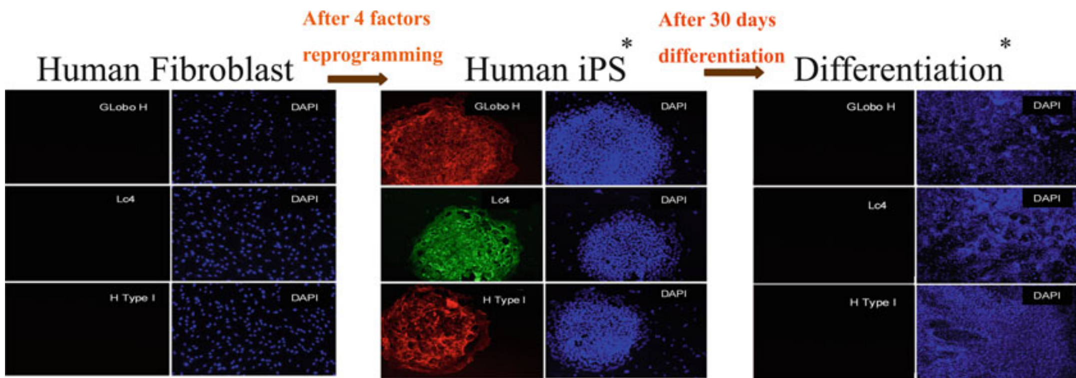


**Fig. 17.3** Expression of GSLs in human iPS and differentiated cells

Collectively, these data point to a strong association of GSL surface markers with the pluripotent state in both ESCs and iPSCs and a contrasting association of GSLs with the undifferentiated state.

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# 18 Differentiation of Human Embryonic Stem Cells into Pancreatic Endocrine Cells

Jennifer E. Bruin and Timothy J. Kieffer

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**Abstract**

Diabetes is a chronic and debilitating disease that results from insufficient production of insulin from pancreatic β-cells. Islet cell transplantation can effectively treat diabetes, but is currently severely limited by the reliance upon cadaveric donor tissue. Human embryonic stem (ES) cells are a promising alternative source of transplantable insulin-producing β-cells, as they are renewable *in vitro* and have the inherent capacity to differentiate into pancreatic endocrine cells. Several strategies have been explored for differentiating human ES cells into pancreatic β-cells *in vitro*, with the most effective to-date being mimicking the key signaling pathways required for pancreas development using small molecules. Although numerous groups have successfully generated insulin-positive cells *in vitro* using step-wise differentiation protocols, as of yet these cells are not mature pancreatic β-cells as they typically express multiple hormones and are not glucose-responsive. Alternatively, others have transplanted immature pancreatic progenitor cells into immunocompromised animals thus allowing endocrine cell maturation to occur over a prolonged period *in vivo*. With this strategy functional islet-like endocrine cells have been generated that express key markers of mature pancreatic β-cells, respond to both meal and glucose challenges, and are capable of reversing diabetes in mice. Although there are still numerous challenges to overcome, these studies demonstrate the feasibility of human

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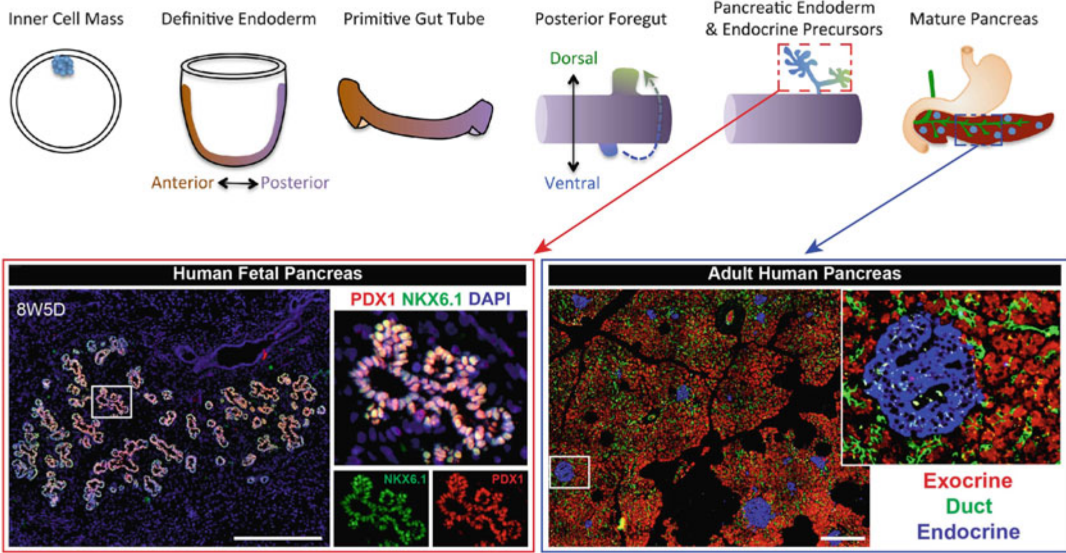


**Fig. 18.1** Summary of the key stages of pancreas development that stem cell biologists have attempted to mimic *in vitro* during the differentiation of human ES cells. Pluripotent cells in the inner cell mass of the embryo develop into three germ layers during gastrulation. The pancreas will eventually arise from the definitive endoderm, which originates from the cells in the primitive streak. As the developing endoderm receives different signaling cues from surrounding tissues, anterior/posterior patterning emerges. The two-dimensional sheet of endoderm cells next forms a primitive three-dimensional gut tube, in which dorsal/ventral patterning becomes evident. The dorsal and ventral pancreatic buds will emerge from the posterior foregut and as the gut tube rotates, the buds eventually fuse to form a single pancreas structure. In the early fetal pancreas, a branching PDX1/NKX6.1 co-positive epithelial network arises, as illustrated in a human fetal pancreas (*red box below*; 8 weeks, 5 days of gestation) by immunofluorescent staining (representative overlay of PDX1, NKX6.1 and nuclei/DAPI, as well as individual PDX1 and NKX6.1 channels are presented). PDX1/NKX6.1 progenitor cells later differentiate into the three main compartments of the pancreas, as illustrated in an adult human pancreas (*blue box below*) by immunofluorescent staining of exocrine (amylase-positive in *red*), endocrine (chromogranin-positive in *blue*) and ductal (CK19-positive in *green*) cells. Scale bars in immunofluorescent images represent 300 μm

ES cells as a potential alternative to cadaveric islets for treating patients with diabetes.

## Introduction

The mature pancreas is composed of three main compartments (endocrine, exocrine and duct) with distinct functions (Fig. 18.1). The bulk of the pancreas is exocrine tissue, containing acinar cells that secrete digestive enzymes into a ductal network that ultimately drains into the duodenum. The endocrine compartment is composed of numerous distinct cell clusters called the islets of Langerhans, which each contain four main cell types, including α-, β-, δ-, and PP cells for secretion of glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. Although the endocrine compartment is only a small proportion of the adult pancreas (~1%), together the islet cells are responsible for maintenance of systemic glucose homeostasis. Hormone secretion from the pancreatic endocrine cells is tightly regulated by numerous physiological stimuli, including local paracrine and autocrine signaling, circulating hormones, the autonomic nervous system and various peripheral nutrient-related signals (e.g., glucose and amino acids) that are delivered to islets by a rich vascular system. For instance, under conditions of high blood glucose (i.e., post-prandial), insulin is released from β-cells into the circulation where it activates

insulin receptor-mediated signaling pathways in peripheral tissues, causing suppression of hepatic glucose output and stimulation of glucose uptake by skeletal muscle and adipose tissue. The first phase of insulin secretion is initiated within minutes of a glucose stimulus and involves the rapid release of primed, pre-docked insulin granules. The second phase of insulin secretion is more prolonged (up to several hours) and involves recruitment of reserve granules stored within β-cells. Overall, only a small percentage of the insulin stores are released in response to a glucose stimulus, and in healthy β-cells this is easily replenished by insulin granule biogenesis. The formation of mature insulin hormone involves translation of a single-chain proinsulin peptide, which is then processed by prohormone convertases (PC1/3 and PC2) and carboxypeptidase (CPE) into equimolar amounts of the mature disulphide-linked insulin along with the cleaved connecting peptide (C-peptide).

Diabetes is a chronic disease with devastating complications and an enormous global impact. In 2011, it was estimated that more than 350 million people worldwide suffered from diabetes (8.3% of adults) and the International Diabetes Federation (IDF) projects that if current trends continue, by 2030 more than 550 million people (one in every ten adults) will have diabetes (www.idf.org). Diabetes is generally characterized by sustained elevation in blood glucose levels resulting from loss of insulin-secreting pancreatic β-cells (Mathis et al. 2001). Patients with type 2 diabetes typically experience an initial phase of hyperinsulinemia, which is a reflection of their β-cells attempting to control glycemia in conditions of increasing peripheral insulin resistance (Rhodes 2005). However, gradual loss of β-cell mass occurs over time and eventually results in hyperglycemia that in approximately one third of cases, requires insulin therapy. Patients with less severe type 2 diabetes are typically treated with anti-diabetic drugs, such as thiazolidinediones (TZDs), Metformin, glucagon-like peptide-1 (GLP-1) mimetics and dipeptidyl peptidase-4 inhibitors, that act to either stimulate insulin secretion or improve insulin action on peripheral tissues. Patients with type 1 diabetes undergo autoimmune destruction of pancreatic β-cells, resulting in severe insulin insufficiency and a requirement for intensive insulin therapy to achieve glucose homeostasis. However, repeated daily insulin injections are a crude and imprecise method for delivering insulin and as such, patients with diabetes remain at risk for dangerous acute episodes of hypoglycemia (Fig. 18.2) and suffer from debilitating chronic health complications as a consequence of chronic hyperglycemia, such as retinopathy, neuropathy, nephropathy and cardiovascular disease.

An elegant solution for treating diabetes would be to simply replace the patient's β-cells, either by stimulating regeneration of endogenous β-cells or transplanting glucose-sensitive β-cells, thus eliminating the need for exogenous insulin therapy. Islet transplantation is a rapid procedure in which a few milliliters of insulin-secreting islets from a cadaveric donor are injected into the portal vein under local anesthesia (Fig. 18.3). Patients are maintained on immunosuppressive drugs to protect the islets from graft rejection, and also recurrent autoimmune attack. The liver has been the most successful site for clinical islet engraftment to date, as it is easily accessible, a major target of insulin action and islets within the liver mimic the secretion of insulin from the pancreas into the portal circulation (Merani and Shapiro 2006). However, islets engrafted within the hepatic vasculature do not experience the relatively high oxygen content that they are accustomed to in the pancreas and as such, there is significant loss of the transplanted islets in the post-transplant period (Merani and Shapiro 2006). There are also concerns that high local insulin levels promote lipid accumulation in surrounding hepatocytes, which can promote islet deterioration (Lee et al. 2007). Despite these challenges, clinical islet transplantation has been demonstrated as a highly effective therapy for diabetes, producing sustained insulin independence or reduced insulin requirements in most patients (Shapiro et al. 2000; Ryan et al. 2001). Moreover, the incidence of life-threatening hypoglycemia is reduced (Fig. 18.2); (Cure et al. 2008)

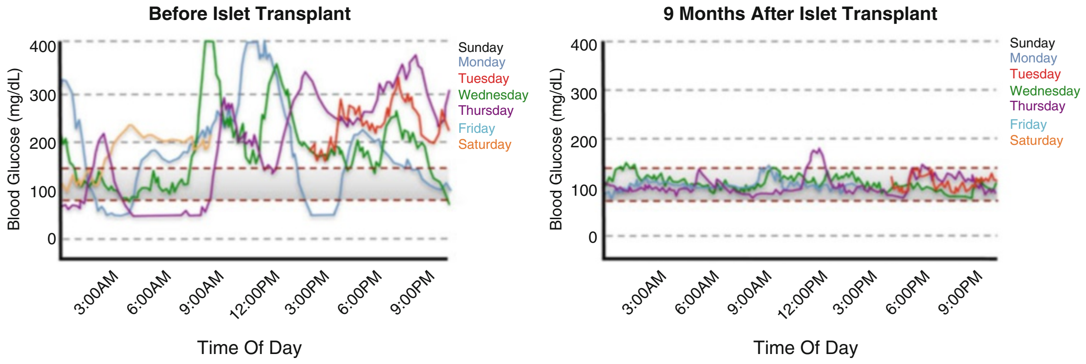


**Fig. 18.2** Continuous blood glucose monitoring from a patient with type 1 diabetes before and 9 months after islet transplantation, demonstrating the efficacy of a cell therapy approach. In particular, note the elimination of hypoglycemic episodes as a result of transplanting glucose-sensitive insulin-producing cells. Clinical data in panel B were kindly provided by Drs. Ron Gill, Alexander Wiseman and Peter Gottlieb at the Colorado Center for Transplantation Care, Research and Education (CCTCARE)

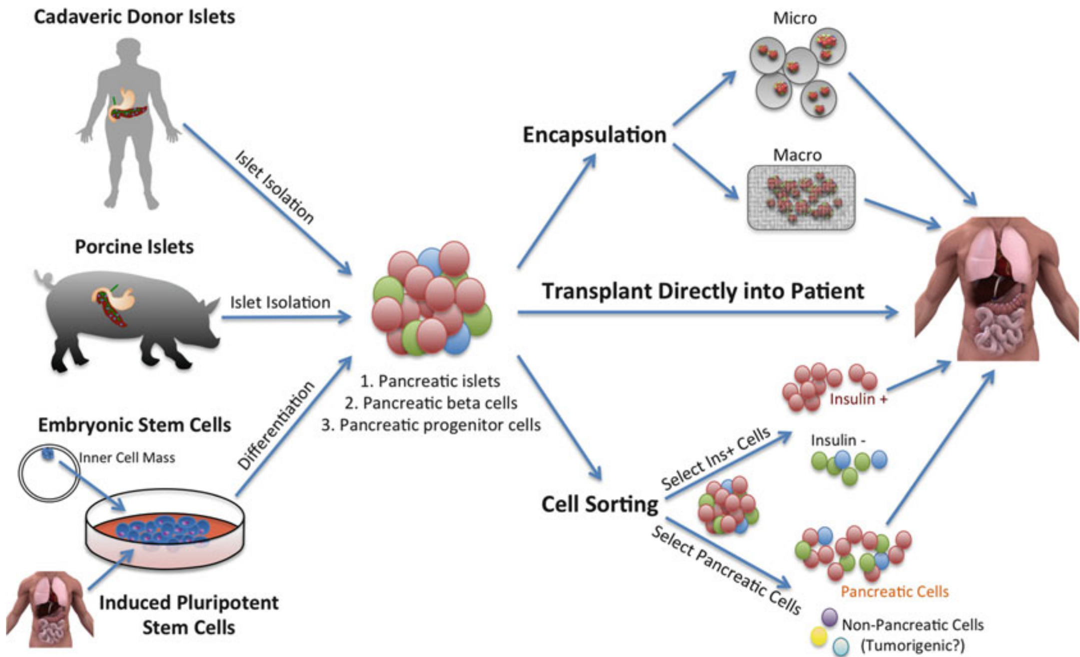


**Fig. 18.3** Possible alternative cell sources for clinical islet transplantation. Currently, islets are isolated and purified from cadaveric human donors and transplanted directly into the portal vein of patients with type 1 diabetes. Potential alternatives to cadaveric islets include isolated porcine islets (fetal, neonatal or adult) and differentiated embryonic or induced pluripotent stem cells. In culture, stem cells may be differentiated into a variety of different endocrine products suitable for transplantation, including whole islets, pure insulin-producing β-cells or pancreatic progenitor cells that would require maturation *in vivo.* Once generated, there are several options for further processing these cells prior to transplantation. Cells can be encapsulated to protect them from the patient's immune system using either microencapsulation (e.g. alginate-based encapsulation of one or more islets per individual bead) or macroencapsulation (e.g. Theracyte$^{TM}$ device encapsulates all transplanted cells in a single pocket) to allow for the passage of signaling molecules, but not cells through pores. Cells may also be sorted prior to transplantation to either select for the cell population of interest (e.g. insulin-positive cells) or to select out the non-pancreatic cells that may contribute to the formation of other lineages and teratomas *in vivo*

and debilitating complications (e.g., nephropathy, retinopathy) can be significantly slowed, even when compared to aggressive insulin therapy (Fung et al. 2007). These remarkable results demonstrate the feasibility of a cell replacement therapy for effectively treating diabetes. Unfortunately there are several roadblocks that prevent widespread adoption of islet transplantation as a clinical therapy, including the shortage of cadaveric islet donors, long-term graft failure over time and the risk of complications associated with chronic immunosuppression. An alternative source of transplantable β-cells could bridge the substantial gap between cell supply and demand for treating diabetes, and also address the concern of graft failure, since repeated engraftments would be an option with an unlimited cell source. However, cultivated β-cells must meet very high safety and efficacy release criteria, with the capacity to store large quantities of insulin and secrete tightly regulated amounts of insulin in response to various secretagogues (e.g., glucose, GLP-1 and arginine). Such cells will have appropriate expression of key transcription factors (e.g., MAFA, NKX6.1 and PDX1), glucose uptake and metabolism machinery (e.g., GLUT1 and glucokinase), depolarization machinery ($K_{ATP}$ and $Ca^{2+}$ channels) and proinsulin processing enzymes (PC1/3, PC2, CPE).

Several promising exogenous sources of glucose-responsive insulin-producing cells have been proposed as potential alternatives to cadaveric donor islets for transplantation, including differentiated embryonic stem (ES) cells or induced pluripotent stem cells (iPSCs) and isolated neonatal or adult porcine islets (Fig. 18.3). In addition, there are numerous conceivable strategies for regenerating endogenous pancreatic β-cells, such as conversion of adult pancreatic cells (e.g., ductal, α- and exocrine cells) and transdifferentiation of various non-pancreatic adult cell types (e.g., liver, umbilical cord blood and gut enteroendocrine cells). This chapter will focus on progress towards the generation of mature, glucose-responsive β-cells from human ES cells and the future challenges that must be overcome before this can be a viable clinical therapy for diabetes.

## Early Efforts to Generate Insulin-Producing Cells from Stem Cells

Initial efforts to produce pancreatic β-cells from ES cells were performed in mouse lines and were based on spontaneous differentiation of ES cell-derived embryoid bodies (EBs) (Soria et al. 2000; Lumelsky et al. 2001; Shiroi et al. 2002). The EB culture method allows for formation of ES cell aggregates in low-attachment plates and is thought to better recapitulate the natural three-dimensional embryonic process of germ-layer formation from the inner cell mass as opposed to the monolayer-format typically used in cell culture. These studies provided valuable evidence that ES cells were indeed capable of generating pancreatic endocrine cells *in vitro* and were a basis from which to pursue further work with human ES cells. Soria et al. (2000) first applied a "cell-trapping" system, which had previously been used with ES cell-derived cardiomyocytes and neural precursors, to select insulin-producing cells from spontaneously differentiating mouse ES cell cultures. In their study mouse ES cells were transfected with a construct containing a neomycin resistance gene under the control of the human insulin promoter for selection of insulin-producing cells (Soria et al. 2000). The insulin-secreting cell clones "trapped" by this system were capable of secreting insulin in response to various secretagogues, including glucose, after a 5-day "maturation" period in low glucose, but no further characterization of these endocrine cells was provided. Interestingly, these cells were capable of temporarily lowering blood glucose levels in diabetic mice following transplantation into the spleen (Soria et al. 2000), although circulating levels of graft-derived insulin were not measured and the engrafted cells were not examined for expression of pancreatic hormones, making these findings difficult to assess. Lumelsky et al. (2001) attempted to improve the spontaneous differentiation of mouse ES cells by adopting a similar strategy to that used for the differentiation of neural cells. Specifically they focused on first

enhancing the production of nestin-positive cells from EBs and introduced the important concept of examining stage-specific markers throughout differentiation, rather than simply examining the end-stage insulin-positive cells (Lumelsky et al. 2001). For instance, they observed the appearance of HNF3β-positive cells that marked definitive endoderm at the end of "Stage 1" and nestin-positive progenitor cells at the end of "Stage 2", which were expanded in later stages by serum withdrawal and addition of B27 supplement/nicotinamide to enhance differentiation of pancreatic endocrine cells in "Stage 5", marked by increased gene expression of insulin (I and II), glucagon, IAPP and GLUT2 (Lumelsky et al. 2001).

These early studies provided sufficient evidence to warrant examining human ES cells for their ability to generate insulin-secreting cells. Assady et al. (2001) demonstrated that human ES cell-derived EBs were capable of spontaneous differentiation into cells that were immunoreactive for insulin and secreted insulin into the media (although not in a glucose-dependent manner). However, the findings from these early studies (Soria et al. 2000; Assady et al. 2001; Lumelsky et al. 2001; Shiroi et al. 2002) were later called into question by subsequent reports that insulin immunoreactivity did not necessarily reflect true insulin biosynthesis within the cells (Rajagopal et al. 2003; Hansson et al. 2004). Rather, the 'differentiated' insulin-positive cells could simply arise from uptake of insulin from the culture medium, such that the genuine differentiation to pancreatic β-cells may have been over-estimated. C-peptide has since been established as a better β-cell marker, as it is not a media supplement, but rather cleaved from the proinsulin precursor protein, stored in the mature insulin granule and co-secreted in equimolar amounts with insulin during exocytosis. It also has a longer half-life in the circulation than insulin (>30 min for C-peptide versus ~3 min for insulin), so it is a useful indicator of insulin secretion (Steiner 2004). The Lumelsky protocol (Lumelsky et al. 2001) has since been applied to human ES cells and reported to produce true insulin-secreting cell clusters, as demonstrated by co-expression of insulin with C-peptide (Segev et al. 2004). Regardless, many groups recognized around this time that spontaneous differentiation of ES cells was unlikely to generate pancreatic β-cells at a sufficient efficiency to be of clinical relevance. Therefore, other strategies were employed with the aim of directing ES cells towards insulin-producing cells.

## Genetic Manipulation to Generate Insulin-Producing Cells

The initial school of thought for guiding pluripotent cells into β-cells was to use genetic manipulation as a means of biasing ES cells towards the pancreatic lineage. Most commonly, a single key pancreatic β-cell gene was introduced into ES cells to produce either constitutive or inducible over-expression throughout the EB-based, spontaneous differentiation culture. Numerous key transcription factors, known to be essential for β-cell development, were examined in this context, including: *Pdx1* (Blyszczuk et al. 2003; Miyazaki et al. 2004; Lavon et al. 2006; Bernardo et al. 2009), *Pax4* (Blyszczuk et al. 2003; Lin et al. 2007; Liew et al. 2008), *Ngn3* (Treff et al. 2006), *Foxa2* (Lavon et al. 2006) and *Nkx2.2* (Shiroi et al. 2005). Initial studies in this area again focused on mouse ES cells (Blyszczuk et al. 2003; Miyazaki et al. 2004; Shiroi et al. 2005; Treff et al. 2006; Lin et al. 2007; Bernardo et al. 2009) and although there were reports of increased expression of pancreatic endocrine-related genes in many studies with transcription factor overexpression, only a few reported enhanced production of insulin-secreting cells relative to spontaneous differentiation (Blyszczuk et al. 2003; Shiroi et al. 2005; Lin et al. 2007).

Regardless, the findings from mouse ES cells encouraged others to apply a similar strategy to human ES cells (Lavon et al. 2006; Liew et al. 2008; Bernardo et al. 2009). *Pdx1* was considered a prime candidate for genetic overexpression, as inactivation of this transcription factor leads to agenesis of the pancreas in both mice (Jonsson et al. 1994) and humans (Stoffers et al. 1997) and thus overexpression may theoretically drive ES cells towards a pancreatic fate *in vitro*. Initially, Lavon et al. (2006) constitutively overexpressed

*Pdx1* and although this resulted in enhanced expression of some pancreatic genes, including somatostatin and pancreatic polypeptide, there was no induction of insulin or glucagon expression. Bernardo et al. (2009) postulated that continuous overexpression of *Pdx1* was likely inappropriate for induction of the pancreatic lineage, given that the timing of gene expression during embryonic development is tightly regulated in a biphasic manner. In mice *Pdx1* expression is initially observed around embryonic day (E) 8.5 in a region of the foregut endoderm that later develops into the pancreatic buds and then is widely expressed throughout the developing pancreatic epithelium until E13.5 (Fig. 18.1); (Jorgensen et al. 2007). After this time, *Pdx1* expression decreases but a second wave of expression is observed in the adult pancreas, restricted to mature β-cells (Jorgensen et al. 2007). Therefore, Bernardo et al. (2009) introduced a tetracycline-inducible *Pdx1* expression construct into human ES cells, and attempted to mimic the biphasic *Pdx1* expression pattern observed during fetal development. As predicted, biphasic induction of *Pdx1* during days 3–6 and again from 11 to 14 of human ES cell differentiation resulted in enhanced gene expression of pancreatic hormones and transcription factors (Bernardo et al. 2009). Although all pancreatic hormones were generally increased with biphasic Pdx1 overexpression, somatostatin expression was most substantially induced (Bernardo et al. 2009), similar to the constitutive overexpression model (Lavon et al. 2006). For that reason, *Pax4* was proposed as an alternative candidate for overexpression, since it is required specifically for the formation of mature β-cells in mice, rather than general formation of pancreatic endocrine cells. Furthermore, it was previously shown in mouse ES cells that constitutive overexpression of *Pax4* enhanced the formation of insulin-positive, but not glucagon-positive cells in EB cultures (Blyszczuk et al. 2003). Liew et al. (2008) demonstrated that constitutive *Pax4* overexpression in human ES cells enhanced *Pdx1* gene expression in mid-stage EBs and insulin expression in late-stage EBs (Liew et al. 2008). Interestingly, this group also demonstrated that *Pax4*-overexpressing cells had an enhanced $Ca^{2+}$ signaling response to KCl stimulation, suggesting that these cells may have improved depolarization machinery, a critical feature of functional β-cells (Liew et al. 2008).

However, despite the reported enhancement of human ES cell differentiation to pancreatic endocrine cells using various genetic modifications, the actual biological relevance of enhancing a spontaneous differentiation protocol was difficult to assess for several reasons. Importantly, the majority of studies did not discuss the efficiency of differentiation to insulin-producing cells, although one study did report that their efficiency increased from 0.1 to 1% with genetic manipulation (Shiroi et al. 2005). These data supported the concept that genetic manipulation could enhance formation of the pancreatic lineage, but also provided clear evidence that this approach would be insufficient for providing clinically relevant numbers of transplantable β-cells for treating patients with diabetes. Furthermore, the insulin-producing cells generated in spontaneous EB cultures were generally inadequately characterized and therefore, their presence controversial. For instance, most studies presented gene expression data, but were lacking evidence of: (a) immunocytochemical staining for protein expression of insulin, C-peptide or other key β-cell markers; (b) C-peptide secretion by differentiated cells in response to appropriate secretagogues; and (c) the presence of insulin granules at the ultrastructural level. Therefore, future work in this field focused on increasing the efficiency of human ES cell differentiation by applying knowledge of pancreatic development and also more rigorously characterizing the end product of the differentiation protocols.

## Generating Insulin-Producing Cells Based on Pancreas Development

In order to successfully direct pluripotent ES cells into functional pancreatic endocrine cells, stem cell biologists next looked to basic developmental biology for knowledge of the signaling pathways involved in pancreatic development. Unfortunately human pancreas development is

poorly understood due to limited access to human fetal tissue, so instead organogenesis was studied in model systems, such as mice (Jorgensen et al. 2007) and zebrafish (Kinkel and Prince 2009). In these models, several key processes in β-cell formation have been identified (Fig. 18.1).

The first important fate decision in early vertebrate development is the process of gastrulation, which begins at approximately E6.5 in mice following the formation of a transient structure called the primitive streak, from which the three germ layers originate, including ectoderm (forms skin and the central nervous system), mesoderm (forms blood, bone and muscle) and endoderm (respiratory and digestive tracts) (Wells and Melton 1999). The endoderm and mesoderm originate from a common cluster of cells called mesendoderm cells that aggregate near the primitive streak node. The fate decision for the formation of endoderm versus mesoderm is controlled by soluble factors that are produced throughout the primitive streak, including fibroblast growth factor (FGF), transforming growth factor β (TGFβ), Wnt growth factor families and retinoic acid (Wells and Melton 1999). In mice, endoderm formation is complete by around E7.5, after which adjacent tissues (early ectoderm and mesoderm) provide specific inductive signals to different regions of the developing endoderm, causing anterior/posterior patterning. For instance, the anterior endoderm receives signals from the adjacent notochord plate and ectoderm, while the posterior endoderm receives signals (such as Nodal) from the lateral mesoderm and primitive streak (Wells and Melton 1999). Next, is the transition from a two-dimensional sheet of endoderm cells at E7.5 into a three-dimensional primitive gut tube by E9.0, with ventral/dorsal patterning in addition to the anterior/posterior axis. Along the anterior-posterior axis of the gut tube are distinct regions (foregut, midgut and hindgut) with developmental potential to form different endodermal organs, including liver, lung, stomach, intestine and pancreas. Mouse pancreas specification occurs at around E8.5 in the posterior foregut and involves independent formation of three primitive pancreatic buds, two in the ventral region (one eventually dominates and the other regresses) and one in the dorsal domain of the foregut. Each developing bud is exposed to distinct signals from their surrounding tissues. For instance, the dorsal bud is in contact with the notochord, which secretes specific growth factors that suppress sonic hedgehog (*Shh*) signaling in the pre-pancreatic endoderm, and the dorsal aorta, which provides endothelial signals for the developing dorsal pancreas (Wells and Melton 1999; Kumar and Melton 2003; Jorgensen et al. 2007). In contrast, the ventral bud is in contact with the developing mesoderm, which provides numerous instructive signals for promoting ventral pancreas development (Kumar and Melton 2003; Jorgensen et al. 2007). Interestingly, the liver is also derived from the same anterior region of the ventral foregut and key signals from the adjacent cardiac mesoderm (including FGFs and BMPs) are thought to be involved in the important fate decision for hepatic versus ventral pancreas formation (Kumar and Melton 2003). At around E12.5 the mouse gut tube rotates and the ventral and dorsal buds fuse, forming a single pancreas structure. In mice, glucagon-positive and insulin/glucagon co-expressing cells are observed beginning at E9.5, during a phase called the "primary transition". Around E13.5 there is rapid and synchronized expansion of the endocrine population during a stage called the "secondary transition", as a result of proliferation and differentiation of PDX1+ endocrine progenitor cells (Jorgensen et al. 2007). During this "secondary transition", between E13.5 and 15.5, all pancreatic endocrine cell types are detectable, the pancreatic ductal network becomes evident and the exocrine compartment also begins to emerge, as indicated by the presence of amylase-positive acinar cells (Jorgensen et al. 2007). The initial endocrine cells that appear in the developing pancreas are scattered, individual cells and only form mature islet structures towards the end of gestation around E18.5 in the mouse (Fig. 18.4). Although the process of pancreas development is largely conserved between species, there are numerous fundamental differences between mouse and human development that must be considered. For instance, pancreas development occurs over a period of several weeks in mice, versus months in humans. Also more subtle, but likely important differences

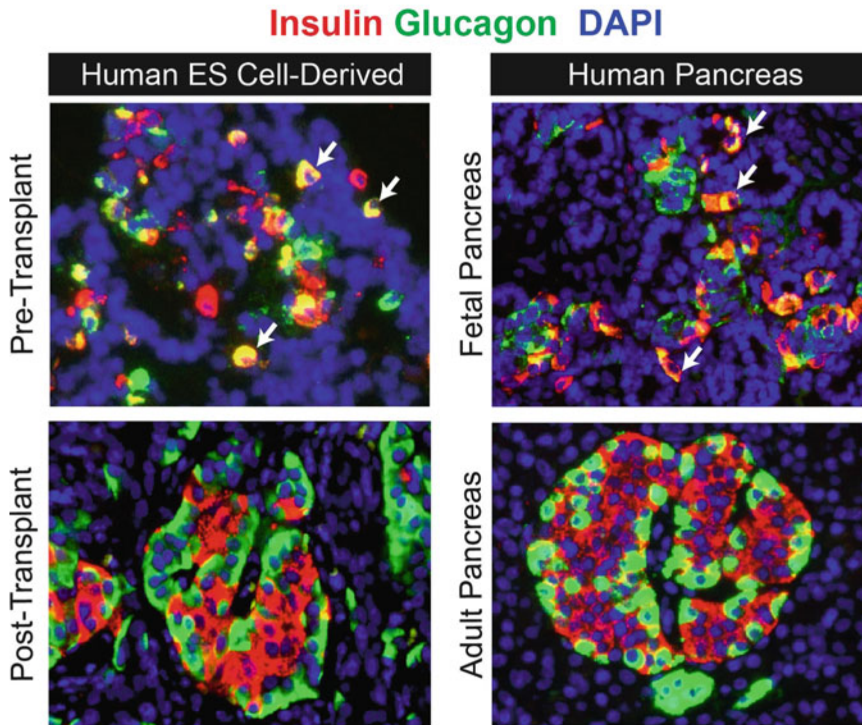


**Fig. 18.4** Human ES cell-derived endocrine cells pre- and post-transplant compared to human fetal (8 week, 5 day) and adult pancreas. Representative immunofluorescent images are provided above; refer to Rezania et al. 2012 for a detailed characterization of human ES cell development and Riedel et al. 2011 for a detailed characterization of human fetal pancreas development. Human ES cell-derived endocrine cells contained numerous insulin/glucagon co-expressing cells (*yellow*, indicated by *white arrows*) prior to transplant, similar to the transient polyhormonal cells observed in human fetal pancreas during early development. Endocrine cells transitioned from scattered, individual cells pre-transplant into islet-like clusters with insulin and glucagon expression in separate cells *in vivo*, similar to the mature adult human pancreas

between species include the order of endocrine cell appearance (insulin-positive cells appear first in humans), architecture of pancreatic islets and relative proportions of endocrine cell types within islets (Jeon et al. 2009; Riedel et al. 2011). The potential consequences of these species differences in islet development and function are still unclear, but may be relevant when translating our knowledge of mouse pancreas development to human ES cell differentiation.

Numerous research groups have effectively applied this knowledge of pancreatic development from model systems to human ES cell culture for the production of pancreatic endocrine cells *in vitro* (D'Amour et al. 2006; Jiang et al. 2007a, b; Shim et al. 2007; Kroon et al. 2008; Chen et al. 2009; Zhang et al. 2009; Cai et al. 2010; Mfopou et al. 2010; Nostro et al. 2011; Xu et al. 2011; Rezania et al. 2012). Novocell Inc. (now ViaCyte Inc.) was the pioneer in this area with their development of novel stepwise differentiation protocols for mimicking pancreatic development in human ES cells without EB formation (D'Amour et al. 2006; Kroon et al. 2008; Kelly et al. 2011). D'Amour et al. (2005) first published a protocol for efficiently differentiating human ES cells into definitive endoderm, the first key fate decision in pancreas development (i.e., gastrulation; Fig. 18.1). In mice, high levels of Nodal signaling specify definitive endoderm, while lower levels specify mesoderm formation; therefore Activin A, a member of the TGFβ family that binds to the same receptors as Nodal, was used to mimic the action of Nodal *in vitro*

(D'Amour et al. 2005). Importantly, D'Amour et al. (2005) not only demonstrated high efficiency with their protocol (>80% cells expressed SOX17, a marker of definitive endoderm), but also temporal gene and protein expression in differentiating human ES cells that corresponded to the transitions observed during vertebrate gastrulation (D'Amour et al. 2005). Nostro et al. (2011) have recently confirmed the importance of generating appropriate progenitor populations at this early stage of differentiation. They carefully demonstrated that the precise timing for induction of Activin/Nodal signaling during the definitive endoderm stage is crucial for downstream development of insulin expression.

Novocell Inc. next published a stepwise 5-stage protocol for mimicking the sequential formation of: (1) definitive endoderm (DE), (2) primitive gut tube, (3) posterior foregut, (4) pancreatic endoderm and endocrine precursor cells and (5) pancreatic hormone-expressing cells (D'Amour et al. 2006). They also carefully characterized each stage of differentiation with several markers of each developing cell population *in vitro*. In the DE stage (marked by *SOX17*, *CXCR4, FOXA2* and *CER*) they improved upon their previous work by addition of Wnt3a for the first day of culture, which increased efficiency of DE specification. The important role of Wnt signaling for promoting the posterior endoderm phenotype in culture and increasing insulin levels at later stages of differentiation was recently confirmed (Nostro et al. 2011). In Stage 2, removal of Activin A was essential for the transition of DE to primitive gut tube (marked by increased expression of *HNF12B* and *HNF4A*). Furthermore, addition of FGF10 increased pancreatic epithelium proliferation and KAAD-cyclopamine inhibited *Shh* signaling, both of which enhanced the downstream generation of insulin-producing cells. During Stage 3, patterning of the gut-tube endoderm cells to a posterior foregut fate was achieved by addition of retinoic acid, along with continued *Shh* inhibition and FGF10 signaling. Retinoid signaling is known to be important for the formation of pancreatic buds and indeed, this was demonstrated by the appearance of *PDX1* expression by the end of Stage 3. These *PDX1*+ posterior foregut endoderm cells were then directed towards the pancreatic and endocrine lineages by the addition of DAPT (Notch pathway inhibitor) and Exendin-4 (GLP-1 mimetic); during Stage 4, co-expression of *PDX1* and *NKX6.1* was indicative of pancreatic epithelium (Fig. 18.1) and the emergence of *NGN3*-positive cells reflected the early stages of commitment to pancreatic endocrine cells. Finally during Stage 5, pancreatic hormone-expressing cells were produced, although these cells resembled immature endocrine cells as they typically expressed multiple hormones and displayed limited responsiveness to glucose (D'Amour et al. 2006). Polyhormonal cells are a transient cell type observed during normal human fetal pancreas development (Fig. 18.4), although their exact role in pancreatic development is an area of controversy (Riedel et al. 2011). In addition, the insulin-positive cells that were produced in Stage 5 did not maintain expression of important β-cell transcription factors, such as PDX1 and NKX6.1 (D'Amour et al. 2006). Regardless, this work was a significant advancement in the human ES cell field, as it reported careful characterization of the differentiating cells at each stage of pancreatic development, including assessment of RNA and protein expression in whole culture homogenates, protein co-expression at the single cell level and ultrastructural morphology by transmission electron microscopy. Furthermore, in addition to examining their cultures for insulin expression, *de novo* insulin synthesis was confirmed by protein expression of C-peptide and pro-insulin (D'Amour et al. 2006).

Several groups have attempted to repeat the original 5-stage Novocell protocol (D'Amour et al. 2006) with other cell lines and either failed to generate pancreatic endocrine cells (Mfopou et al. 2010) or generated insulin-positive cells at very low efficiency (Nostro et al. 2011). This is likely a reflection of the reported variability associated with different human ES cell lines (D'Amour et al. 2006; Osafune et al. 2008; Mfopou et al. 2010; Nostro et al. 2011) and given the limited access to the original Cythera (CYT49) line, it has been challenging to reproduce their *in vitro* findings. Subsequent studies by other groups have instead attempted to improve the efficiency

of this protocol by optimizing specific stages of differentiation. For instance, the formation of *PDX1*+ cells has been considered a key checkpoint during differentiation for assessing the commitment of pluripotent cells towards the pancreas lineage, as opposed to other closely related endodermal tissues, such as liver and intestine. However, studies that have successfully enhanced the efficiency of *PDX1*+ cell formation in culture were not able to demonstrate a substantial increase in the downstream production of insulin-expressing cells as a consequence of enhancing the progenitor pool (Chen et al. 2009; Cai et al. 2010; Mfopou et al. 2010). Nostro et al. (2011) were the first to convincingly enhance the differentiation of insulin-expressing cells beyond the Novocell protocol and they did so by optimizing the early DE population and also by stage-specific inhibition of BMP signaling (Nostro et al. 2011). Furthermore, they demonstrated that different human ES cell lines required variable degrees of BMP inhibition for appropriate commitment to the pancreas lineage. Regardless of the differentiation efficiency, a consistent finding among these and other similar studies was the formation of polyhormonal cells *in vitro*, which represent immature fetal-like endocrine cells rather than mature, glucose-responsive β-cells (D'Amour et al. 2006; Jiang et al. 2007a; Chen et al. 2009; Nostro et al. 2011; Rezania et al. 2011; Xu et al. 2011).

Rezania et al. (2011) recently demonstrated that human ES cell-derived polyhormonal cells were capable of further development into a mature pancreatic endocrine cell type. However, instead of generating glucose-responsive insulin-producing β-cells, this step-wise differentiation protocol converted human ES cells into mature glucagon-secreting pancreatic α-cells, the closest neighbor to pancreatic β-cells. Among several alterations to the Novocell protocol (D'Amour et al. 2006), small molecule screening led to the identification of ALK5 inhibitor II as a potent inducer of insulin and glucagon expression (Rezania et al. 2011). Similar to other studies, this revised differentiation protocol produced primarily polyhormonal cells that co-expressed insulin and glucagon *in vitro*. However, following either extended culture or transplantation, the polyhormonal cells further matured into functional glucagon-secreting α-cells, a finding that has since been confirmed by others (Basford et al. 2011; Kelly et al. 2011). While clearly not the target therapeutic product for treating diabetes, this work represented a significant advance in field. Firstly, the cells arising from the Rezania protocol were the most pure generated to date, with >75% of cultured cells positive for the endocrine marker synaptophysin, >60% positive for glucagon, and cell grafts harvested 4 months post transplant predominantly comprised of glucagon-expressing cells, in the absence of detectable insulin (Rezania et al. 2011). Secondly, this was the first report of a fully differentiated, mature islet endocrine cell type generated *in vitro* from human ES cells and also unlike all previous reports these differentiated cells did not form teratomas following transplantation in mice (Rezania et al. 2011). Furthermore, there is growing evidence from humans and mice that the α-cell and β-cell lineages are closer to each other than previously appreciated. Several high profile publications have recently shown that it is possible to reprogram α-cells to β-cells (Collombat et al. 2009; Liu and Habener 2009; Chung et al. 2010; Lu et al. 2010; Thorel et al. 2010). Therefore, the possibility remains that modifications to this α-cell differentiation protocol (Rezania et al. 2011) may be capable of redirecting human ES cells away from glucagon-producing α-cells and instead towards mature insulin-producing β-cells *in vitro*.

Novocell has also investigated an alternative approach for generating mature β-cells. Rather than attempting to produce mature endocrine cells *in vitro,* they demonstrated that β-cell maturation could be achieved *in vivo* following transplantation of immature human ES cell-derived *PDX1*+ progenitor cells at various sites (subcutaneous, kidney capsule and epididymal fat pads) in immunodeficient mice (Kroon et al. 2008). This strategy had been successfully used with human fetal islets to demonstrate that glucose-responsive insulin-producing cells could be attained after a 12–16 week maturation period under the kidney capsule in nude mice (Hayek and Beattie 1997). Although others had also utilized

this approach with human ES cells, and did report glucose-stimulated insulin secretion *in vivo* (Jiang et al. 2007b; Shim et al. 2007), they also observed the presence of polyhormonal cells (Jiang et al. 2007b) and insulin-positive cells that did not uniformly co-express crucial markers of mature β-cells such as *PDX1*, *NKX6.1* and *MAFA* (Jiang et al. 2007b; Shim et al. 2007). Novocell was the first to provide convincing evidence of β-cell maturity *in vivo*, with glucose-responsive C-peptide secretion and insulin-positive cells that did not co-express other pancreatic hormones but did co-localize with *PDX1*, *NKX6.1*, *MAFA*, C-peptide and pro-hormone processing enzymes (Kroon et al. 2008). Although this study represented an important step forward, it also raised questions about the potential clinical applicability of transplanting a mixed population of immature hESC-derived progenitor cells. First, 15–45% of mice transplanted with pancreatic progenitor cells developed grafts with teratomous elements (Kroon et al. 2008). Secondly, they did not demonstrate maturation of hESC-derived cells in a pre-existing diabetic environment, an important element of any potential cell therapy for diabetes. Instead they showed that once mature, their cells protected mice from hyperglycemia following STZ treatment, a scenario that would not occur clinically. In addition, the *in vivo* development of pancreatic endoderm cells could not be replicated when transplanted in athymic nude rats; only rare islet-like endocrine cells developed and circulating human C-peptide was either undetectable or clinically insufficient and not glucose-regulated (Matveyenko et al. 2010).

In collaboration with an industry partner (BetaLogics), we have recently confirmed the utility of an *in vivo* maturation strategy with a modified version of the 4-stage Novocell protocol (Kroon et al. 2008) that generated a highly enriched population of *PDX1*+ pancreatic progenitor cells from human ES cells (Rezania et al. 2012). Following transplantation under the kidney capsule of immunodeficient mice with pre-existing STZ-induced diabetes, these progenitor cells matured into meal- and glucose-responsive insulin-producing cells that were capable of maintaining normal fasting glucose levels (Rezania et al. 2012). The development of these cells was carefully assessed both *in vitro* and *in vivo* and human ES cells were shown to mature in a manner that was remarkably similar to the human fetal pancreas (Fig. 18.4); (Rezania et al. 2012). For instance, the pattern and timing of endocrine cell clustering in the human ES cell-derived grafts closely paralleled the development of human fetal islets (Fig. 18.4); (Jeon et al. 2009) and the engrafted endocrine cells (insulin+, glucagon+and polyhormonal) expressed the appropriate markers of maturing α- and β-cells that are observed throughout human gestation (Riedel et al. 2011). This study confirmed that human ES cells are indeed capable of maturing *in vivo* into functional pancreatic endocrine cells, although more work is still required to ensure that other cell lineages are excluded from the transplanted cell material.

## Selecting Cells of Interest

To address the ongoing challenge of safely transplanting insulin-producing β-cells derived from human ES cells, a number of groups have proposed cell-sorting strategies to improve the starting material prior to transplantation (Fig. 18.3). This concept has been considered for some time, with even the earliest studies in this field employing a selection strategy. For instance, in their initial experiments with mouse ES cells, Soria et al. (2000) introduced an antibiotic resistance gene under the control of the insulin promoter, in an attempt to select for insulin-secreting clones during spontaneous EB differentiation. In 2004 the same group used a similar "gene-trapping" strategy, but with the *NKX6.1* promoter driving antibiotic resistance, combined with a directed differentiation approach to increase efficiency (Leon-Quinto et al. 2004). Although they claimed that following antibiotic selection, almost 100% of their cells were *PDX1*/*NKX6.1*/insulin-positive, the cells were poorly characterized and therefore this work was difficult to interpret.

More recently, ViaCyte applied a cell selection approach to their previously published protocol for generating pancreatic progenitor cells from human ES cells (Kroon et al. 2008). Kelly

et al. (2011) designed a flow cytometry-based strategy to screen for commercial antibodies that specifically recognized subsets of cells in their mixed population of pancreatic progenitor cells (Kelly et al. 2011). They identified CD200 and CD318 as cell surface markers of endocrine cells (chromogranin-positive subset, largely bihormonal cells) and CD142 as a surface marker of pancreatic endoderm cells (*PDX1/NKX6.1*-positive subset). Using these antibodies, they enriched the human ES cell-derived cultures for each of these subsets of cells prior to transplantation. Interestingly, the CD142-enriched cell population was capable of generating insulin-producing cell clusters *in vivo*, whereas the CD318-enriched hormone-positive population generated mainly glucagon-positive cells *in vivo*. Neither purified cell population was as efficient as the non-sorted, mixed population of endocrine and endoderm cells at developing into functional human C-peptide-secreting cells *in vivo*. This study suggested that both cell populations are required for optimal development of pancreatic endocrine cells *in vivo*, but that the *PDX1/NKX6.1*-expressing pancreatic endoderm subpopulation is likely the source of insulin-secreting cells *in vivo,* rather than the bihormonal insulin/glucagon co-expressing cells generated during the 14-day differentiation protocol (Kelly et al. 2011). Similarly, Basford et al. (2011) used a human ES cell reporter line with the insulin promoter driving GFP expression to purify GFP/insulin-positive cells that were largely polyhormonal (generated with the previously published 'Nostro Protocol', (Nostro et al. 2011)). Following transplantation, the GFP+/polyhormonal cells matured into glucagon-positive, insulin-negative cells *in vivo* (Basford et al. 2011), supporting ViaCyte's cell sorting experiments (Kelly et al. 2011) and also our previous work (Rezania et al. 2011).

## Future Challenges

Numerous challenges remain before human ES cells can be considered as a potential clinical therapy for treating patients with diabetes. First and foremost is the concern of teratoma formation *in vivo*. There are several approaches that may overcome this issue, including: (A) improving differentiation protocols to efficiently eliminate other non-pancreatic cell types from human ES cell-derived cultures; (B) cell sorting to either select for the cell type of interest or select out the non-pancreatic cells prior to transplantation (Fig. 18.3); or (C) introduction of a "suicide gene" that could be used as a safety switch *in vivo*. In this regard, the efficacy of such an inducible suicide gene in patients given a stem cell transplant was recently demonstrated (Di Stasi et al. 2011). A second issue is the requirement for chronic immunosuppression therapy to prevent immune-mediated destruction of transplanted cells. Immunosuppression is associated with an increased risk of wide-ranging toxic side effects and therefore only justified for patients with severely advanced diabetes or those who require immunosuppression for a solid-organ transplant. It is also possible that immunosuppressive therapy may detrimentally impact progenitor cell maturation *in vivo*, but this has yet to be explored. Therefore, the ideal human ES cell therapy approach will address the need for immunosuppression, possibly by implanting either macro- or micro-encapsulated cells to offer protection from the immune system (Fig. 18.3). Notably, "Living Cell Technologies" (http://www.lctglobal.com) is the first company to begin clinical trials testing the therapeutic efficacy of micro-encapsulated porcine islets in humans. Their early work suggesting long-term survival of alginate-encapsulated porcine islets in a type 1 diabetic patient (indicated by detectable urinary porcine c-peptide levels for 11 months post-transplant) supports the feasibility of encapsulation for protecting transplanted cells *in vivo* (Elliott et al. 2007). Alternative strategies for both micro- and macro-encapsulating cells as a way of containing and protecting cells from immune attack have been recently reviewed (O'Sullivan et al. 2011). Finally, human ES cells will eventually need to be cultured and differentiated at a large scale to accommodate the vast numbers of patients who could benefit from this type of cell therapy. These large-scale cultures must be capable of generating pancreatic progenitor and/or endocrine cells consistently and robustly to meet the stringent standards set by federal

regulatory agencies for clinical use (Halme and Kessler 2006). Most importantly, the transplantable cells generated from human ES cells, whether whole islets or purified β-cells, must be demonstrably superior to intensive insulin therapy to justify an experimental cell therapy. These numerous challenges should be tackled collaboratively by both industry and academia, and will require creative teamwork by stem cell biologists in partnership with engineers, immunologists, endocrinologists and developmental biologists. Patients with diabetes are eagerly awaiting what could be stem cell-based relief from their debilitating disease.

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# 19 Transformation of Vascular Endothelial Cells into Multipotent Stem-Like Cells: Role of the Activin-Like Kinase 2 Receptor

Damian Medici and Bjorn R. Olsen

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**Abstract**

Vascular endothelial cells demonstrate remarkable plasticity and differentiate into other cell types during embryonic development and disease progression. This occurs through a process known as endothelial-mesenchymal transition (EndMT) in which endothelial cells acquire properties of mesenchymal stem cells, including multipotency. In this chapter, we will discuss current evidence of EndMT and its contribution to the generation of stem cell phenotype. We will describe the role of EndMT in generating fibroblasts or myofibroblasts in cardiac development, cancer and fibrosis, as well as EndMT-dependent formation of heterotopic bone. Finally, we will discuss the biochemical signaling mechanisms that control EndMT and strategies to inhibit this change in cellular phenotype.


## Introduction

Mesenchymal cells are spindle shaped cells in the stroma of organs and connective tissues (Hay 2005). Epithelial cells have been shown to transform into mesenchymal cells through a mechanism termed epithelial-mesenchymal transition (EMT). EMT is a crucial mediator of embryonic processes such as gastrulation, primitive streak formation, somite dissociation, neural crest cell migration, as well as palate and lip development (Acloque et al. 2009). EMT is also an essential component of fibroblast formation


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during organ fibrosis and wound healing (Kalluri and Weinberg 2009). In cancer, EMT serves as the primary mechanism of metastasis by allowing carcinoma cells to detach from the primary tumor and invade the underlying extracellular matrix (Thiery 2003).

Endothelial cells compose the inner lining of blood vessels. Mural cells such as pericytes and smooth muscle cells wrap around the endothelial tube to provide structure and support during vascular morphogenesis and control constriction of the vessels (Armulik et al. 2005). The endothelium is similar to squamous epithelium in cell shape and polarity. Endothelial cells have been shown to undergo a process similar to EMT called endothelial-mesenchymal transition (EndMT). Like EMT, EndMT plays a critical role in embryonic development, tissue repair and disease (Potenta et al. 2008).

## Endothelial-Mesenchymal Transition (EndMT)

EndMT is characterized by a loss of cell-cell adhesion and change in cell morphology. During EndMT, cells lose apical-basal polarity and reorganize their cytoskeleton to become elongated, spindle-shaped cells. This is correlated with reduced expression of endothelial markers such as VE-cadherin, CD31, TIE1, von Willebrand factor (vWF), and claudin-5, followed by increased expression of mesenchymal markers such as fibroblast specific protein-1 (FSP-1), vimentin, alpha-smooth muscle actin (α-SMA), and N-cadherin (Potenta et al. 2008). These newly formed mesenchymal cells become highly migratory and invade the underlying extracellular matrix by secreting matrix metalloproteinases (MMPs) and laying down a track of fibronectin as they migrate along (Kalluri and Weinberg 2009).

EndMT was originally described as an embryonic mechanism necessary for cardiac development (Markwald et al. 1975). Endothelial cells in the atrioventricular canal and outflow tract undergo EndMT and invade surrounding tissues to form the septa and valves of the heart (Mercado-Pimentel and Runyan 2007). EndMT has also been shown to be associated with human disease. Many of the fibroblasts formed during cardiac, kidney, or pulmonary fibrosis have been shown to be of endothelial origin and arise via EndMT (Zeisberg et al. 2007a, 2008; Hashimoto et al. 2010). In cancer, EndMT has been found to give rise to cancer-associated fibroblasts (CAFs) in the tumor microenvironment where they may play an important role in regulating tumor growth and metastasis (Zeisberg et al. 2007b).

## Endothelial-Mesenchymal Transition Generates a Stem Cell Phenotype

EndMT was traditionally considered to represent a transformation from an endothelial cell to a fibroblast. However, recent evidence suggests that the process might rather be dedifferentiation to a mesenchymal stem cell (Medici et al. 2010). Endothelial cells that undergo EndMT have been shown to express biomarkers found in bone marrow-derived mesenchymal stem cells such as STRO-1, CD10, CD44, CD71, CD90, and CD117. The cells also acquire multipotent differentiation capabilities. Endothelial cells induced to undergo EndMT with growth factors such as transforming growth factor-beta 2 (TGF-β2) or bone morphogenetic protein 4 (BMP4) followed by exposure to osteogenic, chondrogenic or adipogenic culture medium showed successful differentiation into osteoblasts, chondrocytes or adipocytes, respectively. Furthermore, endothelial cells stimulated with these factors in polylactic acid scaffolds subcutaneously implanted in mice showed formation of endothelial-derived bone, cartilage, and fat in the scaffolds (Medici et al. 2010).

EndMT has already been shown to form lesional fibroblasts and myofibroblasts in diseases that cause organ fibrosis (Zeisberg et al. 2007a, 2008; Hashimoto et al. 2010). Additionally, studies have suggested that mural cells (pericytes and smooth muscle cells) might arise by EndMT (Armulik et al. 2005; Potenta et al. 2008). Circulating endothelial progenitor cells have been shown to undergo EndMT and give rise to smooth muscle cell progeny (Moonen et al. 2010). This may be an important mechanism that regulates vasculogenesis.

Similar to EndMT, EMT has been described to give rise to a stem cell phenotype; in particular, the generation of cancer stem cells (Gupta et al. 2009). Mammary epithelium and breast cancer cells induced to undergo EMT express the stem cell marker CD44 and form mammospheres in culture (Mani et al. 2008). Mammary epithelium was also successfully differentiated into osteoblasts, chondrocytes or adipocytes after EMT (Battula et al. 2010). These data suggest that EMT or EndMT might represent a dedifferentiation to mesenchymal stem cells, rather than a direct transformation to fibroblasts.

## Endothelial-Mesenchymal Transition and Heterotopic Ossification

*In vivo* evidence of the EndMT-induced stem cell phenotype has recently been discovered in a rare bone disease called fibrodysplasia ossificans progressiva (FOP) (Medici et al. 2010). In patients with FOP, acute inflammation triggers heterotopic ossification of soft tissues, particularly muscles, tendons and ligaments (Kaplan et al. 2008). In the lesions, a mesenchymal condensation occurs followed by chondrogenesis and endochondral ossification, similar to normal developmental bone formation (Shore and Kaplan 2008). Immuno-histochemical analysis of heterotopic cartilage and bone tissue showed that chondrocytes and osteoblasts in the lesions express the endothelial biomarkers TIE2 and vWF. Furthermore, lineage tracing using Tie2-Cre reporter mice showed that lesional bone and cartilage cells express the enhanced green fluorescent protein (EGFP) reporter, strongly suggesting an endothelial origin of these cells (Medici et al. 2010).

FOP patients carry a heterozygous germ-line mutation (R206H) in a TGF-β/BMP type I receptor called Activin-like Kinase 2 (ALK2) (Shore et al. 2006). R206H is a gain of function mutation that causes ALK2 to be constitutively active. Constitutive phosphorylation of ALK2 and its downstream Smad proteins have been shown with expression of the mutant receptor, independent of ligands (Shen et al. 2009). Expressing mutant (R206H) ALK2 in normal vascular endothelial cells in culture induced EndMT and acquisition of stem cell characteristics. Endothelial cells expressing mutant ALK2 could be successfully coaxed to differentiate into chondrocytes and osteoblasts *in vitro* and *in vivo*. Furthermore, the mesenchymal cells condensing in the early lesions of heterotopic ossification prior to chondrogenesis or osteogenesis in Tie2-Cre reporter mice showed evidence of being of endothelial origin, suggesting that EndMT plays a role in this pathological process (Medici et al. 2010) (Fig. 19.1).

Interestingly, in a study of tumor calcifications, bone-forming cells found in prostate carcinomas expressed the endothelial biomarker CD31, suggesting that they could be of endothelial origin (Dudley et al. 2008). EndMT causes formation of cancer-associated fibroblasts (Zeisberg et al. 2007a), so it is conceivable that EndMT might give rise to osteoblasts that induce calcifications in tumors.

## Endothelial-Mesenchymal Transition Signaling

TGF-β/BMP signaling is the most prominent mechanism that controls EndMT, as confirmed by activating mutations in the ALK2 receptor seen in FOP (Medici et al. 2010). Genetic knockout of ALK2 in mice causes severe heart defects as a result of a lack of EndMT (Wang et al. 2005). Interestingly, when ALK2 is activated by mutation or by ligands, there is an interaction of ALK2 with ALK5. Inhibition of either ALK2 or ALK5 with siRNA is sufficient to inhibit EndMT of cultured endothelial cells (Medici et al. 2010). Furthermore, conditional knockout of the ALK5 gene in endothelial cells is sufficient to inhibit EndMT during heart development (Sridurongrit et al. 2008).

Along with the TGF-β/BMP type I receptors ALK2 and ALK5, the TGF-β receptor 2 (TβRII) and BMP receptor 2 (BMPR2) have been shown to have essential roles in cardiac EndMT (Delot et al. 2003; Mercado-Pimentel and Runyan 2007). Also, both known TGF-β type III receptors called β-glycan and endoglin have been shown to be crucial for EndMT in heart development (Mercado-Pimentel and Runyan 2007).

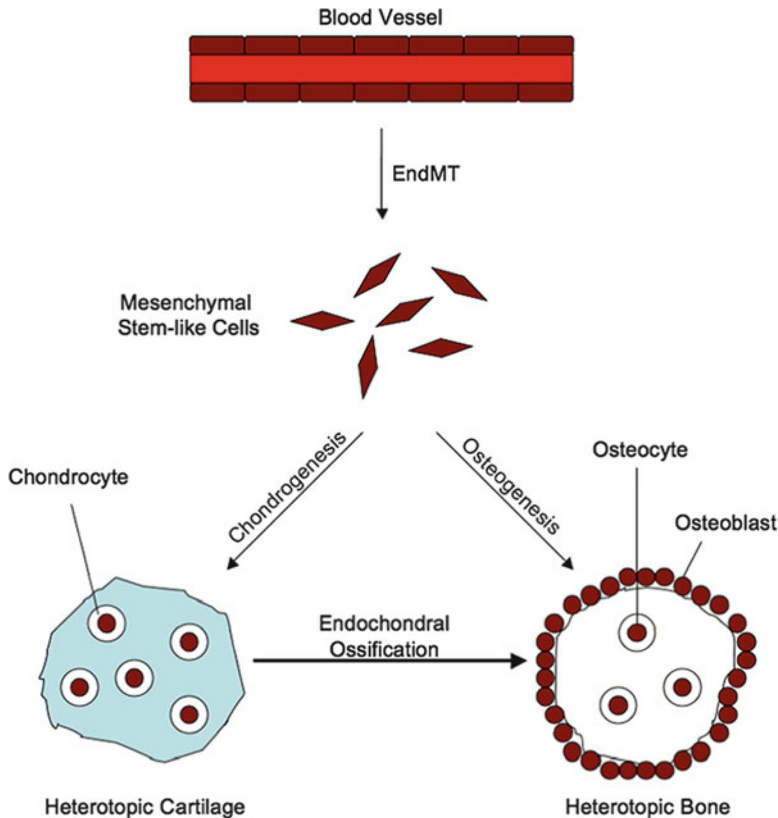


**Fig. 19.1** A schematic overview of the mechanism of heterotopic ossification. Capillary endothelium in soft tissues can be stimulated to undergo EndMT by activation of the ALK2 receptor by ligand activation or mutation. These newly formed stem-like cells can be differentiated into chondrocytes and osteoblasts. Cartilage forms initially, followed by remodeling by endochondral ossification

The signaling ligands BMP2, BMP4 and TGF-β2 have been identified to activate ALK2 (Chen et al. 2004; Medici et al. 2010). Genetic knockout of any of these ligands in mice is sufficient to block embryonic EndMT (Ma et al. 2005; McCulley et al. 2008; Azhar et al. 2009). Interestingly, TGF-β1 and TGF-β3, both of which have been described to induce EMT, have no effect on embryonic EndMT based on the results of genetic ablation (Azhar et al. 2009), suggesting ligand specificity for this process.

The downstream signaling pathways from ALK2 and ALK5 identified for EndMT include Smad-dependent and Smad-independent pathways. Activation of both Smad1/5/8 and Smad2/3 signaling pathways was observed (Medici et al. 2010), as well as the phosphoinositide-3 kinase (PI3K), MEK and p38 mitogen activated protein kinase (MAPK) pathways (Medici et al. 2011). All of these pathways have been linked to expression of an EMT/EndMT-inducing transcription factor called Snail (Medici et al. 2011) (Fig. 19.2).

Various transcription factors that inhibit expression of cell adhesion genes are up-regulated during EndMT, including Snail, Slug, SIP-1, ZEB-1, LEF-1, and Twist (Medici et al. 2010). Currently, only Snail has been shown to be necessary for EndMT by knocking down its expression with siRNA (Kokudo et al. 2008; Medici et al. 2010), but these other factors might also be involved. Over-expression of Snail was shown to be insufficient to induce EndMT (Medici et al. 2011). However, blocking the function of the Snail inhibitor glycogen synthase kinase-3 beta (GSK-3β) (Zhou et al. 2004), which typically occurs through the PI3K or Wnt pathways (Cantley 2002;

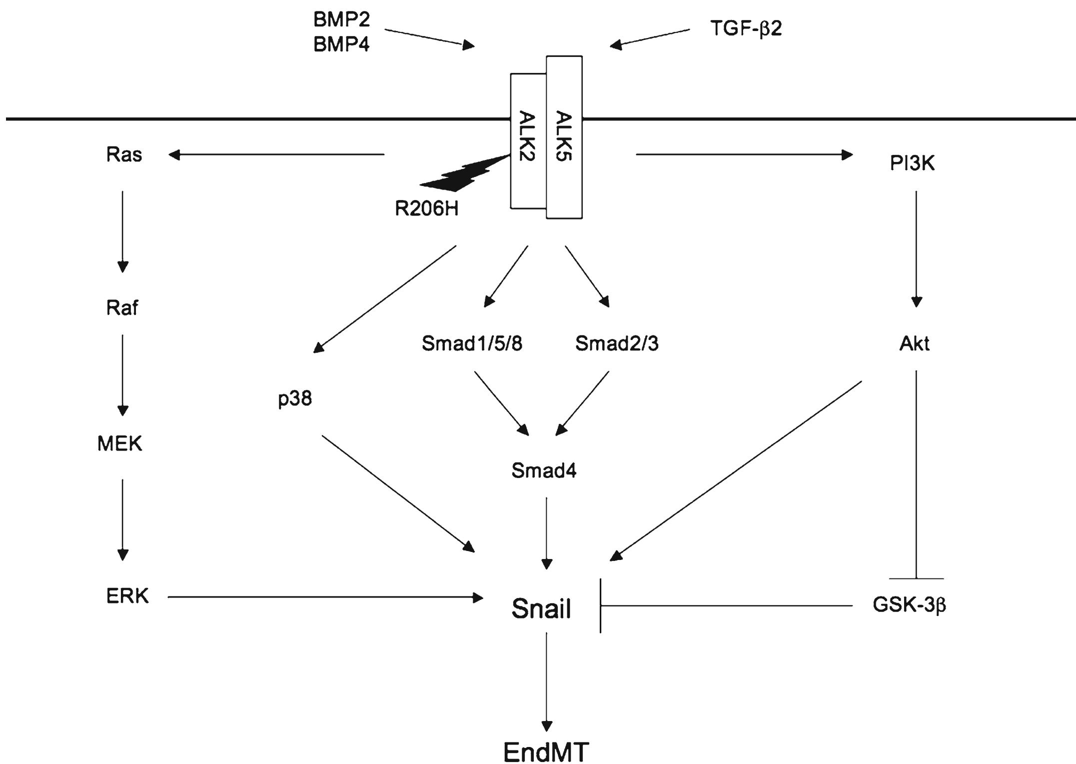


**Fig. 19.2** A diagram of signal transduction pathways that induce EndMT. Activation of the ALK2 receptor by the R206H mutation or by ligands such as BMP2, BMP4 or TGF-β2, cause binding and activation of ALK5. This receptor complex signals through Smad-dependent and Smad-independent pathways that synergistically increase the expression and activity of the EndMT-inducing transcription factor Snail

Logan and Nusse 2004), was sufficient to allow EndMT to be induced by Snail over-expression (Medici et al. 2011). Embryonic EndMT is regulated by Wnt signaling (Liebner et al. 2004), as well as other pathways such as Notch (Chang et al. 2011), yet their roles are not as clearly defined as that of TGF-β/BMP signaling.

## Therapeutic Targeting of Endothelial-Mesenchymal Transition

Inhibiting EndMT should have dramatic implications for reducing the incidence of heterotopic ossification, fibrotic disease, and cancer progression. Therefore, targeting EndMT signaling molecules (Table 19.1) should be a priority for the treatment of such diseases. ALK2, the signaling receptor activated by EndMT-inducing ligands and mutated in patients with FOP, can be inhibited by drugs such as dorsomorphin or LDN-193189 (Yu et al. 2008; Medici et al. 2010). Dorsomorphin has been shown to prevent TGF-β2-induced EndMT (Medici et al. 2010). LDN-193189, although not yet shown to directly inhibit EndMT, has been found to prevent heterotopic bone formation in a transgenic mouse model of FOP with constitutively active mutant ALK2 (Yu et al. 2008).

ALK5 associates with ALK2 during EndMT (Medici et al. 2010), and a chemical inhibitor against ALK5 known as SB-431542 has been successfully used to block EndMT (Moonen et al. 2010). Furthermore, siRNA-mediated knockdown of ALK5 or ALK2 expression prevents TGF-β2- or BMP4-induced EndMT (Medici et al. 2010). Neutralizing antibodies specific for these receptors, or the ligands BMP2, BMP4 or TGF-β2

**Table 19.1** EndMT inhibitors and their targets

| Inhibitors | Targets |
|---|---|
| Dorsomorphin | Activin-Like Kinase 2 (ALK2) |
| LDN-193189 | Activin-Like Kinase 2 (ALK2) |
| ALK2 siRNA | Activin-Like Kinase 2 (ALK2) |
| SB-431542 | Activin-Like Kinase 5 (ALK5) |
| ALK5 siRNA | Activin-Like Kinase 5 (ALK5) |
| LY294002 | Phosphoinositide-3-Kinase (PI3K) |
| U0126 | MEK1/2 |
| SB-202190 | p38 MAPK |
| SIS3 | Smad3 |
| Dominant negative Smad4 | Smad4 |
| Snail siRNA | Snail |
| Bone Morphogenetic Protein 7 (BMP7) | BMP receptors |
| Vascular Endothelial Growth Factor (VEGF) | VEGF receptors |

might also prove beneficial in therapeutic strategies to prevent EndMT.

Targeted inhibition of signaling molecules downstream of TGF-β/BMP receptors has also been successful in preventing EndMT. For instance, chemical inhibitors of PI3K (LY294002), MEK (U0126), and p38 MAPK (SB-202190) were all successful in inhibiting EndMT in cultured endothelial cells (Medici et al. 2011). Expression of a dominant negative Smad4 protein was also able to block TGF-β2-induced EndMT in culture (Medici et al. 2011). In a mouse model of diabetic nephropathy, a chemical inhibitor of Smad3 (SIS3) successfully inhibited EndMT-induced renal fibrosis (Li et al. 2010). siRNA-mediated knockdown of the transcription factor Snail was also sufficient to block EndMT in endothelial cell cultures (Kokudo et al. 2008; Medici et al. 2010).

BMP7 is a known activating ligand of ALK2 (Chen et al. 2004), yet it is also known to be a potent inhibitor of EndMT. Recombinant BMP7 has been shown to block EndMT in culture and in a mouse model of cardiac fibrosis (Zeisberg et al. 2007b). A signaling protein that increases blood vessel growth called vascular endothelial growth factor (VEGF) has also been shown to inhibit EndMT (Paruchuri et al. 2006; Medici et al. 2010). The signaling mechanisms spawned from BMP7 or VEGF signaling that directly perturb EndMT are currently unknown, but recombinant BMP7 and VEGF could be of potential therapeutic use to inhibit EndMT-related diseases.

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# Part V

# Treatment

# 20 Colon Cancer Stem Cells: A Therapeutic Target

Sanchita Roy and Adhip P.N. Majumdar

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**Abstract**

Cancer stem cells (CSCs), that are resistant to chemotherapy, are the major obstacle in treatment of different cancers including colorectal cancer. Recently, markers of CSCs have been identified. CD133, CD44, CD166, ALDH1A1, Lgr5, and several other proteins have been proposed as CSC markers. In this review article, we have discussed several therapeutic strategies that may target the self-renewal pathways, miRNAs and other epigenetic modifiers in colon CSCs to arrest and/or regress recurrent colon cancer.

## Overview of Colorectal Cancer

Colorectal cancer, commonly known as bowel cancer, is a cancer of the colon, rectum, or vermiform appendix. It is the third most common cancer in women and the fourth in men taking 610,000 lives per year worldwide (American Cancer Society 2011). About 72% of the disease arises in the colon and 28% in the rectum. The metastasis of this cancer into the liver is the primary cause of death (Welch and Donaldson 1979). The American Cancer Society has reported 101,340 new cases of colon and 39,870 cases rectal cancers and 49,380 deaths from this disease in year 2011 in the US.

Colorectal cancer evolving from the glandular tissue (Stewart et al. 2006) begins as polyp, the benign growth in the mucosa. Two most common types of polyp are hyperplastic and adenomatous

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polyps. Chances of hyperplastic polyps to become cancerous are very low. Adenomatous polyps are mushroom shaped structures usually grow on a stalk and approximately 5% of these polyps turn into cancer, which could be classified as follows:

Stage 0 – the cancer cells are localized only in mucosa

Stage 1 – the cancer cells are localized in mucosa and invaded submucosa

Stage 2 – Cancer has spread through the muscularis externa to the serosa

Stage 3 – the cancer is spread to the lymph glands on the outside of the colon.

Stage 4 – the cancer has reached other organ/ metastasis

Despite recent advances in cancer treatment, nearly 50% of patients with colorectal cancer show tumor recurrence. Although the underlying cause is not fully understood, one of the reasons for tumor recurrence is thought to be the presence of chemotherapy-resistant cancer stem (CSCs) that retain limitless potential to regenerate (Dean et al. 2005).

## Cancer Stem Cells

The CSC hypothesis was first proposed by German pathologist Rudolf Virchow in 1855. He anticipated that cancer does not arise spontaneously; rather appears on activation of dormant, embryonic-like cancerous cells present in mature tissue (Virchow 1855). Several decades after Virchow, in 1994 Lapidot and coworkers evidentially proved CSC hypothesis. In an experiment, human acute myeloid lymphoma cells which bear the stem cell characters are able to produce leukemia in immune compromised mice. This observation led others to look for CSCs in solid tumors. O'Brien et al. (2007) and Ricci-Vitiani et al. (2007) individually were the first to discover colon CSCs. Ricci-Vitiani et al. (2007) showed that ~2.5% of the tumorigenic cells in colon cancer expressed high CD133. Unlike $CD133^-$ cells, subcutaneous injection of $CD133^+$ colon cancer cells produced tumor in immunodeficient mice. Serial transplantation of such tumor in several generations reproduced tumor without any phenotypic alteration. Moreover, these cells can grow in serum free media *in vitro* with the same antigenic characteristic as found in the original tumor (Ricci-Vitiani et al. 2007). O'Brien et al. (2007) also purified and transplanted $CD133^+$ human colon cancer-initiating cells (CC-IC) into renal capsule of immunodeficient mice to produce tumor. Using limiting dilution analysis it was observed that while $1/5.73 \times 10^4$ unfractionated tumor cells produced tumor in immunodeficient mice, 1/262 of CC-IC in $CD133^+$ cells formed tumor (O'Brien et al. 2007). However, recently, several other markers have been identified for colon CSCs.

CSCs bear the characteristics of normal adult stem cells including self-renewal and multipotency for differentiation into a particular type. Identification and characterization of CSCs remain a technical challenge. Indeed, use of putative stem cell markers to isolate unique subsets of CSC has been employed in recent years.

## Chemotherapy Resistance in Cancer Stem Cells

Chemotherapy is the crucial modality over surgery and radiotherapy for the treatment of solid tumors. However, the resistance to anticancer drugs is still the major hurdle of cancer treatment. Different mechanisms are proposed to be involved in chemotherapy resistances, which are described below.

### ABC Transporters and Multi-drug Resistance

Multi-drug resistance (MDR) limits the effectiveness of chemotherapy. Adenosine tripho-sphate-binding cassette (ABC) transporters, an evolutionary conserved membrane protein can excrete (efflux) toxins from cells utilizing ATP in the liver, kidneys and gastrointestinal tract. ABC transporters can also act as a filter for toxins towards vital organs like brain, placenta and testes. ABC transporters exert chemoresistance by effluxing drugs out of tumor cells consequently decreasing the optimal

intracellular drug concentrations and protecting cells from drug damage. To date, >40 ABC transporter genes have been discovered and are classified into eight subfamilies. A study of more than 400 tumor specimens of colon, renal, adrenal, liver and pancreas, showed that patients with increased levels of MDR1 RNA tended to be more resistant to chemotherapy. CSCs are drug resistant, because they express high levels of ABC transporter proteins. CSCs are known to remain quiescent in the G0 phase over a period of time and following a death stimulus they regenerate tumor cells with chemoresistant characteristic (Prabhudesai et al. 2007).

## Inactivation of Apoptosis

The inactivation of apoptosis is crucial for development of cancer. As apoptosis is the ultimate goal of drug-induced cancer cell death, deregulation of apoptosis may lead the cancer cell to chemoresistance. The apoptosis is conducted by the tumor-suppressor protein p53. Upon DNA damage, oxidative stress or proliferating signals, p53 induces the affected cells to undergo cell cycle arrest (checkpoint function) and/or apoptosis. Most human cancers have either mutations in p53 or defects in p53 regulatory pathways. The common cancer therapies are DNA-damaging agents; hence the cancer cells with impaired apoptotic pathway (p53 mutation or over-expression of anti apoptotic protein BCL-2) are likely to prevent drug-induced apoptosis (Prabhudesai et al. 2007).

## Chemoresistance by miRNAs

microRNAs (miRNA) are evolutionarily conserved, 20–25 nucleotide long, noncoding RNAs that bind to their targets through partial complementary sequence recognition. This results in either degradation of mRNA or inhibition of translation, thus modulating expression of miRNA targets (5). Several hundred miRNAs have been identified in human cells (6). It is estimated that a single miRNA can regulate hundreds of targets, and ≥30% of human mRNAs are regulated by miRNAs (5,6) Therefore, it is not surprising that miRNAs are involved in diverse biological processes, including cell differentiation, proliferation, apoptosis and chemoresistance presumably through a myriad of targets (Kloosterman and Plasterk 2006). Overexpression of miR-140 is reported to inhibit proliferation of colon cancer HCT 116 (wt-p53) cell lines, but less in colon cancer HCT 116 (null-p53) cell lines. Histone deacetylase 4 (HDAC4) was also reported as a target of miR-140. The expression of endogenous miR-140 was significantly elevated in $CD133^{+hi}$ $CD44^{+hi}$ colon cancer stem cells which exhibit slow proliferating rate and chemoresistance. Chemotherapy-resistant colon CSCs became sensitive to 5-FU treatment when endogenous miR-140 was blocked by locked nucleic acid (LNA) modified anti-miR. Taken together, the results suggest that miR-140 is involved in chemoresistance by inhibiting cell proliferation via G1 and G2 phase arrest mediated in part, through the suppression of HDAC4.

## Markers of Colon Cancer Stem Cells

Colon CSCs are currently identified by specific surface epitopes. Recent studies suggest that cells expressing CD44, CD166 and EpCAM/ESA surface markers derived from both CD133+ and CD133− colon cancers have the ability to form tumors. More recently, aldehyde dehydrogenase 1 (ALDH-1), a detoxifying enzyme that oxidizes intracellular aldehydes, has been identified as a specific marker for normal and malignant human colonic stem cells. ALDH1-positive cells, which are sparse and limited to the normal crypt bottom where stem cells reside, increase with progression of normal epithelium to adenoma and carcinoma (Sanders and Majumdar 2011). We have demonstrated that the number of ALDH1-positive cells in the colonic crypt of aged rats increases in response to colonic carcinogen dimethylhydrazine (Sanders and Majumdar 2011). In addition, DCAMKL1, Mushashi-1 and LGR5 have been proposed as makers for both normal and CSLCs in the colon (Levi et al. 2009).

*Wnt* target gene Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), the 7-transmembrane receptor has also been proposed as a promising adult stem cell marker using lineage-tracking experiments.

Doublecortin and CaM kinase-like-1 (DCAMKL-1) have been identified as a putative colonic SC marker. Doublecortin (DCX) is a microtubule-associated protein required for neuronal migration to the cerebral cortex. DCAMKL1 consists of an N terminus with 65% homology to DCX and an additional 360 amino acid C-terminal domain encoding a putative $Ca^{2+}$/calmodulin-dependent protein kinase. Several colon CSC markers are described below in more details.

## CD166

Activated leukocyte Cell adhesion molecule (ALCAM) or CD166, a member of the immunoglobulin super family, is a type 1 transmembrane protein and a ligand for CD6. The extracellular domain of ALCAM contains five Ig-like domains (three Ig-like C2-type domains and two Ig-like V-type domains) of which the amino-terminal V1 domain is essential for ligand binding and ALCAM-mediated cell aggregation. CD166 is expressed on the surface of mesenchymal stem cells and is involved in homotypic and heterotypic adhesion. In 2007, CD166 was proposed as a CSC marker (Dalerba et al. 2007), but its function in normal colon and colon CSC is not fully understood. The choice of CD166 as a marker for CSCs was based on the heterogeneous expression of CD166 in colon carcinoma in combination with the finding that its expression correlates with a shortened patient survival times (Weichert et al. 2004).

## CD44

The CD44, the receptor for hyaluronic acid, is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. It has also been shown to interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). In humans, expression of CD44 is restricted in the crypt base where stem cells reside. Designation of CD44 as colon CSC marker comes from the experiment where SCID mice injected with $CD44^+$ but not $CD44^-$ human colon tumor cells developed tumors. The expression of CD44 has been found to be higher in normal appearing mucosa from patient with adenomatous polyp, where expression directly correlates with age and number of polyps (Patel et al. 2009). In colon cancer cells, Tcf4/β-catenine activates CD44 expression. Increased CD44 expression leads to K-ras, p53 gene modification (Sanders and Majumdar 2011).

## Aldehyde Dehydrogenase 1A1 (ALDH-1A1) and Aldehyde Dehydrogenase 1B1 (ALDH-1B1)

Aldehyde hydrogenases, the tetrameric, NAD (P)+-dependent enzymes composed of 54 kDA subunits. They are responsible for oxidation of endogenous and exogenous aldehydes to corresponding acids. There are three different classes of these enzymes in mammals: class 1 (low $K_m$, cytosolic), class 2 (low $K_m$, mitochondrial), and class 3 (high $K_m$, such as those expressed in tumors, stomach, and cornea). In all three classes, constitutive and inducible forms exist. The high levels of ALDH activity in stem cells have been attributed to ALDH-1A1 expression. ALDH-1A1 is important for multiple biological activities including drug resistance, cell differentiation, and oxidative stress response. High ALDH activity is proposed as a general marker for normal and malignant stem cells. The expression of ALDH-1A1 was found in very few cells of normal human crypts, compared what has been noted for CD44 and CD133. The ALDH1A1 expressing cells were subsequently shown to be the subset of CD44 and CD133 positive cells. However, the expression of ALDH-1A1 is reported to be increased by fivefold in adenomatous crypts. Using flow cytometry based assay purification of viable cells with high ALDH activity has become relatively easy. These observations make ALDH-1A1 as a potent marker (Sanders and Majumdar 2011).

A recent study showed that the ALDH-1B1 was 5.6-fold higher in cancerous tissues than ALDH-1A1. Out of 40, 39 colonic cancer samples have been shown to be positive for ALDH-1B1. The enhanced expression of ALDH-1B1 in the colon adenocarcinoma may have clinical implications and also, suggest this to be a promising biomarker for colon cancer (Chen et al. 2011).

## CD133

CD133 (prominin-1) is an 865 amino acids, 5-transmembrane glycoprotein expressed on the apical plasma membrane protrusions of embryonical epithelial structures surface. CSC forms a variety of tissue including brain, gut, pancreas have been isolated using CD133 as a marker but some differentiated cells like acinar and islet cells in the pancreas, and goblet and columnar epithelial cells lining the colon is shown to be CD133 positive. CD133 is localized in apical/endoluminal membrane of well polarized, differentiated cells, whereas cytoplasmic CD133 is found in cancer cells as evident from CD133 staining. O'Brien et al. (2007) and Ricci-Vitiani et al. (2007) reported low level of expression of CD133 in normal colon tissue, indicating that $CD133^{+}$ cells in cancer samples might result from oncogenic transformation of normal cancer stem cells. However, CD133 has limitations as a CSC marker. It is difficult to fractionate CSC using CD133 because most CD133 antibodies recognize glycosylation-dependent epitopes which vary with the differentiation and transformation status of the cell. In 94% of the cases the level of CD133 expression is found to be the same in both primary colon tumor and metastases. Post-chemoradiotherapy (CRT) residual rectal tumor cells are reported to be positive for both apical/endoluminal membrane and cytoplasmic CD133 staining, while some of them remain negative for cytokeratin 20 (CK20) staining. However, CD133 has failed to show a functional role in tumor progression, i.e., proliferation, migration, invasion, and colonization of distant organs (Papailiou et al. 2011).

## OCT4

OCT4, a major POU-domain transcription factor, is highly expressed in embryonic stem (ES) cells. It is essential for self-renewal and normal pluripotent cell development and maintenance.

Some somatic cancer cells rarely express OCT4. OCT4 is also called as reprogramming genes that induce an ES-cell-like state in fibroblasts commonly known as induced pluripotent stem cells (iPS). Overexpression of OCT4 in the intestine causes dysplasia by inhibiting differentiation in a manner similar to that in the ES (Papailiou et al. 2011).

## SOX2

SOX2, a group B of the Sox family of transcription factor has a role in development, cell differentiation, proliferation. In malignant tissues it is involved in the later events of carcinogenesis like invasion and metastasis, and confers a less differentiated phenotype in tumors. SOX2 is expressed in colon cancer in comparison to their normal tissue counterparts. It is also associated with tumor grade in colorectal cancer as it over expressed at stage T4. Unlike CD133 and OCT4 or SOX2, a strongly positive correlation was reported between SOX2 and OCT4. Post CTR expression of OCT4 and SOX2 decreased in the human specimens. Significant correlation is noted among these stem cell markers in post-CRT residual cancer (Papailiou et al. 2011) (Fig. 20.1).

## c-Myc

c-Myc a transcription factor, high levels of which can block cell differentiation and enhance self-renewal of committed and differentiated cells. During tumor progression, Myc can initiate the formation of cancer initiating cells that retain developmental plasticity and can activate an embryonic stem cell-like program in epithelial cells leading to epithelial tumor-initiating cells. c-Myc is overexpressed in primary colon cancer,

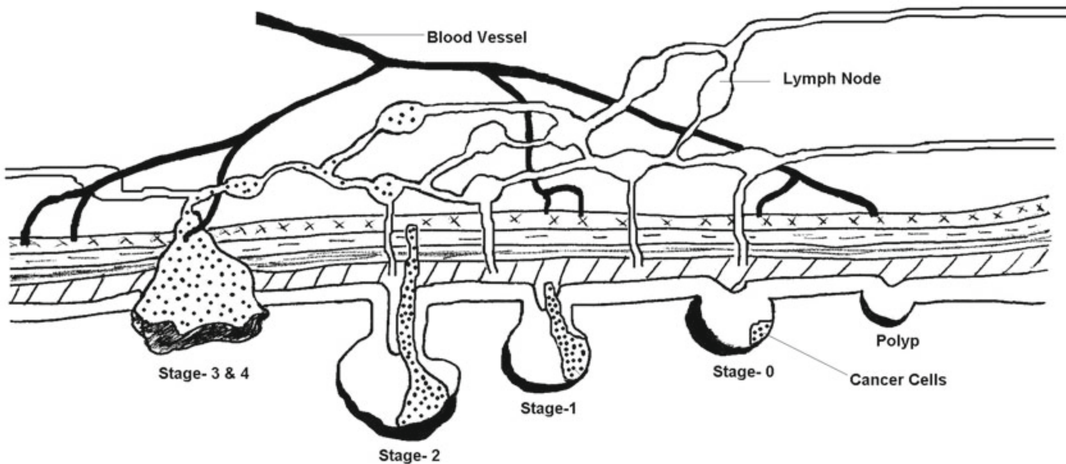


**Fig. 20.1** Stages of colorectal cancer (Illustration adopted and modified: courtesy NCI & JHSPS)

compared to normal adjacent mucosa. Axin, a tumor suppressor protein involved in Wnt pathway, helps in Myc's degradation. Mutations in Axin can directly enhance Myc levels in human tumors. It is degraded quickly by the ubiquitin-proteasome system in normal cells which is attenuated in many tumor cells. This degradation is carried out by Fbw7 ubiquitin ligase, which is reported to be lost in colon carcinomas. A de-ubiquitinating enzyme Usp28, the antagonist of Fbw7 stabilizes Myc is expressed at very high levels in colon cancer (Papailiou et al. 2011).

## ATP Binding Cassette Protein

ATP binding Cassette protein, a drug transporter is abundant in cancer stem cell imparting drug resistance and hence is designated as promising CSC marker. Functional implication of this protein is discussed in previous section.

## Signaling Pathways Regulating Cancer Stem Cell Functions

In normal human colon, intestinal subepithelial myofibroblasts (ISEMFs) located at the base of the crypt provides the niches for stem cells. Apart from helping in organogenesis, repair and defense, ISEMFs can regulate intestinal SC self-renewal and differentiation by secreting hepatocyte growth factor, TGFβ, and keratinocyte growth factor, whose receptors are located on the epithelial cells. Canonical Wnt signaling, Notch, and Sonic hedgehog (Shh), have been identified as key regulators of the SC function (Todaro et al. 2010). Wnt components present at the base of each crypt induce beta-catenin/Tcf-driven transcription in stem cells and Paneth cells. Blocking of Wnt signaling results in loss of the progenitor component as well as defects in Paneth cell maturation. On the other hand constitutive activation of the Wnt pathway causes the expansion of the crypt progenitor cell followed by tumorigenesis (Clevers and Batlle 2006).

Bone morphogenetic protein (BMP) is another regulator. BMP1, BMP2, BMP5, BMP7, SMAD7, and BMP receptor 2 are expressed more in the colon top, whereas the expression of 3 BMP antagonists, gremlin 1 (GREM1), gremlin 2 (GREM2), and chordin- like 1 are high at the basal crypt. Originate from myofibroblasts and smooth muscle cells GREM1, GREM2, and chordin-like 1 help to create the colonic epithelial SC niche by modulating Wnt activity (Todaro et al. 2010).

About 70% of colorectal cancers are associated with homozygous inactivation of the adenomatous

polyposi coli (APC) tumor suppressor gene resulting in activation of the Wnt signaling pathway and the constitutive transcription by the β-catenin/Tcf complex. Loss of APC function is observed from benign adenomas to malignant colorectal cancer and metastasis. Even in dysplastic crypts Wnt pathways remains activated by mutation. APC mutant cells express high levels of EphB2, EphB3 and EphB4 receptors as a consequence of constitutive β-catenin/Tcf activation. Eph receptor tyrosine kinases (RTKs) and their membrane-bound ligands, the ephrins, are essential for embryonic vascular development. EphB and the ephrin-B subfamilies are coexpressed in human colorectal cancer, and ephrin-B2 is expressed at higher levels in human colorectal cancer than in adjacent normal mucosa. These genes target β-catenin and Tcf4 in colorectal cancer. Upon expression of ErpB tumor-initiating cells acquire stem cell properties and repopulate the crypts with their mutant descendants until reaching the surface epithelium where these cells accumulate to form benign adenomas. Contact of tumor cells with normal differentiated cells, which express high levels of ephrinB ligands, results in activation of EphB signaling. Expansion of adenomas is blocked by EphB repulsive signals that limit the spread of tumor. When adenomas become aggressive, the expression of EphB receptors is silenced despite the persistence of Wnt pathway mutations. The mechanism responsible for EphB silencing in malignant colorectal tumors remains unknown; however, promoter methylation as well as point mutations in EphB2 and EphB4 genes are reported in some cases (Clevers and Batlle 2006).

Notch signaling is essential for regulation of differentiation, and maintenance of stem cells. In normal human colon, Notch signaling helps to maintain the crypt compartment. Inappropriate activation of Notch signaling is reported to be associated with the pathogenesis of colon cancers. Significant upregulation of Notch1 and Hes1 has been detected in colon adenocarcinomas, but not in normal differentiated epithelial cells (Miyamoto and Rosenberg 2011).

## Therapeutic Strategies to Target CSCs

A successful therapy needs to be employed to eliminate CSCs, the root of cancer. Targeting key signaling pathways that are active in CSC self-renewal is one of the approaches to therapy. In 2009, two classes of molecules were identified with Wnt inhibitory features. The first class acts on Wnt ligand production by specifically targeting porcupine (PORCN). PORCN is an acyltransferase which adds a palmitoyl group to Wnt proteins, this acetylation is necessary for secretion of Wnt protein. The second class has dual functions- it regulates Axin2 stability and targets β-catenin degradation in the presence of APC mutations. Enzymatic inhibition of poly-ADP-ribosylating enzymes tankyrase 1 and 2 (TNKS) by XAV-939 is reported to stabilize Axin2 and promote degradation of β-catenin. The first class remains unsuitable for treatment of colorectal cancer, because APC mutations sometimes make the tumor Wnt-ligand independent. Blocking of Wnt1 the Wnt ligand with monoclonal antibodies leads to apoptosis in colorectal cancer cell lines bearing APC and β-catenin mutations. Secreted Fzd-related proteins (SFRP) are natural inhibitors of Wnt which structurally mimics Fzd receptors and can prevent binding with Wnt ligands to Fzd receptors and signaling. SFRPs are often methylated and silenced in primary tumors. However, epigenetic reactivation/re-expression of SFRP in colon cancer cells is reported to decrease Wnt activity and augment cell death. Hence an antibody-targeting approach against Wnt ligands and/or blockade of the Frz receptor signaling could be beneficial as therapeutics. Targeting the TCF/β-catenin nuclear complex in cells bearing APC, SFRP mutation is also a promising approach. ICG-001 a TCF/β-catenin inhibitor which inhibits the coactivator CBP induces dose-dependent cell death in colorectal cancer cell lines; interestingly normal colonic epithelial cells are resistant to this compound. ICG-001 will be in Phase-I clinical trials shortly (Felipe de Sousa et al. 2011).

microRNAs (miRNAs) can regulate proliferation and chemoresistance of colon CSCs. One of

miRNAs, the miR-451 was found to be downregulated in colonospheres obtained from different colon carcinoma cells in comparison to parental cells Restoration of miR-451 expression not only decreased self-renewal, tumorigenicity, but also chemoresistance of colonspheres to irinotecan by down regulation of ABCB1. Cyclooxygenase-2 (COX-2) was identified as miR-451 target gene involved in sphere growth. miR-451 down regulation induces the expression of its direct target gene macrophage migration inhibitory factor, which in turn induces of COX-2. COX-2 activates Wnt. miR-451 could be a novel candidate to prevent recurrence and drug resistance in colorectal cancer (Bitarte et al. 2011).

Several clinical trials have evaluated natural agent to prevent colorectal cancer. Among them plant polyphenol compounds EGCG and epigallocatechin (EGC) extracted from green tea and phytochemical curcumin (active compound of turmeric) are predominant. EGCG is reported to decrease the growth of intestinal tumor and inhibit angiogenesis in mice transplanted with human colon cancer cells (Kanwar et al. 2010). Curcumin has a pliotropic effect on colon cancer cells. By blocking EGFR, MAPK, PI3K/AKT pathways it can inhibit cell cycle progression. On the other hand curcumin suppresses angionenesis by blocking VEGF and reduces inflammation by inhibiting NFκB, TNFα, COX2 and induced apoptosis (Kanwar et al. 2010). However, there is limitation to use of carcumin as therapeutic agent since 75% of carcumin is either excreted in the feces or undergoes rapid inactivation due to glucuronidation. Diflourinated carcumin (CDF), an analog of curcumin, exhibits increased metabolic stability. We observed that CDF together with conventional chemotherapeutics could be an effective therapeutic strategy for preventing the emergence of chemo-resistant colon cancer cells by eliminating CSCs (Kanwar et al. 2011).

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# 21 Treatment of Damaged Brain Following a Stroke: New Strategies

Toru Yamashita and Koji Abe

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**Abstract**

Stroke is the second leading cause of death in the world and results in a drastic reduction in the quality of life. New therapeutic strategies for patients suffering from stroke are needed. Possible strategies for treating ischemic stroke include: (i) increasing neuroplasticity (surviving neurons remodel the neuronal network to compensate for impaired neurological function), (ii) enhancement of endogenous neurogenesis (allowing endogenous neural stem cells to give rise to newly born neurons for the repair of disrupted neuronal networks), (iii) stem cell transplantation (supplying exogenous neural stem cells into the infracted lesion for neuronal repair), and (iv) increasing and promoting the mobilization of endothelial progenitors (contributing neuronal repair via enhanced angiogenesis). In the near future, we will combine these strategies to develop more effective therapies for the treatment of strokes.

## Introduction

Stroke is a major cause of death and results in a drastic reduction in the quality of life. Even though the number of stroke patients is continuously increasing, the only effective therapeutic approach is thrombolysis with recombinant tissue plasminogen activator modifying ischemic-related damage, and only in the acute phase of a stroke. A novel treatment strategy for the damaged brain in the sub-acute or chronic phase of a stroke is

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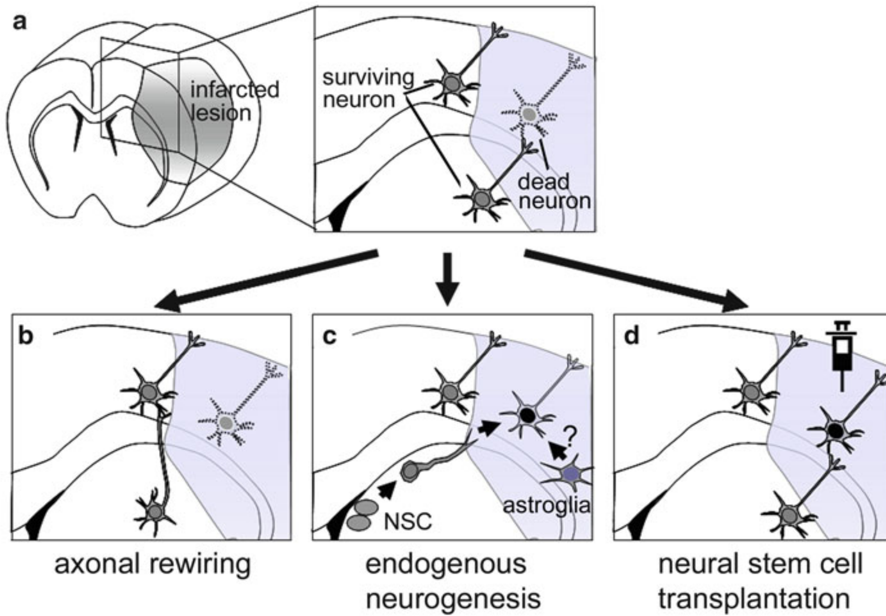


**Fig. 21.1 Potential therapeutic strategy treating the brain damaged after a stroke** (**a**) Schematic diagram of a post-stroke brain. In the infarcted lesion, numerous neuronal cell death occurs, leading to disruption of the neuronal network. (**b**) Diagram illustrating 'rewiring' in the post-stroke brain. After one target neuronal cell dies, the neuronal cells, which have lost their target, are thought to seek new targets and finally form new synapses with other target cells. (**c**) Neural stem cells (*NSC*) located in the subventricular zone can give rise to neuroblasts, which can migrate toward the infarcted lesion, and finally differentiate into mature neurons. (**d**) Exogenous neural stem cells can be transplanted into the infarcted lesion to supply neuronal cells and restore the disrupted neuronal network

thus required. In the infracted brain lesion, multiple neuronal cell death occurs and results in the disruption of the neuronal network, leading to neurological dysfunction (Fig. 21.1a). To remodel the neuronal network, four potential therapeutic strategies are now available. The first strategy is to promote neuroplasticity, in which surviving neurons can reconstruct a new neuronal network to compensate for impaired neurological function through rewiring or axonal sprouting (Fig. 21.1b). The second one is enhancement of endogenous neurogenesis, which allows endogenous neural stem cells to give rise to newly born neurons for the repair of disrupted neuronal networks (Fig. 21.1c). The third strategy is neural stem cell transplantation which supplies exogenous neural stem cells into the infracted lesion for neuronal repair (Fig. 21.1d). In addition, angiogenesis is intimately correlated with neuronal network reconstruction. Therefore, increasing and promoting mobilization of endothelial progenitors can also be a possible therapeutic strategy for stroke. In this chapter, we focus on these four strategies, and discuss the current development in the field with special emphasis on therapeutic applications for strokes.

## Therapeutic Strategy Enhancing Neuronal Plasticity

It is well known that functional recovery can occur spontaneously after an ischemic stroke and that appropriate rehabilitation can enhance this recovery. However, this recovery is often limited and insufficient, resulting in an incomplete return to normal functioning. Many researchers have studied the mechanisms of neuronal plasticity to enhance this recovery. Merzenich et al. (1983) transected the median nerve of adult monkeys and found that the cortical area, which had a matched hand median nerve area, was completely occupied by newly expanded representations of surrounding skin fields 2–9 months after surgery (Merzenich

et al. 1983). Nudo et al. (1996) demonstrated cortical motor function remapping in squirrel monkeys following a stroke. Of note, this drastic remapping was not detected in the control group but rather in the rehabilitative training group (Nudo et al. 1996). Brown et al. (2009) induced focal ischemic stroke in the forelimb sensorimotor cortex of an adult mouse, and found that forelimb-evoked depolarizations reemerged both in the neighboring peri-infarct motor/hindlimb area and in the distant posteromedial retrosplenial cortex by using a voltage-sensitive dye (Brown et al. 2009). These results suggest that the cortex remaps after both deafferentation and stroke in the adult mammalian cortex, and that rehabilitative training can enhance this remodeling. This remapping can arise depending on latent subthreshold inputs to the peri-infarcted region combined with a rewiring of the neural connections (Fig. 21.1b). Moreover, the time frame in which functional recovery occurs after a stroke is also limited. Earlier initiation of rehabilitation therapy is required for a better functional outcome. In an important experiment, rats were exposed to enriched rehabilitation at 5, 14, and 30 days after middle cerebral artery occlusion (MCAO). The animals that received early start rehabilitation (5, or 14 days after MCAO) showed significant recovery whereas those given delayed treatment (30 days after MCAO) displayed little improvement (Biernaskie et al. 2004). Taken together with clinical findings, these results implied that a critical period should exist during which the brain can be modified by rehabilitative experience. What decides a critical period of rehabilitation? One widely held hypothesis is that the balance between positive factors and negative factors for neuroplasticity is able to determine this period. Both glial-derived synaptogenic thrombospondin 1/2 and proteins promoting growth processes such as GAP43, MARCKS and CAP23, which are highly expressed in the subacute phase of a stroke, are regarded as positive factors for neuronal plasticity. On the other hand, inhibitory factors for axonal outgrowth or sprouting such as Nogo-A, myelin-associated glycoprotein (MAG), semaphorin 3A, chondroitin sulphate proteoglycan (CSPG), and neurocan, are regarded as negative factors for neuronal plasticity (Murphy and Corbett 2009). Keeping the time window of neuroplasticity open for a longer period, by suppressing negative factors or up-regulating positive factors for enhancing neuronal plasticity, is now regarded as a possible therapeutic strategy. Rats that received an intracerebroventricular anti-Nogo-A antibody injection starting up to 1 week after MCAO showed evidently greater sensorimotor function recovery compared with an injection containing the control antibody (Tsai et al. 2007). Nogo-A immunotherapy has been reported to result in robust sprouting of new projections from contralateral brain regions into subcortical structures, which are thought to contribute to functional recovery. In addition, both MAG and semaphorin 3A are also thought to be important therapeutic targets contributing to the lack of regenerative capacity of the central nervous system after injury (Kaneko et al. 2006). In contrast, amphetamine, which is a potent psychomotor stimulant inducing neuronal release of norepinephrine/dopamine/serotonin, has been regarded as a candidate neuroplasticity promoter. Amphetamine therapy increases the expression of GAP43 and synaptophysin. Moreover, amphetamine therapy enhances neocortical neuronal sprouting, synaptogenesis, and behavioral recovery in the adult rat (Stroemer et al. 1998). However, clinical trials of humans failed to provide clear evidence for the effectiveness of amphetamine therapy after a stroke (Gladstone et al. 2006). Brain-derived neurotrophic factor (BDNF) has been shown to have a role in homeostatic plasticity and to enhance the intrinsic ability of surviving neurons to newly extend neuronal connections. This BDNF effect was up-regulated by degrading CSPG with chondroitinase ABC (Tropea et al. 2003). The combination therapy, in which negative factors for neuroplasticity are suppressed and positive factors are enhanced, can be a promising therapeutic approach for patients suffering from a stroke.

## Promoting Endogenous Neurogenesis

In the adult mammalian brain, neurogenesis continuously occurs in two restricted regions: the subgranular zone (SGZ) of the hippocampal dentate gyrus (Gage 2000) and the subventricular

zone (SVZ) of the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo 2002). In the SGZ, newly born neurons migrate into the granule cell layer and integrate into the existing neuronal network. In the SVZ, which is a thin cell layer in the lateral walls of the lateral ventricle, neural stem cells produce neuroblasts migrating into the olfactory bulb. We studied the temporal profile of neural stem cell division, migration, and differentiation in the SGZ and the SVZ in the transient forebrain ischemia gerbil model, and evaluated whether the ischemic condition attenuated endogenous neurogenesis. We found that the ischemic condition increased the division of neural stem cells of the SGZ with a peak 10 days after ischemic induction, following which cells migrated into the granule cell layer and differentiated into neurons. We also reported that transient forebrain ischemia enhances neuronal stem cell proliferation in the SVZ with a peak 10 days after ischemia, following the migration of neural precursor cells to the olfactory bulb, suggesting that forebrain ischemia increased neural stem cell number and resulted in increased neurogenesis, mostly in the SGZ and the SVZ (Iwai et al. 2003).

Many researchers reported that newly born neurons can be found in the post-infarcted lesion including the striatum and cortex in another animal model, the transient focal ischemia model. To evaluate whether SVZ neural stem cells supply new neurons to areas damaged by ischemia, we performed region-specific cell labeling and long-term tracing experiments, and found that SVZ-derived neuroblasts migrate towards the injured striatum after MCAO. A long-term tracing study showed that the SVZ-derived neuroblasts differentiated into mature neurons in the striatum, in which they formed synapses with neighboring striatal cells (Yamashita et al. 2006), implying that SVZ neural stem cells can supply newborn neurons to brain lesions injured after a stroke. Recently, neural progenitor cells supplying GABAergic neurons were found, even in the neocortical layer 1 of adult rats, and their proliferation was drastically activated in the ischemic condition (Ohira et al. 2010). Moreover, astroglia seems to be very attractive as another cell source as they can be directly reprogrammed to neurons that form functional synapses *in vitro* (Heinrich et al. 2010).

In the post-stroke brain, newly born neurons can be supplied from the SVZ, the SGZ and the neocortical layer, but this number is too small for recovery of neurological functions. Appropriate interventions are required to enhance the proliferation, survival, and neuronal maturation of endogenous neuronal stem cells and progenitors.

## Stem Cell Transplantation Therapy

Human embryonic stem cells (hES cells) were generated from the inner cell mass of blastocysts (Thomson et al. 1998), and human-induced pluripotent stem cells (hiPS cells) were established by introducing four transcriptional factors, Yamanaka factors, including *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*, into human skin fibroblasts (Takahashi et al. 2007). Both hES and hiPS cells are known as multipotent stem cells with pluripotency and high replication competence. Neural stem cells/neuroblasts derived from hES or iPS cells can be transplanted into the murine brain, and can survive as mature neurons (Chen et al. 2010). Especially, hiPS cells can be produced from each patient's skin fibroblasts, implying that iPS cells do not possess ethical problems found in or associated with hES cells. Therefore, hiPS cells are regarded as a most promising cell source for cell transplantation therapy to supply new neurons to repair a neuronal network disrupted by various kinds of neuronal diseases, such as the ischemic stroke.

To develop iPS cell therapy in a clinical setting, the tumorigenicity of iPS cells is a critical problem that has to be overcome. Germline-competent chimeric mice with iPS cells developed tumors in which the integrated exogenous *c-Myc* gene was re-activated. Attempts were made to establish iPS cells without *c-Myc*, but the induction ratio of iPS cells was drastically reduced (Nakagawa et al. 2008). It has been also reported that Yamanaka four transcriptional factors, which are integrated into the genome by retrovirus vectors, can continuously express in iPS-derived cells, alter their characteristics and also induce tumorigenesis (Soldner et al. 2009). We compared the tumorigenicity of two different iPS cell lines established with or without retrovirus vectors, by

transplanting into the intact or the ischemic murine brain. In the case of virus-free iPS cells, there was no significant difference in tumor volume between the intact and ischemic group. On the other hand, virus-induced iPS cells in the ischemic brain formed significantly larger teratomas than those in the intact brain (Yamashita et al. 2011), suggesting that integrated transcriptional factors could affect cell characteristics and promote the outgrowth of transplanted iPS cells under the ischemic condition. In addition, secondary neurospheres from iPS cells also formed a teratoma in mouse brains at a constant rate (Miura et al. 2009), where a small number of undifferentiated iPS cells were retained to be in a pluripotent state even after a differentiation assay, and formed a teratoma. To realize safe cell transplantation therapy with iPS cells, we have to use an iPS cell line without exogenous gene integration, and should confirm that there are no undifferentiated cells left.

## Role of Endothelial Cells in Ischemic Brain

Endothelial cells are located in most inner parts of blood vessels, directly facing the blood flow. Endothelial cells form a vascular wall with endothelial tight junctions, with both a basement membrane and an astrocyte endfeet link. This endothelial cells-basement membrane-astrocyte structure leads to microvascular integrity, separating blood and the brain extracellular fluid in the central nervous system. At 2 h after the cerebral ischemic induction, dysfunction of the tight junction occurs, resulting in transudation of small molecules, fibrinogen, and plasma from blood to brain parenchyma. Next, loss of integrin receptors occurs on both endothelial cells and astrocytes. Main components of basal membrane, collagen-4, laminin, fibronectine are degraded, leading to the dissociation of each component of the vascular unit (del Zoppo and Mabuchi 2003). Endothelial cells are injured by hypoxia/hypoglycemia under the ischemic condition. Abundant free radicals, which are produced in the ischemic brain, also injure endothelial cells. Seriously injured endothelial cells lose their inherent function keeping their vascular tone and playing an antithrombotic role. This change in endothelial cells results in platelet activation and vasospasm, leading to vascular obstruction with thrombi. Several studies from a human and experimental stroke animal model showed that vascular remodeling occurs in the adult brain after a stroke (Zhang et al. 2000). In the past, the migrating neighboring endothelial cells were regarded as the main cell resource for the regeneration of injured endothelial cells. However, several research groups reported that bone marrow-derived cells can incorporate and differentiate into endothelial cells at the border of the infarct lesion of the focal cerebral ischemia murine model (Hess et al. 2002). Taguchi et al. reported that the systemic administration of human CD34-positive cells, a rich source of endothelial progenitor cells, promoted angiogenesis in a murine model of stroke. Administration of CD34-positive cells after focal ischemia was also followed by a better functional recovery compared with the control group (Taguchi et al. 2004). The results of this report indicate that bone marrow-derived endothelial cells can take part in angiogenesis, contributing to neurological functional recovery. However, an increasing number of scientific reports suggest that there are non-bone marrow-derived cells, which can also give rise to endothelial cells. Recently, tissue-resident stem cells, which were isolated from the heart, are capable of differentiating into endothelial cells (Beltrami et al. 2003). Therefore, at present, it seems unclear which organ is the main resource for endothelial progenitor cells, but, regardless of their origin, the endothelial progenitors circulating in peripheral blood can play an important role in vascular remodeling after a stroke.

## Mobilization of Endogenous Endothelial Progenitors to Angiogenesis

As indicated in the above discussion, there is much evidence to suggest that endothelial progenitor cells contribute to angiogenesis in post-ischemic tissue. Therefore, many researchers have

tried various kinds of agents in the ischemic animal model to test whether these agents can enhance the mobilization of endothelial progenitor cells, leading to augmented angiogenesis. Firstly, vascular endothelial growth factor (VEGF) has been reported to play an important role in angiogenesis through mobilization of endothelial progenitor cells in an animal model and human subjects (Asahara et al. 1999). Zhang et al. reported that administration of recombinant human VEGF at 48 h after the induction of ischemia enhanced angiogenesis in the peri-infarcted lesion and significantly improved neurological recovery in the rat model. These results seem to suggest that VEGF can be a promising agent minimizing ischemic-related injury; however, the issue regarding its utility as a therapeutic agent remains because administration of VEGF at 1 h after ischemia increased blood brain barrier leakage, and significantly worsened brain edema (Zhang et al. 2000). As another candidate, granulocyte colony-stimulating factor (G-CSF) also increased the number of circulating endothelial progenitor cells. We administrated G-CSF in the focal ischemia rat model and evaluated whether G-CSF can promote angiogenesis. We found that newly born endothelial cells were significantly increased in the G-CSF-treated group compared with the vehicle-treated group (Sehara et al. 2007). G-CSF has already been applied for idiopathic or chemotherapy-induced neutropenia and seems to be a well tolerated drug. Moreover, G-CSF can be administrated to patients subcutaneously. Therefore, G-CSF is now regarded as a promising candidate modulating ischemic-related injury. In addition, both erythropoietin and estrogen increase the number of circulating endothelial progenitor cells. Exercise also increases the number of endothelial progenitor cells in mice and in humans, probably through up-regulation of VEGF and angiopoietin (Ding et al. 2004). The mechanism by which these factors augment endothelial progenitor cells is not fully understood. However, it has been reported that VEGF and mobilization of exercise-induced endothelial progenitor cells were blunted in endothelial nitric oxide synthase (eNOS) knock out mouse, suggesting that eNOS plays an essential role in mobilization of endothelial progenitor cells (Aicher et al. 2003).

The first evidence for the pharmacological modulation of circulating endothelial progenitor cells by theroprotective drugs came from studies of HMG-CoA reductase inhibitors (statins). A previous report showed that statins increased mobilization of endothelial progenitor cells and re-endothelialization in the balloon-injured arterial of the rat model (Walter et al. 2002). Chen et al. (2005) reported that 14-days treatment with atrovastatin, which started from 24 h after a stroke, increased VEGF, VEGF receptor 2 and BDNF expression in the peri-infarcted lesion, and showed a significant improvement in functional recovery compared with untreated controls. In addition, statin treatment of human endothelial progenitors was reported to increase the expression of endothelial integrin $\alpha5\beta1$, which was associated with increased adhesive function of endothelial progenitors toward endothelial cells, indicating that statins may promote the homing of endothelial progenitor cells to the site of vascular injury (Walter et al. 2002). Recently, perlecan domain V, which is an extracellular matrix fragment, has attracted attention as another candidate for therapy. Post-stroke domain V administration increased VEGF levels via a mechanism involving brain endothelial cell $\alpha5\beta1$ integrin, promoting angiogenesis in pre-infarcted lesions, and restoring stroke-affected motor function in the murine model (Lee et al. 2011). Perlecan domain V is continuously generated by the post-stroke brain, indicating that even the adult brain has the capacity to regenerate the vascular component to some extent.

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# 22 Parthenogenetic Activation-Induced Pluripotent Stem Cells and Potential Applications

Yu Yin, Na Liu, David L. Keefe, and Lin Liu

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**Abstract**

Parthenogenesis is the process by which an egg can develop into an embryo without fertilization. Parthenogenesis can be induced by artificial activation of an egg in mammals. Parthenogenetic embryos typically fail to develop past mid-gestation, mostly due to insufficient placental development. Therefore they should not be considered as living embryos. However, parthenogenetic embryonic stem (pES) cells can be generated from parthenogenetic embryos. While aberrant genomic imprinting limits development of parthenogenetic embryos, genomic imprinting undergoes reprogramming during isolation and culture of pES cells enabling pES cells developmental pluripotency and extensive differentiation capacity, similar to ES cells. Indeed, pES cells also are designated as parthenogenetic activation-induced pluripotent stem cells (paiPS). pES cells proliferate indefinitely and show genomic stability with minimal tumorigenesis, so they hold great promise for stem cell therapy. Damaged tissues and degenerative diseases could be treated by pES cells derived from patients' own eggs, or from immunocompatible, banked pES cells. Presumably their derivation from a non viable embryo source would raise fewer ethical concerns than ES cells derived from embryos. Also, spare eggs are readily available from fertility clinics or can be retrieved from patients anticipating need for pES cell therapy. Patients facing radiation or chemotherapy could bank eggs to preserve their fertility

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and also produce pES cells for potential cell therapy. This review focuses on the critical step for generation of pES, oocyte activation, reviews the mechanisms of genomic imprinting underlying pluripotency, provides an overview of potential applications of pES for clinical therapeutics, as well as potential drawbacks of pES cells and strategies to overcome those challenges.

## Introduction

Parthenogenesis is a type of asexual reproduction in which the offspring develops without genomic contributions from sperm. Parthenogenesis occurs naturally in some invertebrate animal species but not in mammals. Mammalian parthenogenesis can be induced by activation of oocytes with appropriate methods. Parthenogenesis also provides a model to study early embryonic development. In addition, activation of oocytes has important implications for somatic cloning by nuclear transfer, intracytoplasmic sperm injection (ICSI) and genomic imprinting. Parthenogenesis in mammals results in abnormal development due to the effects of imprinting. Imprinted genes are expressed from only one allele in normal tissues, depending on their parent of origin, so the expression of imprinted genes in parthenogenetically derived tissues is abnormal. Parthenogenetically derived tissues typically lack a paternal genome and have two copies of the maternal genome. Abnormal expression of imprinted genes poses a barrier to normal embryonic development following parthenogenetic activation (Kono 2006).

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocysts. ES cells can generate a wide variety of cell types in vivo and in vitro, as demonstrated by their developmental competence or pluripotency. Human ES cells hold great potential for regenerative medicine and tissue engineering. However, the limited availability of human ES cells and the ethical issues raised by their derivation from potentially viable human embryos constrain their utilization. Parthenogenetic ES (pES) cells are derived from artificially activated eggs, so they generate less controversy, yet they exhibit pluripotency similar to ES cells. pES cells, therefore, provide a promising option for stem cell therapeutics.

## Molecular Mechanisms Underlying Oocyte Activation

Ovulated mammalian eggs are arrested at the second meiotic metaphase II (MII) until fertilization or activation. In MII oocytes, cortical granules (CG) reside beneath the plasma membrane and chromosomes straddle the metaphase plate of meiotic spindles. MII oocytes maintain high levels and activities of c-Mos, mitogen-activated protein kinases (MAPK), and maturation or metaphase promoting factor (MPF), whose up-regulation coincides with the assembly of the new spindles (Tulsiani 2003). Mos/MAPK and the early mitotic inhibitor 2 (Emi2) pathways are the molecular components of cytostatic factor(CSF)and play important roles in MII arrest. MPF is composed of cyclin B and p34cdc2 kinase, and displays a cyclic activity that peaks at metaphase. During egg activation, arrest is released following a sequence of events that leads to the rapid embryonic mitotic divisions, including a transient rise in intracellular $Ca^{2+}$ concentration that initiates the cortical reaction (CR) and resumption of meiosis by decreasing activities of MPF and MAPK, followed by alterations of the zona pellucida (ZP) glycoproteins to block polyspermy. Subsequent events include extrusion of the second polar body, recruitment of maternal mRNAs and formation of pronuclei (Runft et al. 2002). In mammalian oocytes, repetitive increases in the intracellular concentration of $Ca^{2+}$, also called $Ca^{2+}$ oscillations or transients, occur during fertilization. The initial increase in $Ca^{2+}$ appears to be critical for the initiation of egg activation, and the subsequent $Ca^{2+}$ oscillations are essential for full activation of eggs and normal development of the resulting embryos.

The molecular mechanisms whereby sperm induces $Ca^{2+}$ signaling and the response to $Ca^{2+}$ signaling have not been fully defined. Three models have been proposed to explain how sperm

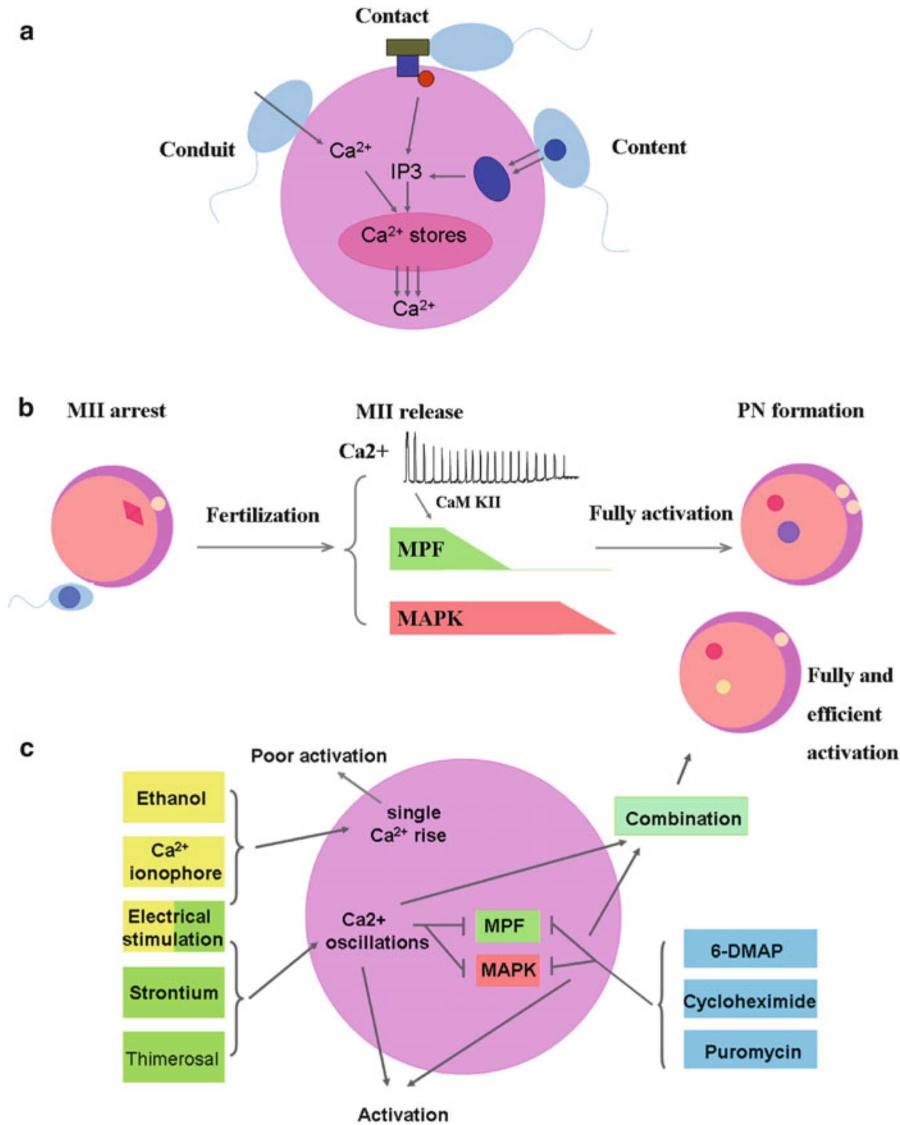


**Fig. 22.1** Mechanisms and artificial methods of oocyte activation (**a** and **b** modified from Liu et al. (2002)). (**a**) Models for $Ca^{2+}$ signaling in mammalian oocytes at fertilization. Conduit, the fusion of plasma membrane leads to $Ca^{2+}$-induced $Ca^{2+}$ release, sperm acts as conduit to transport $Ca^{2+}$. Contact, the binding of a sperm to receptor induces oocyte activation. Content, sperm factors trigger $Ca^{2+}$ release. (**b**) Activation of mammalian oocytes during fertilization. Sperm triggers $Ca^{2+}$ a transient, followed by $Ca^{2+}$ oscillations and inactivation of MPF and MAPK. A sequence of event occurs and leads to fully activation and pronuclear (*PN*) formation. (**c**) Methods for artificial activation of oocytes. Single reagent or stimulation causes single $Ca^{2+}$ rise that leads to poor activation. $Ca^{2+}$ oscillations or the decreases of MPF and MAPK activity induce successful full activation. $Ca^{2+}$ oscillations induce inactivation of MPF and MAPK. Combination of different methods lead to fully and more efficient activation

triggers $Ca^{2+}$ oscillations and activates the oocyte (Fig. 22.1a). One model proposes that the fusion of sperm with the oocyte plasma membrane leads to $Ca^{2+}$-induced $Ca^{2+}$ release. Evidence for this model is provided by the observations that the initial $Ca^{2+}$ transient is more prolonged than subsequent transients and MPF inhibitor abolishes subsequent $Ca^{2+}$ oscillations without suppressing the first one or two $Ca^{2+}$ waves following fertilization, probably through disruption of $Ca^{2+}$

release and refilling of $Ca^{2+}$ store (Deng and Shen 2000). The model hypothesizes that the binding of a spermatozoon to a receptor associated with GTP-binding proteins on the egg plasma membrane induces oocyte activation. This model is consistent with the finding that sperm-oocyte fusion precedes $Ca^{2+}$ release in the oocyte, and with the requirement of G-proteins and IP3 for $Ca^{2+}$ release at fertilization (Miyazaki and Ito 2006). The third model proposes that introduction of a soluble sperm protein into the oocytes during fertilization triggers $Ca^{2+}$ release in the oocyte. Intracytoplasmic sperm injection (ICSI) causes $Ca^{2+}$ oscillations similar to those observed during natural fertilization (Jedrusik et al. 2007). Injecting soluble sperm extracts into oocytes also triggers oocyte activation.

$Ca^{2+}$ oscillations generated during fertilization result in the activation of calmodulin-dependent protein kinase II (CaMKII) (Fig. 22.1b), which is essential for activation of the egg. CaMKII activation decreases MAPK and Cdk1/Cyclin B (a component of MPF) activities to permit resumption of meiosis. CaMKII activation leads to the degradation of Emi2. Cyclin B can be targeted after degradation of Emi2. Defects in CaMKII lead to female-specific sterility (Backs et al. 2009). Fertilization allows completion of meiosis and triggers a coordinated series of events leading to full activation of the oocyte (Fig. 22.1b). Elucidation of the molecular mechanisms underlying oocyte activation, has made it possible to activate oocytes artificially by mimicking the natural process of activation which occurs during fertilization.

## Methods for Artificial Activation of Oocytes

Oocytes are naturally activated by sperm, but artificial stimuli also can trigger oocyte activation and parthenogenetic development. $Ca^{2+}$ oscillations and inactivation of MPF and MAPK are used to evaluate methods of artificial activation. A number of agents have been developed to induce parthenogenesis in an attempt to mimic sperm-induced activation. Stimulating calcium signaling: $Ca^{2+}$ transients are the key trigger for meiotic resumption during fertilization. Calcium transients have been induced artificially by mechanical, chemical and physical stimuli (Fig. 22.1c). Ethanol was the first agent to be used for parthenogenetic activation in mouse oocytes. Exposure of matured oocytes to 7% ethanol for 5–7 min induces activation and pronuclear formation by promoting formation of IP3 and influx of extracellular $Ca^{2+}$ (Presicce and Yang 1994). Ethanol treatment causes a single, sustained, large rise in intracellular $Ca^{2+}$, which derives both from intracellular $Ca^{2+}$ release and extracellular $Ca^{2+}$ influx. $Ca^{2+}$ ionophore A23187 also induces a single $Ca^{2+}$ rise in mammalian oocytes. The calcium ionophore A23187 not only promotes the release of intracellular $Ca^{2+}$ stores but also facilitates influx of extracellular $Ca^{2+}$ (Vincent et al. 1992). Ionomycin, another potent $Ca^{2+}$ ionophore, mobilizes intracellular $Ca^{2+}$ by depletion of $Ca^{2+}$ stores. Electrical stimulation produces transient pores in the oocyte plasma membrane to induce $Ca^{2+}$ influx. A single electrical pulse causes a single, large $Ca^{2+}$ transient, and periodically repeated pulses can induce calcium transients. Though activation can be generated by a single $Ca^{2+}$ rise using ethanol, $Ca^{2+}$ ionophore or electrical pulse, the single $Ca^{2+}$ rise is insufficient for full activation of oocytes, and results in incomplete CG exocytosis, MII arrest and poor preimplantation development(Wang et al. 1998). The initial $Ca^{2+}$ rise inactivates the MPF, but in the absence of $Ca^{2+}$ oscillations, this activity recovers. Moreover, a single $Ca^{2+}$ rise fails to inactivate MAPK, so its activity stays at high levels.

During normal fertilization in mammals the initial $Ca^{2+}$ rise is followed by repetitive $Ca^{2+}$ oscillations. Methods to induce $Ca^{2+}$ oscillations presumably better mimic the physiological processes of fertilization than methods that induce only a single rise in $Ca^{2+}$. Strontium ($Sr^{2+}$) induces $Ca^{2+}$ oscillations at a frequency similar to that of sperm-induced $Ca^{2+}$ oscillations, fully activates oocytes, and produces early embryo development (Liu et al. 2002). $Sr^{2+}$ induces multiple $Ca^{2+}$ rises by displacing $Ca^{2+}$ within the oocyte and inducing intracellular $Ca^{2+}$ release. $Sr^{2+}$ activated oocytes do not exhibit increased rates of chromosome segregation errors, and $Sr^{2+}$ has been used to activate

mouse oocytes after nuclear transfer (Loren and Lacham-Kaplan 2006). Thimerosal, a sulfhydryl reagent, induces repetitive $Ca^{2+}$ oscillations in intracellular $Ca^{2+}$ in mammalian oocytes, and has been used to activate bovine oocytes (Fissore et al. 1995). Thimerosal induces calcium oscillations with smaller peaks and briefer duration compared with those induced by sperm. Disruption of the meiotic spindle and other side effects of thimerosal-incubated oocytes limit its utility for activation (Cheek et al. 1993).

A single $Ca^{2+}$ rise can not induce late events of oocytes activation, such as mRNA recruitment, pronuclear formation, DNA synthesis and cleavage. The decreases in MPF and MAPK activity are critical for resumption of meiosis, chromatin decondensation and transition to interphase. Multiple $Ca^{2+}$ oscillations are believed to maintain low activity of MPF by degradation of cyclin B and/or phosphorylation of p34cdc2 and inactivation of MAPK by ERK2 dephosphorylation (Tulsiani 2003) (Fig. 22.1c). The method to activate oocytes that most closely mimics fertilization would combine $Ca^{2+}$ oscillation and inhibition of MPF and MAPK activities. In MII arrested oocytes, high MPF activity is maintained through continuous equilibrium between cyclin B degradation and synthesis. Inhibition of protein synthesis or protein phosphorylation can enhance oocytes activation by lowering cyclin B levels. A protein serine/threonine kinase inhibitor, 6-DMAP, was shown to enhance the activation of mouse and bovine oocytes. Two inhibitors of protein synthesis, cycloheximide and puromycin, also can activate oocytes. Agents that increase intracellular $Ca^{2+}$ or prevent protein synthesis (phosphorylation) alone result in low cleavage and development rates, but combined treatments are more effective for activation of oocytes. The combination of $Ca^{2+}$ ionophore with 6-DMAP induces high rates of activation, and pronuclear formation without extrusion of a second polar body (Liu et al. 1998), resulting in diploid development. Treatment of $Ca^{2+}$ ionophore A23187 sequentially with cycloheximide induces a high rate of pronuclear formation and development to blastocyst in mouse oocytes (Hagemann et al. 1995), but this treatment leads to extrusion of a polar body and haploid development. Cytochalasin D, an inhibitor of actin filaments, blocks extrusion of the second polar body and generates diploid development. Both the first $Ca^{2+}$ increase and the subsequent inhibition of kinases and proteins are indispensable for full activation of oocytes. Many factors influence the effectiveness of artificial activation, including species differences, oocyte age as well as the method and completeness of activation, and combinations of artificial activators.

## Generation of Pluripotent Stem Cells from Artificially Activated Oocytes

Stem cells have the ability to proliferate indefinitely and to differentiate into specialized cell types. ES cells are pluripotent and have virtually unlimited self-renewal and differentiation potential. They can differentiate into all types of cells or tissues both in vivo and in vitro. Human ES cells hold promise for the treatment of degenerative disorders such as Parkinsons, Alzheimers, and diabetes mellitus. Human ES cell research is fraught with ethical issues, because it typically entails destruction of living embryos, which has prompted the search for alternative sources of pluripotent stem cells. Induced pluripotent stem (iPS) cells are induced by some pluripotent transcription factors and do not involve destruction of viable embryos. The safety issues have to be resolved before iPS cells containing viruses or expression plasmids and oncogene factors are introduced into general use. pES cells are the type of pluripotent stem cells derived from activated oocyte without embryo destruction, and thus avoid many of the most problematic ethical concerns. Like ES cells but unlike iPS cells, pES cells also raise fewer concerns about mutagenesis and tumorigenesis.

The first cell lines derived from parthenogenetic embryos were established from mice nearly 30 years ago. pES cells can be differentiated in vitro when cultured in suitable conditions, form teratomas, and contribute to a variety of adult tissues in chimeras. Thus far, pES cell lines have been derived from several mammalian species, including mice, rabbit, buffalo, primates and

humans. pES cells show extensive differentiation capacity in vitro. Cell lines are stable in culture, maintaining a normal karyotype. However, the differential potential of pES cells remains controversial. In a routinely applied test of stem cell pluripotency, production of chimeras and transmission of cells through the germline of the chimeric animals, pES cells exhibit low chimera production and poor germ line transmission, even after repeated cross-breeding (Hikichi et al. 2007), suggestive of restricted pluripotency.

Previously, most pES cells were derived from oocytes activated by 6–7 % ethanol, which only triggers a single rise in $Ca^{2+}$, show limited pluripotency and exhibit strong tissue preference with limited or no contribution to muscles and gonads in chimeric mice. In contrast, fertilization by sperm induces $Ca^{2+}$ oscillations, which fully activate the oocyte. By mimicking sperm induced $Ca^{2+}$ oscillations, strontium ($Sr^{2+}$) treatment fully activates oocytes, similar to fertilization, and significantly enhances parthenogenetic embryo development. Derivation of pES cells from fully activated oocytes by $Sr^{2+}$ increases the pluripotency of pES cells. Some pES cell lines are indistinguishable from ES cells derived from fertilized embryos (fES cells)- they can contribute to all tissues and organs in chimeras (Chen et al. 2009; Li et al. 2009). Surprisingly, through tetraploid embryo complementation (TEC), these pES cells can develop to full-term without gene modification, showing that pES cells can differentiate into all cell types and functional organs in the body (Chen et al. 2009).

Generation of parthenogenetic activation-induced pluripotent stem cells: Diploid parthenogenetic embryos were produced as previously described (Horii et al. 2008). Artificial activation was performed by brief exposure to $SrCl_2$ and cytochalasin D. After activation, the embryos were cultured until developing to the blastocyst stage. Inner cell mass (ICM)-derived outgrowths were mechanically dissociated into clumps and re plated on mitomycin C-treated feeder layers in ES medium. They were then expanded and passaged as ES cells (Chen et al. 2009; Liu et al. 2011). The first human pES cell line came from one of the failed SCNT hES cells lines reported by Hwang's group, which was actually identified as an human pES cell line through a genome-wide SNP analysis (Kim et al. 2007b). Later, more lines were generated from human parthenogenetic blastocysts (Mai et al. 2007; Revazova et al. 2007). The combination of ionomycin with 6-DMAP has been used to activate human oocytes (Mai et al. 2007). Human pES cell lines then were derived from the ICM's of the resulting blastocysts and cultured like human ES cells. pES cell lines have provided useful models to study the molecular mechanisms underlying stem cell derivation and pluripotency. Also, comparing cell lines generated by different methods has provided information for optimizing protocols, which will definitely improve the potential of pES for clinical applications.

## Molecular Mechanisms Underlying Pluripotency of pES Cells

Parthenogenetic embryos lack full-term developmental potential because they have abnormal expression of imprinted alleles. Global gene expression profiling to compare parthenogenetic blastocysts with blastocysts from normally fertilized embryos show decreased expression of genes important for placental development in parthenote blastocysts. Aberrantly increased Wnt signaling and reduced MAPK signaling was found in early parthenotes. Moreover, as expected some maternally expressed genes were up-regulated (Liu et al. 2010). Increased Wnt and reduced MAPK signaling may facilitate derivation and self-renewal of pES cells.

Unlike parthenogenetic embryos, pES cells derived from parthenogenetic blastocysts show extensive differentiation potential. Many important pluripotent factors expressed in fES cells, such as Oct4 and Nanog, which are essential for ES cell pluripotency, also are expressed in pES cells. Quantitative proteomics analysis did not show significant differences in global protein expression between pES cells and fES cells (Hu et al. 2011), suggesting similar pluripotencies between these two stem cell types. The expression patterns of pluripotent factors and protein

profile are consistent with the high rates of chimera formation and germ line transmission from pES cells.

pES cells can be derived from in vivo ovulated (IVO) mature oocytes and in vitro maturation (IVM) oocytes, and IVM pES cells more closely resemble ES cells from fertilized embryos than do IVO derived pES cells on genome wide gene expression, DNA methylation and protein profiles (Fulka et al. 2011; Hu et al. 2011; Liu et al. 2011).

As described above, parthenogenetic embryos and fetuses fail to express paternally expressed imprinted genes, but pES cells express those genes in a pattern resembling that of fertilized embryos and fertilized embryonic stem cells. The improvement of pES cells developmental potential compared to parthenogenetic embryos may be attributed to the epigenetic reprogramming that occurs during in vitro isolation and culture of pES cell lines. Global demethylation occurs during isolation and culture of pES cells from early embryos. Decreased methylation of *Igf2r*, *Snrpn*, and especially *U2af1-rs1*, was associated with increased contribution of pES cells to chimeras. In vitro culture alters the epigenetic status of imprinted genes (Fig. 22.2) with increases expression of *U2af1-rs1* and *Snrpn* and decreased expression of *Igf2r.* These changes correlate with the pluripotency of pES cells (Horii et al. 2008; Li et al. 2009). On the other hand, imprints in the germ line normally are erased in pES chimeras, suggesting that reprogramming of the germ line is an inevitable event, regardless of the imprint status of primordial germ cells (Horii et al. 2008). Furthermore, by manipulating the expression of two imprinted genes, *Igf2* and *H19*, parthenogenetic development can be extended to term (Kono et al. 2004). Together, correct expression of imprinted genes has been shown to play an important role in the maintenance of parthenogenetic ES cells.

## Gene Imprinting in Parthenogenetic Embryo and pES Cells

The creation of pES cells does not depend on the destruction of viable embryos nor on viruses or expression plasmids to induce pluripotency. pES cells can differentiate into a wide variety of cell types both in vivo and in vitro, thus they hold great promise for the treatment of degenerative disease. Nevertheless, pES cells face a significant hurdle- they must overcome genomic imprinting.

Genomic imprinting is an epigenetic mechanism that, makes certain autosomal genes express in a parent-of-origin dependent manner. Approximately, 100 imprinted genes are known in mammals. Most cluster in specific regions of the genome. DNA methylation is critical for the regulated expression of imprinted genes. Correct regulation of imprinted genes is essential for normal mammalian development. Genomic imprinting appears to be restricted to eutherian mammals, and presumably evolved as a result of conflicting interests of the male and female genomes during the growth process. Imprinted genes play major roles in placental and fetal development. Failure of their regulation leads to perturbed gene expression and can contribute to developmental abnormalities and cancers. Normal mammalian development requires genomic contributions from both the mother and the father. Although oocytes are totipotent in many organisms, this is not the case in mammals, presumably because the maternal genome is epigenetically modified in the germ line to contain only maternal 'imprints', which normally result in repression of certain maternally inherited imprinted genes. A paternal genome is essential to 'rescue' the oocyte, as the maternal genes are imprinted reciprocally to paternal imprints (Fig. 22.2). Early nuclear transplantation experiments demonstrated that androgenotes (two male pronuclei) differ from gynogenotes (two female pronuclei), suggesting that the parental genomes are not functionally equivalent and that the genome is imprinted during gametogenesis. Eggs with two female pronuclei can develop to the 25-somite stage (Fig. 22.3a) but with only meager extraembryonic tissues, suggesting that the paternal genome is essential for the normal development of the placenta (Barton et al. 1984). By contrast, the maternal genome is essential for embryogenesis since androgenetic embryos do not complete normal embryogenesis (McGrath and Solter 1984). More detailed morphological analysis of

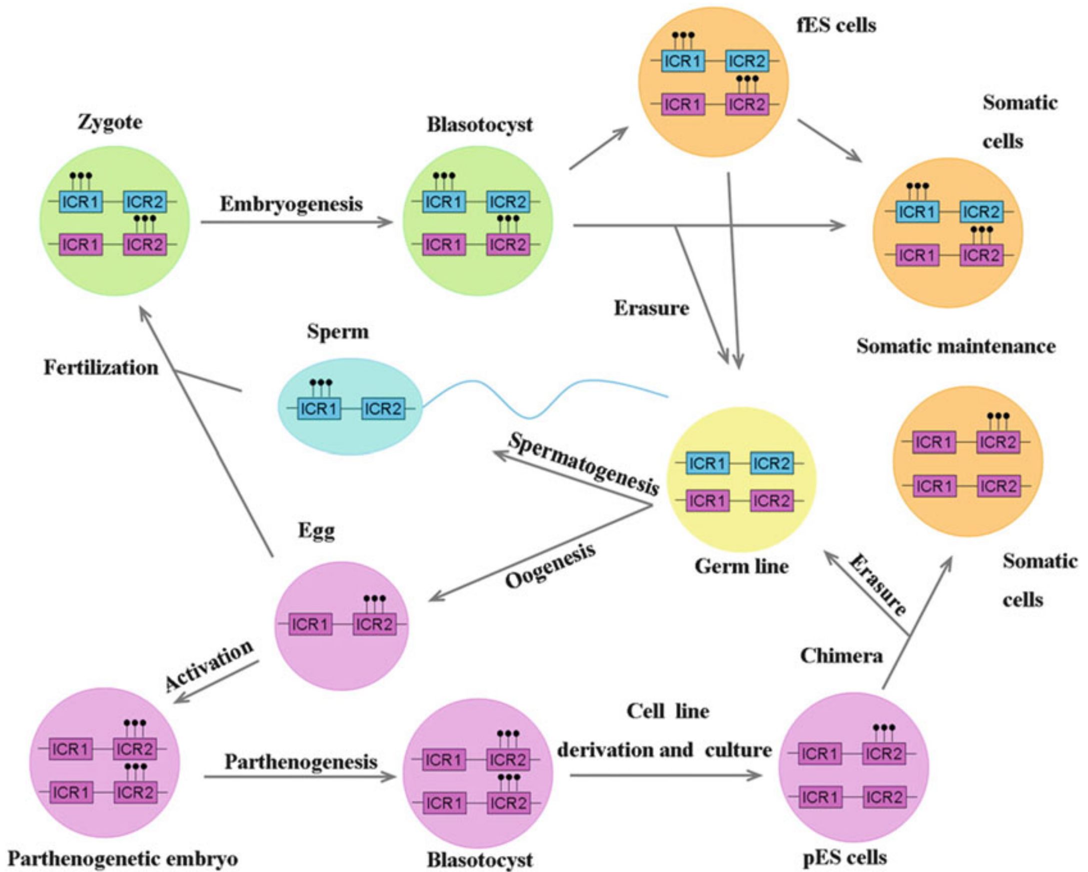


**Fig. 22.2** Imprinting during development. Imprints are established during oogenesis, or spermatogenesis, through mark the imprint control region (*ICR*) differently with methylation. Two examples of ICRs are utilized to show the states of imprints during development. ICR1 stand for ICRs with paternally-derived methylation, while ICR2 stand for ICRs which are methylated during oogenesis. After fertilization of the egg by the sperm, imprints are maintained throughout development, erased and reestablished in germline for the next generation. Imprints are stable in somatic cells and critical for the expression of imprinted genes. Parthenogenetic embryos could not develop to full-term as they lack the contribution from paternal genome. After the artificial activation, ICR1 is hypomethylated while ICR2 is hypermethylated as the duplication of maternal genomic. The epigenetic states of ICRs changed after the derivation of pES cells from parthenogenetic embryo. ICR2 presents decreased methylation after pES cells derivation and culture. After injection into blastocyst to form chimera, pES cells could generate germline chimeras and somatic cells in vivo, and the imprints are maintained or erased normally

parthenogenetic embryos reveals that the failure of parthenotes to develop to term is due to abnormal proliferation and differentiation in both embryonic and extra-embryonic lineages. But if the parthenogenetic mouse embryos carry two sets of haploid genomes from non growing and fully grown oocytes (Fig. 22.3b), they can develop to 13.5 days of gestation with a well developed placenta. In these embryos, the paternally expressed genes, *Peg1/Mest*, *Peg3*, and *Snrpn*, are activated, while the maternally expressed genes, *Igf2r* and *p57Kip2*, are silent in the alleles derived from a non growing oocyte genome (Obata et al. 1998).

Imprinted genes important for placental development, such as *Slc38a4*, *HIF2a*, *Gab1* and *Plac9,* are expressed at decreased levels in parthenote blastocysts. Conversely, maternally expressed imprinted genes which negatively regulate placental growth, such as *H19*, *Tssc3/Phlda*2, *Grb10/Meg1* and *Cdkn1c*, are increased in parthenogenetic

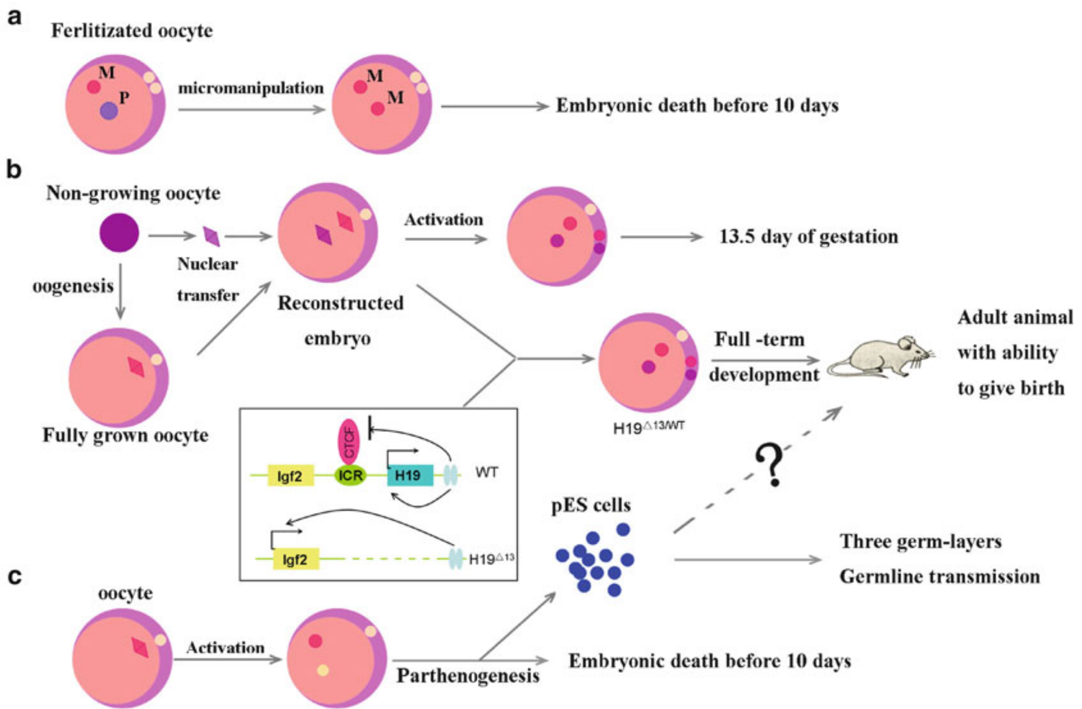


**Fig. 22.3** Parthenogenetic embryo development in mice. (**a**) Embryos with two maternal pronuclear are generated through micromanipulation, and the reconstructed embryos fail to develop to full-term. (**b**) Imprinting occurs during oogenesis, reconstructed embryos that contain the genome from non-growing oocyte and fully grown oocyte can develop to 13.5 day of gestation. Combination of the nuclear transfer and gene manipulation on *H19/Igf2* locus, the embryos get full-term development and the adult give birth normally. (**c**) Parthenogenetic embryos can not develop more than 10 days. However, pES cells generated from these embryos exhibit pluripotency as evidenced by three germ-layers differentiation and germline transmission capacity, and full-term development

blastocysts (Liu et al. 2010). Mammalian parthenogenesis are incapable of full-term development and typically die at mid-gestation (Fig. 22.3c). The reason for this arrest in development is believed to be genomic imprinting (Kono 2006). Imprinting control regions (ICR) regulate the expression of imprinting genes. Methylation of ICRs is established in a sex-specific manner in the germ line. Most ICRs are methylated on the maternal allele, but only a few are methylated on the paternal allele, which include DMRs of *Igf2/H19* and *Dlk1/Gtl2*. After epigenetic reprogramming occurred during isolation and culture of pES cells, pES cells exhibited balanced methylation of *Snrpn*, *Peg1* and *U2af1-rs1*. However the DMRs of *Igf2/H19* and *Dlk1/Gtl2* remain hypomethylated (Li et al. 2009) (Fig. 22.2). Inactivation of *Igf2* and *Dlk1* prevents the birth of parthenote mice. Bi-maternal mice without paternal genomes can develop into viable and fertile female adults through genetically manipulation of two regions: the imprinting centers of *Igf2-H19* and *Dlk1-Gtl2* (Fig. 22.3b). These results suggest that development of mammalian parthenogenotes is limited by imprinted genes. Parthenogenotes and pES cells provide valuable models to study imprinting.

## Potential Applications of pES Cells

Mouse pES cells have been well established, and can differentiate into a variety of cell type. Human parthenogenetic ES cells also have been successfully established. Human pES cells exhibit the ability to self-renew and differentiate, so pES cells hold potential applications for regenerative medicine and tissue engineering. Human pES cells posses several advantages for therapeutic

application compared with human ES cells. pES cells can be derived from IVO and IVM oocytes as above mentioned. Even clinically failed eggs can be used for generation of pES cells through parthenogenetic activation (De Sousa et al. 2009) thus alleviating the limited supply of human eggs available to make human ES cells. Secondly, human pES cells avoid much of the ethical controversy surrounding ES cells. Furthermore, patient-specific pES cells provide stems MHC matched with the oocyte donor, so they may be more suitable for clinical applications (Revazova et al. 2007). It will be important to validate the pluripotency and safety of human pES cells before clinical trials. Similar to human ES cells, human pES cells typically are cultured on mouse feeder layers, which may result in immune reactions and therefore limit their clinical application. Feeder-free culture systems and the use of human feeder cells have been developed for long-term culture of human pES cells (Lu et al. 2010), potentially making human pES more suitable for clinical applications.

pES cells can be induced to differentiate in vitro into many cell lineages, including myogenic, osteogenic, adipogenic, and endothelial. In vivo they can form muscle-like and bony-like tissue Although mouse ES cells can differentiate into functional dopamine (DA) neurons and restore cerebral function and behavior in an animal model of Parkinson's disease, human and non-human primate ES cells do not work as well in in vivo experiments, as shown by teratoma formation and incomplete motor benefits to Parkinsonian. Surprisingly, pES cells from non-human primates restore functional neurons in an experimental model of Parkinson's disease with no teratoma formation (Sanchez-Pernaute et al. 2008), suggesting an advantage of pES cells in the treatment of degenerative disorders.

## Summary and Perspectives

Pluripotent stem cells hold great potential for cell transplantation therapy in patients with regenerative medicine. hES cells have tremendous potential for cell therapy for human diseases such as diabetes and neuro-degenerative disorders. However, human ES cells evoke considerable ethical controversy, because they typically entail destruction of living embryos, hES cells also pose another problem, immuno-rejection. Parthenogenetic ES cells, produced from surplus oocytes, do not depend on destruction of viable, bi parental embryos as do ES cells. In addition, pES cells do not involve infection with viruses or expression of plasmids for their establishment as do iPS cells. pES cells escape immuno-rejection when used for autologous cell transplantation, because when generated from the patient's own oocytes, they are histocompatible with the oocyte donor (Kim et al. 2007a). pES cells, therefore, could provide an important source of histocompatible cell lines for women. Nevertheless, pES cells face another hurdle- genomic imprinting must be reprogrammed and corrected. Recently, however, pES cells were shown to lose maternally methylated imprints and achieve more normal imprint patterns (Horii et al. 2008; Li et al. 2009). Parthenogenetic development can be extended to term by manipulating the expression of two imprinted genes, *Igf2* and *H19* (Kono et al. 2004). More surprisingly, live parthenogenetic pups recently were produced from reprogrammed pES cells through tetraploid embryo complementation (Chen et al. 2009). These encouraging findings suggest that pES cells have more normal pluripotency than the parthenogenetic embryos from which they derive. The pluripotency of reprogrammed pES cells is nearly equivalent to biparental ES cells. Recently, pES cells were successfully generated from aged mice when few oocytes are available (Huang et al. 2010). Moreover, disease allele–free pES cell lines could be developed from the oocytes of affected animals, and even more excitingly, phenotype correction was obtained in donor-genotype recipients after transplantation of in vitro hematopoietic ES cell derivatives (Eckardt et al. 2011). Thus, pES cells could be used to create patient-specific stem cell banks for cell based transplant. Furthermore pES cells also can be used as important tools to study genetic imprinting, homozygosity, heterozygosity, and X-chromosome inactivation. Understanding the mechanisms underlying the

formation of parthenogenetic embryos and parthenogenetic embryonic stem cells will help elucidate the fundamental mechanisms underlying reprogramming and stem cell biology, and ultimately advance cell therapy and regeneration medicine.

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# Neuroprotection for Stroke Using Glial Cell Line-Derived Neurotrophic Factor/Neural Stem Cells Grafting

23

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**Abstract**

Stroke is the common disease among elder people and leading cause of death in the world, while its treatment strategies are very limited. After acute stroke, the survivors still suffer from disability in most cases, resulting in a huge burden for family and society. Thus substantial advances in the prevention and treatment of stroke are of paramount importance. Grafting neural stem cells (NSCs) as cell replacement for neuronal loss due to ischemia has been demonstrated to improve neurological deficits after stroke, while the efficacy of NSCs after transplantation is compromised by the low survival rate and low neuronal differentiation. It has been shown that modifying niche of grafted NSCs can help to increase the survival rate and neuronal differentiation by infusing neurotrophic factors, such as glial cell-derived neurotrophic factors (GDNF); however, direct GDNF injection by intracerebral administration is not an optimal. Recently, the treatment by using of NSCs with overexpressed GDNF ex vivo has been demonstrated to significantly improve therapeutic efficacy, suggesting that it may be a promising approach for neurodegenerative diseases including stroke.

## Introduction

Stroke is the third leading cause of death in the Western world, and the second cause of death in Japan, Korea and China, and it is the leading

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cause of chronic disability. The stroke population is rapidly increasing. The number of individuals over the age of 65 is expected to double between 2005 and 2030. Further, the risk of stroke is more than doubles for each decade of life over age 55. Thus, substantial advances in the prevention and treatment of stroke are of paramount importance.

To date, therapeutic strategies for stroke have been focusing on two main aspects: restoration of cerebral flow and the minimization of the deleterious effects of ischemia on neurons. Intensive research spanning over the last two decades has witnessed significant therapeutic advances in the form of carotid endarterectomy, thrombolytics, mechanical thrombectomy, anticoagulant therapy, antiplatelet agents, neuroprotective agents, and associated risk factors such as hypertension, diabetes and hyperlipidemia. However, current therapeutic protocols remain suboptimal and the development of more effective neuroprotectants is imperative. To date, only the serine protease tissue-type plasminogen activator (tPA) is approved by FDA for ischemic stroke treatment, while the therapeutic window is very narrow in that tPA should be administered within the first 3 h of the onset of ischemia. Although ~30% patients may benefit from tPA treatment, most survivors are subsequently left with significant sensory, motor and/or cognitive disability. Over recent decades, neural stem cells (NSCs) have been found in the adult mammalian brain. They not only can proliferate but also can differentiate into neurons, astrocytes and oligodendrocytes *in vitro and in vivo*. Therefore, NSC-based therapy may be very promising for stroke treatment.

It should be noted that neurotrophins such as nerve growth factor, brain-derived neurotrophic factor, glial-cell derived neurotrophic factor, have exhibited neuroprotection against stroke via pleiotropic effects. These factors may serve as neurotrophic factors for dysfunctional neurons, enhance synaptic plasticity and promote proliferation and differentiation of grafted NSCs as well as induced-stroke NSCs. Recent studies have shown that transplantation of NSCs combined with neurotrophins provides more beneficial treatment for stroke. The current review about stroke treatment will focus on the following aspects: stroke histological features, neuroprotection of GDNF administration, therapeutic efficacy of grafted NSCs, the niche influencing the fate of grafted NSCs, therapeutic efficacy of NSCs overexpressing GDNF.

## Stroke Histological Features

There are two major types of stroke which are ischemic stroke and intracerebral hemorrhage (ICH). Ischemic stroke represents 80% of stroke. It is caused by abrupt and near-total interruption of cerebral blood flow, and produces ischemic changes in the striatum and overlying cortex, leading to long-term sensorimotor deficits. By contrast, ICH is a lethal stroke type, as mortality approaches 50% and neurological disability in survivors is very common.

Several rodent models are available for brain ischemia. Transiently occluding bilateral carotid artery (often 5–10 min.) leads to 'global and/or forebrain ischemia', which selectively destroys specific cell types such as CA1 pyramidal cells in the hippocampus. The more common ischemic model, however, is 'focal cerebral ischemia', most often involving middle cerebral artery occlusion (MCAO), which leads to infarction of the striatum and overlying cortex. Focal cerebral ischemia by MCAO is usually divided into permanent and transient types. Transient cerebral ischemia is performed by occluding artery for a shorter time such as 90–120 min and then recanalizing the occluded artery. Permanent cerebral ischemia is accomplished via ligation, cauterization, or laser-induced photothrombosis, as well as permanent MCAO model.

Once an artery is blocked, its supplied cerebral tissue undergoes different ischemic changes depending on regional cerebral blood flow. In the forebrain ischemia model, CA1 of hippocampus is severely damaged, while the dentate gyrus is almost intact. In the focal cerebral ischemia caused by MCAO, ischemic lesion regions are the supraoptic area, striatum and frontoparietal cortex. The extent of lesion in the supraoptic area is most severe, striatum less severe, and cortex

only has focal or laminar lesions. The lesion cores primarily supplied by the occluded artery, are severely suffered from sharp ischemia and blood flow values are extremely low (<10 ml/100 g per min). Neurons locating in the cores become necrotic and apoptotic at early time of ischemia (≤6 h) at scattered sites. Striatum is marked by appearing spongy and vacuolated tissue. The ischemic core will develop pannecrosis/cavitation typical of infarction over time (≥48 h), where neurons, glial and vascular cells were loss and neuropils are spongy and vacuoles. Infarct exists in the supraoptic area and striatum, but does not exist in frontoparietal cortex in MCAO model. The surrounding areas of cores, where there are rich end-to-end arterial anastomoses between MCA and anterior cerebral artery, are described as penumbra of lesion where regional cerebral blood flow is decreased. The neurons locating in penumbra suffer chronic and mild ischemia and undergo apoptotic changes. The penumbra progresses into infarct with time going, and the size of infarct enlarges within 2 weeks after onset of stroke. Meanwhile, neurological deficits of animals exist over long time after stroke. Over past decades, the treatments for stroke patients, who survive after acute phase and suffer from disability, aphasia and even dementia, aim at the penumbra, providing all kinds of neuroprotective agents for dysfunctional neurons and cell replacement therapy for lost neurons, hoping to reset up the damaged cerebral tissue.

## Neuroprotection of GDNF Administration for Stroke

Glial cell line-derived neurotrophic factor (GDNF) is a glycosylated, disulfide-bonded homodimer that is a distantly related member of the transforming growth factor-beta superfamily. GDNF acts through the extracellular glycosylphospatidylinositol (GPI)-linked receptor, GFRα1, and the transmembrane tyrosine kinase, c-Ret, or via a c-Ret–independent mechanism. c-Ret is common for all the ligands, but GFR shows high affinity for specific ligands. For instance, GFRα1 is the preferred receptor for GDNF, but it can also bind NTN (other member of GDNF family). After binding to its specific receptor complex, GDNF activates several downstream intracellular pathways, including phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and extracellular signal-regulated kinase 1/2-mitogen-activated protein kinase (ERK1/2 -MAPK) pathways. Signals through PI3K/Akt or ERK1/2MAPK pathways lead to different trophic effects. The most widespread paradigm is that the PI3K/Akt pathway is involved in cellular survival, whereas the ERK1/2 -MAPK pathway is involved in differentiation, neurogenesis and the migration of newborn cells. Moreover, phosphorylation of Akt and ERK1/2 promotes synaptic plasticity.

It has been well known that GDNF is a potent neuroprotective agent for dopaminergic neurons. GDNF infusion for Parkinson disease patients has shown early clinical efficacy. Recently, it has been shown that GDNF can protect a variety of neuronal damage both *in vitro* and *in vivo*. Particularly, it is a potent survival factor for motoneurons, which is implicated in therapeutic potential for stroke. GDNF receptor expression has been shown to increase in penumbral areas after MCAO in rats. Accumulating evidence also demonstrates that administration of GDNF decreases cerebral infarct and promotes functional recovery in animal stroke models. For example, topical application of GDNF decreased ischemic brain edema and apoptotic cells. The placement of GDNF-presoaked sponges in contact with the surface of cerebral cortex provided protection for the neurons within the surrounding areas in the rat stroke model. Exogenous GDNF gene transfer reduced the infarct size in rat following stroke. Furthermore, its underlying mechanisms have been involved in blocking ischemia-induced nitric oxide and heat shock protein, inhibiting activation of casapse-3, and potentiating and prolonging phosphorylation-Akt activation (Jin et al. 2003).

In addition, several studies suggest that GDNF may induce neurogenesis in central nervous system. First, overexpression of GDNF in neural progenitors induced genes involved in migration and differentiation. Second, intrastriatal GDNF

infusion increased cell proliferation in the substantia nigra and hippocampal neurogenesis in the intact brain. Third, intracerebral infusion of GDNF promoted striatal neurogenesis after stroke in adult rats (Kobayashi et al. 2006). Conversely, Shang et al. recently declared that topical application of GDNF did not affect neurogenesis after transient middle cerebral artery occlusion (Shang et al. 2011).

Although these findings suggest neuroprotection of GDNF infusion in animal stroke model, a major obstacle in clinical use is the GDNF protein delivery to the brain because of the blood–brain barrier, as well as its sustained bioavailability over time. Generally, the methods to deliver GDNF can be categorized into the surgical route of administration, such as intracerebral or intraventricular, and intra-arterial or intravenous systemic administration. The effects of topical administration of GDNF are transient, and repeated administration is needed. Therefore, the topic administration is not optimal for stroke patients. Gene therapy and the use of viral vectors seem to offer a technique for longevity of GDNF expression within the host brain (Boado et al. 2008); however, the safety of viral vectors remains controversial. Recently, genetically engineered cells with ex vivo method have provided a promising approach to GDNF delivery. Given the migratory potential of NSCs toward ischemic areas, NSCs may serve as appropriate vehicles for delivery of GDNF.

## Therapeutic Efficacy After Transplantation of NSCs

Studies over last decade have proved that NSCs are present in neurogenic regions, such as the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) within the dentate gyrus of the hippocampus and non-neurogenic regions such as cortex and striatum and spinal cord. In human, existence of NSCs with self-renewal and multipotent differentiation capability has also been reported in embryonic and adult brain. NSCs not only can proliferate but also can differentiate into neurons, astrocytes and oligodendrocytes *in vitro and in vivo*. A promising strategy for stroke is cell replacement, using NSCs to restore lost cells and reconstruct the damaged neural tissue. Although strategy that augments intrinsic stroke-induced neurogenesis is a promising approach, we here focus on neuroprotection of grafted NSCs in animal stroke model, including distribution and homing to ischemic regions, survival and differentiation, mechanisms and immunorejection.

### Distribution and Homing to Ischemic Regions After NSC Transplantation

In generally, NSCs may be delivered either systemically into the vasculature or locally into the brain. The migratory properties of NSCs enable them effectively homing to ischemic areas. When NSCs are infused by intravenous vessels, they are transported by blood circulation to the ischemic areas. At initial 3–6 h after transplantation, many NSCs were present in the pial vessel and superficial cortex, corpus callosum and perivascular areas in the parenchyma on the bilateral sides. From 1 to 3 days, NSCs were found throughout ischemic striatum and overlying cortex (Chu et al. 2004a). NSCs were also found sparsely in the contra-lesion side. Additionally, some NSCs were detected in the kidney, lung and spleen (Chu et al. 2004a). Although tissue damage, leukocyte infiltration, and neoplasm growth are not found in these organs evaluated by H&E staining, safety of NSCs by vasculature must be highly considered. It has not been exclusively eliminated that the potential side effects resulting from NSCs infiltrating into other organs for there is no assessment of its safety after a long time observation. Recently, intranasal infusion of NSCs was reported to migrate to ischemic regions after stroke. Compared with brain transplantation, intravenous delivery is not the most efficient method of delivering cells in that it allows NSCs to diffuse into other systemic organs; however, the obvious advantage of intravenous administration is its clinical ease and lack of invasiveness.

By contrast, another administration is that NSCs are directly into ischemic striatum, cortex

or lateral ventricle (Chu et al. 2004b; Lee et al. 2007), which results in tissue damage along the needle passages and limits migration of NSCs. After NSCs were infused into the ipsi-lesion ventricle even if into contra-lesion ventricle, NSCs always migrated toward ischemic striatum along corpus callosum. Majority of grafted NSCs were present in the ischemic regions, and more cells in the striatum than in the overlying cortex were observed (Chu et al. 2004b). In coincidence with this, stroke-induced NSCs from neurogenic subventricular zone (SVZ) migrated preferentially into the severely damaged area of the striatum. The fact that grafted NSCs are preferentially homing to ischemic regions, regardless of intravenous or brain infusion, may ascribe to the ischemic niche. Now, it has been well known that angiogenesis is induced in the ischemic regions after stroke. The microvascular endothelial cells may secrete growth factors and chemokines, which may support and permit grafted NSCs to be selectively homing to ischemic regions. Besides, microvascular vessels may act as scaffolds when NSCs migrate toward ischemic regions. Finally, cytokines and/or chemotactic factors released from activated glial cells may contribute to the migration of NSCs.

## Survival and Differentiation of Grafted NSCs

What is the fate of grafted NSCs after stroke? The longest survival of grafted NSCs was 540 days after transplantation (Chu et al. 2004b). In many experimental designs, NSCs were observed only within few weeks after grafting. Previous study showed that 1–3% grafted cells survived in the transient global ischemia at 10 days after transplantation, and other reports showed that 10–30% grafted human NSCs from striatum and cortex survived at 1 month after transplantation in the rat focal cerebral ischemia(Darsalia et al. 2007). Together, the low survival of NSCs after grafting is well accepted, and suggests that the majority of NSCs are died due to ischemic detrimental niche. As a result, this greatly restricts therapeutic efficacy of NSCs.

Survival of NSCs after grafting is mainly influenced by the local niche. Grafted NSCs, as well as host cells, are suffered from the toxic niche resulted from ischemia. Given this, the time and place of grafting may be keys for survival of NSCs (Darsalia et al. 2011). If NSCs are grafted after acute ischemic period and are placed in the ischemic penumbra instead of ischemic center such as infarct cavity, survival rate of NSCs will be increased. It has well been accepted that the optimal time of transplantation is 2 or 3 days after stroke before the inflammatory response is established. Many groups transplanted NSCs 1–3 days following stroke (Chen et al. 2009; Darsalia et al. 2011), while delayed infusion of NSCs in the stroke model was also reported. Compared with most neuroprotective agents having only a few hours therapeutic time window, transplantation of NSCs seems to be practical and optimal treatment for stroke patients. A few approaches have been considered to increase survival, such as combination of NSCs with neurotrophic factors, neurotrophin-3 (NT-3), GDNF, vascular endothelial growth (VEGF) by synthetic recombination protein or by recombinant virus.

Then, how do grafted NSCs differentiate in the host brain? Previous studies demonstrated that very few (3–20%) of remaining NSCs differentiate into neurons in rats with ischemic stroke by double immunofluorescent staining (Chu et al. 2004a, b; Darsalia et al. 2007). When NSCs were grafted in rats with ICH, most remaining NSCs (55–75%) differentiated into astrocytes, and very few (10–30%) differentiated into neurons in recipient brain (Lee et al. 2007). Astrocytic differentiation of transplanted NSCs took place near the infarct core, and neuronal differentiation occurred mostly in the peri-infarct area. About 40–50% of injected cells were not positive to any cellular marker, and remained only β-gal+, undifferentiated cells (Chu et al. 2004a, b). Furthermore, these new born neurons exhibited mature neuronal morphology and expressed mature neuronal markers such as HuD, calbindin and parvalbumin (Darsalia et al. 2007) and produced neurotransmitters demonstrated by double immunofluorescent staining. Taken together, these studies suggest NSCs in the host differentiate

preferentially into astrocytes not neurons. Contrary to these reports, Darsalia et al. reported that grafted NSCs from human striatal and cortex in stroke-damaged rat striatum differentiated into neurons, and none of the grafted cells differentiated into astrocytes or oligodendrocytes (Darsalia et al. 2007). This great difference should not be neglected before transplantation of NSCs is applied in clinical treatment.

## Immunorejection After Transplantation of NSCs

Transplantation of NSCs can be roughly categorized into allografts and xenografts. Despite the immunological privilege of the brain, allografts and xenografts of NSCs will and can elicit activation of the innate and adaptive immune system. In the central nervous system, microglia and astrocytes have similar roles in mounting an innate immune response evoked in the acute stages of tissue injury. Moreover, blood brain barrier (BBB) is disrupted during ischemic episode, this assisting the recruitment of peripheral components of the immune system. Major histocompatibility complex I (MHC I) is a critical mediator of graft rejection and plays a key role in the ability of cytotoxic T cells and natural killer (NK) cells to recognize "non-self" tissue, including the presence of foreign MHC. Double staining has revealed that astrocytes from NSCs indeed express MHC class I and class II molecules, while neurons from NSCs express only detectable levels of MHC class I. Since NSCs have been demonstrated to constitutively express MHC I, immunorejection after NSC transplantation is considered to be unavoidable. Surprisingly, despite the expression of MHC I on NSCs, NSCs are not recognized by alloreactive lymphocytes *in vitro*. In addition, a series of studies in stroke animal models showed that human or rat NSC transplantation could survive with no signs of immunologic rejection (e.g., lymphocyte infiltration in the systemic organs) without using immunosuppressive agents (Chen et al. 2009; Lee et al. 2007). The newly born neurons from human NSCs have been demonstrated to integrate with rat or mice neural circuits. In spite of these reports, there is wide consensus that exposure to inflammatory cytokines such as stroke-induced IFN-γ and TNF-α stimulates MHC expression in NSCs (Preynat-Seauve et al. 2009), thus indicating a potential risk for immunological rejection due to MHC incompatibility and subsequent requirement of immunosuppressive treatment to avoid rejection. Therefore, caution should be highly paid to ensure that the highest standards of safety and scientific rigor as for the immunorejection before NSC transplantation will be utilized clinically in the future in spite of non-immunorejection after NSC transplantation in animal stroke models.

## How Grafted NSCs Elicit Their Effects

To date, it has been reported that NSC transplantation can promote functional recovery in stroke model. However, the underlying mechanisms are remained controversial. The possible mechanisms may be as follow. First, neurotrophic actions of NSCs have been demonstrated. NSCs constitutively secrete neurotrophic factors (NTs), such as GDNF and NGF, may rescue dysfunctional neurons and promote neurite outgrowth after stroke, thereby preserving existing neural circuitry. Besides, NTs have been shown to promote intrinsic neurogenesis and influence differentiation of NSCs. Second, cell replacement may still contribute to neuroprotection of NSCs against stroke, although it is a very few mature neurons differentiated from NSCs in many experimental paradigm. Third, NSCs may enhance synaptic plasticity, by promoting the formation or maintenance of new connections by host neurons. Expressions of synaptic proteins, such as postsynaptic density-95 and synaptophysin were increased after NSC transplantation (Chen et al. 2009). Recently, Human NSC treatment has been shown to significantly increase dendrite plasticity in both the ipsi- and contra-lesion cortex and this coincides with stem cell-induced functional recovery (Andres et al. 2011). Axonal transport, which is critical for both proper axonal function and axonal sprouting, was inhibited by stroke, but was rescued by NSC treatment, indicating human

NSCs enhance synaptic plasticity (Andres et al. 2011). However, a causal link has not yet been established between functional benefit after cell transplantation and the synaptic integration of grafted cells in the rat stroke model. Fourth, human NSCs and their derived astrocytes do not initiate, but instead suppress an allogeneic response, suggesting a role of NSCs as immunomodulators contributing to improved functional recovery (Lee et al. 2008; Akesson et al. 2009). These all mentioned mechanisms may contribute to the neural behavior recovery after NSC transplantation. Manipulations that are directed at increasing survival and neuronal differentiation of NSCs have been shown to improve functional outcome, including modifying niche of grafted NSCs.

## The Niche Influencing Fate of Grafted NSCs

After grafting, remaining NSCs are capable of differentiating toward neuronal, astrocytic, oligodendrocytic and even endothelial cells, depending on their spatial microenvironment or niche in the host brain. The niche is more complicated and includes intercellular interactions, extracellular matrix proteins, vasculature, host neurons and glial cells, cerebrospinal fluid, and soluble factors such as growth factor and cytokine. The niche ensures the maintenance of self-renewal and the multipotent state of NSCs. This intricate and well-tuned signaling network regulates the transcriptional profile of the NSCs to either keep cells in an undifferentiated state or trigger the precise timing for neurogenesis or gliogenesis to occur. An emerging view points to overlapping pathways of growth factors, metalloproteases, neurotransmitters, and hormones to regulate different aspects of NSCs.

### Growth Factor and Cytokine Niche

It has been noted above that NSCs preferentially differentiate into astrocytes not neurons in the recipient brain. Some studies to date have shown that neurotrophic factors (NTs) and other compounds may promote survival and neuronal differentiation of NSCs. Some receptors of growth factors and NTs have been found to be expressed on NSCs. The specific binding of ligand and receptor can influence differentiation of NSCs. For example, NSCs overexpressing NT-3 was demonstrated to increase the proportion of NSC-derived neurons approximately 20% in the infarction cavity and >80% in the penumbra compared with NSC grafting alone. The new born neurons variously differentiated into cholinergic, GABAergic, or glutamatergic subtypes, appropriate to the cortex. Other factors such as vascular endothelial growth factor (VEGF), which induces both angiogenesis and neurogenesis, and pigment epithelium-derived factor (PEDF), are both known to play a role in self-renewal of NSCs.

In addition, bone morphogenetic proteins (BMPs) are a family of multi-functional growth factors. Among BMPs, BMP4 particularly induces astrocytic and neuronal differentiation of NSCs, depending on the balance between neuronal and astrocytic differentiation pathways. Recently, BMP4 has been shown to induce attachment of neurospheres and astrocytic differentiation of neurosphere NSCs, which is mediated by PI3K/Akt pathway not ERK pathway (Kim et al. 2010). Moreover, interleukin-15 (IL-15) and the IL-15 receptor are expressed in central nervous system and neuronal cell lines. Recent report showed that the α subunit of the IL-15 receptor was expressed in NSCs and differentiating neurons, but not astrocyte or oligodendrocyte progenitors. IL-15 treatment reduced microtubule-associated protein 2 levels in neurons and reduced neurite outgrowth in differentiating neurons but did not affect NSC proliferation, cell proportions and viability of the corresponding lineage cells (Huang et al. 2009).

Finally, hematopoietic factors, including erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), have been considered as a neuronal growth factor in the brain. It has recently been identified that these hematopoietic factors counteract apoptosis, reduce infarct size in stroke models *in vivo* and

drive neurogenesis after stroke. EPO enhanced neuronal differentiation of NSCs *in vitro*, EPO-receptor deficient mice displayed reduced embryonic neurogenesis, mice with a brain-specific knock-down of the EPO receptor showed a decrease in stroke-induced neurogenesis and migration, and exogenously EPO enhanced neurogenesis and behavioural outcome after injury models *in vivo*. In addition, the G-CSF receptor was expressed on NSCs in the brain, and G-CSF stimulated neuronal differentiation of NSCs *in vitro*, and treatment of animal injury models with G-CSF enhanced neurogenesis and functional recovery (Sehara et al. 2007). GM-CSF is an anti-apoptotic and neuroprotective *in vivo*, passes the intact blood–brain barrier. Likewise, recent studies showed that the GM-CSF receptor alpha was expressed on adult NSCs in the rodent brain and in culture. Addition of GM-CSF to NSCs *in vitro* increased neuronal differentiation in a dose-dependent manner (Krüger et al. 2007).

## Vascular Niche

In view of the residence of NSCs in a vascular niche, a network of blood vessels surrounding the SVZ also represents a crucial component of the NSC niche. The cellular adhesion between the NSCs and endothelial cells, which is mediated by laminin receptors in the NSCs, contributes to the proliferation and positioning of NSCs *in vivo*. It was found that SVZ neurospheres cocultured with endothelial cell-conditioned medium generated more immature appearing neurons, oligodendrocytes and astrocytes with radial glial-like/reactive morphology (Plane et al. 2010). Whereas, SVZ neurospheres cocultured oxygen-glucose deprived endothelial cells-conditioned medium stimulated neuroblast migration and yielded neurons with longer processes and more branching (Plane et al. 2010). These data indicate that intact and injured endothelial cells exert different effects on NSCs, and endothelial cells release important factors for maintenance and differentiation of NSCs. Furthermore, it has been also reported that co-transplantation of endothelial cells and NSCs increases survival and neuronal differentiation compared with transplantation of NSCs alone (Nakagomi et al. 2009).

## Extracellular Matrix Niche

Extracellular matrix (ECM) may influence the survival, migration and differentiation of NSCs. Reelin is a large extracellular matrix (ECM) secreted glycoprotein which binds to very low density lipoprotein receptor and apolipoprotein E receptor 2, triggering the adaptor function of the cytosolic protein disabled-1(Dab1) and the phosphorylation of Src family kinases. Recent studies have shown that the absence of Reelin negatively affects proliferation, neurosphere of NSCs-forming ability and neuronal differentiation, but it does not affect astrocytic differentiation of neurosphere (Massalini et al. 2009). Conversely, Kwon et al. reported that expressions of neural and glial markers were not changed in differentiating NSCs by Reelin treatment, indicating that differentiation of NSCs is Reelin-independent (Kwon et al. 2009). Also, it was showed the enhanced differentiation of Dab1$^{-/-}$ neurospheres into GFAP-positive cells and no change of neuronal differentiation of neurosperes compared with normal neurospheres (Kwon et al. 2009). In addition, β-catenin, a protein that functions in both cell adhesion and Wnt signaling, plays a key role in mammalian neural development. β-catenin siRNA decreased the neuron-specific enolase-positive neurons and increased GFAP-positive astrocytes in the NSCs after hyperbaric oxygen therapy *in vitro (*Zhang et al. 2011) .

Matrix metalloproteinases (MMPs) represent a family of enzymes known to play a role in the modification of ECM and can change the cell-ECM substrate interaction. MMPs participate in numerous physiological and pathological processes through the processing of a variety of pericellular substrates including extracellular matrix proteins, cell surface receptors, cell adhesion molecules and growth factors. MMPs are abundantly expressed in NSCs isolated from the human central nervous system and they have regulatory roles during the proliferation and differentiation of NSCs in the embryonic mouse brain. Furthermore,

it was reported that mRNA expressions of both MMP-9 and/or MMP-2 in NSCs of the SVZ were increased several-fold after ischemic insult in adult rats. Recently, it was shown that dynamics of MMPs activation in the dentate gyrus correlated closely with the rate of proliferation and differentiation of NSCs into mature neurons in gerbil global forebrain ischemia. In contrast, lower activity of MMPs paralleled no new born mature neurons in the damaged CA1 of hippocampus (Wójcik-Stanaszek et al. 2011). *In vitro,* MMP inhibitors interfered with both the proliferation and differentiation of the human neural stem cell line derived from umbilical cord blood toward the neuronal lineage (Wójcik-Stanaszek et al. 2011). Taken together, these data suggest that dynamic evolution of MMPs activity matches the progression of proliferation and differentiation of NSCs into mature neurons, highlighting the potential role of these extracellular proteinases in ischemia-induced neurogenesis.

## Glial Niche

In the CNS, glial cells mainly composed of astrocytes and microglia, are the major support for neurons and play critical roles during physiological and pathological conditions. After stroke or other injury, they are activated and secrete neurotrophic factors and cytokines, which can influence the survival and differentiation of NSCs. The importance of astrocytes in the NSC niche was first elucidated using coculture experiments with adult-derived NSCs and astrocytes isolated from different regions of the CNS. Astrocytes, isolated from neurogenic regions of SVZ and SGZ, were able to promote proliferation of NSCs and induce NSCs to differentiate into neurons *in vitro and in vivo.* Whereas astrocytes isolated from the spinal cord or cerebral cortex of adult mice failed to do so (Jiao and Chen 2008). The potential mechanisms may be that astrocytes from SVZ and SGZ can release Sonic hedgehog and Wnt signaling molecules (Jiao and Chen 2008). These indicate that astrocytes throughout different CNS regions exhibit regional heterogeneity with regard to their ability to promote proliferation and differentiation of NSCs. Recently, it has been reported that astrocytes co-incubation with stroke-induced NSCs promote differentiation of stroke-induced NSCs into neurons, astrocytes and oligodendrocytes in the rat stroke model (Nakagomi et al. 2009). Furthermore, astrocytes co-incubation with stroke-induced NSCs increased the number of nestin-positive cells of stroke-induced *in vitro* (Nakagomi et al. 2009). Taken together, these data indicate astrocytes from SVZ and SGZ are capability of enhancing proliferation and differentiation of NSCs.

Microglia is immunocompetent cells of CNS, which are activated in response to injury and diseases and adopt a phagocytic and cytokine secreting phenotype. Microglia are activated following stroke and express a variety of pro-inflammatory cytokines including IL-1β, IL-6 and tumor necrosis factor-a (TNF-a), reactive oxygen species, reactive nitrogen species and nitric oxide (NO). These inflammatory cytokines have been reported to induce in neuroinflammation and neurotoxicity and be involved in cell survival at all stages of differentiation of NSCs. Conversely, microglia are known to produce neurotrophic factors such as GDNF, brain derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), by which microglia can positively influence the survival, proliferation and differentiation of NSCs. Furthermore, human microglia transplantation in rat stroke model induced neuroprotection and behavioral improvement. Together, activated microglia after stroke is beneficial or detrimental depending on diverse microenvironments.

## Cerebrospinal Fluid

Finally, given that fact that NSCs reside in the SVZ and SGZ neurogenic region, growing evidences show that cerebrospinal fluid (CSF) function in survival and differentiation of NSCs. It was reported that NSCs cultured in CSF showed an increased survival rate, a higher number of BrdU positive and DNA synthesizing nuclei compared to standard culture media. Moreover,

CSF increased the survival rate of adult human NSCs; inhibited proliferation of adult human NSCs; CSF increased extension outgrowth velocity from adult human NSCs; CSF facilitated astrogliogenesis but inhibited neurogenesis from adult human NSCs (Buddensiek et al. 2010). In summary, CSF promotes survival and astroglial differentiation of adult human NSCs but inhibits proliferation and neuronal differentiation. Consequently, NSC transplantation by ventricle may not be better way for CSF inhibiting neuronal differentiation.

## Therapeutic Efficacy of NSCs Overexpressing GDNF for Stroke

In spite of improved functional recovery after NSC transplantation in stroke model, the low survival rate of grafted NSCs and low neuronal differentiation are the bottleneck of NSC graft therapy for neurological disorders including stroke. On the other hand, delivery of neurotrophic factors, such as brain-derived neurotrophic factor, vascular endothelial growth factor or glial cell line-derived neurotrophic factor (GDNF), modulates ischemic niche in the brain, and can promote proliferation, differentiation and survival of grafted NSCs. As it is above mentioned, administration of GDNF through intracerebral or intraventricular space has shown neuroprotection against stroke. Whereas, repeated administration of GDNF through this way is required in experimental animals to sustain bioavailability. It has recently been demonstrated that transplanting NSCs modified with GDNF can provide more efficient treatment for stroke.

In ICH mice model, transplanting human NSCs overexpressing GDNF (GDNF/NSCs) showed significantly behavioral improvement by the rotarod test and limb placement test compared with transplanting NSCs alone at 2 and 4 weeks after transplantation (Lee et al. 2009). Grafting GDNF/NSCs significantly increased the number of grafted cells at 2 and 8 weeks post-transplantation compared with grafting NSCs. In addition, the majority of transplanted cells differentiated into neurons or astrocytes (Lee et al. 2009). These data indicate that NSCs overexpressing GDNF promote survival of grafted cells in rat stroke model. The possible mechanisms were shown to reduce the apoptotic cells by decreasing the expression of p53, caspase 9, caspase 3 and Bax (Lee et al. 2009; Chen et al. 2009). It is in agreement with above reports in which GDNF exerts effects by inhibiting apoptosis.

In contrast, in rat MCAO stroke model, transplanting GDNF/NSCs and NSCs significantly promoted neurological behavior recovery by a modified Neurological Severity Score (mNSS) compared with ischemic rats from 1 up to 7 weeks after transplantation, and greater effects of GDNF/NSCs were observed compared with the NSCs alone (Chen et al. 2009). In addition, grafting GDNF/NSCs and NSCs significantly reduced lesion volume. GDNF/NSCs significantly reduced lesion volume at 4 days after transplantation compared with NSCs (Chen et al. 2009). The underlying mechanisms may be that grafting GDNF/NSCs enhances expression of synaptic protein, including synaptophysin and postsynaptic density-95, inhibits apoptosis and increases expression of neurotrophic factors including BDNF and NT-3(Chen et al. 2009).

In summary, the administration of NSCs modified with GDNF shows better neuroprotection for stroke than NSCs alone, suggesting a promising approach to treating stroke patients.

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# 24 Derivation of Germ Cells from Pluripotent Stem Cells: Prospects and Applications

Jinlian Hua, Yue Hu, and Long Wang

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**Abstract**

Germ cells are unique in that they are responsible for passing genetic information to the next generation. Pluripotent stem cells may differentiate into germ cells and even gametes. Here we review mainly about the progresses of pluripotent stem cell differentiation into germ cells and their potential application.

## Introduction

Pluripotent stem cells, which include embryonic stem cells (ESCs) derived from the inner cell mass of an embryo, epiblast stem cells (EpiSCs), embryonic germ cells (EGCs), adult germline stem cells (GSCs), and induced pluripotent stem cells (iPSCs), have unrestricted developmental potential, and thus can give rise to all cell types including germ cells (Hanna et al. 2010). The derivation of human embryonic stem cells (hESCs) and other pluripotent stem cells provides a new era in stem cell research and helps for their therapeutic potential in basic research and regenerative medicine (Hanna et al. 2010; Thomson et al. 1998).

Approximately, one in seven couples suffer from infertility. There are many factors that affect fertility; lack of mature gametes is one main cause. The formation and specification of primordial germ cells (PGCs) and gametes is a basic problem in developmental biology. Germ cells are highly specialized populations of cells that are necessary for the continuation and evolution of

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the species. Mammalian germ cell development is difficult to study, as important early events occur after implantation in the womb. This difficulty is most evident in the human situation because of ethical issues surrounding embryo manipulations (Nagano 2007). Recently, several groups have shown that germ cells can be produced *in vitro* from ESCs (Toyooka et al. 2003; Geijsen et al. 2004; Nayernia et al. 2006; Clark et al. 2004; Kee et al. 2009) and adult stem cells (Dyce et al. 2006, 2011). First live births of offspring from ESC-derived germ cells have been reported (Hayashi et al. 2011). These results suggest that gametogenesis from ESCs may provide an *in vitro* model to investigate germ cell development and facilitate molecular analysis of the germ lineage. Formation of primordial germ cells derived from ESCs is robust, but generating mature germ cells (sperm or oocytes) from pluripotent stem cells remains inefficient and doubtful. Although ESCs are useful for research, clinical usage of stem cell-derived gametes is still a distant prospect. This review focuses on the recent attempts regarding the *ex vivo* development of germ line development from ESCs and the progress and future issues relevant to this field.

## Germ Cell Development

In mice germ cells are specified in the early post-implantation embryo. Inductive signals from the extraembryonic tissues induce primordial germ cell (PGC) precursor cells of germ cells which appear in the proximal epiblast at embryonic day (E) 6.25 (Ohinata et al. 2009). The PGC precursor cells express Fragilis and Blimp1 (Prdm1). These precursor cells give rise to ~40 founder PGCs at E7.25, which express Stella (Dppa3), and are located at the posterior primitive streak. The PGCs then move by active locomotion into the subadjacent endoderm where they begin migration to the gonad. By E8.5, PGCs are found in the hindgut epithelium. At this time, PGCs undergo genome-wide epigenetic reprogramming. At E10.5, PGCs reach at the genital ridges (or fetal gonads). Then, PGC proliferate and increase in numbers, both during migration as well as after genital ridge colonization, until E13.5. After E13.5, male gonocytes undergo mitotic arrest, which lasts until after birth, while female gonocytes undergo meiosis. At E13.5, female germ cells enter prophase of the first meiotic division. They pass through leptotene, zygotene, and pachytene before arresting as diplotene oocytes at the time of birth.

Human PGCs have been identified in the endoderm of the allantois and in the mesenchyme of the stalk at E22. At 4–5 weeks of gestation, the colonization of the gonadal ridges has already formed, and at 7 weeks the testis and ovary appear differentiated. In the human embryo, PGCs differentiate to form oogonia or pre-spermatogonia after incorporation into the gonads and are characterized by a high proliferation rate. The mitotic proliferation of oogonia and prospermatogonia lasts 3–4 weeks. At 11–12 weeks of gestational age, oogonia enter the meiosis cycle (oocyte I), while prospermatogonia cease mitotic division at 18–20 weeks gestation. Human PGCs proliferate actively during migration. In the ovary, the maximal number of oogonia is estimated to be $3\times10^6$ per ovary at 16–20 weeks. A characteristic of human oogenesis over this period is that the developmental stages of germ cells are not synchronized. In fact, until 3 months of fetal life, oogonia and primary oocytes coexist in different stages of meiosis.

The cDNA libraries, microarrays, and expression profiles from PGCs and differentiation stages of germ cells should be valuable resources for researchers in this field. *In vitro* studies have provided important views on the biology of PGCs, and will continue to supply information that can only be obtained by the *in vitro* culture of isolated cells. It is of important to note that germ cell specification and derivation of germ cells from ESCs are feasible in culture.

## Differentiation of Pluripotent Stem Cells (ESCs/iPSCs) to Germ Cells

In recent years, scientists have made significant progresses regarding the establishment of human ES cell lines and their differentiation (Thomson

et al. 1998; Clark et al. 2004). The primordial germ cells (PGCs) and the derived-embryonic germ cells (EGCs) share many properties with ESCs (Shamblott et al. 1998). ESC lines have now been derived from inner cell mass of embryos, and they can differentiate into functional gametes which have the ability to participate in embryonic development, even produce live pups after transplantation in mice (Hayashi et al. 2011). It has been demonstrated that mouse and human embryonic stem cells can differentiate into PGCs and subsequently to early gametes (oocytes and sperm) (Toyooka et al. 2003; Geijsen et al. 2004; Nayernia et al. 2006; Clark et al. 2004; Kee et al. 2009). Immature sperm cells derived from mESCs in culture have been reported to produce live offspring (Nayernia et al. 2006; Hayashi et al. 2011). In addition, preliminary research has indicated that hESCs and iPSCs probably have the potential to differentiate into germ cells by some cytokines, cocultured with specific cells or transfecting germ cell genes (Park et al. 2009; Tilgner et al. 2008; West et al. 2008; Kee et al. 2009; Bucay et al. 2009; Panula et al. 2011; Hayashi et al. 2011). Adult stem cells have also been reported to differentiate into mature germ cells *in vitro* (Dyce et al. 2006). Thus, stem cells may offer a valuable *in vitro* model for the investigation of germ cell development and early stages of human gametogenesis including epigenetic modifications of the germline. Table 24.1 shows the progresses of germ cell derivation from stem cells.

## Conclusions and Perspectives

The presented studies indicated that the derivation of mammalian germ cells, both female and male, is possible *in vitro* from pluripotent stem cells. These *in vitro* systems can be used to improve our knowledge regarding the mammalian germ cell development and also develop novel reproductive technology. An *in vitro* germ cell line derivation system could provide a valuable platform to study some aspects of germ cell development (Fig. 24.1). However, there are three interrelated issues that need to be addressed in order to use this model. The first is that germ cell induction thus far reported appears to be a very rare event. Until now most current methods reported the derivation of eggs or sperm from ESCs as spontaneous and stochastic events or the induction efficiencies were still very low, which makes it difficulty to analyze each of the complex steps leading to the production of germ cells. Therefore, a system is needed which allows directed induction and maintenance of germ cell development. The second problem is that it is yet to be determined if the *in vitro* process faithfully recapitulates normal egg or sperm development *in vivo*. Developing sperm and egg must acquire their characteristic identity tags or imprints, which regulate their functions when embryonic development begins after fertilization. However, it is not yet known whether the gametes derivation *in vitro* have the appropriate imprints. This information is crucial because sperm and eggs seem normal even without the appropriate marks, but their functions will be affected after fertilization when development commences. The third problem is what are the key differences among ESCs, adult stem cells and germ cells, and what is the source of germ cells in adult stem cells? It is difficult currently to explain the derivation of germ cells from adult stem cells. In fact, as described above, the study of *in vitro* germ cell induction is still at a preliminary stage where we apply knowledge obtained in studies *in vivo* to situations *in vitro*, but not *vice versa*. Above all, it is essential to assess whether the three critical steps in germ cell development (specification, migration/proliferation, and colonization/sex-differentiation pre- and postnatal development) do take place in the *in vitro* systems. For the induction of germ cells *in vitro*, specification is the first step, and perhaps the most critical step. Researchers are investigating into the microenvironment including regulatory genes, transcriptional factors responsible for germ cell specification, and the role of local cues from the somatic cells in this process.

Sperm development requires supportive Sertoli cells, while oocyte maturation requires the nourishment from both the theca and granulosa cells. The findings that the accelerated events of the

**Table 24.1** Progresses in production of male/female gametes *in vitro* from stem cells

| Authors | Source | Method | End product |
|---|---|---|---|
| Male gamete production *in vitro* from stem cells | | | |
| Hayashi et al. (2011) | mESC, iPSC | EpiLC induction from ESCs/iPSCs. PGCLCs were induced , Transplantation of the PGCLCs into seminiferous tubules of Neonatal W/Wv Mice,and intracytoplasmic sperm injection (ICSI) | Spermatogenesis and normal offspring from PGCLCs derived ESCs/iPSCs |
| Yu et al. (2009) | 129/SV mESC—XY | Monolayer induction, combined with ectopic expression of Dazl for more than 5 days | Motile tailed-sperm, oocyte-like cells and ovarian follicle-like structures |
| Kee et al. (2009) | hESC-XX,XY(VASA-reporter) | Adherent differentiation combined with over-expression of DAZL, DAZ and BOULE for 14 days | Haploid gametes |
| Aflatoonian et al. (2009) | hESC | EB formation, culture medium with BMP4, RA and neonatal mouse testes conditioned media (14 days) | Round spermatid (1–5%), follicle-like structures, but failed to detect zona pellucida in these structures |
| Yamauchi et al. (2009) | Monkey ESC | EB induction, combined with RA, gonadal cell-conditioned medium and growth factors | Haploid gametes |
| West et al. (2008) | hESC-XY | MEF feeder and basic fibroblast growth factor (bFGF) for 10, 16 and 30 days | Haploid germ-like cells |
| Kerkis et al. (2007) | 129/SV mESC—XY | EB induction, combined with Neurobasal (NB) medium with B27 and 0.1 μM RA | FE-J1$^+$ cells, follicular-like structures, presumptive oocytes and blastocysts |
| Nayernia et al. (2006) | SSC7, SSC12 cell lines derived from ESC | RA induction ($10^{-5}$), FACS selection of Stra8-EGFP$^+$ cells; transfected with the Prm1-DsRed fusion vector, RA induction for 72 h | Prm1$^+$ haploid gametes were derived and live offsprings were got by ICSI |
| Geijsen et al. (2004) | mESC-XX,XY | EB formation, combined with RA induction. On the day 20, FE-J1$^+$ cells were isolated by FACS and injected into oocytes by ICSI | Blastocysts were derived after transplantation (0.01%) |
| Toyooka et al. (2003) | 129/SV mESC-XY | EB induction combined with BMP4-producing cells for 8–10 days, selected MVH-positive cells and transplanted into host testis capsule for 6–8 weeks | Functional sperm |

| Female gamete production *in vitro* from stem cells | | | |
|---|---|---|---|
| Hübner et al. (2003) | mESC-XX, XY (Pou5f1-reporter) | Monolayer, no feeder cells, spontaneous differentiation (43 days) | Oocyte-like cells (20% produced oocytes larger than 40 μm), blastocyst-like structure after parthenogenic activation |
| Clark et al. (2004) | hESC-XX, XY | EB formation,spontaneous differentiation (14 days) | Oocyte-like cells |
| Dyce et al. (2006) | Fetal porcine skin stem cell | Aggregate formation, medium with follicular fluid, gonadotrophins (40 days) | Oocyte-like cells (0.014%) and blastocyst-like structures |
| Danner et al. (2007) | Rat pancreatic stem cell | EB formation, spontaneous differentiation (9 days) | Oocyte-like cells and tissue-like structures |
| Lacham-Kaplan et al. (2006) | mESC-XY | EB formation, conditioned media from testicular cell (7 days) | Ovarian structures (83%) containing putative oocytes |
| Novak et al. (2006) | mESC-XY | Spontaneous differentiation in adherent cultures and through EB formation (20 days) | Ovarian follicles |
| Kee et al. (2006) | hESC-XX | EB formation, growth factors addition (7 days) | Oocyte-like cells |
| Qing et al. (2007) | mESC-XY | EB formation, co-cultured with ovarian granulose cells (10 days) | Oocyte-like cells |
| Salvador et al. (2008) | mESC-XX (Gdf9-reporter) | EB formation, culture medium with LIF (45 days) | Oocyte-like cells but no follicular structures |
| Linher et al. (2009) | Porcine skin stem cells | Monolayer, medium with porcine follicular fluid (50 days) | Oocyte-like cells (1.4%) |
| Nicholas et al. (2009) | mESC-XX(oct4-GFP and stra8-GFP reporter) | EB formation, culture in media containing a germ cell maturation factor cocktail in the absence of feeder layer support (19 days) | Meiotic germ cells, and primary ovarian follicle formation following ovarian niche transplantation under the kidney capsule (21 days) (0.023%) |
| Lavagnolli et al. (2009) | Hybrid cells (ES-SCH): fusion of ESCs and splenocytes from adult female mice | EB formation, culture medium with RA (8 days) | Enlarged cells resembling PGCs on the EB periphery |

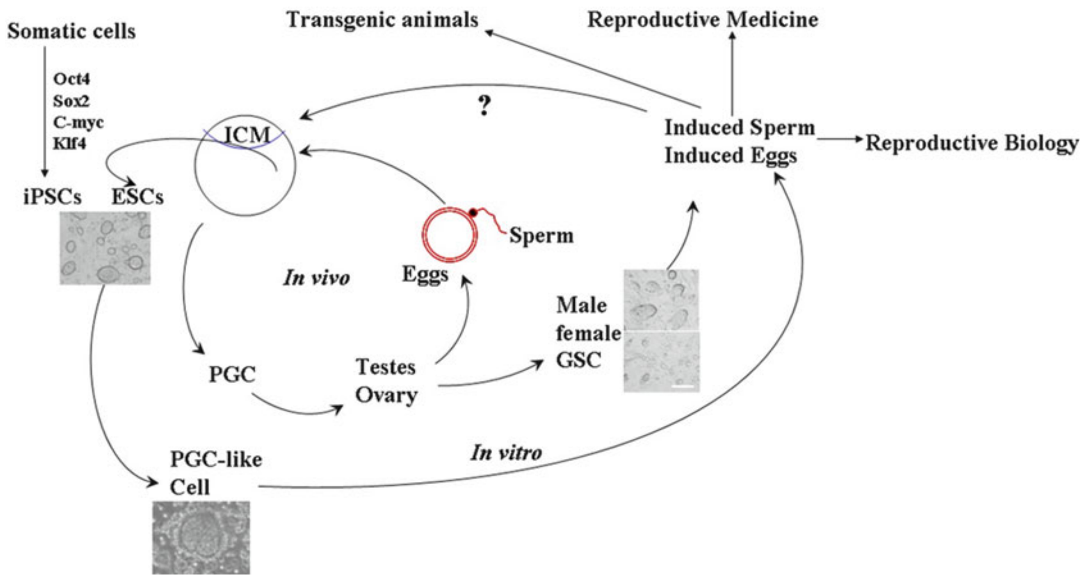


**Fig. 24.1** Derivation of germ cells from pluripotent stem cells, their applications and the cycle of germ cell development. *GSC* germ-line stem cell

derivation of oocyte and sperm from ESCs suggests that either a supportive niche development from ESCs during differentiation or those *in vitro* created germ cells do not need the same support as they do *in vivo*. Studies to identify the presence of the germ cell niche or a means of enhancing their formation from stem cells could contribute towards more efficient and directed strategies for germ cell development. Further proper media conditions, including appropriate nutritional supplements (growth factors, hormone, and extracellular matrix) could help the derivation of germ cells from stem cells (Hua and Sidhu 2008). The emergence of *in vitro* derivation of both germ cells and mature gametes will allow investigations into processes guiding germ cell formation and therapeutic use for these cells. Stem cell derived gametes could be applied in animal breeding and assisted reproductive technology. Additionally, stem cell derived gametes and germ cells may provide an important *in vitro* culture system to study the roles of key genes and complex signaling processes involved in genomic imprinting and reductive division events during meiosis. For example, synthetic sperm and oocyte derived from hESCs could be used to treat male and female infertility. Moreover, if germ cells derived from stem cells can engraft in the host testis and produce viable sperm and oocytes, this could provide insights into spermatogenesis and oogenesis possibly providing new treatments for infertility.

An unlimited supply of stem cells and their derived lineages could have an important impact on medicine. The eggs could be used as recipients for nuclei or genetic material from adult stem cells such as skin stem cells. If these eggs can reprogramme the adult nucleus and successfully develop into the blastocyst stage, patient specific customized hESC lines resulting from nuclear transfer could be created. These customized cells in turn could be used to produce specific cell types for transplantation and to treat specific diseases and allow study of diseases that are currently impossible (Surani 2004). The stem cell-derived oocytes produced for nuclear transfer studies would be a significant advance and may provide an unlimited source of oocytes, which would avoid the problems of scarcity of human eggs and specific ethical considerations.

In conclusion, *in vitro* production of germ cells raises the possibility of deriving mature

gametes from stem cells *in vitro*, providing an option that solves the ethical problems as well as the prospects of unprecedented medical advances. However, the rare events and potential of germ cells derived from stem cells need further research investigations.

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# Purinergic Receptors in Stem Cell Biology

# 25

Henning Ulrich

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**Abstract**

Purinergic receptors belong to the first receptors expressed during embryonic development. As they are expressed by almost every type of cells, it has become clear that these receptors are essential for controlling vital functions of cellular function, much beyond their participation in neurotransmission. In fact, experimental evidence points at regulatory functions of purinergic receptors in proliferation, differentiation, cell death and successful engraftment of stem cells originated from diverse origins. Effects induced by ATP and other extracellular nucleotides depend on the type and differentiation stage of stem cells and environmental conditions of stem cell niches as well as on the subtypes of receptors which have been activated. Metabotropic G-protein-coupled P2Y receptors participate mostly in regulation of proliferation and differentiation; however, besides their functions in stem cell recruitment, these receptor subtypes may also promote inflammatory responses and thereby contribute to disease conditions. Among the ionotropic P2X receptors, the P2X7 subtype is most important for stem cell biology and therapeutic applications due to its induction of pro-inflammatory actions and cell death hindering successful engraftment and differentiation of stem cells. Besides providing promising targets for cell regeneration therapy, purinergic receptors have implications in cancer stem cell growth and differentiation, revealing novel mechanisms for therapeutic intervention.

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## Introduction

During the last decades much effort has been put into the study of developmental processes, including cell proliferation, differentiation, tissue repair and elimination of not any more needed cells. Due to the complexity of elucidating functions of a protein of interest *in vivo*, stem cell models are employed to study mechanisms of differentiation in simplified conditions. Such studies result in the development of *in vitro* protocols for stem cell culture and differentiation into desired cell types with possible therapeutic applications. Moreover, the investigation of stem cell biology will elucidate mechanisms of phenotype determination and control of proliferation and mobilization of endogenous adult stem cells. Such knowledge is important for understanding mechanisms underlying transformation of quiescent stem cells into cancer stem cells as well as the functions of endogenous stem cell niches in tissue repair.

Stem cells reflect specific developmental stages. Pluripotent stem cells (embryonal carcinoma (EC) and embryonic stem (ES) cells) are capable to originate cells of the three primary germ layers of the embryo. Recently, innovative research has revealed that transfection of pluripotency genes into adult somatic cells reconstitutes their embryonic stage. These reprogrammed cells, termed induced pluripotent stem (iPS) cells, with characteristics similar to those of ES cells, were obtained in 2006 from differentiated mouse cells and in 2007 from human cells. The recent described capacity of genetically reprogrammed somatic cells into pluripotent ones could help to overcome current limitations of the therapeutic use of stem cells such as the lack of histocompatibility and ethical concerns.

Stem cells are proliferating by undergoing symmetric cell division and producing two identical cells or by asymmetric division originating a stem cell and another more differentiated cell. Concomitantly, differentiating ES cells loose marker proteins, characteristic for pluripotency, and acquire protein expression profiles of tissue-specific stem cells with limited differentiation capacity. These tissue-specific stem cells are present in the fetal as well as in the adult tissue where they remain throughout life, and are recruited by specific signalling events for replacement of damaged or lost tissue (Glaser et al. 2012).

Adenosine 5′-triphosphate (ATP) was identified in 1970 as the responsible transmitter for non-adrenergic, non-cholinergic neurotransmission in gut and bladder. Purinergic cotransmission was proposed in 1976, and ATP is now recognized as a neurotransmitter in all nerves in the peripheral and central nervous systems (Burnstock 2012). Following an initial slow acceptance, the cloning of various receptor subtypes led to an increase in scientific interest of this topic and turned the study of purinergic signaling into a rapidly expanding field. Almost every cell expresses purinergic receptors pointing at crucial participation of these receptors in diverse cellular functions.

Purinergic receptors are divided into two families based on pharmacological properties. P1 receptors composed of A1, A2A, A2B and A3 subtypes respond to adenosine and its analogues, while P2 receptors are principally activated by ATP and other nucleotides. P2 receptors are further divided into ionotropic P2X and P2Y receptors based on structural differences (Fig. 25.1). Ionotropic receptors are assembled in trimeric form as homo- or heteromeric receptors from P2X1-P2X7 subunits. P2X receptor channels activated upon stimulation with ATP allow the flux of $Ca^{2+}$, $Na^{+}$ and $K^{+}$. The mammalian metabotropic receptor family contains eight P2Y1,2,4,6,11,12,13,14 receptor subtypes. Signal transduction is principally initiated by mobilization of calcium from intracellular stores in case of P2Y1,2,4,6,11 subtypes or inhibition of adenylate cyclase by P2Y12,13,14 receptors (Burnstock 2012).

The availability and life time of ATP liberated in controlled manner for autocrine or paracrine stimulation of purinergic receptors is controlled by a highly efficient enzymatic cascade including ectonucleoside triphosphate diphosphohydrolase (E-NTPDases 1–8), ectonucleotide pyrophos phatases/phosphodiesterases (E-NPPs), ecto-alkaline phosphatases, and ecto-5’-nucleotidase/CD73. These enzymes degrade nucleoside tri- and

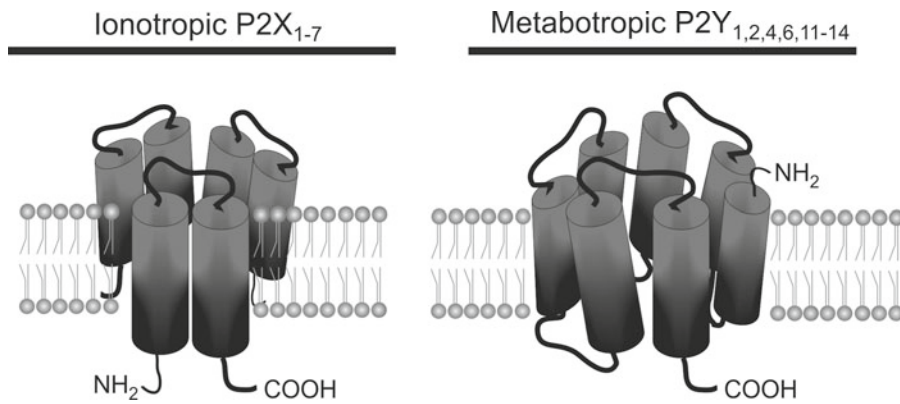


**Fig. 25.1 Division of P2 receptors into P2Y and P2X subtypes based on structural differences.** P2X ionotropic receptors are assembled as homo- or heteromeric receptors from three subunits. P2Y metabotropic receptors couple to G-proteins in order to activate intracellular signal transduction

diphosphates (e.g., ATP and ADP) into nucleosides (e.g., adenosine). Finally, adenosine deaminase (ADA) converts adenosine to inosine for initiation of the purine salvage.

P2 receptors belong to the first receptors expressed in embryonic development and are present in almost every cell type, pointing at their importance in cellular function. Moreover, the importance of purinergic signalling in stem cell biology and development, including regulation of proliferation, differentiation and cell death, has become evident (Burnstock and Ulrich 2011; Glaser et al. 2012). In the following, we will focus on expression and functions of P2 receptors in embryonic and adult stem cells and their participation in induction of proliferation and differentiation of stem and embryonal cancer cell models with emphasis on possible therapeutic applications.

## Regulation of Proliferation and Differentiation by Purinergic Receptors

Stem cells are capable to continuous reproduction by symmetric division or to divide asymmetrically giving rise to a stem cell and another more differentiated progenitor cell. This progenitor cell may persist or differentiate into specialized somatic cell. As we will discuss in the following, distinct purinergic receptor subtypes are involved in regulation of proliferation and induction of differentiation, while other ones are responsible for proliferation blockade and onset of the cell-death program.

Metabotropic receptor subtypes including P2Y1 and P2Y2 receptors are known to promote proliferation of pluripotent stem cell models and subsequent differentiation into neural phenotypes (Resende et al. 2008). Proliferation-inducing effects exerted by P2Y1 receptors have also been described for chick retinal progenitor cells (Ornelas and Ventura 2010). In line with the induction of neurogenic proliferation, proliferation of neurospheres from the subventricular zone of adult murine brain was attenuated after application of the P2Y1-receptor antagonist MRS2179 and in neurospheres from P2Y1-receptor knockout mice.

Moreover, proliferation induction by the P2Y1 receptor is not limited to neurogenic processes. In low-intensity pulsed ultrasound (LIPUS) treated osteoblasts, proliferation was mediated by metabotropic purinergic receptors with principal participation of the P2Y1 subtype (Alvarenga et al. 2010).

On the other side, the exact actions of a nucleotide receptor depend very much on the cellular context. Besides promoting proliferation in many cases, P2Y1 receptor activation may also have contrary effects, such as decreasing the growth of

bone-marrow derived mesenchymal stem cells (Coppi et al. 2007) or inducing apoptosis in astrocytes (Mamedova et al. 2006).

P2Y1 receptors have crucial functions in the formation of a complex neuronal network, such as cortex development, beginning with proliferation and differentiation of neural stem and progenitor cells. Direct evidence for the crucial role of this receptor subtype in cortex development was obtained from the observation that migration of neural progenitor and radial glia cells into the subventricular zone (SVZ) was affected in a mouse model which as consequence of a connexion-43 hemichannel knock-out revealed reduced P2Y1 receptor expression levels (Scemes et al. 2003). As probable underlying mechanism, P2Y1 receptor-mediated calcium wave formation was impaired as well as its propagation to neighboring cells which would occur through connexin 43-hemichannels channels. The defective calcium signalling then affects the release of ATP and neurotrophic factors into the extracellular medium, necessary for stimulation of neighboring differentiating cells. P2Y1 receptor activity was essential for coordination of cortical proliferation, as cells undergoing calcium wave generation were in S-phase (Weissman et al. 2004). Differentiating cells undergo mostly asymmetric neurogenic division, maintaining a pool of undifferentiated cells and promoting neurogenesis. Accordingly, radial glia cells in S-phase release ATP thereby activating metabotropic purinergic receptors on neighboring cells and inducing cell cycle progression in these cells from G1 into S-phase, thus synchronizing subpopulations of cycling cells (Weissman et al. 2004). The hypothesis that initiation of intercellular calcium waves regulates proliferation and neurogenesis of clusters of neighboring VZ and SVZ cells, is in agreement with earlier published results suggesting that opening and closing of hemichannels of differentiating SVZ or VZ cells induces entry or exit from the cell cycle (Bittman et al. 1997).

P2Y2 receptors are known to be involved in inhibition of apoptosis, regulation of proliferation processes and induction of differentiation. P2Y2 receptors together with P2Y1 subtypes were shown to promote proliferation of P19 embryonal carcinoma (EC) cells, and in an advanced stage of differentiation of these cells, at which P2Y1 receptors are not anymore expressed, to induce differentiation into neurons (Resende et al. 2008). Moreover, P2Y2 receptors function in inhibition of apoptosis and increasing cell survival rates of neural cells (Arthur et al. 2006). These neuroprotective features would then result in an increased number of progenitor cells available for differentiation. UTP, activating P2Y2 and P2Y4 receptors, augmented survival rates of mouse fetal midbrain-derived neural progenitors (E13.5), while in the presence of ADPβS, an agonist of P2Y1, P2Y12 and P2Y13 receptors, neuronal cells were eliminated (Delic and Zimmermann 2010). In addition to implications in apoptosis inhibition, P2Y2 receptors are involved in regulation of differentiation. In response to application of all-trans retinoic acid upregulation of P2Y2 receptor expression was visualized normal human epidermal keratinocytes, in agreement with a differentiation-promoting process (Fujishita et al. 2006). Increasing activity of intercellular calcium wave formation by P2Y2 receptors in differentiating osteoblasts points at participation of this receptor in bone-formation (Henriksen et al. 2006).

In addition to P2Y1 and P2Y2 receptor subtypes with already some well-studied functions in stem cell biology, evidence exists for participation of further P2Y receptor subtypes in these processes. For instance, the P2Y14 receptor mediates induction of migration and chemotaxis of bone-marrow hemapoietic stem cells. This receptor was expressed in a limited population of the fetal bone marrow, but not in the fetal liver, that was enriched for G0 cell cycle status, and the responsiveness to bone marrow stromal products underscores the potential of this chemoattractant receptor to participate in bone marrow localization of stem cells (Lee et al. 2003). Moreover, differential expression of P2Y14 and P2Y4 receptors together with the P2X6 receptor was observed during commitment into adipogenic and osteogenic differentiation of adipose tissue-derived stem and ectomesenchymal dental follicle cells (Zippel et al. 2012). NAD+, activating P2Y11 receptors, was released by connexin-43 hemichannels of human bone-marrow stem cells in an autocrine loop to stimulate cellular functions including proliferation, migration and release of prostaglandin E(2) and cytokines

(Fruscione et al. 2011). Nucleotide-induced signaling is also important for hematopoietic stem cell differentiation under the control of cytokine actions. Barbosa and co-workers (Barbosa et al. 2011) showed that *in vivo* administration of ATP resulted in a diminished number of stem cells, common myeloid progenitors and granulocyte–macrophage progenitors (GMPs), while myeloid lineage differentiation was enhanced, allowing the conclusion that purinergic signaling was important for phenotype specification.

P2X receptors participating as ATP-activated ion channels in neuronal transmission are also implicated to possess functions in proliferation and differentiation of embryonic cells. In P19 mouse EC cells, P2X2 receptor activity led to inhibition of proliferation and induction of differentiation into neurons (Yuahasi et al. 2012; Resende et al. 2008) and participated in phenotype specification of differentiated cells, such as functionality of cholinergic and NMDA-glutamate receptors (Resende et al. 2007). P2X7 receptors on the other hand are mostly involved in the induction of cell death and elimination of not anymore needed cells. In this context, it makes sense that induction of differentiation of Neuro-2a neuroblastoma cells by retinoic acid suppresses P2X7 receptor expression (Wu et al. 2009). Moreover, the authors of this paper used down-regulation of P2X7 receptor gene expression as well as pharmacological approaches for receptor inhibition to show that P2X7 receptors halt neuronal maturation, such as neurite extension (Wu et al. 2009). However, P2X7 receptor-mediated action may also include induction of proliferation, such as shown by stable inhibition of expression of this receptor in proliferating P19 cells. P2X7 receptor-mediated increase in gliogenesis was subsequently the consequence of increased proliferation of stem and progenitor cells (Yuahasi et al. 2012). On the other hand, P2X2 together with P2Y2 receptor activity participated in promotion of differentiation into neurons and neuronal phenotype determination (Resende et al. 2007, 2008). Silencing of P2X2 receptor expression along differentiation promoted cell proliferation and an increase in the percentage of cells expressing glial-specific GFAP, while neurogenesis diminished at the same time (Yuahasi et al. 2012) (Fig. 25.2).

## Purinergic Signaling in Tissue Repair

Cell replacement therapy may be based on the isolation of a stem cell source from a donor or the patient, followed by *in vitro* culture and induction to differentiate into cell of the tissue which shall be repaired. Cell death and rejection of transplanted cells are mostly due to immune responses and the absence of adequate stem cell niches at the localization of transplantation. Although underlying mechanisms, by which the local milieu influences stem cell differentiation and tissue engraftment, are far from being understood, recent work suggests that extracellular nucleotides contribute to the appropriate niche and direction of stem cells to the site of injury and tissue repair. For instance, P2Y14 receptors expressed by bone-marrow hematopoietic stem cells induce migration of these cells to the localization of heart injury followed by induction of differentiation at the site mediated by activation of further purinergic receptor subtypes (Lee et al. 2003). P2Y1 and P2Y2 receptors in basal layers of the fetal and adult epidermis (Greig et al. 2003a) are co-expressed with the cell proliferation markers Ki67 and proliferation cell nuclear antigen (PCNA) and induce proliferation in cell cultures of basal keratinocytes (Greig et al. 2003b) suggesting their participation in wound healing and skin regeneration.

Although nucleotides acting through P2Y receptors mostly stimulate proliferation and differentiation of stem and progenitor cells of various origins and contribute in endogenous repair mechanisms, their possible beneficial effects in tissue regeneration could be overlapped by adverse effects at the same time. Depending on the purinergic receptor subtype involved, ATP often exerts pro-inflammatory effects. For instance, the P2Y1 receptor was identified as key player in vascular inflammation (Zerr et al. 2011). Furthermore, therapeutic down-regulation of excitatory signaling for avoiding possible worsening of disease conditions may include inhibition of purinergic signaling. In this context, protective effects in perfused hearts due to inhibition of P2Y receptor activity were observed in the presence

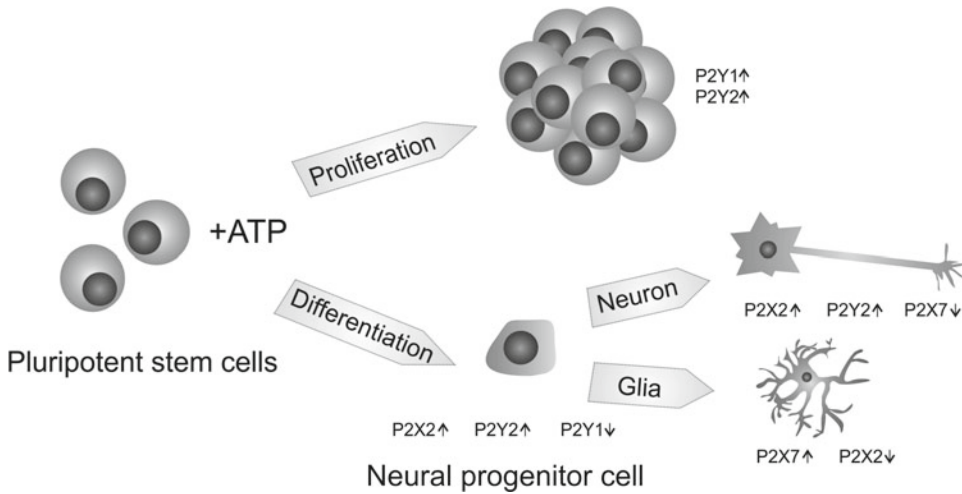


**Fig. 25.2 Pluripotent stem cell proliferation and differentiation into neural cells involves differential purinergic receptor expression and activity.** P19 EC cells used as model for ES cells express functional P2Y1 and P2Y2 receptors, which upon stimulation by ATP promote proliferation. Induction of neural differentiation is accompanied by down-regulation of P2Y1 receptors, while P2X2 and P2Y2 receptors participate in differentiation into neurons (Resende et al. 2007, 2008). Down-regulation of P2X2 or P2X7 receptor expression by RNAi inhibited neurogenesis or gliogenesis, respectively (Yuahasi et al. 2012) (↑, ↓: Increase or decrease of receptor expression and activity, respectively)

of extracellular pyridoxal-5′-phosphate (PLP) (Millart et al. 2009). On one hand, P2Y receptor agonists induce migration and differentiation of neural progenitor cells (Scemes et al. 2003). These effects would be beneficial for recruiting endogenous repair mechanisms as well as for the integration of grafted cells, while on the other hand, inhibition of P2Y receptor activity is required for neuroprotection and limitation of further brain damage by modulating excessive neurotransmitter release in brain disorders (Glaser et al. 2012).

P2X7 receptor expression and activity, often related to cell death in stem cell cultures, has also drawn attention on tissue repair mechanism. This receptor mediates pro-inflammatory actions, excitatory tissue damage and cell death following stem cell engraftment. For instance, in CNS lesions successful engraftment of neural progenitor cells is hindered by the presence of high extracellular ATP concentrations as consequences of the inflammation process which activates P2X7 receptor responses in these cells leading to massive calcium influx and cell death (Delarasse et al. 2009). During human fetal epidermis development, P2X7 receptor expression was detected in periderm cells together with labeling for caspase-3 and TUNEL, markers for terminal differentiation and apoptosis, respectively, suggesting that this receptor eliminates not any more needed cells during final epidermis development (Greig et al. 2003a). Furthermore, the P2X7 subtype is also expressed in stratum corneum in adult epidermis suggesting its participation in apoptotic control. During wound healing processes P2X7 receptor expression was not detected (Greig et al. 2003b), since here P2X7 receptor activity would be damaging to P2Y1, P2Y2 and possibly P2X5 receptor-induced repair mechanisms.

## Purinergic Signaling in Cancer Stem Cells

Regulation of proliferation and differentiation by purinergic signaling has also been documented in tumor cell biology; however, little is known regarding the actions of purines on cancer stem cells. In this context, ATP has been attributed with tumor-promoting or inhibiting roles. The different observed effects probably depend, besides on the subtype of activated receptor and

ligand concentration, also on the percentage of stem cells in the cancer cell population. On one hand, P2 receptors may promote tumor proliferation. For instance, the presence of apyrase, degrading ATP and ADP, diminished glial tumor proliferation, while NTPDase transforming ATP into ADP, an agonist of P2Y1, P2Y12, and P2Y13 receptors, promoted angiogenesis and subsequently growth of glioma tumor models (Braganhol et al. 2009). However, on the other hand, treatment of human gliomas U87 or U343 as well as rat C6 glioma cells with ATP reduced the number of tumor spheres which are characteristic for tumor stem cells (Ledur et al. 2012). Thereby ATP led to reduction of expression of Nanog as well as CD133 and Oct-4, marker proteins of embryonic and cancer stem cells, making purinergic signaling a promising tool for differentiation therapy. These results are in line with the discovery of stem-like cells derived from gliomas which are themselves tumorigenic and capable to self-renewal and multipotency. Purinergic receptor activation forces cancer stem cells into the differentiation pathway, such as observed for untransformed neural progenitors during development and repair of injured brain tissue.

In conclusion, the understanding of purinergic signaling is yet in its beginning. However, experimental evidence points at crucial functions of purinergic signaling in the biology of all type of stem cells. These functions may include the regulation of proliferation and the progress of differentiation, even in complex situations such as brain development. Further suggested implications for purinergic receptor activity include the recruitment of endogenous tissue-specific stem cells as well as their maintenance throughout life. In view of that, activation or inhibition of specific purinergic receptor subtypes could improve tissue repair therapies by stem or pre-differentiated cell engraftment. However, care needs to be undertaken as induction of purinergic signaling can include undesired effects such as pro-inflammatory actions and cell death. The participation of purinergic receptors in tumor cell biology is also not understood; however, initial studies suggest differentiation-inducing effects on cancer stem cells by ATP.

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# Cancer Stem Cells of the Head and Neck

# 26

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**Abstract**

The initiation and metastasis of head and neck squamous cell carcinomas (HNSCC) and other cancers have recently been related to the presence of cancer stem cells (CSC). CSC are cancer initiating, sustaining, and reside mostly quiescently in the tumor. Specific markers and stemness-related genes that characterize putative HNSCC-CSC have been identified. Compared to the bulk tumor mass, CSC are less sensitive to chemo- and radiotherapy and may also have low immunogenicity. Therapeutic targeting of CSC may improve clinical outcome of HNSCC which has two distinct etiologies: infection of epithelial stem cells by high-risk types of the human papillomavirus, or long-term tobacco and alcohol abuse. Recent knowledge on the role of CSC in HNSCC is reviewed and where necessary parallels to CSC of other origin are drawn to give a more comprehensive picture.

## Introduction

Advances in therapy of head and neck squamous cell carcinomas (HNSCC), have improved quality of life but long-term survival rates in patients with advanced stage have remained static over the past three decades (Greenlee et al. 2001). Mortality from HNSCC remains high due to the development of distant metastases and the emergence of eventually inoperable local and regional recurrences that exhibit low responsiveness to

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radiation, chemotherapy, and adjuvant antibody treatment. Even in early stage disease about 10% of the patients eventually relapse with fatal outcome. Only a close analysis of these tumors might yield insights into the underlying biological causes. One might speculate that like in other cancer entities, cancer stem cells (CSC) play a role. It is therefore desirable to gain more knowledge on the biology of HNSCC-CSC. It is important to investigate their sensitivity to current therapeutic strategies as a basis for the development of therapies with greater potential for cure.

As in other solid tumors also in HNSCC-CSC a small subpopulation of cells exhibiting the typical features of CSC are held responsible for tumor maintenance and metastasis (reviewed in Albers et al. 2011) and possibly for resistance towards chemotherapy and radiation therapy. The development of the CSC-theory has prompted a re-examination of current therapeutic concepts aiming at developing therapeutics specifically targeting CSC that might synergize with treatment modalities directed at cancer bulk populations to improve clinical outcome.

This review briefly summarizes recent advances in HNSCC-CSC research.

## Etiology of Head and Neck Squamous Cell Carcinoma (HNSCC)

In most instances, HNSCC is either caused by the spontaneous accumulation of multiple genetic alterations modulated by genetic pre-disposition and chronic inflammation, enhanced by environmental influences such as long-term tobacco and alcohol abuse, or by persistent infection with oncogenic human papillomavirus types (HPV). Carcinogens are regarded the most important factors. Thus, two main etiologies of HNSCC can be defined: tumors induced by toxic substances and tumors induced by the activity of the viral oncogenes of HPV. Both etiologies involve a multistep process and result in alterations affecting two large groups of genes: oncogenes and tumor suppressor genes. HPV-associated HNSCC defines a distinct subgroup. More than 130 human papillomavirus (HPV) genotypes are known to date. Of these, 18 have been shown to be oncogenic in humans. HPV type 16 seems to play the major role in the etiology of HPV-associated HNSCC, particularly those arising in the oropharynx (Leemans et al. 1994). HPV association has been detected in 20–30% of tumors located in all head and neck anatomic subsites and in about 50% of tonsil squamous cancers. For laryngeal cancer, the role of HPV is less clear.

An overall increase in the incidence of HPV-related HNSCC and a decrease of non-HPV-related HNSCC due to changing smoking habits has been reported (Ryerson et al. 2008). However, also regional differences in the prevalence of HPV-related HNSCC have been documented. In the USA the prevalence is higher as compared to Europe. In contrast in Eastern Europe the prevalence is very low. Epidemiologic studies have also shown disparities in the incidence (Auluck et al. 2010) and survival after treatment among different ethnicities in the USA (Ryerson et al. 2008) and a coincidence of cervical cancer and HPV-related HNSCC.

## Human Papillomavirus (HPV) Infects the Basal Cell Layers in Epithelium

It is appealing to speculate that HPV, which primarily infects basal cells in the epithelium, indeed infects epithelial stem cells that are subsequently transformed to become CSC (Martens et al. 2004). This concept suits the highly regulated replication and propagation strategy of these viruses.

The lower rate of carcinogenic risk factors and p53 mutations and a younger patient population suggests that factors, currently unknown, are associated with viral entry, propagation/transformation, and immune evasion in HPV-associated HNSCC patients (Leemans et al. 1994). Failure to clear HPV infection leaves host cells under the influence of the viral oncogenes. For cervical cancers, which are generally caused by high-risk HPV, persistently infected persons can develop clinically or histologically recognizable precancers,

mostly found in the transformation zone of the cervix uteri epithelium that can persist and may develop over time into invasive cancer. For HPV-induced HNSCC a similar sequence of events remains to be demonstrated. The tonsil, where most HPV-related cancers of the oropharynx arise, is composed of crypts that make these studies difficult, although this organ could be easily reached for brushes or biopsies. HPV-derived oncogenes are vital to the tumor cell survival and proliferation and therefore provide a suitable target for anti-tumor vaccination.

Therefore, with these two different etiologies, different treatment options according to the origin of the malignancy, being spontaneously or virally induced, may be developed to target the cancer efficiently.

## Genetic Predisposition to HNSCC

Like other solid tumors, HNSCC are thought to be initiated and to progress through a series of genetic alterations. Individual susceptibility to HNSCC may result from host factors including differences in metabolism, DNA repair, and altered expression of protooncogenes and tumor suppressor genes. These factors influence an individual's phenotype for a number of enzymes (both activating and detoxifying) relevant to that of a single carcinogen or mixtures of carcinogens.

The TP53 gene is the most frequently mutated gene in HNSCC. Alterations are found in 50–80% of the tumors. The TP53 tumor suppressor gene on chromosome 17p13.1 encodes the p53 protein involved in many key events in the cell. The most important are regulation of cell cycle and glucose metabolism in cancer cells, DNA-repair, apoptosis, and senescence. It is induced by various stress signals, including DNA-damage and inflammation. The frequency and type of p53 mutations can act as a fingerprint of carcinogen exposure and may therefore provide information about external etiological agents, intensity of exposure, and host factors affecting the tumorigenic process. In HNSCC, p53 mutations, both the mutation pattern and frequency, have been linked with tobacco smoking and to other environmental exposures while HR-HPV high-risk HPV induced HNSCC regularly shows less p53 mutations (Leemans et al. 1994). Recently, studies of genetic changes causing HNSCC shed new light on the treatment strategy. In these studies, mutations were found in many genes already known, such as TP53, CDKN2A, PIK3CA, PTEN, and HRAS. But most importantly, one new cancer gene, NOTCH1, was identified in HNSCC. Mutations in NOTCH1 occur in 10–15% of the tumors (Agrawal et al. 2011; Stransky et al. 2011). These studies provide strong evidence that NOTCH1 is an important tumor suppressor gene in HNSCC. Like TP53, NOTCH1 shows a higher mutation rate in HPV-negative HNSCC.

It is well known that Notch signalling pathway mainly targets programs of stem and progenitor cell differentiation. Future studies should address the role of NOTCH1 in CSC in HNSCC biology and as a potential target.

Mutations in mitochondria DNA (mtDNA) have been suspected as important oncological biomarkers. They have been identified in both the non-coding and coding regions of human tumors contributing to the tumorigenesis (Lu et al. 2009). Also in HNSCC and their premalignant counterparts mutations were most frequently detected in the displacement-loop region. So their prognostic role is still unknown (Challen et al. 2011).

Some studies have investigated the DNA repair gene polymorphism and the susceptibility to develop primary or secondary HNSCC. For example, The CYP1B1 codon 432 polymorphism (CYP1B1*3) has been identified as a susceptibility factor in smoking-related head-and-neck squamous cell cancer. The impact of this polymorphic variant of CYP1B1 on cancer risk was also reflected by an association with the frequency of somatic mutations of the p53 gene. Combined genotype analysis of CYP1B1 and the glutathione transferases GSTM1 or GSTT1 has also pointed to interactive effects (Thier et al. 2003). Others like regulator of G-protein genes also have been evaluated in HNSCC (reviewed in Albers et al. 2011).

Taken together there is evidence that genetic alterations in a multitude of genes and pathways

can predispose an individual for the development of HNSCC as it does for other cancers as well.

## Definition of Putative Cancer Stem Cells

Similar to physiologic tissues, populations of cells within a tumor are organized hierarchically that exhibit distinct morphologic and functional phenotypes. CSC are viewed as the result of the oncogenic process and the starting point for the formation of tumor and metastasis. CSC exhibit traits such as motility, invasiveness, and self-renewal, and can initiate malignant tumors and drive neoplastic proliferation. According to the CSC model, the phenotypic diversity of cells that form a tumor and its metastases derive from CSC and are organized hierarchically. On a more practical level, one CSC can completely regenerate the tumor it has been taken from. The tumor itself consists of at least two subpopulations: a smaller CSC population and a bulk population of non-tumorigenic cancer cells that have differentiated from CSC and have lost their self-renewing capacity. The concept of this model has been developed in analogy to the renewal of adult tissues like blood that regenerates from a pool of hematopoetic stem cells. Further proof of this model has been provided by demonstrating that selective killing of CSC can stop tumor (Dick 2008) growth.

## Origin of Cancer Stem Cells

It is still uncertain whether a cancer stem cell is the direct descendant of a mutated stem cell or of more mature cells that reacquire stem cell properties during tumor genesis. CSC share important properties with normal tissue stem cells (TSC), including the capacity for self-renewal and the expression of stemness factors. Asymmetric cellular divisions of TSC result in hierarchically organized, irreversibly differentiated tissues with physiologic functions that are under homeostatic control. On the contrary, the result of asymmetric divisions of CSC is a hierarchically organized tumor with a most likely stochastic differentiation that may be reversible in part and results in a disorganized tumor tissue architecture. CSC as compared to TSC have a relatively high division rate. TSC have the requirement for a specific niche. Whether CSC also reside in a specific niche is unknown to date. It is also currently unknown whether normal stem cells are susceptible to cancer-causing mutations that directly give rise to CSC. HPV-related HNSCC might be an exception, since it has been shown that HPV infects the basal layer of the mucosa, a stem cell compartment from which the mucosa is being regenerated. TSC underlie a homeostatic control and divide or differentiate in an ordered fashion. CSC, although sharing characteristics with TSC, support the unlimited growth by replenishing with juvenile tumor cells. They enable the process of metastasis of the tumor by epithelial-mesenchymal transition (EMT) and the reverse process termed mesenchymal-epthelial transition (MET) that allows tumor cell dissemination.

## Comparison of the Hierarchical and Stochastical Tumor Models in HNSCC

HNSCC, as other solid tumors, are histologically highly heterogeneous. It consists of the cancer cells that can be subtyped and grouped into populations with stem cell characteristics and more differentiated cell types that possibly resemble a developmental hierarchy in analogy to normal tissues. Attempts to explain this diversity within a tumor have been made. Currently two competing models exist.

1. The stochastic or evolutionary model uses events like spontaneous shifts in cell phenotypes to explain heterogeneity.
2. The hierarchical model describes a differentiation-like diversification from an initially monoclonal tumor cell lineage.

Many of the biological phenomena occurring in HNSCC can be well explained by this stochastic model. For example, due to the large size of the pre-neoplastic fields expanding beyond typical surgical margins local recurrences or secondary cancers can be explained. The most obvious limitation of

the evolutionary progression model is to explain the cellular heterogeneity observed in tumor nests, which in contrast can be well explained by the hierarchical CSC model. The CSC theory of solid tumors is supported by the observation of cancer cells in close proximity that have different biological behaviour (e.g. to metastasize). This can be explained rather by differentiation than evolution (Fidler and Kripke 1977). Whether this is also the case in HNSCC remains to be shown. Importantly, the metastases, putatively arising from single or few metastasizing tumor cells, also show heterogeneity although exposure to DNA-damaging agents similar to the primary tumor is absent. Zushi et al. successfully developed an in vitro model for recapitulating development and progression of both HPV16-positive and –negative human oral squamous cell carcinomas. The authors transduced HPV16 E6/E7 or mutant CDK4, cyclin D1, and human telomerase reverse transcriptase into primary human tongue keratinocytes, and thereby obtained immortal cell populations named HTK-16E6E7 and HTK-K4DT. When continuously transduced oncogenes like HRAS or EGFR together with MYC in HTK-16E6E7 and dominant-negative P53 expressing HTK-K4DT, these cell populations showed anchorage-independent growth and subcutaneous tumor formation in nude mice (Fidler and Kripke 1977; Zushi et al. 2011).

In conclusion the difference of CSC cells and tumor bulk cells can be demonstrated by their biologic behaviour, which has been used to characterize or identify HNSCC-CSC candidates.

## Formation of Non-HPV Related HNSCC

Heterogeneity in substance abuse-related HNSCC can be well explained by the constant exposure of the oral mucosa to mutagens contained in tobacco products resulting in multiple genetic changes of various degrees in the whole aero-digestive tract until in one area, a point of no return is reached and a pre-neoplastic field develops. This field is of monoclonal origin and expands non-invasively. Clonal divergence and selection within the field leads to the development of cancer (reviewed in Albers et al. 2011; Braakhuis et al. 2005) The phenomenon of tumor evolution by accumulation of stepwise genetic alterations was termed field-cancerization. According to this theory, HNSCC can arise synchronously or metachroneously at different sites. It remains to be demonstrated if differentiated cells during tumorigenesis reacquire stem cell properties or if the genetic alterations have to affect cells in the epitheliums stem cell compartment directly to initiate malignancy.

## Formation of HPV-Related HNSCC

In the case of HPV-induced tumors, these processes can be potentially followed using HPV DNA sequences as a tag of those cells prone to immortalization, tumor progression, and metastasis. HPV infection will form a premalignant field of infected cells (intraepithelial neoplastic lesion) with an extended life span and re-entering of aberrant cell cycles. These cellular fields have a high probability of acquiring more genetic changes that ultimately may give rise to immortalized and transformed cells further progressing to HNSCC. HPV has evolved and adapted to infect stem cells of the epithelium.

These stem cell-like cells are located in the basal layer of the mucosa close to the basal lamina in a quiescent state. Successful infection by HPV therefore requires breaches or micro-injuries of the epithelium, so that the viruses can enter and reach the basal lamina. HPV can attach to the proteoglycans of the basal lamina, which becomes a reservoir for infectious particles. Mircoabrasions and injuries will be closed by activation and proliferation of quiescent (stem)-cells of the tissue. These cells during this physiologic state of proliferating undifferentiated pluripotent cell growth are most probably the target for HPV infection. However, it is not certain yet, that these cells are true pluripotent stem cells.

Upon infection, the viral DNA is maintained in the progeny of this cell for prolonged periods of time, maybe as long as this cell clone is present. Only when these HPV-positive stem cells divide again and finally differentiate to become proliferating suprabasal cells that build up the epithelium,

the HPV switches gene expression to produce genomic copies and structural proteins, which form the viral shell. Finally, viral particles are released passively with descaling cells. This life cycle is dependent on a close interaction of viral and cellular genes and possibly genes regulating the stem cell character of the basal epithelial cell. In terms of field cancerization, HPV initially produces a premalignant lesion of heterogeneous cells with different states of the viral genome. During persistence of the infection and progression towards malignant transformation, the viral genome integrates into the host cell genome at a random position. This integration event henceforth will identify this cells progeny. The integrated HPV-sequences represent a genetic tag of the cell clone, and it turns out that tumors are arising as monoclonal expansion of a single cell. It is currently not known if this happens in a differentiated epithelial cell that subsequently will give rise to new cancer stem cells or if this has to happen in a basal (stem) cell. It is attractive to speculate that in the resulting tumors CSC will be formed from the HPV-tagged differentiated cells and thus are derived from the non-stem cell compartment of the epithelium. Anyhow, this integration event supports the development of a malignant phenotype. Loss or inhibition of viral transcription leads to apoptosis. Therefore, all cells of the tumor, the bulk tumor cells, the metastasizing, and the CSC are tagged with HPV-DNA as a unique marker. Of course over time and during progression to more malignant phenotypes additional genetic and epigenetic changes occur leading to a heterogenization of the tumor mass. The clonal origin, however, will be maintained as documented by the HPV integration site. This may be an analytical advantage over spontaneously arising HNSCC that do not carry a conclusive marker. The "HPV-tagged" tumors may offer an ideal opportunity to investigate and discriminate CSC and bulk tumor cells and their origin and fate.

## Epithelial-Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) is a key step during embryogenesis that enables cells of epithelial phenotype to generate mesenchymal derivatives. Importantly, once the migrating mesenchymal cells have reached their destination, they can undergo a reverse EMT, a mesenchymal-epithelial transition (MET). Therefore, cells may revert back to the epithelial state from the mesenchymal phenotype. Although the EMT program is necessary for normal embryonic development, the aberrant activation of EMT contributes to various pathologic conditions, including fibrosis and carcinoma progression. Recent evidence suggests that cells undergoing EMT acquire stem cell-like properties, and EMT can also induce non-CSC to a CSC-like state (Chaffer and Weinberg 2011). It is supposed that the mesenchymal status seems to be a condition to regain pluripotency. Normal stem cells and cancer stem cells may share a mesenchymal phenotype that enhances their ability to preserve stemness, to regain migratory properties, and to respond to different stimuli during expansion and differentiation. The question arises whether the metastatic cells disseminating from the primary tumor originate from resident stem cells in the tumor or do they derive from somatic tumor cells that have undergone EMT. The role of the EMT in enabling metastatic dissemination remains largely unclear. A recent hypothesis proposed that there are two subtypes of CSC within a tumor. Intrinsic CSC which exist in primary tumors from the very early stages of tumorigenesis may be the oncogenic derivatives of normal-tissue stem or progenitor cells. Induced CSC occur as a consequence of the EMT. In this way, cancer cells can recruit a reactive stroma including fibroblasts, myofibroblasts, granulocytes, macrophages, mesenchymal stem cells, and lymphocytes. These reactive stromal cells release factors like Wnt, TGF-β, or fibroblast growth factor that may cause the neighbouring cancer cells undergoing EMT and reach CSC state (Chaffer and Weinberg 2011).

Accumulating evidence shows a role of EMT in metastatic dissemination of cells with CSC phenotype in HNSCC. Overexpression of receptor tyrosine kinase, TrkB resulted in altered expression of molecular mediators of EMT, including downregulation of E-cadherin and upregulation of Twist. The observation was confirmed in a mouse model by showing that downregulation of

TrkB suppressed tumor growth. Our own data (Chen et al. 2011) show that ALDH+ putative CSC of HNSCC possess upregulated levels of Snail1 and Twist and have significantly increased expression of mesenchymal markers such as alpha-smooth muscle actin and Vimentin. These results directly implicate TrkB in EMT and the invasive behaviour of HNSCC, and correlate with the in vivo overexpression of TrkB in human HNSCC (Kupferman et al. 2010). Recent evidence demonstrated that CSC reside in close proximity to blood vessels and can give rise to tumor endothelium. It was also reported (Kupferman et al. 2010) that endothelial derived factors inhibit anoikis of ALDH+CD44+ head and neck cancer stem cells. In oropharyngeal cancer cell lines it has been shown that β-catenin nuclear accumulation and activation of Wnt signalling pathway is directly E6/E7 dependent (Rampias et al. 2010). MicroRNA-200c is emerging as an important regulator in linking the characteristics of cancer stem-like cells with EMT-like cell signatures. Reduced expression levels of microRNA-200c were found in ALDH1+ /CD44+ HNSCC cells. When upregulated, microRNA-200c could attenuate tumor growth and metastatic capability of EMT in HNSCC (Lo et al. 2011).

The EMT pathway can also be used as target in cancer treatment. Salinomycin, which was identified from a small molecules library, is selectively cytotoxic toward breast cancer stem cells. This pioneer study provides a proof of principle that cancer stem cells exhibiting EMT features can be selectively targeted by drugs (Gupta et al. 2009).

## Putative Markers of HNSCC-CSC

According to the CSC hypothesis, tumors consist of heterogeneous cell populations in distinct phenotypic and functional states that are hierarchically organized. Since cancer cell populations, as it seems, exhibit different susceptibilities to cancer therapy it is an important scientific goal to identify and purify each population to investigate possible weaknesses with regard to therapy and to understand their possibly unique biology. Much effort has therefore been made to identify candidate markers that are either useful for separation of cell populations, for further research, or as target-structures for specific therapies. These markers could be cell-surface markers or molecules involved in specific metabolic or signalling pathways.

Until now, no general CSC marker for solid tumors has been identified. Whether such a marker exists at all remains elusive at this time. It is also possible that CSC markers are tumor specific for the tissue of origin or for the niche from where the tumor is growing. We summarized currently used candidate markers of HNSCC-CSC (reviewed in Albers et al. 2011).

Tissue stem cells (TCS) and CSC share many characteristics, such as self-renewal, and differentiation being the most important. Therefore, they also share some markers. Although the molecular mechanisms of these signal transduction pathways might be the same, their regulation in CSC may be dysregulated and participate in tumor growth.

Comparative studies of TSC and CSC from the same tissues showed that for instance the signalling pathways of Bmi1 and Wnt have similar effects in self-renewal suggesting that common molecular pathways regulate both populations (Pardal et al. 2003).

In head and neck cancer, Prince et al. were the first to demonstrate that the population of HNSCC cells possess the properties of cancer stem cells (Prince et al. 2007), but a relatively high number of CD44+ cancer cells (>5,000 cells) were needed to initiate new tumors in immunodeficient mice. Moreover, in the head and neck CD44s and CD44v6 expression does not distinguish normal from benign or malignant epithelia. CD44s and CD44v6 were abundantly present in the great majority of cells in head and neck tissues, including carcinomas (Mack and Gires 2008). A study found that CD44 was significantly correlated with response to radiotherapy in early stage larynx cancer patients, both at the mRNA and protein levels (de Jong et al. 2010). This finding suggests that CD44 can be a biomarker bearing predictive potential for local tumor control after radiotherapy. Thus, identification of more specific CSC markers for HNSCC is necessary. Recently, high aldehyde dehydrogenase 1 (ALDH1, also known as ALDH1A1) activity was shown to identify the

CSC (Chen et al. 2011). However, in breast cancer the ALDH1$^+$ population shows a surprisingly small overlap with the previously described CD44$^+$/CD24$^{-/low}$ phenotype (0.1–1.2%). The cells bearing both phenotypes appeared to be highly enriched in tumorigenicity, being able to generate tumors from as few as 20 cells (Ginestier et al. 2007). It remains to be determined if there is also a small overlap of stem cell markers in HNSCC. Expression of CSC markers like ALDH1 can directly have an effect on detoxifying molecules in a cell and may explain increased resistance of CSC to cytotoxic reagents.

## Response of CSC to Radiation and Chemotherapy

Compared to many other solid cancers, distant metastases in HNSCC are rarely present at diagnosis, but due to improved local control, the incidence of systemic spread during the course of the disease is increasing. Patients with recurrent or metastatic disease have a poor prognosis, with median survival rates of 6–10 months (reviewed in Albers et al. 2011). In these instances, chemotherapy, apart from antibody-treatment, remains the only systemic treatment option, which is whenever possible combined with surgery and radiation. Clinically, the use of radiation and high-dose chemotherapy results in many instances in a good initial response of the tumor, unless the dosage is limited by co-morbidities and toxicity. Unfortunately, the development of recurrences which are unresponsive to further treatment is frequent and raises the question for the underlying causes. Advances in CSC research may provide some explanation to these phenomena.

In the past few years, studies have begun to investigate the role of CSC for the therapeutic resistance of cancers. In these studies, cell surface markers were used to identify and purify CSC from tumors. It could be shown that the fraction of CSC is enriched in tumor samples or cancer cell cultures after treatment with radiation or chemotherapeutic drugs, and it was therefore proposed that CSC are particularly resistant to radiotherapy and chemotherapy. This resistance might then contribute to treatment failure. Consequently, CSC could represent a novel target for therapeutic treatment.

In a number of studies, evidence for radio- and chemoresistance of CSC has been presented. The mechanisms underlying this resistance are not yet fully elucidated but under investigation. In a study of glioma stem cells with the marker CD133$^+$, after fractionated irradiation ($5 \times 3$ Gy), the CSC-population increased as compared to single dose irradiation ($1 \times 9$ Gy). This can be explained by repopulation or repair after sublethal damage. The authors also found a preferential activation of DNA-damage checkpoints in CD133$^+$ versus CD133$^-$ tumor cells (Bao et al. 2006). Radiotherapy and most of chemotherapeutic agents used in the treatment of HNSCC present their antineoplastic function by disrupting cancer cell DNA integrity. So, the elevated DNA damage response and increased DNA repair capacity in CSC might be the reason of resistance. In HNSCC, recent data show a similar increase in CSC after irradiation. After irradiation, human head and neck cancer xenografts, grown in immunodeficient mice, showed an enrichment in CD44$^+$Lin$^-$ CSC (Diehn et al. 2009). The underlying mechanism might be an increased potential of defence against reactive oxygen species (ROS) in CSC (see below). When using ALDH1 as a marker for CSC in HNSCC, Chen et al. showed that HNSCC ALDH1$^+$ cells were tumorigenic and displayed resistance towards radiotherapy (Chen et al. 2009b). In another study, the authors found silencing of Bmi-1 significantly increased the sensitivity of HNSCC ALDH1$^+$ cells to chemoradiation and the degree of chemoradiation mediated apoptosis (Chen et al. 2009a). CSC showed increased resistance to the chemotherapeutic drug daunorubicin which was connected to an increased expression of multidrug resistance genes (Costello et al. 2000).

In summary, clinical observations and some experimental evidence are available to support the hypothesis that increased resistance and possibly also recurrences and metastases can be attributed to the existence and properties of therapy-resistant

CSC. Nevertheless, further research is needed to provide proof of this concept for HNSCC.

## Role of Oxidative Stress in HNSCC-CSC

Reactive oxygen species (ROS) and nitrogen species (RNS) are collective terms for molecules that exert oxidative stress in cells. They include superoxide, hydrogen peroxide, hydroxyl free radicals, and hydrogen peroxide. ROS are highly active and can cause damage to different cellular components like mitochondrial DNA, proteins and lipids by altering the regulation of various cellular pathways. To counter the damaging effects of ROS, certain defense mechanisms like enzymatic free radical scavengers such as superoxide dismutastes, catalase, glutathione peroxidase, peroxiredoxins, glutaredoxin, and thioredoxin have evolved. ROS have been proposed to stimulate cancer development during initiation, promotion and progression. ROS can also induce DNA damage leading to genomic instability which may in turn initiate tumorigenesis and cancer progression (Lu et al. 2009). However, it might be possible to develop a number of separate therapeutic approaches to target cancer cells by ROS-mediated mechanisms.

Cancer cells may exhibit drug resistance initially or after recurrence following chemo- and radiotherapy. Recent findings suggest that this might be due to CSC with inherent radio- and chemoresistance. The precise pathways leading to this resistance remains yet unclear. To further elucidate this matter, the role of ROS levels in stem cells and CSC in a number of different tissues was explored. It was found that hematopoietic stem cells and mammary epithelial stem cells contained ROS at lower levels than their mature progeny cells. For stem cells, ROS were found to influence the self-renewal and differentiation. Further studies have found that stem cells which reside in a hypoxic environment may be limited in their capability to differentiate and to maintain their stemness (reviewed in Albers et al. 2011). Although it is well known that CSC share some features of normal stem cells, it is unknown if CSC could also be limited in their capability to self-renew and to maintain their stemness in this kind of environment. Further comparative studies of stem cells and CSC are required to address this topic. Similar to normal stem cells, CSC in human and murine breast cancer, contain lower ROS levels than their non-tumorigenic counterparts. It was therefore hypothesized that this contributes to reduced levels of DNA damage after irradiation mediating resistance to this therapy (Diehn et al. 2009) In the same study, a subpopulation of HNSCC with lower ROS concentration than their non-tumorigenic cells populations was described. It is worth noting, that the reduced levels of ROS were associated with induction of antioxidant defenses. The transcription factor, Forkhead Box Os which plays a role in the regulation of an anti-ROS gene expression program in hematopoietic stem cells, was also overexpressed in CSC, possibly leading to increased resistance to oxidative stress and maintenance of stemness (Tothova et al. 2007). Based on recent reports, a correlation between EMT, ROS and CSC has emerged. Kim et al. (2011) reported that $CD13^+$ liver CSC survives in hypoxic lesions after chemotherapy and a TGF-β-induced EMT-like phenomenon is associated with increased $CD13^+$ CSC. Further a study found $CD13^+$ CSC express lower ROS level compared to $CD13^-$ cells, ROS level of $CD13^+$/N-Cadherin$^+$ cells was higher than $CD13^+$/N-Cadherin$^-$ cells, which indicate that the TGF-β-induced EMT process is associated with an increased ROS level. TGF-β plays a role in the reduction of ROS level, promoting the survival of liver CSC (Kim et al. 2011). Since EMT can be affected by many pathways of transcription factors, including Snail, Twist, and Zeb, another signal pathway may be the leading factor of EMT induced by ROS.

Despite tremendous progress in the complex biology of tumor progression, resistance, and metastasis, very little is known about the role of ROS in CSC and their microenvironment. Indentifying targets regulating the ROS levels and redox states in CSC and non-tumorigenic cells will certainly add to our knowledge in the involvement of ROS in CSC-biology and help to identify susceptibilities of these cells increasing the prospect to future therapeutic benefits.

## Clinical Relevance of CSC Research

The increasing knowledge of the existence and biology of CSC led to studies of their specific elimination which could be of potential clinical benefit because an eradication of the replenishing pool of cancer cells would stop tumor growth and lead to tumor involution as bulk tumor cells die off or succumb to therapy. This has been observed in animal experiments where removal of CSC and transplantation of only the non-CSC tumor cells did not lead to sustained tumor growth.

In addition, the evaluation of the frequency of CSC in a given tumor of a patient may be of prognostic value for the overall survival and risk of recurrence. The characteristics of a given CSC population for their marker gene expression and their proliferative state or drug resistance may support a decision for certain treatment options.

Emerging CSC targeted therapies have to overcome three major hindrances: (1) chemoresistance, (2) resistance to radiotherapy, and (3) immune-escape-mechanisms of CSC.

The first two points were already addressed in the chapter “Response of CSC to radiation and chemotherapy”. One very attractive approach of specifically targeting CSC is to develop antitumor T-cell vaccines. The results of these experimental therapies might have been disappointing in clinical studies for the same reasons leading to failure of established therapeutic modalities: Resistance of CSC. One could speculate that if immunotherapies could specifically target CSC, these limitations could be overcome and clinical success could be achieved. One potential target in HNSCC is the recently described CD8 defined T-cell epitope of ALDH1 or the development of a CSC-dendritic cell vaccine. Success of these potential therapies will depend on how well immunological responses to CSC can be modulated for example by vaccine adjuvants upregulating antigen-processing and presentation.

A different immunotherapeutic approach to target HNSCC is the use of monoclonal antibodies. Different strategies have thus far entered the clinic: (1) antibodies directed against tumor surface antigens that trigger immune effector cells that cause tumor cell death, (2) antibodies that are conjugated to cytotoxins or radiation emitters causing cell damage directly upon binding and (3) antibodies blocking or inhibiting cellular pathways after binding to the respective receptor. These strategies have resulted to various degrees in an improved prognosis and survival but not yet in cure. This variable success can be explained with tumor immune-escape (e.g. downregulation of the target) and a heterogeneous expression of the antibody targets in the tumor. A number of studies investigating the use of antibodies targeting CSC of solid cancers are underway.

In HNSCC to date no antibody selectively targeting CSC has been described yet but candidates are under investigation. Currently, a chimeric human/murine monoclonal anti-EGF-R antibody (Cetuximab) is in clinical use that showed in preclinical studies three different mechanisms affecting tumor cells: (1) enhanced tumor cell apoptosis, inhibition of proliferation and invasiveness by blocking the tyrosine-kinase mediated pathways, (2) antibody-dependant cell mediated toxicity and (3) blockage of the nuclear import of EGFR preventing activation of DNA repair. A specific affinity of these antibodies towards CSC has not been described. In vitro testing, however, showed that activation of EGFR in HNSCC leads to an increased side population (SP) as defined by HOECHST dye, and conversely, inhibition of EGFR leads to a decrease in SP implicating a possible role of EGF-R in regulating HNSCC-CSC (Chen et al. 2006). CD44v6 antibodies either radiolabeled or coupled with a cytotoxic drug entered phase I clinical testing in patients suffering from HNSCC. As discussed before, the role of CD44 to identify CSC in head and neck cancer remains controversial. Nevertheless in a phase I dose escalation study the treatment showed promising anti-tumor effects. One patient, however, developed toxic epidermal necrolysis and died, indicating that perhaps anti-CD44v6 was not exclusively targeting the CSC or even bulk tumor respectively. In late dose-escalation studies the antibody was coupled to mertansine, a cytotoxic drug, but toxic effects were severe (Riechelmann et al. 2008). This observation may be supported by immunohistologic studies showing

that the expression of CD44v6 is not exclusively restricted to the tumor (Mack and Gires 2008).

Whether single antibody treatment will be sufficient to eliminate CSC or if combining different antibodies that aim at separate CSC targets will be necessary for successful elimination of CSC remains open until they become available to clinical testing. Nevertheless, since antibody treatments show a toxicity profile different from cytotoxic agents or radiotherapy they can be combined with these treatment modalities and may therefore provide an additional treatment option in the future.

In conclusion, initiation of malignant tumor growth depends on the transformation of somatic cells. Necessary features are acquisition of immortality, self-renewal, and a dedifferentiated phenotype. These resemble abilities much like normal those stem cells have. The concept is that (i) initially somatic stem cells are being transformed and give rise to tumor cell heterogeneity, or (ii) a tumor cell reacquires stem cell-like features. There is some evidence arguing for the CSC model due to observations of a monoclonal tumor origin from a stem cell and subsequently development of heterogeneity during growth of the tumor mass.

The detection of CSC in certain malignancies has fostered studies to identify and characterize equivalent cells in many tumor entities. It turned out that such cells can be found but to date the markers available to identify CSC remain rather specific for the tissue of origin than for the CSC per se. A generic marker has not been found so far.

Due to their high tumorigenicity and drug resistance, CSC are thought to be responsible for tumor regeneration and regrowth after chemotherapy. It can be expected that a tumor with a high content of cancer stem cells may be rather resistant to chemotherapy. In turn the proportion of stem cells may increase due to positive selection after chemotherapy because of their resistance. Clinical observation supports that this may lead to a more aggressive tumor-phenotype.

As in other malignancies the presence of cancer-initiating and sustaining cells can be postulated also in head and neck cancer. Initial experimental results demonstrate that CSC-like cells can be isolated and further characterized from HNSCC tumors and cell lines. A definite marker for HNSCC stem cells has not been described at present, but markers have been identified that can be used to enrich for tumor cells with CSC-properties.

The identification of conclusive CSC markers and definition of the impact on therapeutic outcome ultimately may lead to diagnostic procedures for evaluation of CSC content and adequate therapeutic strategies. Furthermore, specific CSC markers could also serve as potential targets for upcoming therapies.

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# 27 Functional Restoration of Salivary Glands After Radiotherapy: Roles of Wnt and Hedgehog Pathways

Fei Liu

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**Abstract**

Many head and neck cancer survivors treated with radiotherapy suffer from permanent impairment of their salivary gland function, which significantly compromises their quality of life. This condition is caused by lack of regeneration following radiation damage due to loss of functional stem/progenitor cells and deterioration of the microenvironment. Current treatments such as artificial saliva and saliva secretion stimulators can only temporarily relieve the symptoms. Wnt and Hedgehog intercellular pathways are highly conserved during evolution and regulates regeneration or repair of various tissues after injury. We found recently that both Wnt and Hedgehog signalling activities are marginal in adult salivary gland but significantly upregulated during their functional regeneration after physical injury and promote expansion of salivary stem/progenitor cells, while radiation does not activate Wnt pathway in salivary gland. Transient activation of Wnt pathway prevented radiation-induced hyposalivation through inhibition of apoptosis and maintenance of salivary stem/progenitor cell populations.

## Introduction

Head and neck cancer (HNC) is the fifth most common cancer with 49,260 estimated new cases in 2010 in USA (Jemal et al. 2010). Radiation therapy is the most common form of treatment for HNC, and nondiseased salivary glands are

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often exposed to radiotherapy. Due to the exquisite radiosensitivity of salivary glands, irreversible hyposalivation is common (60–90%) in HNC survivors treated with radiotherapy (Wijers et al. 2002). The irreversible hyposalivation after radiation is caused by loss of functional glandular stem/progenitor cells that normally continuously replenish aged saliva producing cells (Konings et al. 2005). Current treatments such as artificial saliva and saliva secretion stimulators can only temporarily relieve the symptoms, while regenerative strategies targeting salivary stem/progenitor cells have shown promises for functional restoration in animal models (Lombaert et al. 2011). However, little is known about the molecular control of adult salivary stem/progenitor cells. We found recently that both Wnt and Hedgehog signalling activities are marginal in adult salivary gland but significantly upregulated during their functional regeneration after physical injury and promote expansion of epithelial salivary stem/progenitor cells (Hai et al. 2010). Radiation does not activate Wnt pathway in salivary gland, while transient activation of Wnt pathway in mice prevented radiation-induced hyposalivation through inhibition of apoptosis and maintenance of salivary stem/progenitor cell populations (Hai et al. 2011).

## Wnt/β-Catenin Pathway and Tissue Regeneration

The Wnt (wingless/int)/β-catenin intercellular signalling pathway, also known as the “canonical Wnt pathway”, is highly conserved during evolution and plays essential roles in regulating the differentiation, proliferation, death and function of many types of cells. In this pathway, secreted Wnt proteins bind to their receptors Frizzled (FZD) and co-receptor LRP5/6 (Lipoprotein Receptor-related Protein), resulting in inactivation of a complex of proteins that normally processes cytoplasmic β-catenin to initiate its degradation; then accumulated cytoplasmic β-catenin translocates into the nucleus, where it forms active transcriptional complexes with members of Tcf/Lef family of DNA-binding factors (Huang and He 2008) (Fig. 27.1). The Wnt/β-catenin pathway is modulated by many endogenous secreted inhibitors, such as Dickkopf (Dkk) that promotes internalization of co-receptor LRP5/6. A novel family of secreted proteins, R-Spondin (RSpo), interferes with Dkk1-mediated internalization of LRP6, thus relieves the inhibition DKK1 imposes on the Wnt pathway.

For various organs, Wnt/β-catenin appears to be a central regulator of regeneration or tissue renewal, especially in that of epithelial organs such as airway, liver and intestine. The adult intestinal epithelium undergoes tremendous self-renewal through active proliferation in crypt stem cell compartments, inhibition of Wnt signalling with Wnt antagonist Dkk1 in adult mice markedly inhibited proliferation in intestine, accompanied by progressive architectural degeneration with the loss of epithelial structures; whereas lift of this inhibition at later time points was followed by epithelial regeneration, indicating a striking reliance on Wnt/β-catenin pathway for the renewal of the adult intestine epithelia. During the regenerative processes in airway and liver after injuries, Wnt activity is remarkably increased in the stem cell compartments; these regeneration processes are severely impaired by inhibition of Wnt pathway, and significantly enhanced by forced activation of Wnt signalling (Goessling et al. 2009). For instance, in animal models of chemotherapy or radiation-induced oral mucositis, derepression of Wnt/β-catenin pathway with RSpo1 enhances basal layer epithelial regeneration and accelerating mucosal repair.

Besides the direct effects on adult stem/progenitor cells, Wnt/β-catenin pathway may also promote neovascularization during regeneration. After experimental myocardial infarction, level of cytosol β-catenin protein is up-regulated in vascular endothelial cells during neovascularization, indicating activation of Wnt/ β-catenin pathway; ectopic expression of β-catenin or Wnt1 stimulates proliferation of human umbilical vein endothelial cells (HUVEC) in vitro, while expression of Wnt inhibitor SFRP-1 reduces proliferation of cultured endothelial cells; moreover,

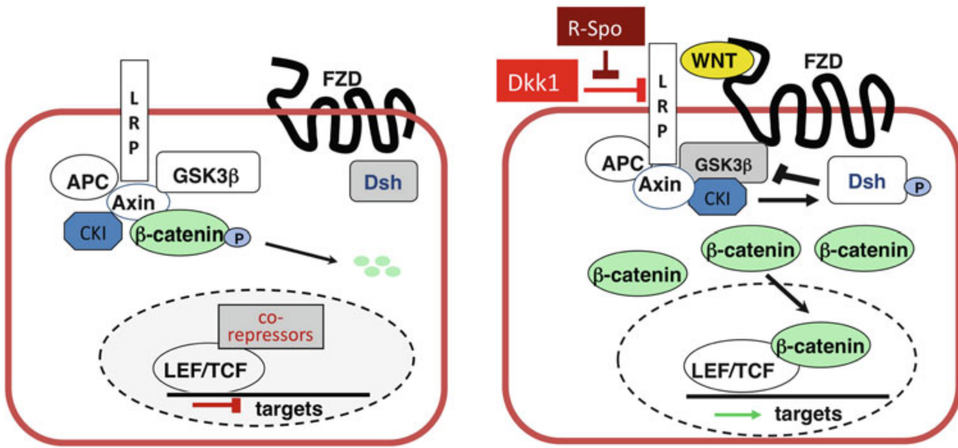


**Fig. 27.1** Simplified scheme of the Wnt/β-catenin pathway. *Left panel*: In the absence of canonical Wnt signalling. *Right panel*: In the presence of canonical Wnt signalling

Wnt/β-catenin pathway appears to regulate transcription of several known angiogenic regulators, such as vascular endothelial growth factor (VEGF) and Interleukin-8 (Zerlin et al. 2008).

## Hedgehog Pathway and Tissue Regeneration

Hedgehog (Hh) signalling is pivotal in maintenance of adult tissue postnatal development and tissue repair or regeneration. Three hedgehog ligands are present in mammals, including sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh). Among these three hedgehog family members, Shh is the most widely expressed. The activation of hedgehog signalling is initiated through the binding of the ligand to a 12 transmembrane protein receptor, Patched (Ptc), which acts as a negative regulator of a seven transmembrane protein, Smoothened (Smo). Hh exerts its biological influence through a signalling cascade that culminates in an alteration of the balance between activator or repressor forms of the Gli zinc-finger transcription factors ($Gli^A$ or $Gli^R$). In the absence of Hh, Ptc blocks Smo activity, and full-length Gli proteins are proteolytically processed to generate C-terminally truncated $Gli^R$

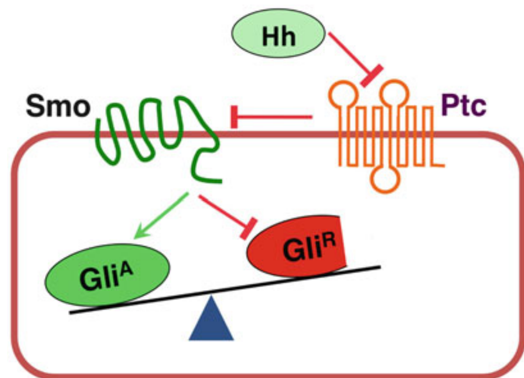


**Fig. 27.2** Simplified scheme of Hh pathway

that actively represses a subset of Hh target genes. Hh binding to Ptc unleashes Smo activity, which blocks $Gli^R$ production and promotes $Gli^A$ activation (Jiang and Hui 2008), thus activates expression of target genes, such as *Gli1* and *Ptc1* (Fig. 27.2).

Hh pathway is crucial for epithelium regeneration of stomach, airway, prostate and exocrine pancreas (Jiang and Hui 2008). During gastric regeneration following induction of gastric ulcer, the expression of Hh pathway components de-creases at first, then increases gradually after

several days; blockade of Hh signalling significantly represses the differentiation of gastric gland cells, but increases the proliferation of gastric progenitor cell. During acute exocrine pancreatic injury mediated by Cerulein and following regeneration, Hh pathway is activated as shown by the up-regulation of multiple pathway components and expression of a *Ptc-lacZ* reporter gene; while blockade of Hh signalling impairs epithelial regeneration in injured pancreas; notably, in this regeneration process, blockade of Hh signalling does not affect the formation of de-differentiated epithelial progenitors (metaplastic lesions), indicating that Hh signalling is only necessary for differentiation of the metaplastic epithelium into mature acinar cells. Taken together, these data indicate that Hh pathway is required mainly for functional differentiation in regenerative processes.

On the other hand, Hh pathway is sufficient to promote therapeutic blood vessel growth in various adult other tissues including heart, cornea, skin, skeletal muscle, and peripheral nerve (Lavine and Ornitz 2007). Hh signalling promotes vascular growth by inducing expression of VEGF and angiopoietin (ANG) molecules. But different from VEGF induced uniform increase in capillary-sized blood vessels, growth of multiple blood vessel types is promoted by Hh activation. So far, activation of Hh pathway has shown its efficiency in treating myocardial infarction, stroke, skin and peripheral nerve complications of diabetes via neovascularization in animal models. These data implicate the Hh signalling pathway as a useful target for therapeutics aimed to minimize ischemic damage and improve tissue function in conjunction with both macrovascular and microvascular disease. In radiated salivary glands, damages on both capillary segments and larger blood vessels are present and contribute significantly to the development of hyposalivation, so it would be worthy to examine whether activation of Hh pathway can promote the repair of vascular damage in irradiated salivary glands too.

## Cross-Talks Between Wnt and Hedgehog Pathways During Regeneration

During regenerative processes of various tissues, Wnt and Hh pathways are activated simultaneously in overlapping cell populations and interact at multiple levels. The net outcome of these cross-talks seems to be dependent on the cellular context and tissue/stage specific. In many tissues Hh and Wnt signalling promote cell proliferation in a cooperative or interdependent manner. For instance, in injured bladder, Shh expression in epithelial stem cells increases and elicits increased stromal expression of Wnt protein, which in turn stimulates the proliferation of both epithelial and stromal cells to support regeneration (Shin et al. 2011). Other mechanisms may also account for the synergism between Wnt and Hh pathways: $Gli^R$ can block Wnt signalling by binding β-catenin and inhibiting its transcriptional activator activity; while a genome-wide *in silico* study has predicted a large number of mammalian enhancers harbouring both Gli and Tcf binding sites (Hallikas et al. 2006). On the other hand, in renewal of distal colon epithelium, activation of Hh pathway limits Wnt activity in the crypt base, the putative stem cell niche, possibly via induction of Wnt inhibitor SFRP1 and down-regulation of Tcf4 and β-catenin expression (van den Brink et al. 2004). Compare the roles of Wnt and Hh pathways in salivary gland regeneration side by side will facilitate our insight of both the molecular control of the regenerative processes and their intertwined cross-talks.

## Wnt Pathways in Development and Regeneration of Salivary Glands

During mouse salivary organogenesis, expression of Wnt receptor Frizzled-6 increases gradually, while Wnt/β-catenin pathway is activated firstly in the mesenchyme and later, at the time of lumen formation, in the ductal epithelium except the endbuds (Patel et al. 2011). Mesenchymal

Wnt/β-catenin signalling induces expression of ectodysplasin (Eda) to trigger activation of Edar/NF-κB pathway and expression of Sonic Hedgehog in epithelium, EDA is a type II membrane protein of TNF (Tumour Necrosis Factor) superfamily, and shed from the cell membrane to bind as a trimer to its trimerized cognate receptor Edar and activate canonical NFκB pathway. Inhibition of mesenchymal Wnt/β-catenin signalling impairs SMG branching morphogenesis *ex vivo* (Haara et al. 2011). On the other hand, ectopic activation of Wnt/β-catenin signalling in epithelium blocks branching morphogenesis *ex vivo*, while non-canonical Wnt signalling promotes ductal maturation possibly by regulation of ductal maker Cp2l1, and the lack of both Wnt/β-catenin signalling and noncanonical Wnt signalling activities in endbuds is mediated through FGF-mediated upregulation of SFRP1, a secreted inhibitor of Wnt signalling (Patel et al. 2011).

In normal adult human salivary glands, both Wnt5a and Wnt signalling modulator Secreted frizzled-related protein 2 (SFRP2) are expressed at different levels. In adult mouse SMGs, Wnt4 is highly expressed, while Wnt/β-catenin signalling is marginal but activated significantly in epithelium during functional regeneration after ligation of main excretory ducts (Hai et al. 2010). Ectopic activation of epithelial Wnt/β-catenin signalling promoted expansion of putative c-Kit$^+$/Sca-1$^+$ salivary stem cells (Hai et al. 2010). Interestingly, radiation damage does not significantly affect activity of Wnt/β-catenin pathway in salivary gland (Hai et al. 2011). Activation of Wnt/β-catenin pathway in various tissues after injury is mediated through cAMP/PKA signalling triggered by Prostaglandin E2 (PGE2) produced locally as a universal response to tissue damage, which may be an evolutionary conserved mechanism to rapidly upregulate cellular proliferation to foster organ repair (Goessling et al. 2009). However, in saliva of patients received $^{131}$I for the treatment of thyroid diseases, the level of bicyclo-PGEm, a stable metabolite of PGE2, is significantly decreased compared with that in healthy controls (Rodrigues et al. 1998), suggesting the PGE2/β-catenin pathway is down-regulated in irradiated salivary gland.

Concurrent transient activation of Wnt/β-catenin pathway in epithelium prevented radiation-induced salivary gland dysfunction, likely by both suppressing apoptosis and preserving functional salivary stem/progenitor cells (Hai et al. 2011). However, similar Wnt activation 3 days before or after radiation did not show such beneficial effects, possibly due to intensive induction of mitosis in epithelial cells or missing the critical treatment window right after radiation to inhibit apoptosis respectively (Hai et al. 2011); in addition, the ectopic Wnt activation in epithelium after radiation may also impair acini differentiation during tissue regeneration similarly as during embryonic branching morphogenesis (Haara et al. 2011).

## Hedgehog Pathway in Development and Regeneration of Salivary Glands

In epithelium of embryonic salivary gland, the expression of Sonic Hedgehog (Shh) is induced by Edar/NF-κB pathway downstream of mesenchymal Wnt-EDA pathways (Haara et al. 2011). Shh may act within the epithelium in a juxtacrine manner to promote proliferation and differentiation of epithelial cells (Jaskoll et al. 2004). Branching morphogenesis of SMG is promoted by Hh activation *ex vivo*, and impaired by Hh inhibition *in vivo* and *ex vivo*, which could be rescued by FGF8 peptide supplementation *ex vivo*, demonstrating that FGF pathway functions in parallel or downstream of Hh pathway in this process (Jaskoll et al. 2004). In addition, Hh activation promotes cell polarization and lumen formation in developing SMG *ex vivo* (Hashizume and Hieda 2006).

In adult salivary gland Hh signalling is marginal but activated significantly during functional regeneration in together with Wnt signalling (Hai et al. 2010). Targeted expression of Gli1 in the salivary glands promoted epithelial proliferation,

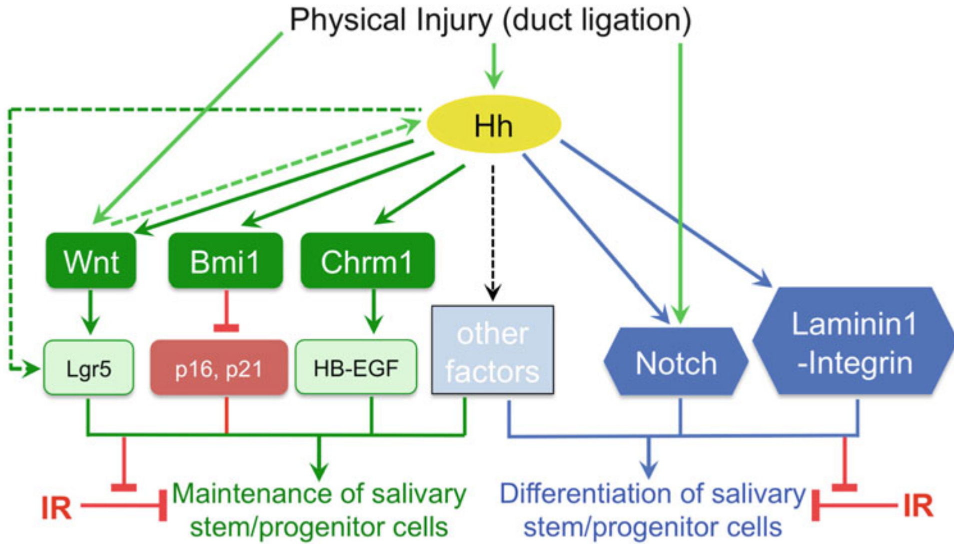


**Fig. 27.3** Hypothesis on the roles and interactions of signaling pathways during salivary gland regeneration after injury. *IR* Irradiation. *Dashed line*: Non-determined interactions

and the salivary glands become histologically normalized after withdrawal of Gli1 expression (Fiaschi et al. 2011).

## Targets of Hedgehog Signalling Related with Regeneration of Salivary Glands

In normal human mammary stem cells, Hh signalling upregulates Bmi1 (B lymphoma Mo-MLV insertion region 1 homolog) expression to promote self-renewal (Liu et al. 2006). The polycomb gene Bmi1 is a transcriptional repressor that regulates stem cell self-renewal through repression of important cell cycle regulatory genes in the INK-4A/ADP ribosylation factor (ARF) complex, p16INK4A, p19ARF and p21Waf1; recently, Bmi1 is also found to protect normal neural stem cells and human keratinocytes from radiation damages through recruitment of the DNA damage response machinery or epigenetic silencing of oxidase genes such as Lpo, p22phox, GP91, and p47phox.

CHRM1 (Muscarinic M1 receptor) is the major muscarinic receptor in the embryonic SMG epithelium, and acetylcholine signalling via CHRM1 promotes epithelial morphogenesis and proliferation of Keratin5$^{+}$ progenitor cells by transactivating Heparin-Binding Epidermal Growth Factor (HB-EGF) pathway during salivary organogenesis (Knox et al. 2010). CHRM1 signalling transactivates HB-EGF pathways by matrix metalloproteinase (MMP)-mediated cleavage of proHB-EGF to release the N-terminal ectodomain HB-EGF and the carboxyl-terminal fragment (CTF) of HB-EGF from epithelial membrane. HB-EGF induces membrane type 2 (MT2)–MMP and FGF receptor (FGFR) expression via EGFR pathway, and MT2-MMP-dependent release of bioactive NC1 domains from collagen IV promotes SMG branching morphogenesis via Integrin β1 and PI3K-AKT signalling pathway (Rebustini et al. 2009). Meanwhile HB-EGF-CTF moves into nucleus to promote cell proliferation by binding and exporting nuclear promyelocyticeukemia zinc finger (PLZF) protein, a transcriptional repressor, to allow expression of target genes such as cyclin A. In adult SMG acini cells CHRM1 is not ubiquitously expressed and only plays a minor role in cholinergic stimulation of salivary flow, which is mainly mediated by ubiquitously expressed CHRM3 under physiological conditions. Keratin5$^{+}$ cells increase during regeneration of adult SMGs after duct ligation when the innervation to the gland is intact (Hai et al. 2010),

and postnatal epithelial regeneration of salivary glands may require muscarinic stimulation of the Keratin5⁺ progenitor cells in a similar manner, as indicated by the *ex vivo* culture of denervated lobules of adult SMGs with muscarinic agonist or antagonist or EGFR antagonist (Knox et al. 2010). Hh signaling is known to promote innervation or regeneration of various peripheral nerves directly or via induction of neurotrophic factors such as Brain-derived neurotrophic factor (BDNF), hence functions through Chrm1/HB-EGF pathway to regulate salivary progenitor cells.

Another conserved intercellular signalling pathway, Notch pathway, is also closely related with morphogenesis and regeneration of salivary gland. Expression of activated Notch4 (int-3) transgene interferes with epithelial differentiation in mouse salivary glands, and salivary glands contained proliferating immature ductal cells and multiple poorly differentiated adenocarcinomas (Jhappan et al. 1992). On the other hand, during development of drosophila salivary gland, Notch ligand Serrate directs formation of actin rings in the salivary duct. Normal human SMGs express Notch 1–4, Jagged 1 and 2, Delta 1, and HES1, with nuclear localization indicating Notch signalling *in vivo*; in rat SMG, Notch pathway was activated during regeneration of after duct obstruction; in human salivary gland cell line HSG, Notch signalling is critical for growth and differentiation (Dang et al. 2009). Notch ligand Jagged2 (Jag2) and Notch target gene HEY2 are both direct target genes of Hh pathway, while muscarinic activation increases expression of Notch receptor Notch1 in oligodendrocytes, and activity of ADAM17 protease, which cleave Notch1 to facilitate Notch signalling (Bozkulak and Weinmaster 2009).

The branching morphogenesis and differentiation of salivary epithelial cells depends on interactions between extracellular matrix (ECM) proteins and their integrin receptors similarly as in other branched organs. Laminin-111 (laminin-1/LM-111), a heterotrimeric ECM protein essential for basement membrane formation, and its receptor Integrin α6β1 are required for branching morphogenesis of mouse SMG *ex vivo* (Kadoya et al. 1995). Consistently, interaction between laminin-1 and Integrin α6/β1 are required for *ex vivo* differentiation of human salivary gland cell HSG, which functions through upregulation of metallothioneins, a group of cystin-rich proteins with various functions including metal metabolism and homeostasis (Hecht et al. 2002). Other laminins and integrin receptors are also important for salivary gland development and homeostasis. Mutation of Integrin α3 leads to defects in the apical-basal polarity axis and in the basement membrane of mouse SMG epithelial cells, which may be through regulation of Cdc42 and RhoA. Laminin α5 controls SMG epithelial morphogenesis through β1 integrin signalling by regulating FGFR expression, which also reciprocally regulates the expression of Laminin α5; interestingly, loss of both integrin α3 and α6 resulted in a similar phenotype to that of Laminin α5 knockout, suggesting that interactions between α3β1 and α6/β1 integrins with laminin 511 are required for SMG development. In adult salivary gland, rodent and human salivary progenitor cells express intracellular laminin and its receptor integrins such as α6 (CD49f), β1(CD29) and β4 (CD104) (Sato et al. 2007); and integrin α6β1-expreessing cells isolated from rodent salivary glands have stem cell capabilities including colony formation and multipotent differentiation. Sjögren's syndrome is characterized by low levels of acinar compartment-specific laminin α1, and signalling mediated by integrin α1β1 and α2β1 is necessary for salivary gland remodelling by inducing differentiation of intercalated duct progenitors to acinar cells (Porola et al. 2010). These data suggested that Laminin/Integrin pathway is also essential for homeostasis of salivary gland, and provides a promising target for regenerative therapy to restore salivary function. Hh signalling activates Lama1 expression during assembly of the myotomal basement membrane, and activates Integrin α2 (Itga2) expression during osteoblast differentiation of human bone marrow Mesenchymal stem cells.

In conclusion, our recent findings in mouse models suggested that transient activation of Wnt or Hh pathways are promising strategies to prevent or rescue salivary gland hypofunction after radiotherapy, and the interactions between Hh,

Wnt and several other conserved intercellular signalling pathways during salivary gland regeneration worth further exploration to improve the efficacy and safety of these approaches.

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# Neural Stem Cell Migration: Role of Directional Cues and Electric Fields

28

Miguel Arocena and Jon Martin Collinson

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**Abstract**

The migration of neural stem cells (NSCs) is a key component of their therapeutic potential. NSCs are among the potential tools for cell-based therapies directed at CNS repair, and a better understanding of their capacity to respond to directional cues can contribute to their improved targeting to injured regions. These responses are also essential for the observed ability of NSCs to closely track brain tumor cells *in vivo*, which has significant clinical potential as well. Recently, it has been shown that NSC migration *in vitro* can be precisely controlled by the application of an external electric field (EF). EFs have been widely studied as directional cues *in vitro*, and their application to control cell migration *in vivo* as well as their use in clinical settings is beginning to be developed. Controlling neural stem cell migration by using diverse directional cues, among them EFs, will contribute to their use as therapeutic tools.

## Introduction

Neural precursors have remarkable migratory capacities, which play important roles during CNS development and which underlie cell transplantation therapies in animal models of neurodegenerative disease. Neural stem cell activity is found initially in the apical progenitor cells of the neural tube. As the neural epithelium thickens during early embryogenesis, the apical progenitors

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(radial glia) elongate and span the ventricular (apical) to pial (basal) surfaces of the CNS. The radial glia are the neural stem cells during embryonic neurogenesis and these cells may produce intermediate (basal) progenitors and neurons that migrate radially, away from the subventricular zone (Noctor et al. 2008). Most radial glia terminally differentiate or apoptose towards the end of CNS development, but some neurogenic progenitors persist into adult life. In the course of adult neurogenesis, these neural stem cells can still be found in the subventricular zone of the cortex (at least) and subgranular zone of the dentate gyrus. Subventricular cortical stem cells give rise to neuroblasts, which will then undergo a remarkable process of migration anteriorly across the brain, along a structure named the rostral migratory stream, to finally reach the olfactory bulb (Ghashghaei et al. 2007). These migratory events rely on diverse types of directional cues for precise targeting to the appropriate regions. For instance, neural stem cells have been observed to migrate radially across the cortex along the extended processes of other cells (Noctor et al. 2008), whereas neuroblasts in the rostral migratory stream respond to combinations of, among others, Slit-mediated chemorepulsive signals and netrin-1, GDNF- and BDNF-mediated chemoattractant signals (Chiaramello et al. 2007).

NSCs can be grown *in vitro* in large quantities, particularly via the formation of cell aggregates known as neurospheres from cortical, hippocampal or spinal cord explants, and can then be transplanted. Both *in vitro* and *in vivo*, neurosphere-derived cells show great capacity for differentiation into a wide variety of glial and neuronal cells types, consistent with a stem cell origin and indicating clinical potential for CNS repair. Transplanted NSCs have shown extensive migratory behaviors in several experimental models of brain and spinal cord pathology (Lindvall and Kokaia 2006), and they have also been demonstrated to have remarkable tropism towards brain tumors (Aboody et al. 2000). Transplanted NSCs also respond to different types of guidance cues to reach their targets. Recently, NSCs have been shown to display a strong directional migratory response when exposed to electric fields (EFs) both *in vitro* and in the environment of an *ex vivo* brain slice model (Arocena et al. 2010; Meng et al. 2011). EFs have been shown to be strong directional cues for a wide variety of cell types *in vitro* (McCaig et al. 2005), and their ability to control NSC migration could have potential clinical applications. This review will focus on the directional cues that guide NSC migration in the context of brain repair therapies and brain tumor targeting, and in the use of EFs to control cell migration, particularly NSC migration.

## Directional Cues for Neural Stem Cell Migration in Brain Repair Therapies

Chemotactic signals are among the main cues that guide NSCs and more committed neural precursors during development, and this chemotactic response is mirrored when NSCs are transplanted in animal models of brain injury. For instance, in a mouse model of stroke, it was shown that in the cerebral hemisphere where ischemic injury is induced the cytokine SDF-1 is markedly up-regulated, and that NSCs transplanted to the contralateral hemisphere migrated towards high SDF-1 areas in the injured hemisphere (Imitola et al. 2004). NSCs expressed the SDF-1 receptor, CXCR4, and exposure to a chemical gradient of SDF-1 *in vitro* led to a marked increase in transmigration in a Boyden chamber. Moreover, when NSCs cultured as neurospheres were co-cultured with ischemic brain explants, NSCs migration out of neurospheres towards explants was remarkably stimulated compared to control explants, but this increase was abrogated by treatment with a blocking antibody against the SDF-1 receptor. SDF-1 is up-regulated by astrocytes and endothelial cells that are activated by the inflammatory milieu of the ischemic brain. As an inflammatory signature is common in different brain pathologies, gradients of cytokines produced in this process could become major directional cues for transplanted NSCs, contributing to the remarkable homing of NSCs to injured brain areas. A recent study has provided additional

support for this concept, by showing that directed migration of NSCs transplanted into the dentate gyrus of mice with kainic acid-induced seizures is dependent on the cytokine CXCL12 (Hartman et al. 2010). Also, *in vitro* assays further confirm that many other cytokines, such as VEGF, PDGF-BB, RANTES and M-CSF, are chemoattractants for NSCs (Zhang et al. 2003; Schmidt et al. 2009).

Besides chemotactic signals, the topography of the brain environment could also be a source of directional cues for NSCs. For example, endogenous neural progenitors have been shown to move along blood vessels in the course of their migration towards damaged areas in brain slices from a mouse model of stroke (Kojima et al. 2010). *In vitro*, NSCs are responsive to engineered topographic cues, for example aligning in parallel to fibers with diameters ranging from 0.3 to 1.5 μm (Yang et al. 2005). *In vivo*, topographic cues could cooperate with other directional signals to help migrating NSCs maintain their course over long distances, enabling their migration to areas of brain pathology.

The extracellular matrix plays critical roles in cell migration, and there is evidence that its interactions with NSCs can modulate the directedness of their migration in the injured brain. In a mouse model of ischemia where neurons in the CA1 region of the hippocampus are selectively lost, a marked difference in directed migration of two neural stem cell clones after hippocampal transplant was observed, with one of the clones being much more effective at repopulating areas of neuronal death (Prestoz et al. 2001). The same difference in migratory performance was observed in assays *in vitro* with various extracellular matrix substrates, but it was abolished when interactions between these substrates and integrins were inhibited. As both clones expressed similar levels of several integrins, it was suggested that variations in integrin signaling could underlie their different migratory capabilities. It is therefore conceivable that interactions between the extracellular matrix and NSCs could act in combination with other guidance cues to promote directed NSC migration *in vivo*.

## Directional Cues for Neural Stem Cell Migration Towards Brain Tumors

The phenomenon of NSC tropism towards brain tumors was originally described for intracranial glioma (Aboody et al. 2000), but has since been extended to medulloblastoma, neuroblastoma and melanoma brain metastases (Kim 2011). Directed migration towards tumors is robust, as NSCs transplanted intracranially at distant sites from the tumor mass or delivered intravenously were able to reach it and, remarkably, they were also observed to closely follow small groups of tumor cells migrating away from the main tumor mass (Aboody et al. 2000). As in the case of NSC migration to injured brain areas, the large amount and variety of cytokines produced in tumor areas constitutes a fundamental source of guidance cues for NSCs. *In vitro*, NSCs show strong chemotactic responses to cytokines produced by glioma cell lines, particularly VEGF and HGF (Kendall et al. 2008). The cytokine SDF-1 also plays an important role in glioma tropism, as NSCs migrate *in vivo* towards areas of the tumor with high levels of this cytokine, in particular the tumor border and hypoxic regions within the tumor (Zhao et al. 2008). Hypoxia leads to upregulation of SDF-1 and VEGF in glioma cell lines, whereas reactive astrocytes that accumulate at the tumor border might be another source of SDF-1 (Zhao et al. 2008). Therefore, a variety of diffusible signals produced by tumor cells, either normally or in response to hypoxia, and also by cells from the tumor stroma, can be exploited by NSCs as guidance cues, which contributes to the persistent tropism they display towards brain tumors.

Tumors also produce abundant extracellular matrix, which can profoundly alter the microenvironment surrounding tumor cells. Gliomas remodel the pre-existing extracellular matrix, synthesizing in particular large amounts of vitronectin, fibronectin and tenascin. All of these substrates promote NSC migration and, importantly, they induce high levels of NSC transmigration when they are used to coat the underside of a Boyden chamber filter (Ziu et al. 2006),

showing that NSCs are capable of haptotaxis -the directed migration of cells in gradients of substratum-bound ligands- in response to extracellular matrix produced by gliomas. It is therefore conceivable that the local enrichment in certain adhesive molecules in the brain tumor microenvironment could operate as an additional directional cue for NSCs, cooperating with other guidance signals to strengthen their long distance migration towards the tumor mass.

## Electric Fields as Directional Cues

EFs, defined as gradients of voltage across space, are vectors, and can orient charged particles, as can be observed in the classic images of the patterns formed by charged threads placed in EFs. Already by the end of the nineteenth century it was known that single-celled protists could migrate parallel to an EF vector. This directed migration phenomenon was termed galvanotaxis, or electrotaxis. Since then, many studies have reported the electrotactic behavior of a wide variety of cell types, from amoeboid single cells of *Dictyostelium*, to vertebrate cells such as keratinocytes, fibroblasts and neutrophils (Erickson and Nuccitelli 1984; Zhao et al. 2006). Different cell types have been observed to migrate either towards the cathode (the negative pole) or towards the anode (the positive pole). For instance, *Dictyostelium*, keratinocytes, and neutrophils normally migrate towards the cathode (Zhao et al. 2006), whereas Schwann cells and retinal pigment epithelial cells move towards the anode (Mckasson et al. 2008; Gamboa et al. 2010). These results show that the final orientation of the electrotactic response is not uniquely determined by the EF, but also by how different cell types interact with it.

When cells are exposed to an EF, charge movement is generated on the outer surface of the cells, where $Na^+$ and other cations accumulate around negatively charged sugar moieties of transmembrane proteins and glycolipids. Cations will drag along water, generating an electro-osmotic flow, which has been shown to asymmetrically redistribute membrane proteins to the cathode-facing side of cells (McLaughlin and Poo 1981).

Cathodal redistribution of membrane receptors is an early event after EF exposure. For example, in keratinocytes, the epidermal growth factor (EGF) receptor localized preferentially on the cathodal cell membrane as early as 5 min after the beginning of EF exposure (Fang et al. 1999), and fast cathodal redistribution of the EGF receptor has also been demonstrated in corneal epithelial cells (Zhao et al. 1999). Inhibition of the EGF receptor reduced its cathodal accumulation and subsequent cell electrotaxis, suggesting that asymmetric signaling from this receptor is required for electrotaxis (Fang et al. 1999).

The phosphatidylinositol-3-OH kinase (PI3-K) pathway has also featured as an important component of the signaling events that lead to electrotaxis. Keratinocytes, fibroblasts and neutrophils from mice deficient in the p110γ catalytic subunit of PI3-K showed markedly impaired electrotactic responses (Zhao et al. 2006). Conversely, cells from mice deficient in the PI3-K antagonist PTEN display enhanced electrotaxis. Also, the same study showed that, similar to what occurs in chemotaxis, the PI3-K product PIP3 accumulates at the leading edge of cells undergoing electrotaxis.

A model of electrotactic migration has been proposed in which cathodal accumulation of activated receptors such as the EGF receptor would initiate localized signaling events such as activation of the PI3-K pathway, which in turn would stimulate protrusive activity, transforming the cathodal side of cells into the leading edge and directing cell migration towards the cathode (McCaig et al. 2005). Intracellular calcium can participate in sensing electric gradients; for instance blocking calcium influx with calcium channel blockers impaired keratinocyte electrotaxis (Fang et al. 1998). It has been proposed that preferential calcium entry to one side of the cell initiates electrotaxis in that direction (Mycielska and Djamgoz 2004). Other mechanisms are also likely to contribute to electrotaxis, because cathodal migration can occur without cathodal receptor accumulation (Finkelstein et al. 2007) or calcium entry (Brown and Loew 1994).

Although electrotaxis has been mostly studied in *in vitro* or *ex vivo* settings, there is evidence that applying EFs *in vivo* can also trigger electrotactic

cell migration. In a mouse model in which CD4 and CD8 T cells express green fluorescent protein (GFP) an EF was applied in the peripheral tissue of the ear in living mice on the stage of a confocal microscope, and the migration of T cells in the ear tissue was recorded (Lin et al. 2008). When exposed to an EF, T cells migrated towards the negative electrode implanted in the ear, moving directionally as much as 100 μm in 1 h. Also, the strength of the EF applied *in vivo* was between 200 and 500 mV/mm, which is within the same range as EF strengths used in *in vitro* experiments (McCaig et al. 2005).

Although the use of EFs to guide whole cell migration has not yet reached clinical applications, EFs are actively researched in pre-clinical and clinical settings for their ability to promote nerve outgrowth. EFs have been shown to promote cathodal growth of neurites from frog and mammalian neurons in culture, and subsequently were shown to promote axonal regeneration in animal models of spinal cord injury (McCaig et al. 2005). These results led to a phase 1 trial in humans with spinal cord injury, in which a device generating an oscillating EF was implanted in the site of the wound (Shapiro et al. 2005). The aim of the oscillating device was to promote bidirectional regeneration of nerve fibers, and it reversed the polarity of the EF every 15 min, a time calculated to promote nerve growth towards the cathode on one side of the lesion, but not long enough to induce retraction of nerves facing the anode on the other side. This study led to significant clinical improvement in sensory and motor symptoms, although additional improvement over standard treatments has not been achieved (McCaig et al. 2005).

## Neural Stem Cell Migration in Electric Fields

One of the first studies of NSC electrotaxis analyzed the migratory behavior of embryonic neural progenitors in applied EFs (Li et al. 2008). The methodology used was to subject embryonic forebrain explants to EFs, and to analyze the spatial distribution of cells migrating out of the explant. The results obtained indicated that cells leaving the explant were stimulated to migrate with high directionality towards the cathode in the presence of EFs, and that inhibition of the N-methyl-D-aspartate (NMDA) receptor, a glutamate ionotropic receptor, impaired their electrotactic behavior. EF exposure increased a physical association between the NMDA receptor and Tiam1, an activator of the Rac subclass of Rho GTPases, and also increased the phosphorylation levels of Pak1, a kinase involved in actin polymerization downstream of Rac. Therefore, components of the actin remodeling machinery are activated by EFs in a population of neural progenitors, which in turn have a strong directional response to EFs. In this study, a high number of cells co-expressed nestin, a marker of NSCs, and doublecortin, a marker of neuroblasts not found in undifferentiated neural progenitors such as radial glial cells (Noctor et al. 2008), suggesting that the cell population in the embryonic forebrain explants used was a mixture of neural progenitors in different stages of differentiation, most of which seemed nevertheless highly responsive to EF exposure.

Two other studies of NSC electrotaxis (Meng et al. 2011; Arocena et al. 2010) have used both primary cultures of murine embryonic neural progenitors grown initially as neurospheres, which is one of the hallmarks of NSCs, and an extensively studied clone of adult neural stem cells derived from rat hippocampus, which has been previously shown to be able to differentiate into neurons, astrocytes and oligodendrocytes (Kuwabara et al. 2004). Both types of NSCs showed marked electrotactic responses when exposed to EFs of 250 and 500 mV/mm (Meng et al. 2011), migrating towards the cathode. When the polarity of the EF was switched *in vitro*, cells very quickly reversed course and began migrating in the opposite direction, suggesting that EFs can precisely steer NSC trajectories. Importantly, both electrotaxis and electrotactic reversal were recapitulated in an *ex vivo* model by transplanting embryonic NSCs into organotypic spinal cord slices and exposing them to EFs, which indicates that environments approaching the complexity of the CNS can support electrotactic migration of NSCs.

Both growth factors and PI3-K signalling are important for NSC electrotaxis (Meng et al. 2011). In the absence of growth factors, electrotactic migration was abolished, although a reduced response remained at higher EF strengths for adult NSCs. There were differences in the growth factor requirements of embryonic and adult NSC electrotaxis, with the former depending on both EGF and FGF, and the latter on FGF only. These are also the growth factors used for routine maintenance of each cell type. Treatment with the PI3-K inhibitor LY294002 markedly reduced electrotaxis in both NSC types, and embryonic NSCs derived from mice lacking the p110γ catalytic subunit of PI3K had a much diminished electrotactic response compared to wild type embryonic NSCs.

A detailed analysis of the migration of adult NSCs in the presence or absence of EFs showed that these cells moved by dynamically extending and retracting protrusions in all directions, but that after an EF was applied protrusion extension was remarkably biased towards the cathode, although this bias was attenuated by treatment with LY294002 (Arocena et al. 2010). This suggested that exposure to EFs was inhibiting the extension of protrusions towards the anode, which was further supported by the observation that protrusions extending towards the cathode retract when the polarity of the EF is reversed. A simple model of NSC electrotaxis based on the experimental data was able to reproduce the migration patterns of NSCs, and suggested that PI3-K functions in NSC electrotaxis mainly, but not only, by controlling the orientation of protrusions.

The propensity of NSCs to migrate in electric fields may represent a potential strategy for clinical targeting towards sites of brain injury, but it is not yet clear for most diseases how this could effectively be achieved *in vivo*. Nor is it fully apparent under which conditions NSCs could produce clinically significant amelioration of symptoms once they have reached the sites of brain lesion, especially when newly differentiated neurons would have to project and make multiple synaptic connections to restore CNS function. However, sites of brain lesion, which may be associated with perturbation of ion flow (e.g., from damaged cells) and vascularisation, hence producing their own endogenous electric fields, may act as in vivo guidance cues. The response of NSCs to endogenous electric cues can be manipulated genetically as described above and it may be possible therefore to bias their migration clinically. Furthermore, diseases such as multiple sclerosis, that require therapeutic NSCs only to differentiate into oligodendrocytes, offer a more immediate prospect of cell-based therapy based on successful targeting of NSCs to the site of disease.

The main conclusion from these studies is that NSCs are highly responsive to EFs, both *in vitro* and *ex vivo*. It is conceivable therefore that devices capable of delivering EFs *in vivo*, such as the one designed for spinal cord injury treatment, could be used to guide NSC migration, initially in animal models of brain injury, and eventually in clinical settings. NSCs exploit combinations of several directional cues to sustain their remarkable tropism to damaged areas in the CNS. EFs could become a valuable addition to the array of chemotactic, adhesive and topographic signals that NSCs recognize, focussing their migration towards specific targets.

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# ABL-Kinase Mutations in Progenitors and Stem Cells from Chronic Myeloid Leukemia Patients

29

Jean-Claude Chomel, Nathalie Sorel, and Ali G. Turhan

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**Abstract**

Tyrosine kinase inhibitors (TKIs) have profoundly changed the natural history and prognosis of chronic myeloid leukemia (CML). However, a small proportion of patients develop a resistance towards targeted therapies. Missense mutations located within the kinase domain of the BCR-ABL oncogene (also referred to as BCR-ABL mutations or ABL-kinase mutations) are the most common mechanism of resistance. Although it has clearly been established that genetic instability inherent to *BCR-ABL* expressing leukemic cells predisposes the latter to the acquisition of mutations, the hierarchical distribution of these mutations in stem cells was not known until recently. There is now evidence suggesting that ABL-kinase mutations occur in hematopoietic progenitors and stem cells expressing the *BCR-ABL* oncogene, adding therefore an increased level of complexity to the phenomenon of TKI-resistance in CML stem cells.

## Introduction

Chronic myeloid leukemia (CML), characterized by a clonal proliferation of myeloid progenitors originating from a single malignant stem cell, represents the model of myeloproliferative neoplasms. A t(9;22)(q34;q11) translocation, occurring in a primitive hematopoietic stem cell (HSC), gives rise to the Philadelphia chromosome (Ph1) and fuses the 5′ portion of the breakpoint cluster

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region (*BCR*) gene in-frame to most of the *ABL* gene except the first alternative exon. The BCR-ABL fusion oncoprotein, generated by this translocation, displays a deregulated tyrosine kinase (TK) activity and is directly responsible for the phenotypic alterations associated with CML, including an increased cell proliferation, a dysfunction in cellular adhesion/migration and a resistance to apoptosis (Goldman and Melo 2003). Historically, in the absence of efficient anti-leukemic treatments, and despite cytoreductive therapy (hydroxyurea), the disease progressed from a chronic phase (in which hematopoietic differentiation is not altered) to an accelerated phase and finally to a blast crisis (or acute phase) characterized by the blockage of terminal myeloid differentiation.

Over the last three decades, significant improvements have been accomplished in the therapy of CML. Allogeneic stem cell transplantation was the first potentially curative treatment for patients in chronic phase. Interferon-alpha, alone or associated with cytarabine, displayed real efficiency in a small number of patients, allowing durable complete cytogenetic remissions (absence of Ph1-positive metaphase in conventional cytogenetic analysis) in patients responding to the treatment. More recently, tyrosine-kinase inhibitors (TKIs) have been developed to target the BCR-ABL oncoprotein through its enzymatic activity. The introduction of imatinib mesylate (an ATP-competitive inhibitor) in the early 2000s has profoundly modified the natural history of CML and dramatically improved patient survival (Druker et al. 1996). Indeed, the overall survival of CML patients is ~90% after 5 years of imatinib first-line therapy (Druker et al. 2006). Nevertheless, a small percentage of patients (15–20%) develop resistance. Second generation TKIs such as dasatinib or nilotinib, were then designed to overcome imatinib resistance or intolerance (Shah et al. 2004; Weisberg et al. 2005). Used as second line therapies, these drugs are particularly useful to target CML cells harboring mutations within the BCR-ABL kinase domain. Recently, they have been found to be highly effective in the first-line setting, displaying a more rapid effect on hematological and molecular responses (Kantarjian et al. 2010; Saglio et al. 2010).

Resistance toward targeted therapies (based on imatinib experience) is characterized by hematologic, cytogenetic and molecular criteria, as suggested by European LeukemiaNet recommendations (Baccarani et al. 2009). Primary or immediate resistance is defined as a failure to achieve predefined levels of response to therapy. On the other hand, a loss of an established response reflects a secondary resistance. Biological mechanisms of resistance are drug-dependent, BCR-ABL-dependent or BCR-ABL and drug-independent (Apperley 2007). A comprehensive review of these different mechanisms has to include other common causes of "resistance" such as patient non-compliance, individual variability in gastrointestinal absorption and hepatic metabolism, interactions with other medications or foods (such as grapefruit juice) that can alter drug metabolism. Concerning imatinib-dependant resistance mechanisms, it is obvious that a decreased intracellular concentration of the drug leads to a reduced activity. In this context, plasma sequestration of imatinib by α1-acid glycoprotein, down-regulation of OCT-1 uptake transporters and up-regulation of ABCB1 (MDR1) and ABCG2 efflux pumps greatly affect drug intracellular availability. The BCR-ABL oncogene itself can confer TKI resistance according to two mechanisms; mRNA transcript over-expression caused by genomic amplifications, or development of acquired point substitutions within the tyrosine kinase domain. Although ABL-kinase mutations only account for approximately 25% of cases, they are the most studied mechanism of resistance. Finally, genetic instability, clonal chromosomal evolution, leukemic stem cell quiescence and activation of alternative signaling pathways represent other mechanisms of TKI resistance. All these mechanisms have been implicated in imatinib resistance, but their precise contributions remain to be established. It is noticeable that primitive quiescent leukemic stem cells are intrinsically refractory to all tyrosine kinase inhibitors (Graham et al. 2002; Copland et al. 2006; Jorgensen et al. 2007). In addition, recent data suggest that the leukemic stem cell

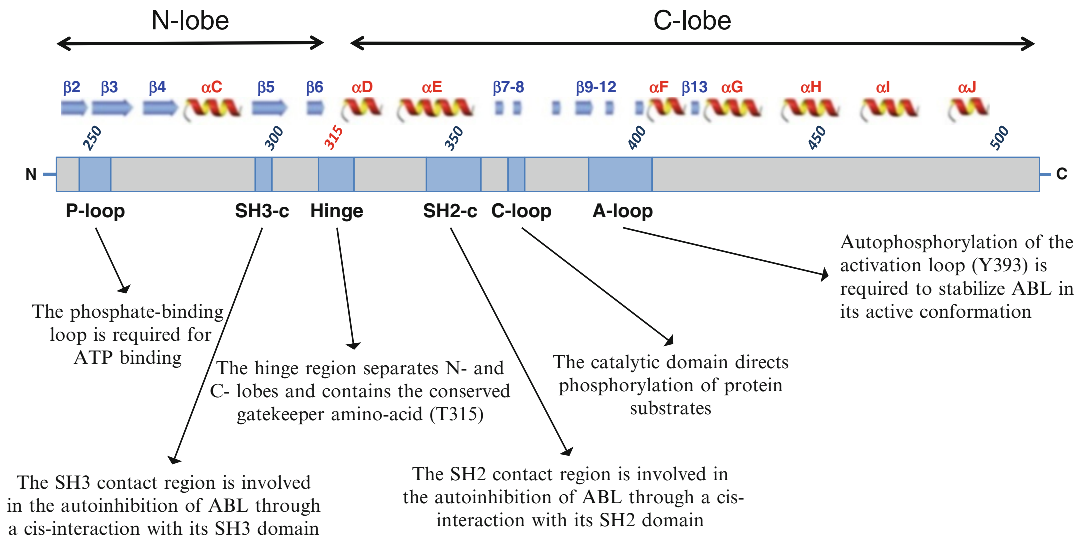


**Fig. 29.1 Schematic representation of the ABL tyrosine kinase domain.** ABL tyrosine kinase domain contains a succession of α-helix and β-sheet structures and adopts a two-domain architecture with small N-terminal and larger C-terminal lobes. The distinct regions of the tyrosine kinase domain are shown (P-loop, SH3-contact, Hinge, SH2-contact, C-loop and A-loop) along with their respective functions

niche could play a prominent role in the innate resistance of primitive hematopoietic stem cells towards TKIs, by promoting quiescence or cell survival (Schmidt et al. 2011).

## ABL-Kinase Point Mutations

Tyrosine kinases (TKs) catalyze the phosphorylation of protein substrates on a tyrosine residue. These proteins are involved in signal transduction and thus play an essential role in cell biology. Approximately 100 TKs have been identified within the human kinome including receptor or non-receptor proteins (such as SRC, ABL or BCR-ABL). Whatever their location, TKs are all characterized by the presence of a well-conserved kinase domain consisting of a succession of α-helix and β-sheet structures (Nagar et al. 2002). The TK domain can be divided in carboxy- and amino-terminal lobes (C and N lobes) tethered by the hinge region. N lobe contains the P-loop (phosphate-loop or nucleotide-binding loop) and C lobe includes the catalytic (C-loop) and the activation loop (A-loop) with the major site of auto-phosphorylation (Fig. 29.1). In the active form of the kinase, A-loop adopts an extended conformation to allow ATP and substrate binding. Conversely, inactive conformation of A-loop impairs these bindings. Distinct regulation domains, located along the TK domain, contribute to the auto-inhibition of the kinase. Concerning ABL, cis-interactions between SH2/SH3 (SRC homology 2/3) regulatory domains and the TK domain stabilize the inactive state of the kinase. More specifically, these interactions occur with SH3-contact site located within N-lobe (interaction with proline residues) and SH2-contact site located in the C-lobe (through hydrogen bonds).

Imatinib can bind reversibly the inactive conformation of several tyrosine kinases such as ABL, BCR-ABL, KIT, PDGFRA and PDGFRB. This small drug targets the ATP-binding pocket located between the two lobes of the kinase domain. In this situation, the imatinib/BCR-ABL complex is stabilized through hydrogen bonds (with E286, T315, M318, I360, H361 and D381 ABL amino acids), several van der Waals contacts (with L248, Y253, V256, A269, K271, V289, M290, V299, I313, F317, G321, R362, L370, A380, F382 ABL amino acids) and some water-mediated ionic interactions (Nagar et al. 2002).

**Table 29.1** ABL-kinase mutations identified in imatinib-resistant CML patients

| | Mutations | | | Mutations | | | Mutations | |
|---|---|---|---|---|---|---|---|---|
| | A217P | | SH3-c | L298V | | | V379I | |
| | Y232H | | SH3-c | V299L | W | | A380T | W |
| | **M237V** | | Hinge | F311L/I/V/Y | | | F382L | W |
| | I242T | | Hinge | T315A | H | A-loop | L384M | |
| | **M244V** | | Hinge | **T315I** | H | A-loop | L387M/F/V | |
| P-loop | L248V | W | Hinge | **F317L/V/I/C** | W | A-loop | M388L | |
| P-loop | **G250A/E/V/R** | | | L324Q | | A-loop | Y393C | |
| P-loop | Q252E/H/R | | SH2-c | Y342H | | A-loop | **H396P/R/A** | |
| P-loop | **Y253F/H** | W | SH2-c | M343T | | A-loop | A397P | |
| P-loop | **E255K/V** | | SH2-c | A344V | | | S417F/Y | |
| | E258D | | SH2-c | A350V | | | I418S/V | |
| | W261L | | SH2-c | **M351T/V** | | | A433T | |
| | L273M | | SH2-c | **E355D/G/A** | | | S438C | |
| | E275K/Q | | | **F359V/I/C/L** | | | P441L | |
| | **D276G** | | C-loop | D363Y | | | E450K/G/A/V | |
| | T277A | | C-loop | L364I | | | E453G/K/V/Q | |
| | E279K | | C-loop | A365V | | | E459K/V/G/Q | |
| | V280A | | C-loop | A366G | | | M472I | |
| | V289A/I | W | | L370P | W | | P480L | |
| | E292V/Q | | | V371A | | | **F486S** | |
| | I293V | | | E373K | | | E507G | |
| | | | | H375Y | | | G514S | |

W and H represent the amino acids involved in van der Walls and hydrogen bindings to imatinib respectively. Mutations in red account for more than 85% of cases

Up to now, more than 100 missense mutations affecting approximately 70 amino acids of the TK domain (amino acids 200–515) have been identified in CML patients with imatinib resistance (Apperley 2007; Soverini et al. 2011) (Table 29.1). Schematically, the presence of these amino acid changes modifies the protein structure and induces either a conformational change of the kinase domain or a disruption of the interactions between imatinib and BCR-ABL (affecting hydrogen or van der Waals bonds). Consequently, drug binding is impaired or totally abolished. Mutations of 13 amino acid residues, mainly located within the functional regions of the TK domain, account for more than 85% of cases. For unknown reasons, P-loop (amino acids 248–256) represents the most frequently mutated area (G250A/E/V/R, Y253F/H, E255K/V mutations). The Y253F/H mutation favors the transition from inactive to active state of the kinase to which imatinib cannot bind. G250E mutation obstructs the access of the drug to the ATP-binding pocket (Gasser 2005). The well-known T315I substitution, located in the hinge region, accounts for ~15% of acquired resistance cases due to a mutation. One of the consequences of

T315I is the disruption of a hydrogen bond that seems critical for the binding of imatinib. M351T (SH2-contact site) and H396P/R (A-loop) mutations contribute to a destabilization of the inactive form of the kinase through distinct mechanisms. It must be noted that a strategy of *in vitro* imatinib resistance screening of randomly mutagenized BCR-ABL has allowed the identification of about 80 amino acid substitutions within the kinase domain (some of them already found in patients) and approximately 20 others in SH2 and SH3 domains (which have not been observed in patients) (Azam et al. 2003).

ABL-kinase mutations are rarely found in patients with primary resistance using direct sequencing techniques (sensitivity 15–20%). From a functional point of view, the majority of mutations only leads to moderate or intermediate resistance toward imatinib except some P-loop mutations (such as G250E, Y253F/H, E255K/V) and the T315I gatekeeper substitution. Second generation TKIs, dasatinib and nilotinib, have been shown to overcome the majority of BCR-ABL mutations except T315I. Nilotinib, as imatinib, can only binds the inactive conformation of the BCR-ABL kinase and targets the same proteins. On the other hand, dasatinib (a SRC/ABL inhibitor) is functional on inactive and active forms of various targets including ABL and BCR-ABL. However, some particular mutations appear specifically resistant to dasatinib (V299L, T315A, F317L/V/I/C) or nilotinib (Y253H/F, E255K/V, F359V) supporting the requirement of a mutational evaluation before deciding to switch from a TKI to another. The T315I mutation confers an absolute resistance to all ATP-competitive BCR-ABL inhibitors used in clinical practice (imatinib, dasatinib and nilotinib). Recently, a pan-BCR-ABL inhibitor (ponatinib) was shown to be active against the gatekeeper mutation (O'Hare et al. 2009).

## Mutations in the Progenitor/Stem Cell Compartment

The occurrence of ABL-kinase mutations in Ph1 stem cells is highly suggested by clinical experience, as well as by experimental data using hematopoietic stem cell purification strategies. The detection of these mutations in a high proportion of leukemic cells during disease progression is in favor of their occurrence at the stem cell level. Analysis of the HSC compartment in CML patients can be performed by the purification of primitive hematopoietic populations using phenotypic characteristics ($CD34^{+}/CD38^{-}$ cells) followed by functional tests, such as long-term culture-initiating cell (LTC-IC) or NOD/SCID mice repopulating cell (RC) assays. It should be noted that the evaluation of *in vivo* NOD/SCID–RC assays has been difficult in the past due to low engraftment efficiency. From a practical point of view, LTC-IC assays are currently the most stringent functional test for *in vitro* evaluation of primitive CML stem cells.

The presence of BCR-ABL domain mutations was first reported in HSCs from three CML patients with late chronic or acute phase (Sorel et al. 2004). These patients harbored a Q252E or a M351T mutation at the time of imatinib resistance. Denaturing Gradient Gel Electrophoresis (DGGE) experiments on RT-PCR products were performed on peripheral blood samples, on $CD34^{+}/CD38^{+}$ and $CD34^{+}/CD38^{-}$ purified cells, as well as on pooled clonogenic progenitors originating from LTC-IC assays started with $CD34^{+}/CD38^{+}$ and $CD34^{+}/CD38^{-}$ cells. The ABL-kinase mutation initially detected in patient blood was found to be present in $CD34^{+}/CD38^{+}$ but not in $CD34^{+}/CD38^{-}$ purified primary cells. However, when the latter cell population was analyzed in LTC-IC assays, the non-adherent cells from the 5-week old cultures, as well as their clonogenic progeny, were found to harbor the initial mutation (as the more differentiated cell compartments) (Sorel et al. 2004). These data demonstrated that a BCR-ABL mutation could occur in a small population of leukemic stem cells, which can undergo *in vitro* amplification and differentiation (required to reveal them). Subsequently, other groups have also shown that ABL-kinase mutations could arise in $CD34^{+}$ cells from CML patients who achieved complete cytogenetic response on imatinib therapy (Chu et al. 2005). Using a sensitive subcloning-PCR protocol, the authors identified mutations already reported in clinical studies (L248P, D276G), but also substitutions only observed in *in vitro* screenings.

These findings confirm that mutation within the BCR-ABL kinase domain (with or without clinical relevance) can arise in the hematopoietic stem cell compartment.

Amongst ABL-kinase mutations occurring in *BCR-ABL*-expressing cells, the T315I substitution has been a challenge as it confers resistance to all three TKIs in clinical use. Recently, several inhibitors (such as ponatinib) have been developed for targeting leukemic cells harboring this mutation. However, in order to be fully efficient, drugs need to target the most primitive leukemic stem cells. As the gatekeeper substitution (like other ABL-kinase mutations) arises in a leukemic cell harboring native *BCR-ABL*, it was of significant interest to accurately quantify T315I *BCR-ABL* mRNA transcripts in order to estimate the amount of mutated Ph1 cells amongst those without mutation. For this purpose, we have developed a quantitative RT-PCR assay using an allele-specific LNA (locked nucleic acid) primer (Chomel et al. 2009). Using this procedure combined with stem cell detection and quantification methods, we have addressed the question of the hierarchical level of HSC involvement in a CML patient whose blood leukemic cells harbored the T315I substitution at the time of TKI-resistance. The occurrence of the T315I substitution was studied in hematopoietic progenitors present in blood and bone marrow samples. CFU-C (colony forming units in culture) assays were performed on mononuclear and $CD34^+$ cells in order to identify committed hematopoietic progenitors. Long-term cultures were initiated from $CD34^+$ cells, and after 6 weeks, LTC-IC content was analyzed using their clonogenic progeny (Chomel et al. 2010). The gatekeeper mutation was shown to occur in a very primitive Ph1 stem cell (LTC-IC) without altering its myeloid (and erythroid as well) terminal differentiation potential since the mutation was found in CFU-GM (colony forming unit-granulocyte macrophage), CFU-GEMM (colony forming unit-granulocyte erythrocyte monocyte megacaryocyte) and BFU-E (burst forming unit-erythroid) subpopulations. As in non-mutated Ph1 stem cells, a progressive decline of CML cells expressing T315I *BCR-ABL* mRNA is observed in long-term culture. In the same study, a complete screening of the tyrosine kinase domain by DGGE was carried out on 110 individual hematopoietic colonies. In addition to the T315I substitution, 15 other mutations, some of them with clinical relevance (E255K, Q252H, F359L and H396P) were also identified in a few colonies (Chomel et al. 2010). The identification of such alterations in progenitor assays could highlight the genetic instability of CML stem cells. A previous work had already shown the instability of *BCR-ABL* gene in $CD34^+/CD38^-$ sorted cells and CFU-C assays from chronic phase imatinib-naïve CML patients (Jiang et al. 2007a). Using allele-specific RT-PCR or direct sequencing, the authors characterized many ABL-kinase point mutations (>75), some of them with clinical interest in terms of TKI-resistance. The recurrent presence of nucleotide abnormalities already identified in some screening experiments is a strong argument in favor of BCR-ABL-induced genetic instability in the stem cell compartment. In the same way, comparative genomic hybridization experiments have shown the presence of genomic alterations in $CD34^+$ purified cells from imatinib-resistant CML patients (Joha et al. 2011). The exact mechanisms leading to genetic instability in CML stem cells and the question of whether the abnormalities already identified in bulk leukemic cells (such as increased oxidative stress and altered DNA repair pathways) are present in stem cells remains undetermined at the present time.

The demonstration of greater levels of BCR-ABL oncoprotein in $CD34^+/CD38^-$ as compared to $CD34^+/CD38^+$ cell populations (Jiang et al. 2007b) and of reactive oxygen species (ROS) in quiescent CML stem cells (Koptyra et al. 2006) indicates that the same abnormalities will be operational at the stem cell level. We have previously shown the occurrence of *de novo* ABL-kinase mutations in murine HSCs after bone marrow transduction with a native BCR-ABL-expressing vector transfer and later *in vitro* selection in the presence of imatinib (Flamant and Turhan 2005). The demonstration of the occurrence of mutations within an inert BCR-ABL plasmid suggests the mutagenic role of BCR-ABL TK activity in the context of any eukaryotic cell, compatible with

the concept of a self-mutagenesis of the ABL-kinase domain in CML patients. Consequently, the hematopoietic stem cell compartment might be particularly prone to *BCR-ABL*-associated genetic instability.

Overall, it is now clear that BCR-ABL point mutations conferring TKI resistance arise in a hematopoietic stem cell with classical self-renewal and differentiation capabilities. In addition, genetic instability of primitive leukemic cells seems to be the major cause predisposing to the acquisition of such kinase mutations. In this context, the Ph1 stem cell compartment might serve as a "reservoir" for resistant mutants. However, the possibility that mutations occur in a more committed cell is not excluded; this phenomenon could thus explain transient detection of mutated *BCR-ABL* mRNA in clinical laboratory monitoring.

## Emergence of ABL-Kinase Mutations

A critical issue concerning ABL-kinase point mutations is related to the time of their acquisition in Ph1 stem cells and their subsequent amplification during targeted therapy. Two hypotheses can be considered including the selection of pre-existing mutations by imatinib (or second generation TKIs) or the generation of such genetic abnormalities under targeted therapies. The first hypothesis refers to the concept of selective pressure under antibiotic therapies and is largely supported by the cancer cell resistance phenomenon observed in clinical oncology. The alternative hypothesis is the generation of *de novo* mutations under the influence of TKI therapies as previously discussed in a BCR-ABL plasmid model (Flamant and Turhan 2005). At the present time, no experimental data exist in favor of one of these two plausible hypotheses, as genetic instability is a common event in both.

In the clinical setting, mutational analysis of the BCR-ABL kinase domain is usually performed on blood samples, at the time of resistance, using direct sequencing or screening methods such as DGGE, DHPLC (denaturing high performance liquid chromatography) or HRM (high resolution melting). Sensitivity of these methods varies from 15 to 20% (direct sequencing) to 1–5% (screening methods). As a result, the identification of potential rare pre-existing mutated leukemic cells can only be done by the use of sensitive methods in the peripheral blood or classical techniques on individual clonogenic colonies.

The presence of ABL-kinase mutations antedating imatinib treatment has been studied in patients in whom RNA samples taken prior to the therapy were available for analysis. In this particular setting, it has been shown that mutations found at the time of resistance were also detectable at diagnosis using allele-specific RT-PCR (Roche-Lestienne et al. 2002). These findings support the existence of BCR-ABL mutants prior to imatinib and their subsequent clonal amplification under targeted therapy. It is therefore possible that ABL-kinase mutations might occur during the chronic phase of CML in the absence of any treatment, and imatinib (and probably other TKIs) could then select out those conferring resistance to the therapy (Shah et al. 2002) (Fig. 29.2). In this case, two conditions must be theoretically fulfilled. Firstly, a mechanism promoting the occurrence of mutations within the BCR-ABL kinase domain is required. Secondly, in the absence of selection pressure (tyrosine kinase inhibitors), mutant Ph1 cells should only survive if they present a biological advantage.

CML leukemic cells are endowed with an exclusive mutator phenotype due to various mechanisms including the accumulation of ROS and the inhibition of DNA repair mechanisms such as mismatch repair (Koptyra et al. 2006) or nonhomologous end joining pathways (Deutsch et al. 2001). Genetic instability initiated by *BCR-ABL* gene expression could then promote point mutations restricted to its own locus (Yuan et al. 2010). Indeed, in the context of imatinib resistance, nucleotide changes have only been found at the BCR-ABL locus, more precisely within the tyrosine kinase domain. The presence of ABL-tyrosine kinase mutations in the stem cell compartment (in the presence or absence of TKIs) clearly highlights the instability of the BCR-ABL gene itself (Jiang et al. 2007a). Thus, the acquisition

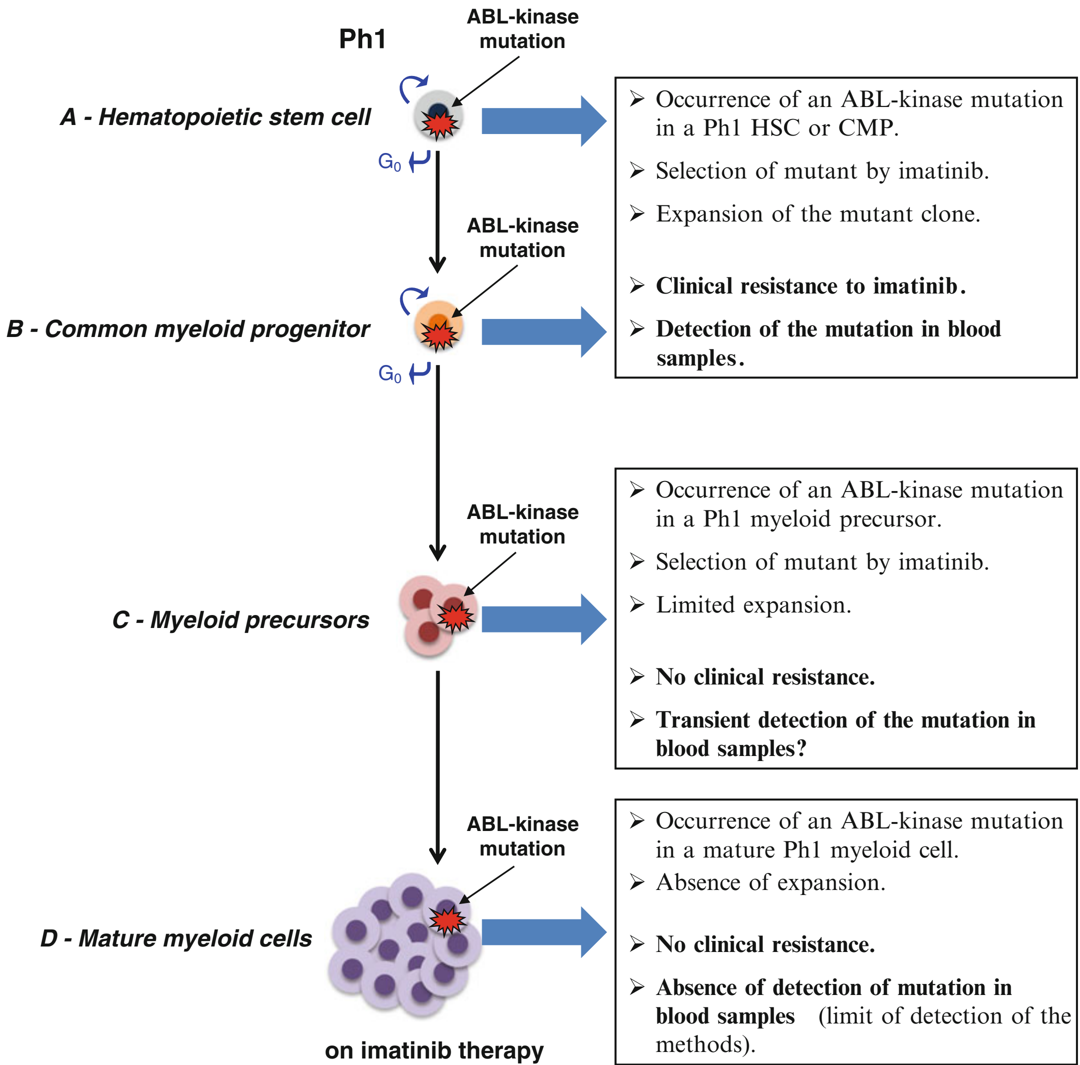


**Fig. 29.2 Selection and expansion of imatinib-resistant mutants.** Ph1 hematopoietic stem cells (*HSCs*) and common myeloid progenitors (*CMPs*) retain the capability to differentiate, to self-renew and to remain quiescent (in the $G_0$ phase of the cell cycle). The acquisition of the t(9;22) translocation in a hematopoietic stem cell or CMP is the consequence of a genetic instability phenomenon and is directly responsible for leukemia phenotype. The Ph1 chromosome does not only activate many oncogenic signaling pathways, but favors an additional genetic instability of the BCR-ABL locus itself. ABL-kinase point mutations can thus develop at any stage of the hematopoiesis process (*A*–*D*). Under exposure of imatinib, only mutated BCR-ABL clones conferring drug resistance are finally selected. Among them, only HSCs and CMPs (*A*–*B*) are able to give rise to a sufficient amount of mutant cells to initiate a clinical resistance to the therapy

of TK point mutations seems to be greatly related to the genetic instability phenomenon. When occurring in a primitive Ph1 stem cell, a BCR-ABL mutation should confer a slight growth and/or survival advantage over other precursors in order to persist without targeted therapy selection pressure. Nevertheless, an effective biological advantage for mutated leukemic cells has not clearly been established so far (Griswold et al. 2006; Miething et al. 2006). The question remains thus open regarding the presence of pre-existing mutations later selected by TKIs. If this proposition

appears attractive, it has recently been proposed that the presence of mutation antedating the treatment could not be required to their development under targeted therapy (Yuan et al. 2010). In this situation, the generation of missense mutations could depend on *BCR-ABL* gene expression and the high mutagenesis potential of the BCR-ABL locus.

One other relevant issue concerns the time needed to obtain, under targeted therapy, a sufficient amount of mutated hematopoietic stem cells (with proliferative potential) to achieve a clinical resistance. Routinely, the monitoring of CML patients on TKI treatment is based on hematological, cytogenetic and molecular responses. The regular molecular follow-up every 3 months in blood samples allows the determination of *in vivo* kinetics of response to targeted therapy. In case of TKI failure (primary resistance) or loss of drug response (secondary resistance), the screening for ABL-tyrosine kinase mutations is required. Once a mutation has been detected in a patient, highly sensitive mutation-specific assays, can be used for studying patient samples retrospectively, in order to establish the first occurrence of the mutated clone and to define the kinetics of its appearance. Generally, the kinetics of emergence of mutated clones appears to be heterogeneous in imatinib-resistant patients and, therefore, should be analyzed with caution. Nevertheless, in the case of resistance due to an ABL-tyrosine kinase mutation, we can postulate that the mutation can be detected in blood samples after approximately 1 year of treatment (Gruber et al. 2005; Chomel et al. 2009). For *in vitro* experiments, the development of BCR-ABL mutations has been observed in 3–6 weeks on CFU-C assays from primary CML cells, and in only 2 weeks in a CML cell line model (Jiang et al. 2007a; Yuan et al. 2010).

In patients harboring a BCR-ABL mutation clearly involved in imatinib resistance, a switch to second generation tyrosine kinase inhibitors (dasatinib or nilotinib) is mandatory. However, it has been reported that these patients display an increased probability to develop newly acquired mutations such as the T315I substitution or mutations specific to second line therapy (V299L, T315A, F317L/V/I/C for dasatinib or Y253H/F, E255K/V, F359V for nilotinib) (Soverini et al. 2009). TKI-resistant mutations selected or unselected by different TKIs used in sequential therapies, reflect an increased genetic instability. Moreover, the delay of emergence of *BCR-ABL* mRNA transcripts harboring the second mutation (e.g., T315I) might be shortened to a few months. This assumption, based on allele-specific quantitative RT-PCR experiments, supports the parallel between an increased selective pressure induced by more potent drugs and a rapid emergence of novel mutations initiated by genetic instability.

In conclusion, chronic myeloid leukemia is a unique paradigm of human oncogenesis and a model for targeted cancer therapies. The introduction of imatinib as a first line treatment has revolutionized the management of the disease and enhanced the overall survival of patients. However, the great efficacy of this TKI is, in a significant proportion of patients, limited by the acquisition of drug resistance. Missense mutations located within the BCR-ABL tyrosine kinase domain represent the most common and most studied mechanism of resistance. Approximately, 100-point mutations located within the BCR-ABL tyrosine kinase domain have been detected in CML resistance.

Current clinical and experimental data suggest that genetic instability induced by BCR-ABL, associated with alteration of DNA repair mechanisms, is responsible for the development of nucleotide variations in the BCR-ABL locus itself, through a “self-mutagenesis” phenomenon, which can confer imatinib resistance. ABL-kinase mutations can occur theoretically in any cell and any stage of hematopoietic hierarchy, but only stem cells or progenitors capable of self-renewal are able to sustain the mutant clone. Analysis of cultured primary cells from CML patients harboring a BCR-ABL mutation in blood samples has definitively demonstrated the occurrence of such substitution in the primitive stem cell compartment that could serve as a reservoir for these mutated Ph1 subclones. The issue of hematopoietic cells harboring ABL-kinase mutations prior to imatinib treatment remains a subject of discussion. Nevertheless, once generated,

resistant clones can be selected by the therapy. Overall, ABL-kinase mutation could occur in proliferating stem cell or progenitor early in the disease course. In addition, mutations do not seem to promote a real survival advantage until exposure to imatinib therapy.

Second generation tyrosine kinase inhibitors are effective on most of mutated leukemic cells. Nevertheless, a few mutants resist specifically to nilotinib or dasatinib or to both. Consequently, sequential therapies (imatinib then second generation TKI after imatinib failure), can lead, in rare cases, to the selection of a restricted number of mutations and finally to the emergence of the T315I gatekeeper substitution. In this situation, the kinetics of emergence of these genetic abnormalities seem influenced by the existence of a previous imatinib-resistant mutation. Selecting/deselecting cycles induced by the use of different TKIs highlight the fact that ABL-kinase mutations are the consequence and the marker of an increased genetic instability phenomenon.

The recent development of a panel of tyrosine kinase inhibitors potentially efficient on all reported mutations (including the T315I substitution with ponatinib) could thus underestimate the importance of BCR-ABL point mutations in drug resistance. However, it must be emphasized that these mutations can occur at the stem cell level and that these cells are intrinsically refractory to all known ATP-competitive tyrosine kinase inhibitors. Consequently, BCR-ABL point mutations remain undoubtedly relevant in the context of resistance toward tyrosine kinase inhibitors.

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# Part VI

# Transplantation

# Transplantation of Stem/Progenitor Cells: Potential Treatment for Erectile Dysfunction Following Radical Prostatectomy

**30**

Muammer Kendirci

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**Abstract**

As the maintenance of a satisfactory quality-of-life is recognized as the principle concern for men and sexual dysfunction is an independent determinant of a worse general health-related quality-of-life, practicing urologists have focused on the understanding of the pathophysiology of erectile dysfunction (ED) following radical prostatectomy (RP) with the concepts of instituting prophylactic measures for prevention and early recovery from ED. ED following RP is often the result of inadvertent injury to the cavernous nerves that course along the prostate capsule and innervates the corpora cavernosa of the penis. The introduction of the nerve-sparing procedures to preserve erectile function has been embraced globally and has made for greater acceptance. However, as unassisted nerve regeneration is a slow process, denervation-induced damage, including cavernosal fibrosis and cavernosal smooth muscle apoptosis frequently develops following RP. Pharmacological penile rehabilitation postoperatively with oral or intracavernosal vasoactive drugs is theorized to be of benefit in most patients, allowing for faster and more complete recovery of erectile function. The current belief is that penile rehabilitation programs with available measures maintains erectile tissue integrity and prevents corporal smooth muscle atrophy and collagen production. Studies on neuroprotection and neuroregeneration will help to preserve erectile function following RP.

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Emerging evidences from preclinical studies using mesenchymal stem cells (MSCs) have been providing significant data for the future. Although MSCs were originally believed to provide for tissue regeneration through engraftment and long-term survival in injured tissues via their presumed plasticity, accumulating findings suggest a plethora of additional mechanisms, including paracrine mechanisms. In spite of the fact that MSC therapy has shown efficacy in animal models of cavernous nerve injury that reflects the nerve injury following RP and other pelvic surgeries, further research is warranted to overcome a number of translational issues on the path towards clinical application of MSCs for the treatment of post-prostatectomy ED.

## Introduction

Prostate cancer is the most commonly diagnosed and treated solid malignancy of male. It has a significant impact on men's health, with 217,730 new cases diagnosed each year, and 32,050 annual deaths attributed to this disease (Jemal et al. 2010). The lifetime probability of developing prostate cancer is estimated to be 20% (Bianco et al. 2005). Although several treatment options available for prostate cancer including hormonal ablation, external beam radiation, cryotherapy and brachytherapy, radical prostatectomy (RP) is the recommended treatment for most cases of organ-confined disease. Cancer-specific survival after RP has been reported as 95% at 15years (Bianco et al. 2005).

As the maintenance of quality-of-life is the principle concern for about half of patients who select RP for the treatment of prostate cancer, an increasing focus on understanding the pathophysiology of post-prostatectomy ED and the idea of instituting prophylactic measures for prevention and early recovery from ED. Furthermore, sexual dysfunction has been reported to be an independent determinant of a poorer general health-related quality-of-life at 2years after primary treatment for prostate cancer (Penson et al. 2003).

The incidence of ED in men following RP has been reported to range from 16 to 82% depending on a number of factors including cancer stage and grade of prostate cancer, patient age, comorbidities, preoperative erectile function and degree of cavernous nerve preservation during RP (Kendirci and Hellstrom 2004; Kendirci et al. 2006a). The *Prostate Cancer Outcomes Study* reported that only 22% of men who had undergone RP could achieve sufficient erection for sexual intercourse without medical aid at 24 months following the procedure (Stanford et al. 2000). These patients may also exhibit other sexual dysfunctions, such as ejaculatory failure, shortening of the penis, fibrotic changes in the penis, and orgasmic disorders.

Despite the refining in the techniques of RP in time, there is no guarantee that erectile function will be preserved. Better understanding on cavernous nerve anatomy and accessory pudendal arteries and evolving technologies such as magnifying surgical area and nerve stimulations during RP have contributed to preserving the nerves and arteries that could results in better potency rates following the procedure.

## Mechanisms of Erectile Dysfunction in Post-radical Prostatectomy

RP causes ED by damaging the neurovascular mechanisms that initiate the erectile response. After bilateral nerve-sparing RP, erectile function is impaired in the early postoperative period because of "neuropraxia," a relative trauma invoked during surgical dissection of the neurovascular bundles. The mechanism of cavernous nerve-fiber-injury involves in part Wallerian degeneration with a loss of normal nerve tissue connections to the corpora cavernosa and associated neuroregulatory functions, both causing cavernosal tissue degeneration and atrophy (Kury et al. 2001; User et al. 2003).

Temporary bilateral cavernous nerve injury may induce significant apoptosis of smooth muscle cells of the corpora cavernosa, particularly in the subtunical area, which in turn hypothetically leads to cavernosal insufficiency and veno-occlusive

dysfunction (User et al. 2003). Hence, chronic hypoxia and denervation initiate apoptosis, and increase the deposition of connective tissue (Klein et al. 1997). Numerous investigations support the concept that post-prostatectomy ED diminishes the circulation of arterial blood through the corpora cavernosa, which leads to reduced oxygen content, increased production of TGF-β, and accelerated corporeal fibrosis (Mulhall et al. 2002).

Human corpora cavernosal biopsies revealed that trabecular elastic and smooth muscle fibers were diminished, and collagen content was significantly increased at 2 months following RP compared with preoperative biopsies. These changes contributed to further deterioration of cavernosal histology compared to early postoperative biopsies at 12 months, supporting the progressive cavernosal fibrosis that occurs in humans following RP (Iacono et al. 2005).

Molecular consequences of RP are also theorized to cause a reduction in penile dimensions. A prospective study measuring preoperative and postoperative penile lengths in 124 men with prostate cancer reported a significant decrease in penile size after RP (Savoie et al. 2003). These animal and human data support measures either to preserve erectile function preoperatively or early postoperative rehabilitation in order to recover erectile function faster.

## Postoperative Rehabilitation of Erectile Function

Due to the type, such as thermal damage, ischemic injury, mechanically induced nerve stretching, and the local inflammatory effects of surgical trauma, and the degree of cavernous nerve injury, the exact recovery time for return of erectile function after the procedure is unpredictable and maximal erectile recovery is not witnessed until a mean period of 18 months following surgery (Walsh 2000). In effect, the cavernous nerves may be functionally inactive for as long as 2 years after surgery, even if the nerve-sparing technique is correctly executed. These observations encourage clinicians to employ penile rehabilitation for early recovery of erectile function.

The clinical strategy of postoperative penile rehabilitation after RP arose from the concept that induced early sexual stimulation and augmented blood flow oxygenating erectile tissue would facilitate the return of natural erectile function and resumption of medically unassisted sexual activity (Kendirci et al. 2006a). This oxygenation has been shown to lead to an increase in the production of intracavernous prostanoids, which are thought to be protective for cavernosal smooth muscle. Schwartz et al. (2004) demonstrated the protective effects of sildenafil on intracorporeal smooth muscle. The authors documented preserved cavernosal smooth muscle content with sildenafil. Other possible beneficial effects of type 5 phosphodiesterase (PDE5) inhibitors in penile rehabilitation following RP are the promotion of neurogenesis, the protection of cavernosal endothelium, and antifibrinogenetic activity. An additional psychological benefit is that penile rehabilitation soon after RP may allow for the resumption of sexual activity and improve quality-of-life for the couple.

Although there are yet to be a consensus regarding the implementation of penile rehabilitation programs; its initiation time, the frequency of application, the type of vasoactive agents, and the dose regimen to be used, some recent studies have reported a number of approaches by success. After first report on intracavernosal alprostadil injections to improve the recovery of spontaneous erections, several authors employed erectogenic rehabilitation programs including oral PDE5 inhibitors, vacuum erection devices and intracavernosal vasoactive agent injections (Kendirci and Hellstrom 2004; Kendirci et al. 2005b, 2006a).

In a recent prospective study, Mulhall et al. (2005) evaluated the use of an erectogenic pharmacotherapy regimen following RP whether it improves recovery of spontaneous erectile function. These authors reported improvements in a number of outcomes including the proportion of men who had recovery of spontaneous functional erections, the ability to respond sildenafil, the time course to respond sildenafil, the ability to respond to intracavernous injections, the percentage of men who had normalization of the erectile function domain scores, the number of men left

with severe ED post-RP, and the dose of medication required to obtain a penetration rigidity erection.

Based on few available findings, either intracavernous injections or a vacuum erection device can be recommended as a first-line option for the first few months postoperatively, as their mechanism of action does not require intact neural transmission. Thereafter, conversion to PDE inhibitor therapy may be a reasonable option for those patients who can achieve at least partial tumescence (Kendirci et al. 2006a).

## Treatment of Erectile Dysfunction After Radical Prostatectomy

Active surveillance and motivation of subjects for ED following RP is as important as early penile rehabilitation. Besides their use in penile rehabilitation protocols, PDE5 inhibitors, vacuum erection devices, and intracavernous/intraurethral application of vasoactive agents can be used to treat patients with ED after RP. Oral PDE5 inhibitors are recognized as the first-line treatment options in post-prostatectomy ED patients. The success rates vary depending on patient age, preoperative erectile function, comorbidities and degree of cavernous nerve preservation. Early postoperative prophylactic administration of alprostadil injections appears to improve subsequent responses to oral sildenafil, with lower doses of the drug being necessary.

Patients who do not respond to PDE5 inhibitors or are contraindicated to have them prescribed are candidates for the second-line approaches of intraurethral or intracavernosal administration of vasoactive agents. Despite low compliance by patients, vasoactive agent applications provide significant success rates. Vacuum erection devices, provide a safe, cost-effective, non-invasive, nonmedical alternative to intracavernosal injection therapy in the post-prostatectomy ED patient with varied satisfaction rates. A combination therapy of an oral PDE5 inhibitor and abovementioned tools can be performed when one approach is failed (Kendirci et al. 2006c). The placement of a penile prosthesis provides a potentially definitive permanent therapy for ED following RP and it is generally reserved for those who fail to respond to the less-invasive pharmacologic therapies or vacuum erection devices.

## Basic Science Studies on Cavernous Nerve Injury-Induced Erectile Dysfunction

In addition to the clinical penile rehabilitation attempts, several studies employing methods, which provide neuroprotection or stimulation of neurogenesis, that have documented restoration of cavernous nerve function after RP. In cavernous nerve injury models, a number of neurotrophic factors (such as, nerve growth factor, acidic- and basic-fibroblast growth factor) and several potential neurotrophic effectors (including neurtrin, immunophilins, growth hormone, sonic hedgehog protein, vascular endothelial growth factor, brain-derivated neurotrophic factor, insulin-like growth factor-1, and prostaglandin $E_1$) in the corpora cavernosa have been investigated either alone or in combination with nerve grafting and demonstrated improvement in erectile function after cavernous nerve injury (Kendirci and Hellstrom 2004; Kendirci et al. 2005b). Gene therapies with various viral vectors, tissue reconstruction, and tissue engineering have recently been used to deliver neurotrophic factors (Kendirci et al. 2005a, 2006d). Immunophilin ligands, PARP inhibitors and erytropoetin are studied in nerve injury models in animals in order to either preserve nerve degeneration or neuronal regeneration with promising results (Allaf et al. 2005; Kendirci et al. 2005b; Sezen et al. 2009). Another attempt by May et al. (2004) demonstrated that Schwan cell-seeded tube repair restored erectile function after cavernous nerve injury.

## Stem/Progenitor Cells in Erectile Dysfunction

There have been a small number of studies on use of stem cells in ED, including diabetes-related ED, age-associated ED and cavernous-nerve-injury-induced ED in animal models. Song et al. (2007) transplanted immortalized human

fetal MSCs into the healthy corpora cavernosa of the penis of male rats under immunosupression, and evaluated whether these cells were able to differentiate into endothelial and smooth muscle cells. However, these authors concluded that immortalized cells from human fetuses do not replicate easily in applications that will be used in future clinical practice. Bivalacqua et al. (2007) investigated the feasibility of injecting MSCs alone or eNOS-modified MSCs for the treatment of age-associated ED. They found improvements in erectile function at evaluations of 7 and 21days after injection of eNOS-modified MSCs as evidenced by cavernous nerve electrical stimulations *in vivo*. They also reported that these improvements in erectile functions were associated with increased eNOS protein, NOS activity, and cGMP levels in the penis. These authors concluded that injected MSCs would be candidate in ED due diabetes. In a similar aged-associated ED model, Abdel Aziz et al. (2010) reported on the effects of intracavernous injection of MSCs for ED. They confirmed previous findings regarding recovery of erectile function both in short-term (3–4 weeks) and long-term (3–4 months) follow-ups. Although they found increased levels of cGMP and alterations in the extracellular matrix leading to markedly dilated sinusoidal vascular bed, they were unable to show the morphological changes of transplanted cells and co-localization with smooth muscle or endothelial markers.

Diabetes is known to be a major risk factor for ED. Diabetic ED men are the difficult-to-treat ED population due to its multifactorial origin and severity of ED. Garcia et al. (2010) evaluated the effects of MSC therapy on erectile function in type II diabetic rats. They used intracavernous injection of autologous undifferentiated adipose-derived stem cells (ADSCs) and found improvements in intracavernous pressure in response to cavernous nerve stimulation in transplanted animals compared to diabetic-controls measured at 21 days after injection. Although there was no significant engraftment of stem cells, they reported significant increase in nNOS in the penile dorsal nerve and in the number of endothelial cells in the corpora cavernosa of the treated animals.

Kendirci et al. (2006b) studied the feasibility of treatment with *ex vivo* expanded MSCs genetically-modified with eNOS on improving erectile function in an animal model of streptozotocin-induced diabetes. After isolating bone marrow from rat and expanding *ex-vivo*, they transduced mesenchymla stem cells (MSCs) with genes coding for lacZ (for tracking purposes) and eNOS, and transplanted these cells into diabetic rat corpora cavernosa. They observed significantly increased levels of eNOS protein and activity from cultured cells. Two days after intracavernous injection, lacZ-positive cells were detected in the corpus cavernosum. In diabetic animals injected with eNOS transfected MSCs, cavernous nerve electrostimulation caused intracavernous pressure to be significantly higher than in either diabetic controls or in animals injected with lacZ-transfected MSCs. The authors demonstrated that intracavernous transfer of MSCs genetically enhanced to express eNOS improved diminished erectile activities in type I diabetic rats.

## Stem/Progenitor Cells in Cavernous Nerve Injury-Induced Erectile Dysfunction

As any type of surgery for prostate cancer is associated with nerve-induced ED, regardless of the degree of cavernous nerve preservation, there have been a number of animal models to mimic this prostatectomy-induced ED, including nerve resection, nerve freezing and nerve crush. Among these animal models, nerve-crush injury seems to be the best model reflecting post-prostatectomy ED. Due to the nature of the injury during RP, preclinical studies have focused on the nerve-crush injury model for studying surgery-induced ED.

The first attempt in restoring erectile function following cavernous nerve injury using stem cells was performed by Bochinski et al. (2004) who transplanted neural embryonic stem cells either into the corpus cavernosum or adjacent to the major pelvic ganglia, the injury site. They found significant improvements in erectile function in response to cavernous nerve stimulation. They also observed neurofilament and nNOS staining

resulted in increased neuroregeneration or nerve preservation compared in stem cell transplanted animals compared with injured controls. Similar to previous findings, they were unable to find any direct evidence of engrafted stem cells in the harvested tissues. They concluded that transplanted stem cells do not require prolonged presence in tissue to exert function, but their mechanism of action might have occurred through growth factor secretion. Even though this study did not employ MSCs, these findings provide a relevant clue on the method stem cells or transplanted cells in general heal injured tissues.

Recently, Fall et al. (2009) tested whether intracavernous transplantation of adult bone marrow cells could improve erectile function following bilateral cavernous nerve injury. These authors performed cavernous nerve resection for the model, and found corporeal denervation, diffuse apoptosis of smooth muscle and endothelial cells in erectile tissues. After transplantation of stem cells into the penis, they observed decreased apoptotic cell number, normalization of nNOS and eNOS levels, and partially restored erectile responses 5 weeks after injections. It is not known whether improvements in erectile function was due to stem cell engraftment or paracrine mechanisms induced by stem cells, as shown by increased NOS levels.

In another recent study by Albersen et al. (2010), the applicability of intracavernosal ADSCs injection was tested for ED following crush injury of the cavernous nerves, as a model for neuropraxia. Interestingly, they observed, based on absence of incorporation for transplanted cells, beneficial effects ADSC lysate on erectile function and partially restored smooth muscle content in the penis, decreased corpora cavernosal fibrosis, and importantly, restored nNOS expression in the dorsal penile nerves. However, it was not elucidated whether the observed effects were a result of local or systemic distribution, and whether retrograde axonal transport of lysate molecules may have played a role in conferring benefit. The lack of engraftment provided evidence for paracrine interactions between stem cells and host tissue. In order to answer this issue, they further examined in culture studies. Results from the experiments indicated that stem cells might secrete substances that induce nerve regeneration and axonal sprouting.

Recently, Kendirci et al. (2010) isolated bone marrow MSCs from transgenic GFP-rats, and selected a subpopulation for p75 low affinity nerve growth factor receptor (p75NGFR). These cells were injected into the corpus cavernosum immediately after bilateral CNI (crush), followed after 4 weeks by intracavernous pressure measurement in response to cavernous nerve electrostimulation. Intracavernous injection of p75NGFR-MSCs resulted in a significantly higher intracavernous pressure measurements compared to all other groups except the sham-operated group. Rats injected with typical unselected MSCs had partial erectile function rescue compared with animals that received p75NGFR-MSCs. Fibroblast (cell control) and phosphate buffered saline (vehicle control) injection did not improve erectile function. Rare surviving engrafted MSCs and p75NGFR-MSCs had a fibroblastic rather than a neuronal morphology. No GFP positive cells were detected in the penile tissue of rats receiving fibroblasts. The rare long-term engraftment of MSCs and p75NGFR-MSCs, and their morphology *in vivo* indicated that the beneficial effects of stem/progenitor cells administration were not likely the result of cell replacement by engraftment and differentiation, e.g. as neurons. In studies with cultured cells, the authors examined the secretion of bFGF, NGF, BDNF, VEGF and IGF-1 by p75NGFR-MSCs, growth factors previously reported associations with ED in rats with cavernous nerve injury. Of all secreted proteins, bFGF was the growth factor secreted most by p75NGFR-MSCs compared with the other cell types. This growth factor is a promising candidate to further investigations, since it is well-known to provide neuroprotection in the central and peripheral nervous systems and bFGF was identified as a principal neurotrophic factor in the penis.

## Future Directions and Translational Issues

Besides advancements in our knowledge pertaining to the modes of action of injected MSCs, there

are a number of translational issues that need to be elucidated regarding the potential clinical use of cell-based therapies in ED following RP: Firstly; what type of stem cells would be the most appropriate candidate for the purpose of preserving and/or restoring erectile function? Although the answers are yet to be answered, both MSCs and ADSCs seem to be convenient based on accumulating animal data. Furthermore, these cell types can be used in an autologous fashion and are not exposed to the ethical repercussions embryonic stem cells poses. Several other factors to determine the type of stem cells used include cost, ethical issues, ease of isolation and culturing, risks, effectiveness and abundance from the source.

Secondly; it is currently unknown for what period stem cells could survive in the target tissue after implantation or injection. Current data support that there is a time-dependent decline of stem cells after implantation into the target tissues (Fandel et al. 2011). Recent studies using quantitative analysis demonstrate that injected stem cells could survive for a couple of days after implantation, and disappear soon after (Huang et al. 2010; Fandel et al. 2011). This is not only the case in ED models but has also with other disease models, such as myocardial infarction. If disappearance of cells follows implantation, then incorporation and differentiation of stem cells may not be the only mode of action that could restores erectile function. Recently, researchers have postulated that paracrine mechanisms are involved in restoration of erectile physiology (Albersen et al. 2010; Kendirci et al. 2010). A number of growth factors secreted from the implanted stem cells could be responsible for restoration of erectile function following cavernous nerve injury. Furthermore, injected stem cells targeting the injured tissues were recruited towards the major pelvic ganglia following cavernous nerve injury. Why stem cells target certain tissues following injury, and whether stem cells mobilize and home under steady-state conditions remains a topic of debate. Elucidation of the mode of action of MSCs is a major hurdle that needs to be delineated before advances and clinical application.

Thirdly; unwanted side effects of stem cell transplantation in this specific setting need to be defined. Specifically, tumorogenicity and differentiation of injected cellular preparations in unwanted directions. Lin et al. (2010) proposed that ADSCs might influence growth of prostate tumor cells in a nude mouse model. This issue needs to be further elucidated before we can apply stem cell therapy in men who may have residual tumor cells at section margins or in circulation. Another issue is where these cells end up after disappearing from the major pelvic ganglia, to determine what changes take place in these tissues.

Last but not least, stem cell use in humans is lacking. To the best of our knowledge, the only study that has used stem cells to treat ED in a clinical setting is by Bahk et al. (2010) from Korea. These researchers implanted umbilical cord stem cell into the penis of seven men with diabetes-related ED and reported that six regained morning erections within 6 months. However, despite increased penile rigidity, none of the men were able to achieve vaginal penetration unless aided by PDE5 inhibitors before sexual intercourse. In post-prostatectomy patients, no clinical trials to date have been initiated.

In conclusion, as the maintenance of a satisfactory quality-of-life is recognized as the principle concern for men and sexual dysfunction is an independent determinant of a worse general health-related quality-of-life, practicing urologists have focused on the understanding of the pathophysiology of ED following RP with the concepts of instituting prophylactic measures for prevention and early recovery from ED. The introduction of the nerve-sparing procedures to preserve erectile function has been embraced globally and has made for greater acceptance. Additionally, pharmacological penile rehabilitation postoperatively with oral or intracavernosal vasoactive drugs is theorized to be of benefit in most patients, allowing for faster and more complete recovery of erectile function. The current belief is that penile rehabilitation programs with available medications, including oral PDE5 inhibitors, intraurethral and intracavernosal vasoactive agents, and VED, maintains erectile tissue integrity and prevents corporal smooth muscle atrophy and collagen production. Studies on

neuroprotection and neuroregeneration will help to preserve erectile function following RP.

Emerging evidences from *in vitro* and *in vivo* preclinical animals studies using MSCs have been providing significant data for the future. Although MSCs were originally believed to provide for tissue regeneration through engraftment and long-term survival in injured tissues via their presumed plasticity, accumulating findings suggest a plethora of additional mechanisms, including paracrine mechanisms. In spite of the fact that MSC therapy has shown efficacy in animal models of cavernous nerve injury that reflects the nerve injury following RP and other pelvic surgeries, further research is warranted to overcome a number of translational hurdles on the path towards clinical application of MSCs for the treatment of post-prostatectomy ED.

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# Collection of Peripheral Blood Stem Cells from Healthy Family and Unrelated Donors for Haematopoietic Stem Cell Transplantation

31

Isabel Leal Barbosa

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**Abstract**

Mobilized Peripheral Blood Stem Cells (PBSC) are increasingly used in allogeneic stem cell transplantation (SCT), due to their relative easiness to be collected compared with those from bone marrow (BM). PBSC are mobilized from BM to the peripheral blood (PB) with growth factors, usually Granulocyte Colony-Stimulating Factor (G-CSF), and because by using this method, higher stem cell (SC) doses, also known as CD34+ cells are collected, this is reflected in faster engraftment (Brissot E, Chevallier P, Guillaume T, Delaunay J, Ayari S, Dubruille V, Gouill SL, Mahe B, Gastinne T, Blin N, Saulquin B, Flandrois G, Devys A, Stocco V, Cesbron A, Dehaut F, Moreau P, Harousseau J-L, Mohty M, Bone Marrow Transplant 44:613–615, 2009). The aims of this review are: to assess the efficacy of PBSC mobilization in family and unrelated donors, to evaluate the existence of predictive factors for collection, to review the safety and efficacy of collection procedures, to analyse the adverse effects throughout the various phases of these procedures, to follow up donors, and to discuss second donations.

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**Fig. 31.1** World location of unrelated donor registries (Petersdorf 2010)

## Historical Need of Stem Cells for Haematopoietic Transplantation and Donor Registries

After the success of the first human BM transplant, performed with a sibling donor more than 40 years ago, the potential use of haematopoietic stem cell from unrelated donors begun to be explored to increase the donor pool (Petersdorf 2010; van Rood and Oudshoorn 2008). Enthusiasm for the recruitment was sparked by Shirley Nolan in the United Kingdom, in her search to find a donor for her son (born in 1971), who suffered from Wiskott-Aldrich syndrome. He unfortunately died in 1979, without finding a suitable donor to perform a transplant. Shirley Nolan created the first registry of unrelated donors in 1974, the Anthony Nolan Register, to promote access to Human Leucocyte Antigens (HLA) matching donors worldwide (Cleaver 1993).

Registries of unrelated stem cell donors began to appear, namely the Europdonor Foundation, launched by Jon van Rood in Leiden, Netherlands. In 1988 this organization became known as the Bone Marrow Donors Worldwide (BMDW) and continued the effort to collect HLA phenotypes and other relevant data from volunteer stem cell donors, and is responsible for the coordination of their worldwide distribution (Oudshoorn et al. 1994). Participants include 66 stem cell donor registries from 47 countries, and at the end of 2011 the number of donors in the BMDW database is almost reaching 19 million. There are currently 785 users from 486 organisations authorized to access the online BMDW services (//www.bmdw.org).

In 1994, the World Marrow Donor Association (WMDA) formalised the international activity to promote the exchange of donors across international borders (Petersdorf 2010). The National Marrow Donor Program (NMDP) is a non-profit organization of volunteer haematopoietic cell donors, founded in 1986 and based in Minneapolis, Minnesota, USA (Bolan et al. 2008). Now called be the Match Registry, it has grown to more than nine million donors, the largest and most racially and ethnically diverse registry of its kind in the world (Karanes et al. 2008; Johansen et al. 2008).

The NMDP cooperates with BMDW and world registries. Most national registries, including the Match registry, have access to these worldwide volunteer donors, either through the BMDW or through individually arranged agreements. More than 50% of the transplants arranged by the NMDP involve either a foreign patient or a foreign donor. The WMDA's original goal was to "facilitate efficient, timely, and reliable transfer of marrow and other forms of haematopoietic stem cells collected for transplantation of patients resident in other countries" (Gahrton et al. 2003), and it is still a major aim for all registries worldwide (Fig. 31.1).

## Selection of Family and Unrelated Donors

Issues related to children and adolescent family donations will not be discussed in this chapter, as they represent special situations that have to be analysed and resolved with medical and family consent. Initially only transplants between relatives, using BM cells, were performed. In the last 20 years the source of cells for transplantation rapidly changed from BM to mobilised PBSC. One of the first growth factors to be used for this cell mobilization was G-CSF (Anderlini and Champlin 2008).

The use of PBSC has outgrown the use of BM cells, although there are specific pathologies in which it is still preferred to use BM. In the early 1990s, it became clear that with various cytokines it was possible to mobilize SC from BM to the PB, and these cells became known as PBSC (Holig et al. 2009).

Several associations namely, WMDA and The Joint Accreditation Committee-ISCT (Europe) & EBMT (JACIE), were implemented, so as to establish criteria for cell donations. JACIE was founded by the European Group for Blood and Marrow Transplantation (EBMT) and the International Society for Cellular Therapy (ISCT) (Hurley et al. 2010). These are two leading scientific organizations involved with HSC transplantation in Europe. Their primary aim is to promote high quality patient care and laboratory performance in haematopoietic stem cell collection, processing, storage, and transplantation centres, through an internationally recognized system of accreditation.

## Donor Registry and Selection Steps

With time the need for allogeneic transplants has been increasing rapidly. However, the numbers of patients without available related donors rise very quickly in the last two decades, due to a higher number of families with few children. The selection and management of unrelated donors and family donors have some overlapping aspects (van Walraven et al. 2010). During these phases of donor recruitment and selection, information must be given to the donor regarding all the phases leading to the graft donation. Unrelated donors must, when registering, sign a 1st informed consent. When activation of a registered donor is requested by a transplant centre, a 2nd informed consent is signed in accordance with the Helsinki declaration. Cooperation and good coordination between donor registry (and the collection centre) and the transplant centre are essential in all phases leading up to donation (Foeken et al. 2010).

Before mobilization, all donors have a medical check up to assess medical suitability, transplantation-transmissible infectious diseases, and contraindications (e.g., pregnancy, autoimmune disease, history of thromboembolic disease) for G-CSF administration (Pulsipher et al. 2009). Donors should be informed of the possibility of a second donation either because of failure of the graft or because the recipient is involved in a program that might demand an additional donor collection such as donor lymphocytes (van Walraven et al. 2010).

## Mobilization

G-CSF is a standard stimulation cytokine and appears to be well tolerated without known long-term effects. The short-term safety profile of G-CSF is well known and the dose of 7.5–10 μg/kg body weight (b.w.) given subcutaneously, is widely accepted as a mobilization regimen (Anderlini and Champlin 2008). The kinetics of white blood cells (WBC) and SC under G-CSF treatment is quite uniform, although a remarkable variability sometimes occurs amongst individuals, usually with peak of CD34+ cells on the 5th day of mobilization (Lysák et al. 2011).

The predominant adverse effects that occur during mobilization, as described by several groups, include bone pain, headaches, flu like symptoms, fatigue, and fever. Some donors have to stop their daily routines by day +4 of G-CSF administration due to some of these symptoms (Pulsipher et al. 2009). Thus, to reduce adverse

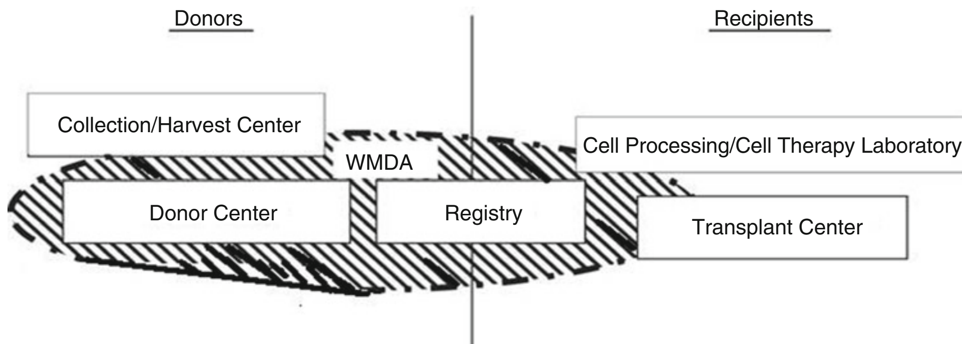


**Fig. 31.2** Collaborative effort between donor registry/collection centre and transplantation centre (Adapted from Hurley et al. 2010)

effects, the mobilization should be done with the lowest dose of growth factor possible (Miller et al. 2008). Although there may arise concerns involving the use of G-CSF in healthy donors, it is important to recognise that G-CSF is a natural occurring cytokine whose levels are known to increase under physiologic status and primary infection.

## Collection

There has to be very concerted actions between donor registry/collection centre and transplantation centre to make the arrival of the graft possible and in due time to perform the transplant within 3 days of stem cell collection (Fig. 31.2).

Collection of allogeneic haematopoietic grafts from family and unrelated donors has become a standard procedure for the many donor registries working worldwide. Some of the issues raised by these donations have been the safety of donors and their follow-up (Lysák et al. 2005).

### Monitoring of PBSC Donors

Complete blood counts and differentials are performed at the initial consultation (2–4 weeks before donation), and then before and after each aphaeresis. During and immediately after the aphaeresis, vital signs and adverse events are documented by the medical staff (Brissot et al. 2009).

### Aphaeresis

The first aphaeresis is usually performed on day 5 of mobilization. The major aim of these procedures is to obtain a sufficient number of CD34+ cells for transplant, with the minimum number of aphaeresis. If the required number of $CD34^+$ cells per recipient body weight is not collected, G-CSF administration is repeated, and a second PBSC collection is performed on day 6 (Hölig et al. 2009). PBSCs are collected by a continuous-flow blood cell separator (Cobe Spectra; Caridian BCT) via bilateral peripheral venous that is removed after completing the procedure. The processed blood volume ranges between 2 and 3 times the donor's total blood volume (TBV) and it takes between 2 and 3 h to complete (Holig et al. 2009).

### Efficacy of PBSC Mobilization: Donor Age and Gender

The success of this procedure is dependent on various factors. There are conflicting reports regarding the influence of gender, age, weight, and other biological parameters in the outcome of CD34+ cell collection. Regardless of weight or other parameters, younger donors present a superior PB CD34+ cells count on day 5. Furthermore, while the CD34+ cell number in the PB decreases with age, the PB WBC count is higher in older donors (Lysák et al. 2005). Usually men are better mobilizers than women (Fig. 31.3) (Holig et al. 2009).

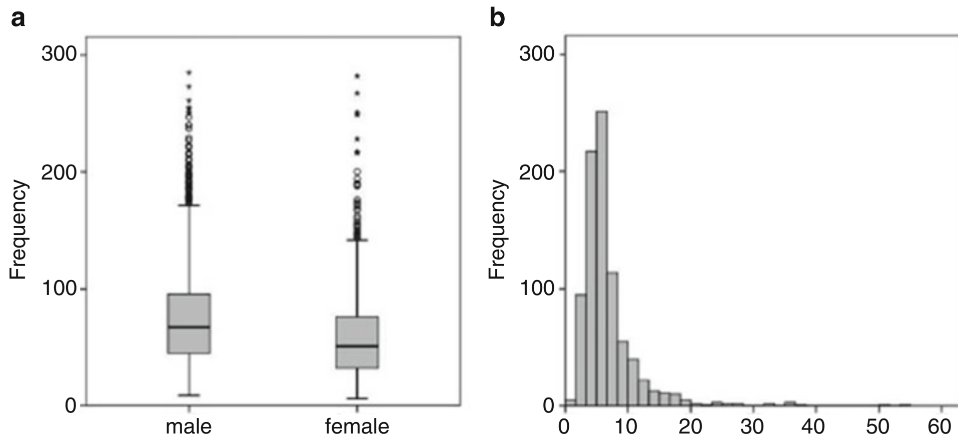


**Fig. 31.3** Efficacy of CD34+ mobilization and PBSC yield (Hölig et al. 2009). (**a**) CD34+ cells in peripheral blood at day 5 of G-CSF application; (**b**) Cumulative CD34+yield per Kg recipient body weight

The increasing age of patients submitted to SCT implies that old siblings are selected for donors. These donors generally mobilize less CD34+ cells and are submitted to a median number of two aphaeresis (Lysak et al. 2011).

In 2005, the boards of several associations namely EBMT, FACT, JACIE, NMDP, and WMDA issued a consensus statement in support of the use of a common coding of haematopoietic progenitor cells and other therapeutic cell products. This led to the establishment of a well structured terminology for haematopoietic progenitor cells, which has been adopted as the formal terminology and makes the exchange of cell products worldwide easy.

## Adverse Events for Donors During Apheresis and in the Immediately Follow Up

During aphaeresis there are usually no severe complications. One of the more frequent adverse effects associated with collections is paraesthesia associated hypocalcaemia. This adverse event is easily controlled with the administration of intravenous calcium.

Several groups have performed post-donation follow-up of donors and have shown drops in platelet counts ($<50\times10^9$/L). To avoid any risks to donors some groups reinfuse autologous platelets separated from the first aphaeresis if the counts fall $<50\times10^9$/L. Amongst some PBSC donors there is a persistent lymphopenia with a normal leukocyte count, since the first control, but they do not show clinical signs. The meaning of these haematological changes must, however, be evaluated.

## Second Donations

Recently, WMDA published a special report guideline for subsequent donations following initial PBSC collection (Confer et al. 2011). Overall, between 5 and 10% of donors are asked to provide a subsequent donation of PBSC or therapeutic cells (TCs); the latter are usually requests for donor lymphocytes. However, novel strategies mean that other products (for example, mesenchymal stem cells) may increasingly be requested (Confer et al. 2011). There are a few reports evaluating the mobilisation and collection of PBSC after two cycles of G-CSF administration. One group reviewed data from the Spanish National Donor Registry (De la Rubia et al. 2002) and another group from the German Bone Marrow Center (Platzbecker et al. 2005) and both reported that the yields of CD34+ cells were significantly lower in the second donation.

There are also some immunological changes especially after second mobilization. One report evaluated T, B, and NK cells in the peripheral blood of nine sibling PBSC donors, 1 year after their first donation (Storek et al. 2000). Their sole finding was a modest lymphocytopenia at 1-year post-donation, of uncertain clinical significance. All other cell populations including naïve CD4+ and CD8+ cells were normal.

In conclusion, there has been a significant evolution in the stem cell transplantation knowledge since the early days, including the discovery of a new source of stem cells, mobilised PBSC. The less invasive nature of this collection in comparison with BM collections has turned this into the main product used to treat haematological malignancies. Treatment of patients older than 65 years of age also became possible due to the possibility of mobilization and collection of PBSC from their sibling with similar age (Lysák et al. 2011).

After concerns regarding donor's health were raised linked supposedly to the administration of G-CSF, several groups have published articles highlighting donor's follow ups that have shown that although some of them seem to have a persistent lymphopenia that needs to be monitored, there is no reason for alarm. On the other hand, the use of cytokines to mobilize the cells from the bone marrow into the blood stream allows the obtention of higher cell numbers and has been linked to faster recoveries in transplanted patients; however, in some cases, finding a matching donor is a problem especially for ethnic minorities or those who do not have a matching sibling/related donor. For that reason International registries play an essential role in allogeneic transplantation by recruiting donors all over the world and keeping records of HLA typing for all of them. By doing so, they created a worldwide database that makes finding donors (for patients that do not have a matching sibling/related donor) easier and also establish an essential connection/bridge between collection centres and transplant centres.

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# 32 Effect of Smoking on the Outcomes of Cancer Patients After Hematopoietic Stem Cell Transplantation

Shawna Ehlers and Carrie Bronars

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**Abstract**

Tobacco use is the leading preventable cause of death in the United States. Recent research in Hematopoietic Stem Cell Transplant (HSCT) populations has begun to examine smoking cigarettes as a risk factor for adverse transplant outcomes. Based on these early studies, estimated prevalence rates vary widely. Lifetime history of smoking among HSCT patients likely approximates that of the general population, while current use appears less than the general population. Smoking has been associated with multiple adverse HSCT outcomes, including shorter duration of disease-free and overall survival; higher treatment related mortality; higher pulmonary infection and respiratory failure rates; higher rates of disease recurrence; increased risk for cardiovascular events; and longer duration of hospitalization. Potential mechanisms are likely many, and include impaired bone marrow hematopoiesis, inflammation, impaired pulmonary function, and also significant associations with other maladaptive health behaviors know to adversely affect general population health outcomes, but understudied in HSCT populations. Future directions for tobacco research in HSCT should focus on use of behavior science methodology to minimize biases common to self-report methodology, and analyzing the effects of both current and lifetime use. Current use is a modifiable risk factor. Cancer diagnosis and HSCT serve as "teachable moments" during which patient

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motivation to quit tobacco use is increased. Survivor care plans serve as a timely platform for integration of evidence-based tobacco dependence treatment into a comprehensive care plan that targets all known significant risk factors to optimize HSCT outcomes.

## Introduction

Tobacco use is the leading preventable cause of death (ACS 2011). It is responsible for one out of five deaths in US (Mokdad et al. 2004; Steenland et al. 2004) and accounts for at least 30% of all cancer deaths (USDHHS 2010). Recent evidence suggests that tobacco use is linked to worse cancer patient outcomes after hematopoietic stem cell transplantation.

## Tobacco and Hematologic Malignancies

A 2004 meta-analysis of smoking and cancer (Levitz et al. 2004) confirmed an etiological link between smoking and increased risk of leukemia, especially among individuals who have acute myeloid leukemia. Hypothesized mechanisms (Cooke and Bitterman 2004; Geyer et al. 2010; Gritz et al. 2008; Sandler et al. 1993; USDHHS 2010) include direct carcinogen impact, activation of other carcinogens through induction of metabolizing enzymes, influence on immunologic functioning (increasing pro-inflammatory cytokines and T-cell function), and impact on the prevalence of the translocation and over expression of bcl-2. (Varadarajan et al. 2010) found decreasing rates in leukemia mortality coincided with declines in population based smoking rates. Smokeless tobacco use has not been studied in relation to hematologic malignancies.

## Prevalence of Tobacco Use in Hematopoientic Stem Cell Transplant Populations

A lifetime history of smoking has been documented in 10–62% of hematopoietic stem cell transplant (HSCT) candidates (Ehlers et al. 2011; Tran et al. 2011), while 0–17% of candidates reported smoking in the year prior to transplant (Chang et al. 2004a, b; Ho et al. 2001; Marks et al. 2009; Miceli et al. 2006; Savani et al. 2005; Sharma et al. 2005; Thompson et al. 2002; Wilczynski et al. 1998). This range of lifetime smoking prevalence rates documented among HSCT candidate studies varies widely, but surrounds the general population prevalence rate. Current use ranges slightly lower than the general population prevalence. The 2009 general population prevalence of smoking is estimated as 19.3 % current use and 57.1% of US adults report smoking more than 100 cigarettes in their lifetime (CDC 2009, 2011).

The prevalence of smoking and smoking relapse in the post-transplant period has been examined by a prospective and a retrospective cohort study, yielding estimates of 0–17%. Chang et al. (2004a) examined smoking prospectively among CML patients undergoing allogeneic HSCT. They documented a 62% lifetime prevalence of smoking with 0% smoking 1 year after transplant. One study has examined smoking prevalence among a retrospective cohort of HSCT survivors, documenting a 17% prevalence 2–22 years post-transplant (Bishop et al. 2010). The similarity to smoking prevalence among HSCT candidates suggests that the prevalence of smoking is stable into the survivorship period. However a cohort bias may be inflating the survivorship estimate, given that current smoking rates have decreased from over 42.4 % in 1965 to 19.3% in 2011 (CDC 2011). Prospective cohort studies are needed to understand the course of smoking across the HSCT care continuum.

## Outcomes Associated with Tobacco Use in HSCT Populations

### Survival

Recent studies have linked smoking to worse survival after HSCT, focused on allogeneic HSCT recipients treated for acute and chronic myelogenous leukemias (Chang et al. 2004a, b; Ehlers et al. 2011; Marks et al. 2009). Marks et al. (2009)

studied sibling allograft recipients treated for CML. They documented that lifetime heavy smoking (>10 pack years, >20 cigarettes per day) was associated with reduced disease free survival and overall survival compared to non-smokers. Five-year survival rates were reduced by 18% (68% among never smokers vs. 50% among high dose smokers). Additionally, treatment related mortality was 50% for heavy smokers versus 28% in non-smokers. The treatment related mortality rate for low-dose smokers was not significantly different (32%). Treatment related mortality for unrelated donor transplants was 68, 50, and 49% for high-dose, low-dose, and never smokers. Similarly, Hoodin et al. (2004) found that lifetime smoking history before transplant was associated with decreased survival rates among a mixed sample of HCST recipients. Savani et al. (2005) also associated a lifetime history of smoking with a fivefold increase in the risk of transplantation-related mortality from pulmonary causes. Ehlers et al. (2011) examined current and lifetime smoking history. They documented that current smoking of any quantity (defined as use during the year prior to transplant) was associated with lower rates of survival post transplant compared to never-smokers, among a HSCT recipients treated for acute leukemias. The effect for lifetime smoking history on survival was not statistically significant in this study of 152 HSCT recipients. Null results are documented by Chang et al. (2004a); smoking behavior was not found to affect 1 year survival rates among patients with CML who underwent HSCT. We could not locate published studies on the impact of tobacco use among other disease-specific HSCT populations. Notably, smoking has been found to negatively impact overall survival rates among Non-Hodgkin's lymphoma patients (Geyer et al. 2010).

## Disease Remission and Recurrence

At least four studies have examined the impact of smoking on recurrence among allogeneic HSCT recipients. Marks et al. (2009) found that smoking 10 or more years (lifetime) was associated with a higher relative risk of CML relapse among allogeneic recipients. Multivariate analysis showed LOWER dose smokers were more at risk for relapse, which the authors attributed to higher mortality rates among high dose smokers. Chang et al. (2004b) also led two studies of recurrence associated with smoking among allogeneic HSCT recipients treated for chronic myelogenous leukemia. She found a 1.7% increased risk of recurrence associated with each lifetime pack-year of smoking after allogeneic HSCT. In a later study, she documented a threefold increased risk of disease recurrence for a person with any lifetime smoking history (Chang et al. 2004b). Tran et al. (2011) documented a higher risk of relapse in a mixed disease sample of allogeneic HSCT recipients related to a twofold increase in lifetime pack-years, however this was not statistically significant in their sample of 845 recipients with 85 smokers. One study reported no difference in disease remission between smokers and non-smokers pre-transplant treated for acute myelogenous leukemia (Chelghoum et al. 2002)

## Transplant Complications

Infection is a common complication of HSCT. One study found that pulmonary infections were associated with smoking during the aplastic period following chemotherapy for acute myeloid leukemia pre-transplant (26 smokers vs. 18% non-smokers), (Chelghoum et al. 2002). Miceli et al. (2006) documented that active pre-transplant smoking was also associated with increased risk of pulmonary infection post-transplant, with 37% of tobacco users versus 27% of non-users experiencing infection (60 days post follow-up). Graft versus host disease is also a common complication of allogeneic HSCT. One study (Marks et al. 2009) found no difference in the incidence of acute and chronic GHVD in past or present smokers compared to non-smokers.

Notably, smoking has a dose-dependent effect on bone loss which increases fracture risk (Ward and Klesges 2001). Bone grafts also have a lower success rate among past and current smokers compared to non-smokers (Spear et al. 1999). The association between smoking and bone health deserves further study among HSCT recipients,

especially those treated for multiple myeloma who commonly experience bone lesions and fractures.

## Comorbidities

A few studies document the pulmonary effects of smoking among HSCT recipients as at least one probable mechanism of increase mortality risk. As above, Savani et al. (2005) associated a lifetime history of smoking with a fivefold increase in the risk of transplantation-related mortality from pulmonary causes. Ehlers et al. (2011) found that pulmonary/respiratory causes of death was higher in previous and current smokers (20, 26%) compared to never smokers (6%). Similarly, Tran et al. (2011) found that each doubling of lifetime pack-years smoked was associated with a 33% increased risk of early respiratory failure, independent of pre-transplant lung function.

Reduced pulmonary function associated with smoking has also been documented among HSCT candidates and recipients pre-transplant. Ehlers et al. (2011) documented reduced FEV1 and DLCO associated with smoking pre-transplant. Post-transplant, smoking has been associated with increased diffuse alveolar damage (Sharma et al. 2005). A study by Ho et al. (2001) resulted in null findings in an examination of severe pulmonary complications limited to the period 60 days post-transplant. Two other studies found null results in examination of a possible association between lifetime smoking and lung function. Notably the prevalence rates of smoking in these samples were much lower than expected based on general population prevalence rates (6.7 and 34%) (Thompson et al. 2002; Wilczynski et al. 1998).

Published examinations of non-pulmonary comorbidities associated with smoking among HSCT recipients are rare, and deserve further exploration as possible contributing mechanisms to explain increased mortality risk. Tichelli et al. (2008) examined the occurrence of cardiovascular events after allogeneic HSCT with a prevalence of 3.6% in the 7 year time period (range from 1 to 13 years). They found that 41% of people who had an arterial event (MI, Chronic coronary heart disease, angina) post-transplant smoked cigarettes currently. Though not studied among HSCT recipients, smoking has also been associated with multiple other comorbidities (Ehlers et al. 2011; Savani et al. 2005; Tran et al. 2011).

## Health Care Utilization and Costs

Given the increased risk of HSCT mortality, pulmonary risk, and general smoking related comorbidities, it is reasonable to hypothesize that higher health care utilization and costs are associated with smoking. One study (Ehlers et al. 2011) examined this question, finding that current smokers averaged 21 more days hospitalization in an outpatient-based HSCT program in the 1 year post transplant compared to never smokers. Though not calculated within their study, they cite national average charges for hospitalization with leukemia as primary diagnosis that translated to average hospitalization charges $126,434 greater for a patient smoking pre-transplant within the first year post-HSCT.

## Potential Mechanisms

Zhou et al. (2011) hypothesized that cigarette smoking may impair bone marrow hematopoiesis and induce inflammation, documenting that marrow from healthy individuals exposed to cigarette smoke extract had significantly diminished CFU-E, BFU-E and CFU-GM. Cigarette smoke extract inhibited the growth of erythroid and granulocyte-macrophage progenitors. Additionally, health behaviors cluster, such that people who smoke are more likely to engage in other maladaptive health behaviors that may additively (Blanchard et al. 2008; Coups and Ostroff 2005) or synergistically affect outcomes.

## Limitations of Studies Cited and Future Directions

The true prevalence of lifetime smoking and smoking prior to hematologic diagnosis among HSCT candidates is likely at least as high as the

general population. Many of the studies cited here report lifetime prevalence rates that are much lower, thus the possibility of biases (e.g., self-report, retrospective recall, selection, abstraction methodology) resulting in under-reporting should be considered and addressed in future studies. Additionally, the range of smoking prevalence post-transplant likely reflects a self-report bias, namely under-reporting related to social stigma and a desire to please clinician teams who provided potentially lifesaving treatment. Underestimated prevalence of smoking likely translates to reduced precision and effect sizes in the above literature, thus under-estimating the true effects of smoking on HSCT outcomes. Additionally, several studies are underpowered to detect differences among smokers (i.e., not enough smokers in these studies to detect differences) (Marks et al. 2009), and follow-up may not be of sufficient duration to detect effects (Thompson et al. 2002).

When possible, standardized self-report queries with normative references should be employed in prospective cohorts. Population norms should be referenced when reporting smoking prevalence rates to estimate possible bias and missing data. Within retrospective cohorts, vague or absent tobacco information should be categorized as missing data. Future studies utilizing biochemical validation of self-report (e.g., cotinine as a metabolite of nicotine) could aid in elucidating the true prevalence of active smoking, but cannot estimate history of smoking. This would help address potential self-report biases, such as fear of reporting tobacco use in the context of HSCT candidacy evaluation or follow-up.

Additionally, current smoking should be differentiated from lifetime smoking, as the former is a modifiable risk factor that can be targeted within patient care. Most current studies focus on lifetime smoking. Current smoking should be liberally defined (e.g., within the year prior to transplant) to account for self-report biases that likely increase surrounding the time of evaluation and treatment. Smokeless forms of tobacco use are understudied at least partially due to lower prevalence rates, but deserve further research attention nonetheless.

## Smoking Cessation Among HSCT and Cancer Patient Populations

Smoking cessation has not been studied among HSCT patients, beyond above documented change in prevalence rates. The work of Gritz et al. (2006) suggests that cancer diagnosis is a teachable moment; the sooner after diagnosis smoking cessation intervention is delivered, the more personally relevant and potentially effective. Observational studies also link cancer diagnosis to increased quit attempts (Cooley et al. 2011; Hsu et al. 2011). Relapse rates among cancer patients vary, but generally range from 13 to 60% 1 year after treatment (Gritz et al. 2006).

## Clinical Practice Guidelines

Evidence based practice guidelines for the general population utilize the "5 As" (Ask, Advise, Assess, Assist, and Arrange) (Fiore et al. 2008). This brief intervention approach focuses on systematically identifying tobacco use, providing professional advice to quit using tobacco, determining motivation/willingness to quit, providing counseling and/or pharmacotherapy, and including as a topic of follow up visits. The guidelines are based on behavioral science, including motivational interviewing principles and problem solving/skills training. The American Cancer Society guidelines support application of this approach to cancer patients (Doyle et al. 2006).

## Limitations in Smoking Cessation Trials

Smoking cessation trials among cancer populations focus primarily on head and neck and lung cancer populations, in which the disease is highly correlated with tobacco use. These intervention studies often utilize small sample sizes and thus may be under powered. Additionally, many of the interventions take a general approach and may not be addressing specific needs of patients.

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# Ex Vivo Expansion of Stem and Progenitor Cells Using Thrombopoietin

33

Zoran Ivanovic and Marija Vlaski

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**Abstract**

Although most researchers predicted that thrombopoietin (TPO) would be a lineage specific regulator, with physiologic effects limited to megakaryocytes and platelets, it became clear that it is also an important growth factor of hematopoietic stem cells. This property of TPO qualified it for ex vivo expansion procedures aimed to amplify committed progenitors and stem cells, or, at least, to maintain the latter. During more than one decade of experimental work and preclinical development, the first efficient expansion procedures appeared enabling an acceleration of hematopoietic reconstitution after transplantation. All successful procedures were based on a cytokine cocktail containing TPO among other cytokines. The action of TPO on stem cells seems to be related to its hypoxia-mimicking properties.

## Thrombopoietin: Much More Than a Lineage-Specific Growth Factor

Long time ago thrombopoietin was suggested to have a role in the regulation of platelet production analogous to that of erythropoietin (EPO) in erythropoiesis. More than three decades of work were necessary to get the purified molecules of animal and human origin (Kaushansky and Drachman 2002). The TPO molecule was named differently according to the actions of this molecule revealed in various systems: megakaryocyte growth and development factor (MGDF), megapoietin,

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c-Mpl ligand. In fact, "c-Mpl ligand" is often used as a synonym for thrombopoietin although this generic term describes a family of ligands which bind to c-Mpl that was found to be the TPO receptor. These ligands include endogenous thrombopoietin, recombinant human thrombopoietin (rHuTPO), pegylated recombinant human MGDF (PEG-rHuMGDF), thrombopoietin peptid mimetics – agonists. The name c-Mpl comes from the fact that this receptor is encoded by a retroviral oncogen that caused a myeloproliferative leukemia (thus "Mpl") in mice (Souyri 1998). The functional regions of thrombopoietin are analogous to those of human growth hormone and erythropoietin and the signal transduction pathways involve JAK, STAT, MAP kinase as well as some other intracellular mediators.

Two recombinant forms of human molecules exhibiting TPO functions exist: (1) rHuTPO which is identical to the native human glycosylated molecule and (2) PEG-rHuMGDF composed of first 163 amino acids of thrombopoietin bound a 20 kDa polyethyleneglycol moiety, non-glycozylated. The common functional unit – erythropoietin-like domain is identical in these two molecules (Kuter and Begley 2002).

It was widely expected that TPO would display a lineage specific action, similar to EPO in erythropoiesis. Indeed, TPO is the most important regulator of platelet production as it was shown both in vitro and in vivo. It prevents apoptosis of megakaryocytes and increases their number, size and ploidy. So, TPO serves to amplify a basal megakaryocyte production mechanism and is crucial for growth of megakaryocyte progenitors (CFU-Mk). An inverse relationship between platelet levels and plasma TPO concentration exists and the platelet production is highly stimulated upon TPO administration to both normal and myelo-suppressed animals. Further research, however, showed that thrombopoietin is much more than a lineage-specific megakaryocyte growth factor (Kaushansky 1997). Rapidly, it became obvious that TPO affected the generation and development of early and late erythroid progenitors both in vitro and in vivo, and was leading to enhanced erythropoietic recovery after myelo-suppressive therapy. Furthermore, the TPO action also extends to the granulocyte monocyte lineage (Kaushansky et al. 1996). As a matter of fact, this action was not at all limited to committed progenitors: TPO acts on cell population enriched in quiescent hematopoietic stem cells (bone marrow of five fluorouracil-treated mice) enhancing the numbers of hematopoietic colonies developed in semi-solid cultures including CFU-GEMM (Ku et al. 1996). This effect was obtained in synergy with either Interleukin – 3 (IL-3) or Stem Cell Factor (SCF). These data were corroborated by the data of the same group demonstrating that addition of TPO to either IL-3 or SCF to CD34+/CD38$^{lo/-}$/c-kit$^{lo}$ cells led to an enhanced production of committed progenitors in suspension culture (Kobayashi et al. 1996). Similarly, in experiments with Lin$^{-}$/Ho$^{lo}$/Rh$^{lo}$ which express c-Mpl whose survival was supported by TPO. In fact, in single cell experiments, TPO alone was able to support the survival of a fraction of these cells and synergized with that respect with IL-3, SCF and Interleukin-6 (IL-6) (Kaushansky 1997). Altogether, these data, based on phenotypically defined cell population enriched in stem cells, implied the role of TPO in stem cell maintenance that will be later fully confirmed (Huang and Cantor 2009). Soon, remarkable data appeared with cord blood cells demonstrating that FLT-3 ligand (FL) synergizes with TPO to maintain, without presence of stromal cells, for more than 6 months, an inexhaustible production of committed hematopoietic progenitors together with a massive expansion of LTC-IC considered to be a sub-population of stem cells (Piacibello et al. 1997). Traditionally, the role of TPO in medicine was figured out in the context of its stimulation of megakaryocytopoiesis and platelet production or a substitutive therapy (non myeolo-ablative treatment, myelo-ablative treatment, myelo-dysplastic syndrome, HIV, liver disease), as well as in stem and progenitor cell mobilization. Since it was confirmed by using functional stem cell assays *in vitro* and *in vivo* (LTC-IC and SRC) that TPO amplifies a stem cell sub-population ex vivo (Piacibello et al. 1997, 1999), new perspectives for TPO use appeared: its role in ex vivo expansion of stem and progenitor cells for cell therapy.

## The Role of Thrombopoietin in Protocols for *Ex Vivo* Expansion of Hematopoietic Grafts

The necessity to amplify committed progenitors and stem cells ex vivo is related to the source and level of primitiveness of hematopoietic cells aimed for transplantation: (1) to overcome a limited number of these cells (cord blood, apheresis product of "bad mobilizers", a limited number of bone marrow punctions); (2) to shorten the period of post-transplant agranulocytosis related to immaturity of progenitor and stem cell sub-populations. Any culture system aimed to expand hematopoietic cells for engraftment results in the production of precursors and mature cells and, in most cases, in the simultaneous amplification of committed progenitors. The first result relies upon the fact that the differentiation of committed progenitors is enhanced in ex vivo cultures, and the second is based upon two simultaneous events: the amplification of committed progenitors by their own division, and by their production from more primitive cells differentiating rapidly in culture and, hence, exhausting themselves.

Due to this premise, TPO was, and still is, extensively studied as a cytokine with major role in cultures designed to produce the pure megakaryocytes and consequently, platelets. This approach tends to solve the problem of prolonged post-transplant thrombocytopenia and hence to decrease the dependence on platelet transfusion (Panuganti et al. 2010). It will not be extensively discussed in this chapter which is focusing on the role of TPO in the engineering of the complete hematopietic grafts. A complete hematopoietic graft should contain both committed progenitors and stem cells to ensure not only rapid but also lifelong hematopoietic reconstitution. This second demand means that the expansion procedure at least should not impair stem cell activity with respect to the native graft. In fact, with respect to the generation of committed progenitors from stem cells it is just opposite to the first one. In order to achieve a good compromise between these two opposite demands, the different cytokine cocktails were studied to improve the efficiency of ex vivo expansion cultures. Since 1997 (Piacibello et al. 1997), TPO is almost regularly used for this purpose. The same authors demonstrated later that the addition of KL and IL-6 to FL and TPO could enhance the prolonged (7 months culture) output of committed progenitors (CFU-GM, CFU-Mk and BUF/CFU-GEMM) (up to 100 million fold) without impairing LTC-IC expansion (up to 270,000-fold) (Piacibello et al. 1998). In their next study, the question of stem cells was addressed by an in vivo approach: engraftment to NOD/Scid mice – Scid Repopulating Cells (SRC) (Piacibello et al. 1999). By limiting dilution analysis, it was shown a 65 and a 78-fold expansion of SRC (after 7–8 and 9–10 weeks, respectively) without impairing the massive expansion of clonogenic progenitors and LTC-IC. It should be stressed that similar culture systems without TPO resulted either in a modest expansion of stem cells (including SRC) or even their decline during the culture.

In order to get a clinically relevant expansion protocol, during a decade TPO (MGDF) was tested in cultures with other cytokines, with different media, with or without serum, with various durations of cultures. These studies were performed on murine bone marrow cells, human bone marrow cells and those mobilized in peripheral blood, as well as on cord blood cells. This research confirmed the important role of TPO for expanded graft quality with respect to both primitive stem cell and committed progenitors content. Although TPO alone enhances cell survival and proliferation of hematopoietic cells in culture the presence of other cytokines is necessary to moderate its pro-differentiation (to megakaryocyte line) action. Thus, for expansion of hematopoietic grafts, TPO is most frequently associated with FL, SCF, IL-6. Some protocols also used are G-CSF, EPO, and IL-3.

From nowadays viewpoint, we could conclude that clinically successful protocols of ex vivo expansion based on cytokine stimulated cultures necessarily contain TPO (MGDF) associated at least with SCF or FL or both. Indeed, the first pre-clinical studies on baboons (Norol et al. 2000) as well as the first clinical study demonstrating statistically significant acceleration of short

term granulocyte recovery (Reiffers et al. 1999) were based on CD34+ cells mobilized in peripheral blood and expansion protocols using TPO (MGDF) with other cytokines. Studies on baboons, performed with cellular product obtained after 6 days of culture supplemented with SCF, FL, TPO, and IL-3 demonstrated a consistent acceleration of hematopoietic reconstitution (but not total abrogation of post-transplantation neutropenia). The clinical trial of Reiffers et al. (1999), based on a 10 days culture supplemented with SCF, GCSF and MGDF, however, demonstrated an abrogation of post-myeloablative chemotherapy neutropenia. In this first cohort, the expanded cells were engrafted together with non-expanded cells, but similar results were obtained when only expanded cells were injected to patients (Boiron et al. 2006). The same technical approach was used for expansion and consequent autologous transplantation of CD34+ cells of breast cancer patients (11 patients transplanted with expansion product plus non-manipulated cells) to obtain a positive effect on the duration of post-transplantation neutropenia (McNiece et al. 2000). These results were confirmed by an Australian study which design was more complex. Nevertheless, using the same approach to expand the cells, the authors demonstrated an acceleration of hematopoietic reconstitution with ex vivo amplified cells as well as a reduction of the incidence of febrile neutropenia (Prince et al. 2004). It should be stressed that no clinical trial, based on cytokine-stimulated expansion of hematopoietic cells in liquid cultures demonstrated a statistically significant acceleration of hematopoietic reconstitution before including TPO (MGDF) in cytokine cocktail.

## Thrombopoietin, "Oxygen Issue" and Clinical-Scale Expansion Protocols

A quarter of century ago, Dello Sbarba concluded that stem cells relied on an anaerobic metabolism (Dello Sbarba et al. 1987) and a decade ago, we suggested for the first time that the hematopoietic stem cell renewal was related to hypoxia (Ivanovic et al. 2000). In the meantime, a large series of papers appeared, clearly demonstrating that stemness is really related to anaerobiosis, whatever stem cell model studied: adult stem cells, embryonary stem cells, induced pluripotent stem cells (Ivanovic 2009; Varum et al. 2011). Furthermore, the pro-differentiation effect of a hyperoxic environment (considering here the ambient air-O2 concentration (20–21%) which is de facto hyperoxia with respect to tissue O2 concentration which are much lower), could be explained by our concept of "oxygen stem cell paradigm" through the evolutionary prism (Ivanovic 2009). This concept considers the self-renewal as a simple proliferation without differentiation which is a result of expression of ancestral primitive genome while the other genes, encoding the extensions and functions accumulated during the evolution, were locked. This way, the self-renewal looks like the simple proliferation of primitive anaerobic eukaryots while the differentiation assumes unlocking of more or less O2 dependant i.e. energy demanding functions. So, to preserve stem cells functions in course of an ex vivo expansion, the pro-differentiation influence of hyper-oxygenation should be avoided. As a matter of fact, the low O2 environment is recognized to be an important part of stem cell niche and it should be reproduced ex vivo in order to maintain stem cells in spite of heavy cytokine stimulation. This viewpoint was confirmed with bone marrow cells of murine and human origin as well as with those mobilized to peripheral blood (Ivanovic and Boiron 2009). All these data demonstrated that low O2 concentration (1–1.5%) maintained better the capacity of proliferating stem cells in course of ex vivo expansion. They have been done without TPO but in presence of SCF among other cytokines. In conditions compatible with clinical use (serum free medium and cytokine cocktail already used for clinical grade expansion and transplantation – MGDF, SCF, G-CSF in short-term liquid cultures) we demonstrated on CD34+ cord blood cells that an appropriately low O2 concentration (3%) could enable a massive expansion of clonogenic progenitors combined with maintainance of stem cells (SRC) (Ivanovic et al. 2004). In these conditions, a

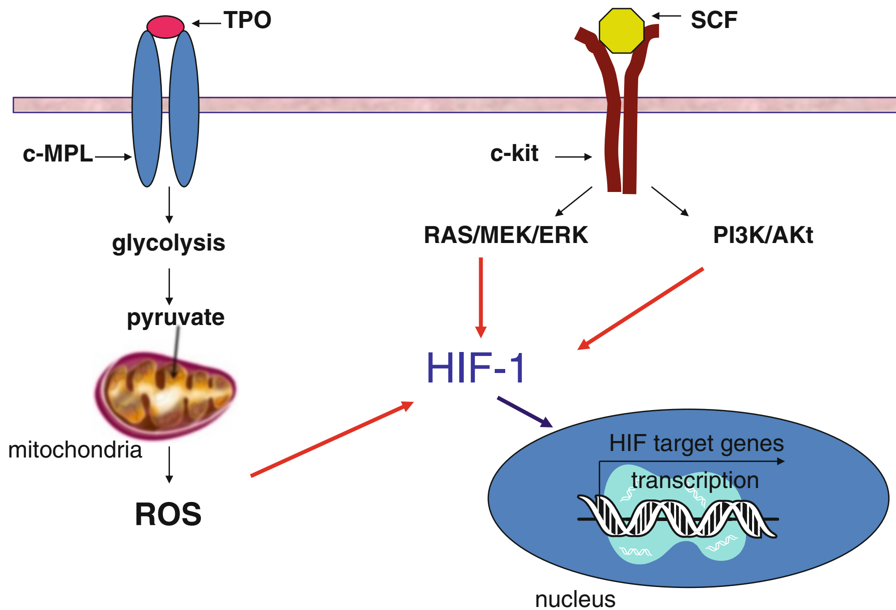


**Fig. 33.1 Oxygen independent TPO and SCF induced HIF-1α expression**. Binding of TPO to its receptor c-Mpl triggers the signaling cascade stimulating glucose uptake and glycolysis. It leads to the increased ROS production by the mitochondria, stabilizing HIF-1α. Activation of tyrosine kinase receptor, c-kit, upon binding of SCF elicits two majors signaling pathways: PI-3-kinase/Akt and Ras/MEK/Erk. Their activation is necessary to achieve high levels of HIF-1α protein expression. Stabilized HIF-1α as a part of HIF-1 transcription factor stimulates expression of HIF-1 targets genes

low dose of IL-3 turned out to synergize with low O2 concentration in maintenance of stem cells. The literature data concerning IL-3 seem to be confusing with respect to this point. An appropriate analysis, however, suggests the importance of absence of serum in culture for this effect as well as a necessity of a low concentration of this cytokine – otherwise the IL-3 does not stimulate only proliferation but also differentiation of stem cells (Ivanovic 2004).

A posteriori, major breakthrough in ex vivo expansion of hematopoietic stem cells could be connected with neutralization of ambient air hyperoxia ex vivo, not only by decreasing oxygenation of cultures but also by enhancing their anti-oxidative capacities (Fan et al. 2008). Avoiding or limiting hyperoxygenation by decreasing O2 concentration limits possibility to generate ROS. Thus, some biotech companies developed/improved anti-oxidative systems in expansion media, a principle which was confirmed independently (Fan et al. 2008). A new generation of media appeared, enabling to enhance fold expansion of progenitors and to attenuate significantly the exhaustion of stem cells during expansion culture even at 20% O2 (Ivanovic et al. 2006; Fan et al. 2008). The effect of a cooper chelator tetraethylenepentamine (TEPA) could be also, at least in part, related to the diminution of oxidative stress (Prus and Fibach 2007).

In this context, the positive impact of TPO (MGDF) on the maintenance of stem cell activity in course of expansion is probably related to the fact that it regulated positively Hypoxia-Inducing Factor 1- alpha (HIF- 1α), the crucial factor enabling cellular functions at low O2 concentration (Yoshida et al. 2008) (Fig. 33.1). Indeed, the HIFs system, apart his role in the regulation of anaerobic metabolism, is directly or indirectly related to a major part of genes and pathways considered today as factors of stemness. Some of them are presented in Fig. 33.2 (the data issued from publications concerning adult stem and tumoral cells).

SCF also induces HIF-1 α in hematopoietic cells at 20% O2, although by a different mechanism (Fig. 33.1). In addition, a synergistic effect of

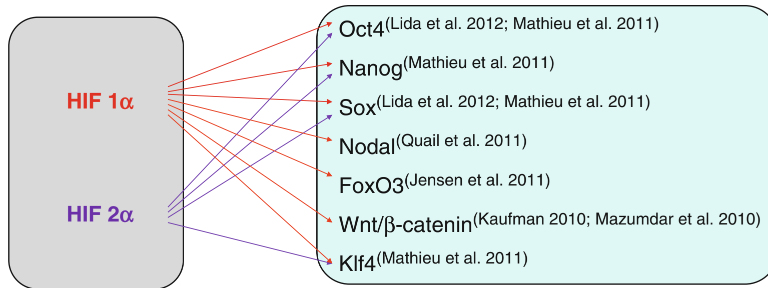


**Fig. 33.2 HIFs mediated expression of stemness regulators in the adult stem and tumoral cells.** In the variety of tumor cell lines, HIF-1 and HIF-2 stimulate expression of "self renewal genes", Oct4, Nanog, Sox2 and Klf4. In addition, expression of factors associated with pluripotency (Nodal and FoxO3A), is depending and regulated by HIF-1. In the adult stem cells, low oxygen mediated signalling via HIF-1 contributes to the their maintenance by direct activation of Wnt/B-Catenin pathway

IL-6 with SCF and/or TPO observed in some studies could be related to the context of "hypoxic response". For example, we demonstrated that a low O2 concentration (1%) synergizes with IL-6 in elaboration of this effect (Kovacevic-Filipovic et al. 2007). So, the expansion medium with antioxidative capacities combined with cytokines which are mimicking hypoxia i.e. normoxia in situ (low O2 concentration with respect to the ambient air) (as TPO and SCF) seems to compensate the negative effects of ambient air hyperoxygenation (20–21% O2).

On the basis of these principles, we improved the initial expansion procedure (Reiffers et al. 1999; Boiron et al. 2006): the new generation medium (HP01, Macopharma, Tourcoing, France) has been validated in clinical-grade cultures (Ivanovic et al. 2006). Due to unavailability of PEG-rHuMGDF on the market, it was replaced by rHuTPO in cytokine cocktail (TPO, SCF, G-CSF, 100 ng/ml each) (Duchez et al. 2011), demonstrating that the "pegylation" was irrelevant in ex vivo situation. Using these modifications, mobilized peripheral blood CD34+ cells from 19 multiple myeloma patients were expanded ex vivo and transplanted in an autologous context. Comparing to control group composed of 38 patients matched for age, sex, stage of the disease, first line hemotherapy, status of the disease at time of transplant, year of transplant, time between diagnosis and transplant, CD34+ mobilization techniques, and the median number of total nucleated cells and CD34+ cells collected, the important and statistically significant acceleration of poly-morphonuclear and platelet recovery was obtained (Milpied et al. 2009, manuscript in preparation).

Concerning ex vivo expansion of cord blood hematopoietic cells, the analysis of several clinical trials realized so far revealed one of several reasons for their inefficiency: (1) low ex vivo expansion fold of progenitors and stem cells; (2) an exhaustion of stem cells in course of expansion due to the culture conditions; (3) A shift of 8–12 days between the injection of non-expanded and expanded fraction which does not allow to appreciate the effect of expanded fraction; (4) only a fraction of the graft was expanded ex vivo; (5) The combination of points (1), (2) and (4) results in insufficient number of cells belonging to the populations critical for hematopoietic reconstitution. Unlike the trials that did not use TPO in cytokine cocktail (Ivanovic and Boiron 2009), Koegler et al. (1999) used TPO to expand 1/8 fraction of a cord blood unit with a good efficiency (600-fold for total cells, 21-fold for progenitors and 3.9-fold for LTC-IC) that was transplanted 24 h after non expanded fraction (7/8 of cells) to a child suffering from acute lymphoid leukemia with infectious complications. Even if a very small number of cells was amplified, the authors estimated that expanded cells contributed to the acceleration of granulocyte reconstitution. Two other clinical trials, although using both SCF and TPO (MGDF) (Shpall et al. 2002; de Lima et al. 2008) failed to

demonstrate a substantial acceleration of post-transplant blood reconstitution most probably due to the fact that only a fraction of cord blood unit was expanded, thus, not providing a sufficient quantity of critical cell populations.

On the basis of our experience acquired during the studies of low O2 issue and development of the expansion procedure for autologous transplantation, we developed a procedure for expansion of stem and progenitor cells starting from cord blood CD34+ cells. The cytokine cocktail (SCF, FL, TPO, G-CSF), showed to maintain the stem cells capable of long-term lympho-hematopoietic reconstitution of NOD-Scid mice (Kobari et al. 2000) was adopted. Original concept of one-step culture (Kobari et al. 2000) was replaced by a two-step culture (14 days) allowing the dilution of culture at Day 6 with fresh cytokine supplemented medium that was performed with CD34+ cells isolated after thawing of previously cryopreserved CBU. Association of this cytokine cocktail containing MGDF with HP01 medium resulted in massive expansion of total cells (several hundred fold) as well as of committed progenitors and CD34+ cells (~100-fold) without impairing the stem cell capacity as proved by SRC assay and the capacity of a secondary engraftment of NOD-Scid mice (Ivanovic et al. 2011). Again, to upscale our ex vivo procedure at clinical scale (with a modification: duration of culture reduced at 12 days), the results obtained with MGDF were reproduced with rHuTPO which is the only clinical grade-TPO molecule available on the market at the moment (submitted). Based on this technology, a clinical trial started on adult patients suffering from hematopoietic malignancies in Hematology Department of Bordeaux University Hospital (Eudract 2008-006665-81, Clinicaltrial. NCT 01034449) directed by Professor Noël Milpied. For this trial CD34+ cells were selected after thawing of CBU in Bordeaux Centre of French Blood Institute (EFS) and CD34 negative fraction was cryopreserved again, to be thawed and injected at Day 12, 3 h after expanded product. So far, during 15 months, seven patients were transplanted with the cell product expanded from CD34+ cells and non-expanded CD34+ fraction of the same CBU. Apart one patient who failed to engraft, six other patients demonstrated a rapid (time to reach 500 poly-morphonuclear cells was 7 days (6–19)) and long-term (a lympho-hematopoietic donor chimerism for more than 1 year so far) hematopoietic reconstitution (Milpied et al. 2011). This clinical trial, if the results of the second half of study confirmed those obtained so far, might prove that only one CBU which CD34+ cells were appropriately expanded ex vivo could allow a rapid and sustained allogenic engraftment of adult patients.

## Conclusion and Perspectives

The review of data concerning the experimental studies and the clinical trials based on cytokine-stimulated expansion of hematopoietic cells aimed to enable rapid and sustained hematopoietic reconstitution point to the important role of TPO (MGDF) molecule. Indeed, all clinical studies that could be considered efficient were based on expansion procedures using TPO in cytokine cocktail. An analysis of EPO action on stem cells in the context of recent data confirming relationship between anaerobic aspects of stem cells and the phenomenon of self-renewal as well as a hypoxia-like effect of TPO (resulting in stabilization of HIF-1α) reveal that this could be the major way by which TPO operates to maintain the primitiveness of stem cells in expansion cultures. The same analysis also explains the synergistic effect of SCF, IL-6 and anti-oxidants.

Since the stimulation of c-Mpl i.e., thrombopoietin receptor on stem cells seems to be crucial for an appropriate outcome of expansion culture concerning the balance between self-renewal, commitment and differentiation of stem cells (also depending on a simultaneous action of some other cytokines and their receptors) it could be figured out that TPO could be replaced in cultures by the small-molecule agonist of c-mpl that could have some advantages with respect to a protein produced by a recombinant technology (as rHuTPO and PEG-rHuMGDF) for ex vivo engineering and consequent clinical application of cellular products (low cost, security, immunogenicity…).

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**ERRATUM**

# Role of Reactive Oxygen Species Formation from Oxidized Low Density Lipoprotein in Bone Marrow Stem Cells

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