Stem Cells and Cancer Stem Cells 10
Therapeutic Applications in Disease and Injury

M.A. Hayat
Editor

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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 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 (Martens 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 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 a man presenting with a PSA above this level is likely to require a rectal biopsy, but only in 25 % of men with serum levels of PSA between 4 ng and 10 ng/ml have cancer (Masters 2007). The PSA threshold currently being used for biopsy ranges between 2.5 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 (4–10 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, to help identify 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 of 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, or 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 problems 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 non-communicable 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 sequelae. 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

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

This is volume 10 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.

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 66 contributors representing 13 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 five subheadings: Mesenchymal Stem Cells, Induced Pluripotent Stem Cells, Neural Cells and Neural Stem Cells, Role of Stem Cells in Disease, and Stem Cell 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 my students for their help in many ways in completing this project.

M.A. Hayat

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Part I

Mesenchymal Stem Cells

Mesenchymal Stem Cells in Bone Regeneration

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Contents

Abstract

Since the discovery that certain bone marrow cells are capable of ectopic bone formation in 1963, scientists scented new therapeutic strategies to overcome clinical problems of bone defects unable to heal or fuse spontaneously. Today, these cells are defined as Mesenchymal Stem Cells (MSCs) and beside bone marrow other tissues emerged as rich sources for this valuable cell type. Even if their role in endogenous bone regeneration is still under debate, exogenous supply already revealed promising results. Due to proper isolation, proliferation and differentiation into the osteogenic lineage, MSCs were capable for bona fide bone formation *in vitro* as well as *in vivo*. Nevertheless, obstacles remain, as clinical utilization of MSCs has not fulfilled the expectations based on animal studies yet. Application of MSCs has the theoretical potential to promote bone healing. Though, further investigations are required in order to implement transplantation of MSCs as a standard procedure in orthopedic and reconstructive surgery.

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Introduction

Although there is still much to be learned, our knowledge about bone regeneration has rapidly evolved over the last decades. In contrast to most human tissues, bone has an outstanding regenerative potential, highlighted by its capacity to heal without fibrous scar formation. Besides numerous cells and genes involved in this complex process, Mesenchymal Stem Cells (MSCs) play a pivotal role. Capable to differentiate into the osteogenic lineage, MSCs form osteoblasts and thus new bone tissue. Due to this capacity, MSCs were utilized for tissue engineering purposes to overcome impaired healing of bone defects, which are otherwise unable to heal or fuse despite adequate treatment. Combined with growth factors and scaffolds, MSCs may accelerate, enhance or even replace endogenous bone formation, assuring its regenerative potential.

The following chapter should give an insight into the role of MSCs in the complex biology of bone regeneration as well as their therapeutic utilization regarding current tissue engineering principles and future directions.

Control of Osteoblast Development

Verification of osteogenic differentiation is the principle baseline to identify cells involved in the complex process of bone regeneration. With respect to osteoblasts, several genes have been identified including *Runx2*, *Osterix*, *Collagen 1*, *Osteopontin (OPN)*, *Bone Sialoprotein (BSP)* and *Osteocalcin (OCN)*. These genes are expressed in a chronological sequence, allowing MSC differentiation via osteoprogenitors, preosteoblasts, osteoblasts and finally differentiated osteocytes. Activation of *Runx2* (formerly called *Cbfa1*), which represents the earliest marker of osteogenic differentiation (member of the runt homology domain transcription factor family), seems to be crucial to initiate osteogenesis. While *Runx2* is postnatally expressed in osteoblasts, periosteocytes and perichondrocytes, deletion of *Runx2* leads to absence of ossification and skeletons that only consist of chondrocytes and cartilage. In addition, heterozygous Runx2-mutants reveal hypoplastic clavicles and open fontanelles, mimicking the severe disease pattern of cleidocranial dysplasia, a hypothesis, which could soon be verified in humans. In summary, there is high evidence to recognize *Runx2* as a mastergene of osteogenic differentiation.

Another important factor crucial for bone formation is *Osterix*. *Osterix* is a zinc finger proteinase and member of the SP family of transcription factors. Its expression occurs subsequent to *Runx2* and deletion is likewise associated with an absence of ossification. Even if detailed mechanisms are currently unclear, *osterix* seems to direct the commitment of preosteoblasts to osteoblasts; away from the chondrogenic lineage.

In contrast to *Runx2* and downstream *Osterix*, whose expressions remain stable until the end of osteoblastic differentiation, other osteogenic markers are expressed asynchronously and inconstantly, thus up- or downregulated as the progenitor cells differentiate and the matrix matures and mineralizes. For instance, the ectoenzyme *alkaline phosphatase (ALP)*, whose activity increases and then decreases, when mineralization is well progressed. Nevertheless, the analysis of individual osteoblasts in 21-day fetal rat calvaria demonstrated that *ALP* was ubiquitously expressed in all osteoblasts, whereas other genes were only detectable in subsets of osteoblasts, indicating a global role of *ALP* in bone regeneration. Verifying this aspect, mineralization defects were detected in all bones after deletion of *ALP* in knockout mice.

Other osteogenic markers include extracellular matrix proteins like *Collagen1*, *OPN*, *BSP* and *OCN*. Even if their genes all contain binding sites for *Runx2* in the promotor region, expression levels vary even more compared to *ALP*. Whereas *OPN* and *BSP* peak twice, *OCN* is only upregulated in postproliferative osteoblasts and thus in later stages of osteogenic differentiation. Furthermore, their role in bone formation is not completely understood yet, since *OPN*, *BSP* and *OCN* are not globally expressed by all osteoblasts and deletion of *OCN* and *OPN* does not result in a general failure of osteoblast differentiation and maturation, even if differences to normal bone were detected.

This may further indicate currently unknown pathways for osteogenic differentiation of *MSCs* or the possibility to skip specific factors under particular conditions.

Endogenous Bone Regeneration

Every weight bearing leads to microscopic fractures, which initiate remodeling and thus new bone formation. This mechanism triggered by physical forces occurs constantly in the healthy skeleton and is essential to warrant lifelong strength and stability (Herman et al. 2010). In contrast, if physical forces increase or remodeling capacity exhausts, macroscopic fractures emerge leading to pain and dysfunction. In the majority of cases however, bone regeneration occurs within a few weeks after adequate fracture treatment. Irrespective of the distinctive pathways, which are currently known, MSCs play an outstanding role in the complex process of bone regeneration. For instance, indirect fracture healing, the most common form of fracture healing, occurs after conservative as well as operative fracture treatment without anatomical reduction of the fracture ends. Consisting of both intramembranous and endochondral ossification, the latter outweighs in indirect fracture healing leading to callus formation, mineralization and subsequent new bone formation (Gerstenfeld et al. 2006). Several proinflammatory molecules are released by the fracture hematoma, stimulating cartilaginous callus production, angiogenesis as well as differentiation of osteoblasts (Marsell and Einhorn 2011). Therefore, MSCs have to proliferate and differentiate into chondrogenic, osteogenic and angiogenic cells. The origin of MSCs involved in this process is still under debate. Besides local bone marrow, periosteum and endosteum, several studies observed migration of MSCs over the blood stream, which suggest recruitment of MSCs in remote tissues. Colnot assessed the role of local osteogenic progenitors in Rosa26 mice and described additional chondrogenic differentiation, important for callus formation by periosteal cells, whereas endosteal and bone marrow cells were restricted to bone regeneration through means of intramembranous ossification, *i.e*. direct bone formation (Colnot 2009). Another local group of MSCs potentially affecting bone regeneration reside in the adjacent soft tissues like muscle, fat and tendon. Even if their capacity to differentiate into the osteogenic lineage is well established *in vitro*, no evidence is given for a direct participation in the normal repair process *in vivo*. This lack of evidence is based on the problem, that differentiated osteogenic cells express markers, which are lineage specific, but not tissue specific, making it difficult to deduce their origin.

A same problem emerges in defining the source of MSCs invaded from remote tissues. An increase of circulating osteoblastic precursors following bone injury as well as migration to fracture site has been assessed in several studies. Currently, known molecular events mediating their recruitment include BMPs, HIF-1alpha and SDF-1/CXCR-4. Especially, stromal cell-derived factor-1 and its G-protein coupled receptor CXCR-4 represent a well-examined factor for homing specific MSCs to the site of trauma (Granero-Molto et al. 2009). While SDF-1 expression increases at the fracture site, circulating MSCs only home if they are CXCR-4 positive (Kitaori et al. 2009). Accordingly, fracture healing was impaired utilizing SDF-1 antagonists or manipulation of SDF-1 and CXCR-4 gene expression. At the fracture site MSCs support bone regeneration, even if their specific role is not completely defined yet. Utilizing a parabiosis mouse model, Kumagai et al. observed migration of cells positive for the osteoblast marker alkaline phosphatase to the fracture site, but not their integration as osteocytes (Kumagai et al. 2008). Taguchi et al. revealed similar results after transplantation of GFP bone marrow into wild-type mice (Taguchi et al. 2005). While GFP-/Osteocalcin-positive cells were found on fracture callus surface, incorporation could not be determined. Thus, it remains unclear if circulating osteogenic cells support natural osteogenesis through bone formation or secretion of osteoinductive factors. However, currently more data are emphasizing the importance of residual MSCs in bone regeneration (Fig. 1.1). Nevertheless, it is undeniable that MSCs, irrespective of their origin, are crucial for endogenous bone regeneration.

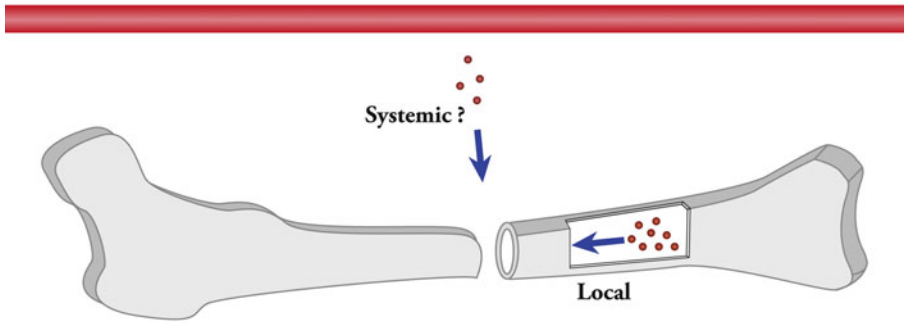

Fig. 1.1 Contribution of local and systemic stem cells to bone regeneration. Systemic stem cells could migrate through the vascular system, whereas local stem cells could originate from the bone marrow, periosteum and endosteum

Bone Tissue Engineering

In spite of the aforementioned regeneration capacity, in some cases bone healing reveals insufficient. By exceeding a critical defect size, which is not only defined by the space between two fragments, but also by individual conditions like minor perfusion or presence of infection, bone tissue is unable to heal or fuse, leading to non-union and pseudo-arthrosis. Furthermore, the incidence increases with a more aged population suffering from osteoporosis, diabetes and vascular disease, making critical sized defects a big challenge to the reconstructive and orthopedic surgeon.

Current treatment options include autologous and allogeneic bone grafts, bone graft substitutes, growth factors as well as complex surgical procedures, all of which can be implemented solely or even combined. This multitude of alternatives available, however, reflects both the inadequacy of any single approach and the dire need for improved strategies to reconstruct critical sized bone defects. Therefore, therapeutic utilization of MSCs might be a promising strategy to overcome problems of bony defects.

In the middle of the 1960s, Friedenstein isolated a population of cells from bone marrow capable for ectopic bone formation (Friedenstein et al. 1966). Transplanted in a certain density into a subcutaneous chamber haematopoesis ceased and osteogenesis occurred. These findings highlighted the presence of specific precursor cells as well as the possibility to utilize their osteogenic potential, initiating the era of cell-based principles for bone healing purposes. Today, it is known, that the isolated cells responsible for osteogenesis were MSCs and their bone regenerative potential has been assessed in numerous studies. Combined with osteoconductive and osteoinductive supplements MSCs accelerated, enhanced or even replaced endogenous bone formation. In this context osteoconduction describes the ability to provide attachment and migration of cells as well as protected void space in which new bone tissue can form. In general, the osteoconductive component is delivered by synthetic or allogeneic materials, formally known as scaffolds. In contrast, osteoinduction is provided by growth factors, which induce undifferentiated cells to differentiate into the osteogenic lineage. Beside bone morphogenetic proteins, as the most established osteoinductive supplement, latest studies assessed Fibroblast Growth Factor -9 and -18 as well as vascular endothelial growth factor A (VEGFa) capable to stimulate osteogenic differentiation (Behr et al. 2010b, 2011).

However, regarding successful bone grafting approaches, osteoconduction and osteoinduction, provided by scaffolds and growth factors, play an important but more supportive role, whereas osteogenic cells are theoretically capable to create new bone independently. With respect to this unique feature, gaining osteogenic cells play a paramount role for bone regenerative purposes.

Current options include osteoblasts, embryonic stem cells (ESCs), induced pluripotent stem cells (iPS) and other postnatal mesenchymal cells.

Whereas mature osteoblasts are limited in availability, as their harvest requires loss of bone at the donor site, embryonic stem cells show unregulated differentiation and formation of teratomas and teratocarcinomas. Furthermore, ESCs rose ethical concerns regarding fetal tissue harvest (Wu et al. 2007). In contrast, adult stem cells and progenitor cells are likewise capable to differentiate into the osteogenic lineage omitting the aforementioned bottle necks. In this context MSCs represent a well-studied adult stem cell population. MSCs are identified by specific characteristics, like plastic adherence, distinctive surface markers or the proof of their differentiation potential. Even if formation of osteoblasts, adipocytes and chondrocytes is sufficient for characterization of MSCs, they are also capable to differentiate into other cell lineages beneficial for skeletal tissue engineering purposes. Regarding the promising potential of MSCs, it is important to point out that the extend of their abilities is critically affected by specific conditions, especially the harvest site.

Sources of Mesenchymal Stem Cells

MSCs have been isolated from bone marrow, umbilical cord blood, adipose tissue, dental pulp and periosteum (Seong et al. 2010). Interestingly, tissue origin of MSCs seems to be a major determinant of progenitor characteristics. For example, colony-forming frequency was lowest in stem cells derived from umbilical cord blood and highest in adipose tissue derived stem cell populations, although the proliferation capacity was highest in umbilical cord blood stem cells (Kern et al. 2006).

With respect to endogenous bone formation, bone marrow and periosteum reveal the only proven sources part taking in this process. Bone marrow derived stem cells (BMSCs) are the most utilized stem cell source for both clinical and tissue engineering purposes. Even if bone formation with means of BMSCs is well established *in vitro* as well as *in vivo*, some obstacles remain as bone marrow availability is limited and their regenerative potential decreases with increasing patient age (Muschler et al. 2001). Periosteum derived MSCs (PDMSCs) show high osteogenic potential, however, harvest and isolation is difficult, resulting in severe donor site morbidity (Zhang et al. 2005).

Therefore MSCs should fulfill several requirements proposed for successful clinical use in regenerative applications (Gimble 2003):

1. They should be found in abundant quantities (up to billions of cells).
2. They should be harvested with a minimally invasive procedure.
3. They should be able to differentiate along multiple cell lineage pathways in a controllable and reproducible manner.
4. They should be safely and effectively transplanted into an autologous or allogeneic host.
5. They can be manufactured in accordance with good manufacturing practice guidelines.

Since 1964, when Rodbell reported the existence of MSCs in adipose tissue, adipose tissue derived MSCs (ASCs) became a promising cell source for tissue engineering purposes (Rodbell 1964). ASCs can be readily harvested during a minor liposuction procedure under local anesthesia, in contrast with the significant pain experienced by patients undergoing bone marrow, periosteum or dental pulp stem cell harvest. Compared with bone marrow, adipose stem cell concentration reveals equal but is more readily available in large quantities, potentially rendering a period of *in vitro* expansion unnecessary (Behr et al. 2010a).

Even if evidence about their osteogenic potential shows controversy, ASCs have been successfully used in regenerative applications in numerous animal models especially in bone and cartilage repair. For example, they have been shown to facilitate chondrogenesis in a rabbit condylar defect model (Nathan et al. 2003). Their osteogenic potential has been demonstrated in a murine calvarial defect model (Cowan et al. 2004). Adipose tissue derived stem cells seeded onto apatite-coated poly-lactic-co-glycolic acid scaffolds were implanted into 4 mm critical-sized calvarial defects and demonstrated improved healing compared with non adipose tissue derived stem cell seeded implants.

In addition, supportive but important abilities of ASCs to enhance bone formation were highlighted

in recent studies. In clinical practice it is well known, that immunoreactions, due to infections or foreign body reactions by implanted materials, critically compromise the healing potential of large scaled bone defects or even simple fractures. Therefore ASCs show an immunosuppressive effect on local host tissue (McIntosh et al. 2009). By an interaction with different cell types of both innate and adaptive immunity, ASCs are able to decrease the inflammatory response leading to better conditions for bone regeneration. Furthermore, by combining osteogenic cells with scaffolds and growth factors, numerous studies have shown that angiogenesis is essential for successful bone grafting approaches (Das and Botchwey 2011). Providing nutrition supply as well as evacuation of cell debris, vascularization enhances bone formation by delivering osteogenic cells and osteoinductive proteins to the defect site. In this context ASCs promote angiogenesis in a paracrine fashion as well as by differentiation into the endothelial lineage, paving the way for higher vascularity and thus enhanced bone regeneration capacity (Fraser et al. 2006). Current techniques to facilitate adequate vascularization include in vitro prevascularization of the graft in co-cultures of endothelial and osteogenic cells as well as additional supply of angiogenic factors, especially of VEGFA. Whereas, prevascularization in co-cultures requires additional *in vitro* cultivation compromising the clinical approach as a single-stage procedure, VEGFA reveals auspicious capabilities as it promotes both angiogenic and osteogenic differentiation to facilitate bone healing. For instance, human ASCs supplemented with VEGFA increased bone formation as well as angiogenesis in murine calvarial defects through cell-autonomous and paracrine effects (Behr et al. 2011). Another crucial aspect for future clinical applications of MSCs was recently illuminated by Mirsaidi et al. (Mirsaidi et al. 2012). Whereas BMSCs decrease in numbers as well as in regeneration and osteogenic differentiation potential, ASCs maintain telomere length, telomerase activity and osteogenic differentiation in aged osteoporotic mice. Regarding an increasing population age and senile osteoporosis incidence, both affecting occurrence of critical sized defects, ASCs seem to be the leading autologous cell source for the development of novel bone regenerative therapeutic strategies. Nevertheless, some authors declared critical disadvantages of ASCs for bone tissue engineering purposes. Compared to other sources osteogenic differentiation capacity as well as bone quality was minor in several studies (Kern et al. 2006). In addition no study could demonstrate bone marrow derivation from transplanted ASCs, thus no complete bone formation. Other authors even question if ASCs are at all capable to form bone *in vivo*, diminishing aforementioned beneficial aspects of ASCs especially compared to BMSCs. The latter are easily extractable, capable to form bone marrow and their osteogenic capacity is well established in numerous studies, potentially preponderating their age-depending lack of proliferation and differentiation capacity as well as their limited availability.

Clinical Use

Despite a fair amount of experimental data suggesting the potential of MSCs in bone regeneration, clinical use of MSCs is still limited to few clinical trials and case series. For instance, an obstacle in using BMSCs is the low cell rate in bone marrow aspirates, resulting in the need for centrifugation. In one study, *ex vivo* cultured autologous BMSCs were combined with shaped hydroxyapatite scaffold to treat bone defects of 4–7 cm in three patients (Quarto et al. 2001). 2 months postoperatively, the graft was well integrated into the bone, demonstrating a distinct acceleration as opposed to conventional grafting. In another study, tibia nonunions were treated with BMSCs in 60 patients by percutaneous autologous bone marrow grafting. Healing was successful in 53 of the patients 4 months after surgery. Importantly, the authors concluded that the concentration of BMSCs needed to be larger than 1,000/cm^3 to achieve union, since the seven patients that failed union were treated with less than 1,000/cm^3 BMSCs (Hernigou et al. 2005). These data have implications for the routine use of this method since aspirates may contain less BMSCs than the suggested concentration. Gan and coworkers further refined this concept. They performed perioperative enrichment of MSCs and combined these cells with β-tricalcium phosphate for posterior

spinal fusion (Gan et al. 2008). 95% of their 41 patients showed good spinal fusion results after 34 months. In addition, the authors confirmed that young patients (<40 years) had higher quantities of BMSCs than patients older than 40 years.

Clinical experience with ASCs in bone regeneration is limited to case reports. For instance, human ASCs have been shown to heal or stimulate healing of a calvarial defect in combination with fibrin glue (Lendeckel et al. 2004). Moreover, hASCs have been shown to heal a large maxillary defect when combined with β-tricalcium phosphate and BMP-2 in a microvascular ectopic bone flap 8 months post-operatively (Mesimaki et al. 2009).

Summarized, these studies illustrate the potential of MSCs for bone regeneration in clinical practice, though many aspects such as impact of patient's comorbidities still have to be learned.

Future Directions

Utilization of MSCs in combination with a suitable scaffold holds great promise in bone tissue regeneration. However, the perfect combination of cells and scaffolds has not been determined yet. Moreover, it is disputable whether this combination can be solely detected in animal studies or whether controlled clinical trials are necessary to achieve this aim. The specific drawbacks of different MSC types might be overcome by combination with growth factors. In addition, it would be desirable to engineer bone tissue, which has the capacity to produce stem cells itself. In turn, this is a prerequisite for regeneration in case of subsequent injuries. From a surgical perspective, the ultimate goal would be to fulfill this in a single-stage procedure (Fig. 1.2).

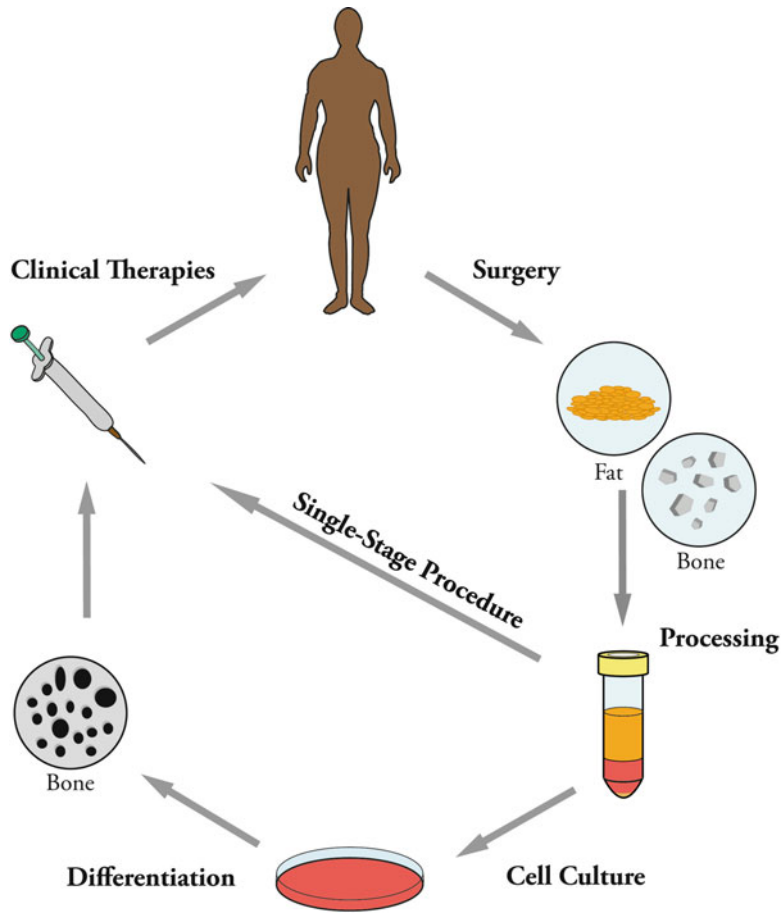

Fig. 1.2 Reconstructive cycle. Mesenchymal stem cells are isolated from the patient and differentiated for later autologous transplantation. Alternatively, mesenchymal stem cells could be applied in a single-stage procedure

We have learned a lot about the use of stem cells during the last decades and physicians have only started to translate this knowledge to clinical situations, *i.e.* the reconstruction of critical sized bone defects.

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2 Experimental (Preclinical) Studies and Clinical Trials of Adipose Tissue-Derived Mesenchymal Stem Cells for Autoimmune Diseases

Eun Wha Choi

Contents

Abstract

Mesenchymal stem cells (MSCs) are considered attractive therapeutic tools for the treatment of various diseases, including autoimmune disease. MSCs display many advantageous properties such as immunomodulation, capacity for differentiation and transdifferentiation, homing activity, and paracrine effects. Further, adipose tissue-derived MSCs (ASCs) are regarded an ideal source of these stem cells because of their plentiful supply, availability, non-immunogenic properties, and minimal ethical considerations as well as the fact that their capacity for proliferation and differentiation is less likely to be affected by aging. In the few preclinical studies and clinical trials that have been performed, treatment with ASCs have ameliorated the clinical symptoms of various autoimmune diseases by reducing inflammatory responses, improving Th1/Th2 balance, and inducing regulatory T cells. ASCs are also an ideal vehicle to deliver genes into target tissues for gene therapy because of their unique biological and immunological properties. Using ASCs as a vehicle to insert appropriate therapeutic genes into cells could prove a novel method for immune modulation in autoimmune disease.

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Introduction

Autoimmune disease occurs when the body tissues are attacked by its own immune system because of an inappropriate immune response directed toward self-antigens. Over 60 autoimmune diseases exist affecting about 6% of the population (Siatskas et al. 2006).

Current systemic therapies using steroids or immunosuppressant drugs are not always effective and are rarely curative for patients with severe autoimmune disease. Furthermore, they are associated with side effects and substantial toxicity (Jantunen and Myllykangas-Luosujärvi 2000). Recent advances in the understanding of the pathogenesis of autoimmune diseases have given rise to many targeted approaches for the treatment of these diseases, including the use of biological agents that target cytokines and immune cells, and stem cell therapy.

Mesenchymal stem cells (MSCs) are non-hematopoietic progenitor cells with multi-lineage differentiation potential. Recently, MSCs have been considered as natural immune modulators, and therefore, research has been directed at using MSCs for the treatment of immune-mediated diseases. In addition to immunomodulation, MSCs also hold potential for the repair of various defects because of a number of attractive properties, including differentiation capabilities, paracrine effects, and their capacity for directional migration (Wang et al. 2011). MSCs were first isolated from the bone marrow, and later from other tissues, including the placenta, muscle, cartilage, and fat. Bone marrow MSCs were the first to be used for therapy. However, collection from the bone marrow is a painful, invasive procedure that generates relatively low yields.

Adipose tissue is becoming an alternative source of MSCs, and it is readily available and easy to obtain. Research focusing on ASCs has intensified with many preclinical studies and clinical trials (Choi 2009). This review provides an overview on the etiology, classification, and experimental animal models of human autoimmune disease. It will highlight the advantageous properties of ASCs and the application of these cells in the treatment of autoimmune diseases.

Etiology and Classification of Autoimmune Diseases

The etiology of autoimmune diseases is unknown, but is thought to result from a combination of genetic factors, inappropriate immune regulations, and hormonal and environmental factors (Choi 2012). The underlying mechanism is generation of an adaptive immune response against self-antigens (Siatskas et al. 2006). This autoimmunity can be induced by many factors, including failure of clonal anergy or clonal suppression of self-reactive lymphocytes, release of sequestered antigens, molecular mimicry between microbial antigens and host proteins (self-antigen), inappropriate expression of major histocompatibility complex (MHC) class II molecules, cytokine imbalance, dysfunction of the idiotype network regulatory pathways, general regulatory T-cell defects, and polyclonal B cell activation due to infection by bacteria or viruses (Kuby 1994).

Autoimmune diseases can be broadly classified as organ-specific or systemic depending on the location of the target antigen and clinical features (Siatskas et al. 2006). Common examples of systemic autoimmune diseases include systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, ankylosing spondylitis, and polymyositis. Organ-specific autoimmune diseases include type 1 diabetes, Addison's disease, Hashimoto thyroiditis, Graves' disease, Sjögren's syndrome, vitiligo, pernicious anemia, glomerulonephritis, myasthenia gravis, Goodpasture's syndrome, autoimmune hemolytic anemia, idiopathic thrombocytopenia purpura, and pulmonary fibrosis (Kuby 1994).

Advantageous Properties of Mesenchymal Stem Cells

MSCs are considered promising therapeutic tools because of their capacity for differentiation and

transdifferentiation and their paracrine effects. MSCs release paracrine factors, including trophic (anti-apoptotic, supportive, and angiogenic), chemoattractant, anti-scarring, and immunomodulatory factors. Ponte et al.'s study (2007) showed that BM MSCs express many receptors, including the tyrosine kinase receptors, platelet-derived growth factor receptor (PDGF-R)α, PDGF-Rβ, and insulin-like growth factor receptor (IGF-R), regulated upon activation, normal T-cell expressed and secreted receptor (RANTES) as well as the macrophage-derived chemokine (MDC) receptors, C-C motif receptor (CCR)2, CCR3, and CCR4, and the stroma-derived factor (SDF)-1 receptor, C-X-C chemokine receptor (CXCR)4. BM MSCs migrate in response to many chemotactic factors such as PDGF-AB, IGF-1, RANTES, MDCs, and SDF-1. Baek et al.'s study (2011) using flow cytometry and reverse-transcriptase polymerase chain reaction revealed that ASCs express CCR1, CCR7, CXCR4, CXCR5, CXCR6, epidermal growthfactor receptor (EGFR), fibroblast growth factor receptor 1 (FGFR1), transforming growth factor beta (TGF-ß) receptor 2, tumor necrosis factor (TNF) receptor superfamily member 1A, PDGF-Rα, and PDGF-Rβ. The authors also reported that the migration of ASCs is controlled by various growth factors and chemokines. Molecules that mediate immunomodulatory effects of MSCs include prostaglandin E2 (PGE2), transforming growth factor-β (tumor growth factor-β, TGF-β), hemoxygenase-1 (HO-1), hepatocyte growth factor (HGF), 2, 3-dioxygenase (IDO), interleukin (IL)-10, human leukocyte antigen G (HLA-G), and leukemia inhibitory factor (LIF). These molecules produce anti-proliferative effects on T cells or natural killer (NK) cells, PGE2 modulates the secretory profile of dendritic cells and macrophages, and HO-1 and LIF induces the generation of regulatory T cells (Meirelles Lda et al. 2009). Further, MSCs have low immunogenicity because they lack MHC class II or co-stimulatory molecules such as B7, CD40, and CD40L, and as such are poorly recognized by T cells. MSCs are, therefore, an attractive alternative source for treatment of tissue injury and immune-mediated diseases. As described above, ASCs are considered as an ideal source of stem cells for regenerative medicine because they are abundant, easily available, and non-immunogenic with minimal ethical objections to their clinical use. Further, several recent studies revealed that ASCs are less affected by aging and multiple passaging events compared to BM MSCs, as measured by p21 gene expression, telomerase activity, and senescence-associated β-galactosidase activity (Chen et al. 2012). Therefore, ASCs represent a promising autologous cell source for cell-based therapy and cell-based gene therapy in various patients, even in elderly patients.

Experimental Animal Models of Autoimmune Diseases

Animal models for autoimmune diseases have provided an understanding of the mechanism underlying autoimmunity and a valuable insight into potential treatments (Kuby 1994). Animal models for autoimmune diseases are broadly divided into spontaneous, experimentally induced, and genetically engineered animal models (Taneja and David 2001). Two major multigenic spontaneous mouse models of human autoimmune diseases are F1 hybrids of New Zealand Black and New Zealand White mice (NZB/W F1) and non-obese diabetic (NOD) mice. NZB/W F1 mice develop a spontaneous autoimmune disease with striking similarities to human systemic lupus erythematosus (SLE). In female NZB/W F1 mice, anti-nuclear antibodies, including anti-dsDNA antibodies, are produced, and develop severe immune complex-mediated glomerulonephritis. All mice die from renal failure by 10–12 months of age (Wang et al. 1996). Genetic analysis of these NZB/W F1 mice have provided very important information on the genetic mechanisms involved in the development of SLE (Taneja and David 2001). NOD mice spontaneously develop diabetes that resembles human type I diabetes mellitus (Type I DM). Human type I DM and NOD mice share a similar pathology and

immunological basis. They have islet-reactive CD4 + T and CD8+ T cells and the predisposing MHC class II molecule (HLA-DQ8 and I-Ag7) genes (Taneja and David 2001). Other spontaneous animal models include the obese-strain chicken that resembles Hashimoto's thyroiditis.

Experimentally induced animal models can be induced in various species by immunization with potential known autoantigens in complete Freund's adjuvant. Well-known autoantigens include myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein, which are used to induce autoimmune encephalomyelitis that resembles human multiple sclerosis. Other common autoantigens include thyroglobulin, type II collagen, acetylcholine receptor, and interphotoreceptor retinoid-binding protein that are used to induce models of Hashimoto's thyroiditis, human rheumatoid arthritis, human myasthenia gravis, and human uveitis, respectively (Kuby 1994). The advantage of experimentally induced animal models is the control over the onset and progression of disease.

Genetically engineered animal models include MHC-transgenic models, TCR-transgenic models, cytokine knockout models with/without cytokine receptor, and TCR knockout models. For example, the HLA-DQ8 rat insulin promoter (RIP).B7-1-transgenic mice are an excellent model for human type I DM. HLA-DQ8 RIP.B7-1- transgenic mice are produced by crossing HLA-DQ8 (type 1 diabetes-predisposing MHC class II molecule) transgenic mice with RIP.B7-1 transgenic mice, which express the co-stimulatory molecule B7-1 in the β cells of islets (Wen et al. 2001). Crossing KRN mice that express rearranged specific TCR genes with a NOD strain (H-2Ag7) induces spontaneous systemic arthritis that resembles rheumatoid arthritis (Matsumoto et al. 1999). IL-2, IL-2R α, IL-10, and TCR-α knockout models develop spontaneous inflammatory bowel disease, resembling human Crohn's disease, and IL-10 knockout models develop chronic enterocolitis that resembles human inflammatory bowel disease (Kühn et al. 1993).

Therapeutic Application of Adipose Tissue-Derived Mesenchymal Stem Cells in Experimental Animal Models of Autoimmune Diseases

The efficacy of ASCs has been studied in experimentally induced animal models of autoimmune diseases, including autoimmune encephalomyelitis, type II collagen-induced autoimmune arthritis, inflammatory bowel disease, and autoimmune thyroiditis.

Multiple sclerosis is a multifocal inflammatory disease of the central nervous system that leads to a broad spectrum of clinical signs. The main mechanism of the development of disease is the release of sequestered self-antigen such as myelin basic protein in oligodendrocytes caused by trauma to tissues following an accident or bacterial or viral infection (Kuby 1994). This leads to a lethal demyelinating attack, scarring, progression to physical and cognitive disability such as paralysis and sensation, and visual and sphincter problems. Activated T cells in the cerebrospinal fluid infiltrate the brain tissue and spinal cord, producing an inflammatory response and destruction of the myelin (Kuby 1994). Constantin et al. (2009) studied the effect of syngeneic ASC transplantation on experimental autoimmune encephalomyelitis. Chronic experimental autoimmune encephalomyelitis was induced in female C57BL/6 mice at 6–8 weeks of age by subcutaneous immunization with 200 μg of myelin oligodendrocyte glycoprotein (MOG_{33-35} peptide) in incomplete Freund's adjuvant containing 0.8 mg/ml *Mycobacterium tuberculosis*. Pertussis toxin (50 ng) was also injected at the day of immunization and after 48 h. Intravenous administration of murine (syngeneic) ASCs before disease onset significantly reduced the severity of autoimmune encephalomyelitis and decreased spinal cord inflammation and demyelination. Intravenous administration of murine (syngeneic) ASCs in chronic experimental autoimmune encephalomyelitis also significantly reduced axonal loss and demyelination

and induced Th2 cytokine shift. ASC inhibited MOG-specific T-cell proliferation; suppressed the production of IFN-γ, GM-CSF, IL-17, IL-4, and IL-5; and induced IL-10 production in vitro. Ex vivo analysis showed that peripheral lymph node cells isolated from mice treated with ASCs displayed suppressed MOG-specific T-cell proliferation and decreased production of both proinflammatory and anti-inflammatory cytokines (Constantin et al. 2009). Constantin et al.'s study showed that the migration of ASCs to the lymph nodes was significantly higher when the ASCs were injected shortly after the induction of the autoimmune response. It was also suggested that chronic inflammation and expression of VCAM-1 on the brain endothelium in mice with encephalomyelitis can recruit ASCs expressing α4 integrin from the blood.

Rheumatoid arthritis is characterized by chronic joint inflammation, subsequent cartilage destruction, and bone erosion. Rheumatoid arthritis develops following the activation and expansion of autoreactive Th1 and Th17 cells that modulate the release of proinflammatory cytokines and chemokines, promoting the activation and infiltration of neutrophils and macrophages. González et al. (2009) studied the effects of human and murine ASCs in DBA/1 mice with collagen-induced arthritis. In this study, collagen-induced arthritis was induced in DBA/1 mice at 7–10 weeks of age by subcutaneous immunization with 200 μg of chicken type II collagen emulsified in Freund's complete adjuvant containing 200 μg of *Mycobacterium tuberculosis*. This was followed by a subcutaneous booster injection with 100 μg of chicken type II collagen emulsified in Freund's complete adjuvant. Administration of human ASCs into mice with collagen-induced arthritis decreased Th1-mediated autoreactive response, Th17 production, and inflammation. The authors also reported significantly decreased concentrations of TNF-α and IL-1ß in sera as well as of TNF-α, IL-6, IL-12, IFN-γ, IL-1ß, IL-17, and RANTES in joint protein extracts. Increased levels of IL-10 were found in the joint protein extracts, and significantly greater numbers of CD4 + CD25 + FoxP3+ regulatory T cells were found in both draining lymph nodes and synovium. Intraperitoneal administration of 10^6 human ASCs at the onset of disease completely prevented the progression of arthritis, but the administration of human ASCs into mice with severe collagen-induced arthritis minutely attenuated the clinical signs. Both syngeneic (ASC from DBA/1 mice) and allogeneic (ASC from C57BL/6 mice) ASCs had beneficial effects on collagen-induced arthritis (González et al. 2009).

Inflammatory bowel disease is a group of inflammatory conditions and chronic idiopathic tissue destructive diseases of the colon and distal small intestine that is caused by dysfunctional mucosal T cells and cellular inflammation. The major types of inflammatory bowel disease are Crohn's disease and ulcerative colitis. Activated Th1 cells promote the activation and infiltration of neutrophils and macrophages in both Crohn's disease and experimental colitis. Cytokines and free radicals produced by infiltrating cells and resident macrophages play a pivotal role in tissue inflammation and destruction (Fiocchi 1998). Gonzalez-Rey et al. (2009) studied the effects of human and murine ASCs in C57BL/6 mice with experimental colitis. Colitis was induced in 7-week-old C57BL/6 mice by administering dextran sulfate sodium (DSS) in drinking water *ad libitum*. Acute colitis was induced by administering 5% DSS in drinking water from day 0 to day 7, and chronic colitis was induced by administering 3% DSS in drinking water in a cyclic manner (2 cycles of 7 days with DSS, followed by 10-day period without DSS supplementation). In their study, intraperitoneal administration of ASC significantly attenuated clinical signs, induced histological improvement, and prevented weight loss, diarrhea and inflammation. Treatment of ASCs decreased Th1-derived inflammatory response and the level of inflammatory cytokines in the colon. ASC treatment also increased IL-10 production in the colon and mesenteric lymph nodes, and the number of CD4 + CD25 + FoxP3+ regulatory T cells in the mesenteric lymph nodes. Both syngeneic (ASCs from C57BL/6 mice) and allogeneic (ASCs from BALB/c mice) ASCs had beneficial effects on experimental colitis (Gonzalez-Rey et al. 2009).

A study on the effect of ASC transplantation in mice with experimental autoimmune thyroiditis is

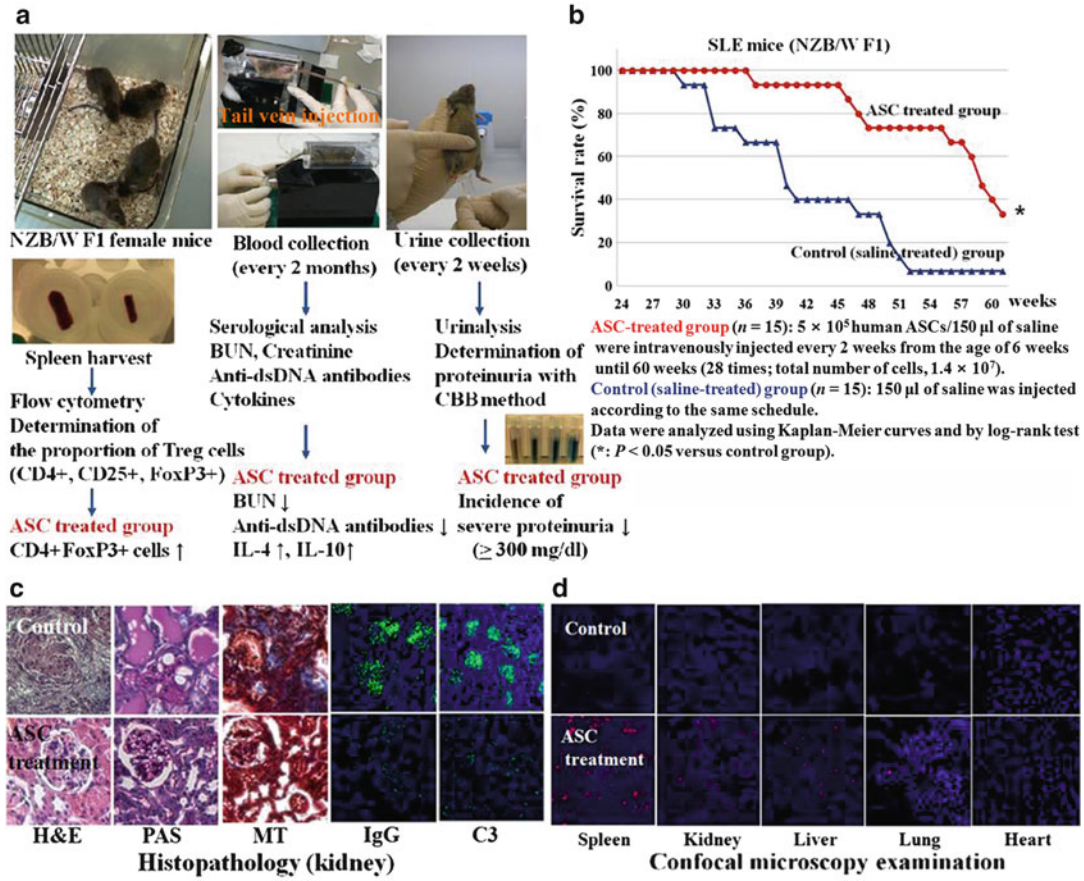

Fig. 2.1 Treatment of female F1 hybrids of New Zealand Black and New Zealand White (*NZB/W F1*) mice, a murine model of human systemic lupus erythematosus (*SLE*), with human adipose tissue-derived mesenchymal stem cells (*ASCs*). (**a**) Overview of experimental methods and serological, urological, and immunological results; (**b**) Survival in NZB/W F1 mice; (**c**) Hematoxylin and eosin (*H&E*), periodic acid-Schiff (*PAS*) reagent, Masson's trichrome staining, and immunofluorescence (using FITC-anti IgG and FITC-anti C3) of the kidneys obtained from the mice at the age of 40 weeks; (**d**) Confocal microscopy examination of CM-DiI-labeled ASCs (Choi et al. 2012)

described in the next section "Genetically engineered ASCs for autoimmune disease."

The efficacy of long-term serial human ASC transplantation was studied in NZB/W F1 mice (Fig. 2.1a), a spontaneous murine model of human SLE (Choi et al. 2012). Human ASCs (5×10^5) were intravenously administered every 2 weeks from the age of 6 weeks until 60 weeks [the total number of human ASCs injected to each surviving mouse of the human ASC-treated group at the end point was 1.4×10^7 (5×10^5 per injection, 28 times)]. Serial ASC transplantation before disease onset significantly improved survival rate (Fig. 2.1b), histopathology of kidneys (Fig. 2.1c), and immunologic abnormalities. ASC treatment also significantly decreased the concentrations of anti-dsDNA antibodies and blood urea nitrogen (BUN) as well as the incidence of proteinuria. Treatment of ASCs significantly increased the levels of GM-CSF, IL-4, and IL-10 in the serum and the proportion of CD4 + FoxP3+ cells in the spleen. Systemically infused ASCs labeled with red fluorescent tracker dye were mostly present in the spleen, with many also being evident in the kidney and liver (Fig. 2.1d). There was little evidence of fluorescent-labeled cells in the lung and heart (Fig. 2.1d).

Clinical Trials of Adipose Tissue-Derived Mesenchymal Stem Cells to Treat Autoimmune Diseases

Autologous ASC transplantations have been performed in patients with refractory autoimmune diseases (Ra et al. 2011). Patients were intravenously administered 1–5 $\times$ 10^8 ASCs around one to six times, with a multiple sclerosis patient receiving additional intrathecal injections (1×10^7 ASCs per injection, three times) and one of rheumatoid arthritis patients receiving additional intraarticular injections (1–1.5 $\times$ 10^8 ASCs per injection, two times). Immunological parameters measured from one patient with polymyositis, and three patients with rheumatoid arthritis showed decrease in inflammatory responses. Further, clinical signs were improved in all patients treated with autologous ASCs. A 19-year-old female patient with autoimmune inner-ear disease, who had severe progressive hearing loss, showed improved hearing, with the hearing in her right ear restored to normal. A 46-year-old woman with multiple sclerosis displayed improved clinical signs as measured by the "expanded disability status scale" (EDSS), which is a method of quantifying disability in multiple sclerosis. A 35-year-old woman with polymyositis, who was unable to walk slope, was able to climb stairs following this treatment. A 50-year-old woman with rheumatoid arthritis showed improved arthritis index, and two other female patients with rheumatoid arthritis, who were unable to stand or walk, were able to do both following ASC treatment; in these two cases, steroid treatment was also discontinued.

Genetically Engineered Adipose Tissue-Derived Mesenchymal Stem Cells for Autoimmune Diseases

Genetically engineered MSCs can further improve the therapeutic effects of MSCs by the overexpression or suppression of the target genes and by providing additional control over disease progression or relapse. MSCs are an ideal vehicle to deliver genes into the target tissues because of their unique biological and immunological properties such as homing activity and immunoprivileged status (Baksh et al. 2004). MSCs have been modified by various non-viral or viral transduction methods. When permanent expression of the therapeutic gene is required, integrating viruses such as lentivirus or retrovirus are preferred because of their capacity for long-term expression. On the other hand, when transient expression of the therapeutic gene is preferred, a non-viral gene delivery system or non-integrating vectors such as adenovirus are the method of choice (Park et al. 2003). Lentiviral systems are advantageous in the treatment of autoimmune diseases by using cell-based gene therapy. First, lentiviral systems can efficiently be applied to a broad range of cell types, including non-dividing, senescent, and terminally differentiated cells (Naldini 1998). Second, lentiviral system has a capacity for long-term expression as described above. Further, transduction with lentiviral vector did not adversely affect the morphology, viability, and differentiation potential of MSCs (McGinley et al. 2011).

As adipose tissues are an alternative source of MSCs, treatment with genetically engineered ASCs that can modulate immune responses was also attempted in experimental animals with autoimmune disease. Choi et al. (2011) studied the effect of ASCs overexpressing CTLA4Ig on the development of experimental autoimmune thyroiditis by using a lentiviral system. Antigenic stimulation of T cells generally requires the presence of two signals provided by an antigen-presenting cell (APC) and cytokines (Gimmi et al. 1993). The first signal is mediated via the T cell receptor/CD3 complex and an antigenic peptide presented by a MHC molecule, and the second signal is mediated via B7:CD28 co-stimulation that induces the proliferation of T cells. Blockade of the B7:CD28 co-stimulatory interactions with soluble CTLA4Ig fusion protein has been shown to inhibit humoral immunity (Linsley et al. 1992), graft rejection (Lenschow et al. 1992), and graft-versus-host disease (Blazar et al. 1994), and to ameliorate

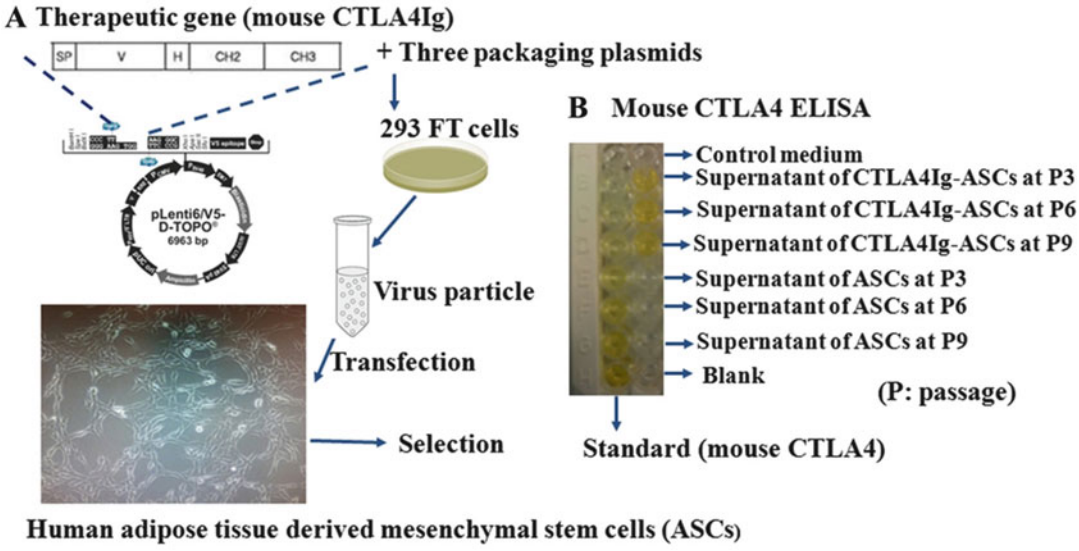

Fig. 2.2 Construction and confirmation of human adipose tissue-derived mesenchymal stem cells transduced with mouse CTLA4Ig. (**a**) Therapeutic gene was composed of the extracellular domain of mouse CTLA4 (V: NM_009 843, 258-629) to inhibit the B7:CD28 co-stimulatory signal and the hinge and CH2-CH3 domains of the human immunoglobulin gamma 1 constant region (H-CH2-CH3: J00228, 503, 892-936, 1055-1384, and 1481-1803) to prolong the half-life of the therapeutic protein in vivo. The human oncostatin M signal sequence (SP: NM_020 530, 53-127) for the secretion to body fluid was ligated to the therapeutic gene. Mouse CTLA4Ig gene transduction into MSC was conducted by ViraPower^{TM} Lentiviral Expression Systems (Invitrogen). The ViraPower packaging mix and Mouse CTLA4Ig-pLenti6/V5 TOPO expression plasmid were co-transfected into the 293FT cell line by using Lipofectamine 2000. Virus particles were collected and transfected to human adipose tissue-derived MSCs. Selection was performed using blasticidin (5 μg/ml). (**b**) To confirm whether CTLA4Ig-transduced ASCs expressed CTLA4 and secreted CTLA4 effectively into the extracellular space, cell culture supernatants were collected to determine the concentration of mouse CTLA4 by ELISA (Choi et al. 2011)

autoimmune diseases (Finck et al. 1994; Choi et al. 2008). Transplantation of ASCs or CTLA4Ig gene-transduced ASCs reduced inflammatory immune response and improved Th1/Th2 balance in experimental autoimmune thyroiditis. Moreover, CTLA4Ig-ASC transplantation showed superior results in reducing the concentration of serum anti-thyroglobulin autoantibodies compared to treatment with ASCs only. Transduction with lentiviral vector in ASCs did not significantly affect the immunophenotype of ASCs, which showed long-term expression of therapeutic gene (Fig. 2.2), These findings suggest that target gene transduction using lentiviral vector system is well matched with ASCs and can elicit therapeutic potential without significantly changing the innate characteristic of ASCs (Choi et al. 2011).

Insertion of inducible regulatory genes within ASCs by non-toxic and effective new gene transfer systems may hold potential as a novel method for immune modulation in autoimmune diseases. By determining the appropriate gene targets for the treatment of autoimmune diseases, stem cell-based gene therapy will be able to facilitate the synergic effect of gene therapy and stem cell therapy. Before application in clinical settings, the genetically engineered cells will have to be extensively tested at the genetic, molecular, and cellular levels.

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Validity of Markers for Epithelial Cells and Mesenchymal Cells

3

Jianyuan Chai

Contents

Abstract

Stem cells are best known for their potential of differentiation. However, those so-called differentiated cells are not exactly lack of plasticity. This can be easily seen during wound healing, in which a lot of epithelial cells transform into fibroblasts or myofibroblasts to repair the tissue damage. Once the wound is healed, these cells can return to their original identity. This is commonly called epithelial-mesenchymal transition (EMT) or vice versa. Normally, these events are highly regulated and coordinated at the molecular level, so that tissues and organs can maintain their normal functions afterwards. However, when these processes are out of control due to various pathological reasons, adverse events like fibrosis can happen, or even worse, cancer cells can also take this advantage and become metastatic. Therefore, it is critical to identify these signs as early as possible, so that corrections can be made when it is necessary. Dozens of molecules have been used as markers to distinguish epithelial cells from mesenchymal cells, while emerging evidence questions their validity. The truth of the matter is each of these markers can be expressed in both groups at certain time point. EMT or vice versa is a progressive and highly dynamic process, and therefore, the molecular phenotype of a cell is volatile. It is the cumulative effect of all these so-called "markers" that finalize the cell identity, epithelial or mesenchymal.

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Introduction

One of the main characteristics of stem cells is cell plasticity, capability to differentiate into various cell types in response to environmental signals. However, those so-called differentiated cells, e.g., epithelial cells, are also capable to transform into other cell types when they receive certain molecular stimulus. Epithelial-mesenchymal transition (EMT) or vice versa MET plays critical roles in embryonic development, wound healing as well as in cancer metastasis. Epithelial and mesenchymal represent two of the main differentiated cell types. The distinctions between these two lines of cells were already established 200 years ago. In principle, epithelial cells are characterized by their: (1) strong intercellular connection; (2) keratin-based cytoskeleton; and (3) clear cell polarity. These cells connect to each other to form single or multiple layers to cover both the external and internal surfaces throughout the body, serving the purposes of protection, absorption, secretion and sensation. In contrast, mesenchymal cells usually have no intercellular connections with each other, contain a highly developed vimentin-based cytoskeleton suitable for locomotion, and have no clear distinction of either apical, lateral or basal side when they are in rest. However, more and more evidence shows that these two types of cells are not as rigid as they are commonly thought. Both epithelial and mesenchymal cells possess some degree of plasticity *in vitro* and *in vivo,* namely, they are interchangeable.

Our knowledge on EMT/MET can be traced back to 1908 when Frank R. Lillie described the phenomenon in his embryological book '*The Development of the Chick*' (Lillie 1908); however, it only became a hot topic approximately 15 years ago when its involvement in chronic progression of fibrotic disorders and cancer metastasis gained recognition (Radisky et al. 2007; Ruiz and Günthert 1996). Now we know that EMT and MET are not only essential in embryonic development and morphogenesis, but they also have been well kept by most vertebrates throughout their adulthood as key mechanisms for wound healing and tissue repair. Although these differentiated cells are not as much versatile as real stem cells, epithelial and mesenchymal cells by inter-conversion can compensate for stem cell shortage in tissue repair. Inadvertently, these beautiful features of differentiated cells are sometimes hijacked by "evil forces" when they are deregulated (Ruiz and Günthert 1996). Fibrosis is a typical example in which EMT is overwhelmed and, as a result, normal tissue structure is replaced by excessive mesenchymal cells and their products (Radisky et al. 2007). Cancer is another example. In this case, some genetically altered epithelial cells have acquired mesenchymal features to move around in our body, invade our healthy organs and break down their normal functions (Ruiz and Günthert 1996).

In order to prevent and treat undesirable incidences associated with EMT or MET, such as fibrosis and cancer, it is often required to detect cell type changes as early and precisely as possible. Through the literature, dozens of molecules have been claimed or used as markers for either epithelial cells or mesenchymal cells, and it happens more often than anticipated that researchers claim EMT or MET when one or two of these markers have been switched. For instance, a change from keratin expression to vimentin expression, or a change from E-cadherin to N-cadherin, is often considered as a gold standard to define EMT by many researchers. However, an increasing number of exceptions raise doubts about the validity of these markers. Where to draw the line? This chapter will evaluate these molecular markers based on *in vitro* and *in vivo* evidence, compare them in epithelial cells versus mesenchymal cells, and provide our advices for those in this line of work.

Epithelial Markers

As discussed above, the most prominent feature of epithelial cells is their cohesion and tendency to form continuous cell layers regardless of *in vitro* or *in vivo*. Adjacent epithelial cells tend to connect to each other through multiple

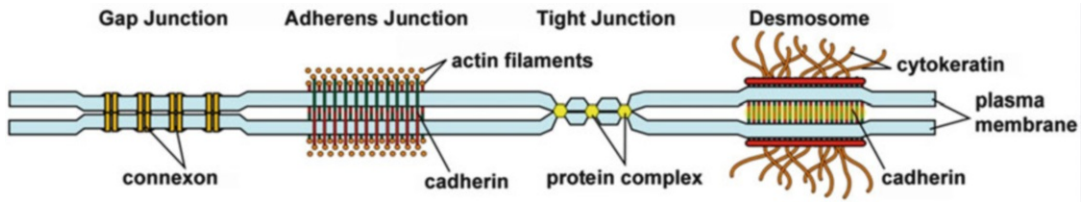

Fig. 3.1 An illustration of different types of intercellular connections

intercellular locks, including Tight Junctions, Adherens Junctions, Desmosomes and Gap Junctions, which further connect to the intracellular cytoskeleton (Fig. 3.1). For this reason, the molecules constituting these membranous structures are commonly used as markers to identify epithelial cells.

Tight Junctions

Tight Junctions (TJ) are located in the most apical lateral regions of epithelial cells and thus they are also indicators for cell polarity. The main components of a TJ include transmembrane proteins Claudin (23 members known in human), Occludin, Junctional Adhesion Molecule (JAM-A, -B, and -C) and intracellular adaptor protein Zona Occludin (ZO-1, -2, and -3) that connects a TJ to the actin cytoskeleton (Balda and Matter 2009). These proteins together form an impermeable barrier to prevent ions and molecules to pass through intercellular space, so that the only way for them to enter tissue is to pass through epithelial cytoplasm, usually by diffusion or membrane transporters.

However, an increasing amount of evidence shows that these molecules are not always unique to epithelial cells. For instance, Claudin-5 has been identified in cardiomyocytes (Sanford et al. 2005), JAM-A and JAM-C have been reported in fibroblasts derived from various tissues including derma, lung, cornea, and embryo (Morris et al. 2006), and ZO-1 is not only associated with cell membrane of corneal fibroblasts, but it can also translocate to the nucleus to serve as a transcription cofactor in case of corneal injury (Benezra et al. 2007). Our study showed that, at the transcriptional level, all TJ components can be detected in fibroblasts (Chai et al. 2010a). Moreover, the level of TJP1, the gene coding for ZO-1, is highly expressed in colon fibroblasts. These molecules can also be detected in both epithelial and mesenchymal cells at the translational level, although they are usually more localized to the membrane in epithelial cells, compared to mesenchymal cells (Fig. 3.2).

Adherens Junctions

Adherens Junctions (AJ) are specialized subapical structures that form stable cell-cell contacts in essentially all types of tissue (Niessen and Gottardi 2008). Molecules that constitute an AJ include transmembrane protein E-cadherin and intracellular adaptors α-, β-, and δ-catenin (p120), which in turn provide anchorage to the actin cytoskeleton.

Among AJ components, all three catenins (α, β, and δ) have been documented in fibroblasts (Gurung et al. 2009). As shown in Fig. 3.3, both β- and δ-catenin (p120) are expressed at considerable levels in fibroblasts and localized predominantly to the cell membrane like epithelial cells, although nuclear and cytoplasmic expression are also very common, in which case, they might play regulatory roles in gene transcription. E-cadherin is not unique to epithelial cells either. E-cadherin expression is commonly considered a hallmark of epithelial cells, because its expression is mainly controlled at the transcriptional level and it is normally inactivated in mesenchymal cells (van Roy and Berx 2008). Modulation of E-cadherin expression levels has been vastly used as a key theme of epithelial plasticity and cancer metastasis. However, E-cadherin expression is still

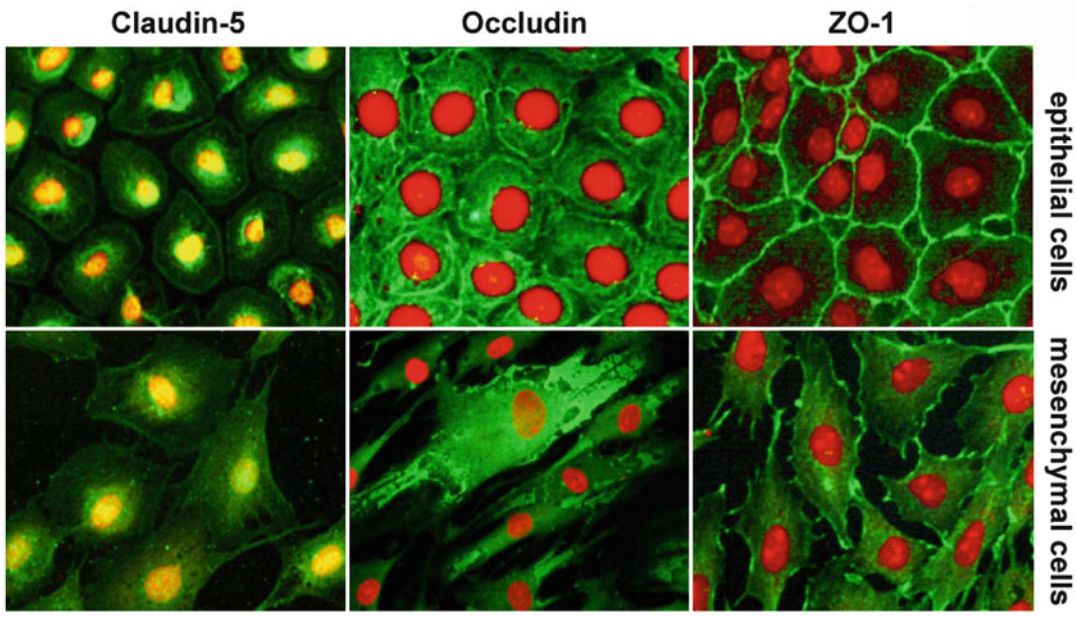

Fig. 3.2 Comparisons of molecular components (FITC-labeled) of Tight Junctions in epithelial cells versus fibroblasts. Nuclei are counterstained with propidium iodide

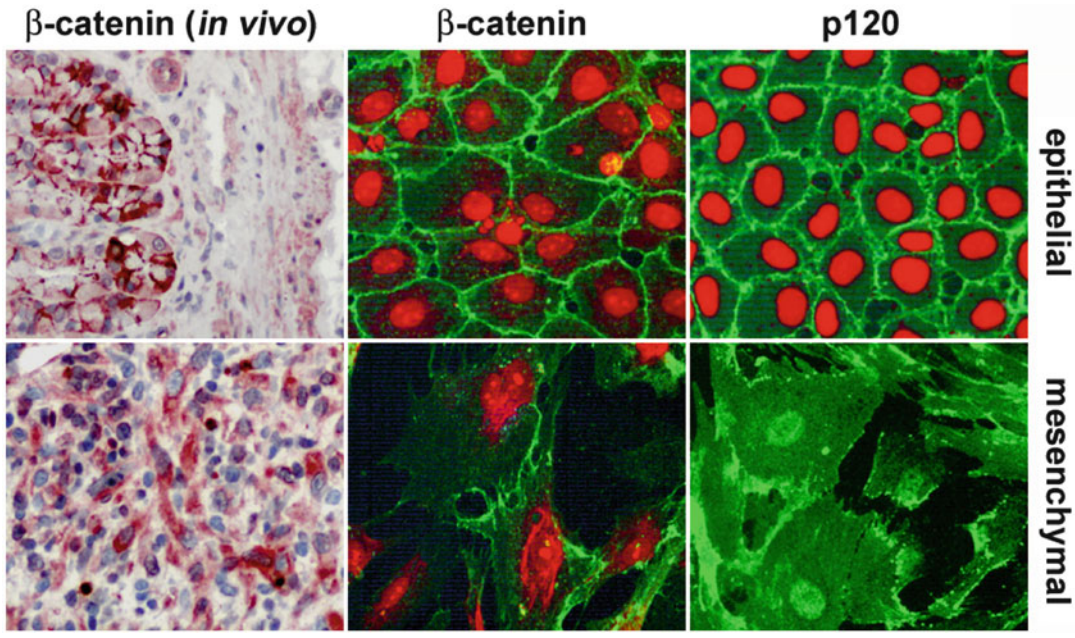

Fig. 3.3 Comparisons of molecular components of Adherens Junctions in epithelial cells versus fibroblasts. *In vivo*, β-catenin is expressed in both epithelial cells (gastric mucosa) and fibroblasts (submucosa). *In vitro*, catenins are labeled with FITC and nuclei are counterstained with propidium iodide

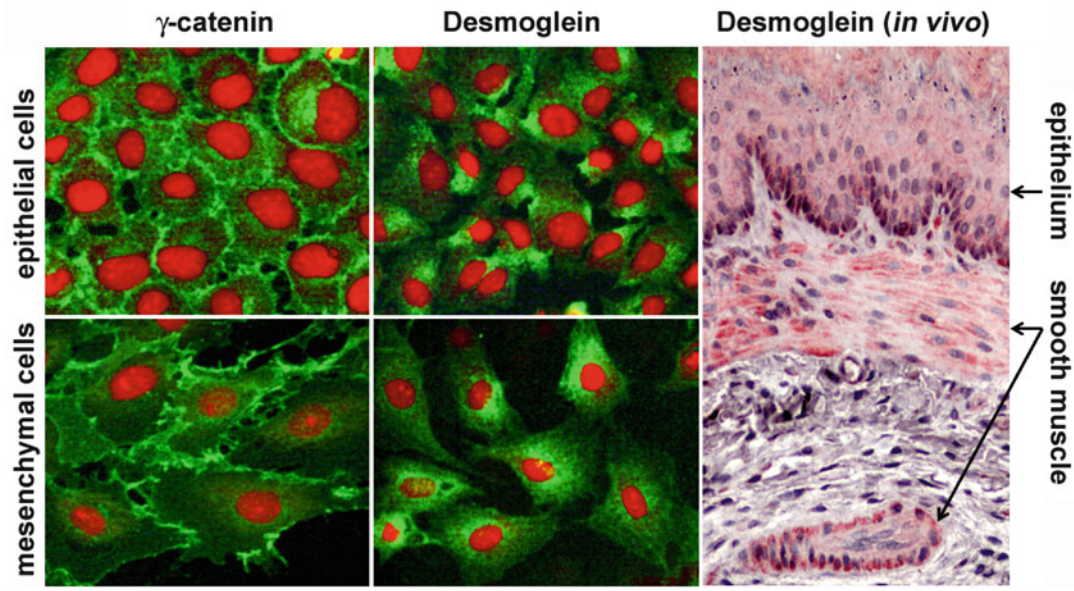

Fig. 3.4 Comparisons of molecular components of Desmosomes in epithelial cells versus fibroblasts. *In vitro*, these components are labeled with FITC and nuclei are counterstained with propidium iodide. *In vivo*, Desmoglein is expressed in both epithelial cells (gastric mucosa) and smooth muscle cells (muscularis propria and blood vessels)

maintained in most of differentiated tumors, including carcinomas of the skin, head and neck, esophagus, breast, lung, liver, colon and prostate. Furthermore, our study showed that E-cadherin is not only expressed in rat embryonic fibroblasts, but its level in these cells is consistently higher compared to rat gastric epithelial cells (Chai et al. 2010a). As shown in Fig. 3.6, E-cadherin expression can also be found in smooth muscle cells sometimes.

Desmosomes

Desmosomes are like buttons joining the lateral edges of adjacent epithelial cells through cadherin molecules such as Desmoglein and Desmocollin, which link with cytokeratin fibers through desmosomal plaque proteins such as Desmoplakin and Plakoglobin (γ-catenin).

All of the desmosome proteins have been reported in fibroblasts of periodontal ligament (Yamaoka et al. 1999) and dental pulp (Sawa et al. 2005) or other non-epithelial cells, and are also very flexible in selection of cytoskeletal partners. For example, Desmoplakin interacts with cytokeratin filaments in epithelial cells, but can also bind to vimentin in fibroblasts (Stappenbeck et al. 1993) and to desmin in cardiomyocytes (Kartenbeck et al. 1983). Desmoglein expression can be found in fibroblasts as well as smooth muscle cells, with no clear distinction in localization patterns between epithelial and mesenchymal cells. Like other catenins, γ-catenin is not only highly expressed in mesenchymal cells, but also predominantly localized to the plasma membrane in both cell types (Fig. 3.4).

Gap Junctions

Gap Junctions are channel-like structures each composed of two connexons which connect across the intercellular space and regulate trafficking of small molecules (<1 kDa) between adjacent cells. Each connexon is a pore through the cell membrane and is formed by a ring of six connexin proteins (>20 isoforms identified). Connexins are expressed by virtually all types of cells, including fibroblasts, except sperms

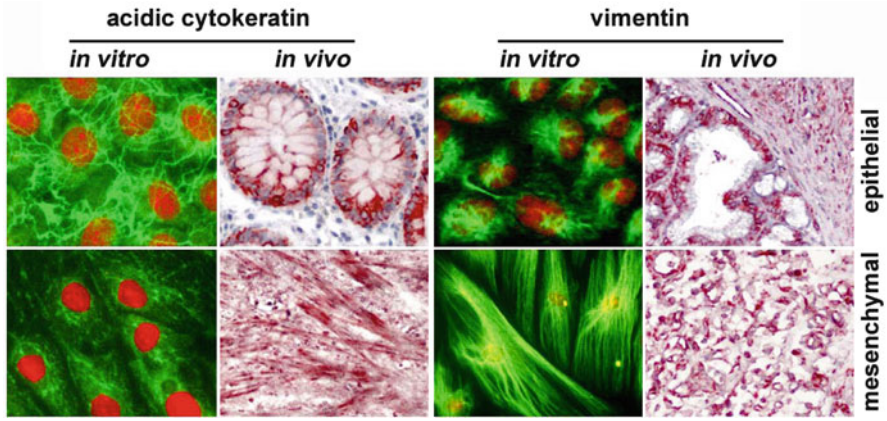

Fig. 3.5 Comparisons of intermediate filaments in epithelial cells versus mesenchymal cells. Keratins are found at low levels in fibroblasts but at higher levels in smooth muscle fibers *in vivo*. On the other hand, vimentin is found in both epithelial cells and fibroblasts *in vitro* and *in vivo*. *In vitro*, these molecules are labeled with FITC and nuclei are counterstained with propidium iodide. *In vivo* expressions are shown in epithelial cells of gastric glands and in smooth muscle cells and fibroblasts of submucosa

and erythrocytes (Li et al. 2003). In our study, Connexin-43 (GJA1) expression was found higher in some mesenchymal cells than in epithelial cells (Chai et al. 2010a).

Cytokeratins

Cytokeratins are intermediate filament proteins commonly found in epithelial cells. There are at least 20 different isoforms, which are generally classified in two groups: the low weight, acidic type I keratins (CK1-9) and the high weight, basic or neutral type II keratins (CK10-20). Inside of cells, these two types of keratins exist as heterodimers and their different combinations usually determine the subtypes of the epithelium. For instance, CK4 pairs with CK13 in esophageal squamous epithelium, while CK7 is usually in partnership with CK20 in intestinal columnar epithelium. For this reason, expression of CK7 or CK20 in esophageal epithelium is considered as a sign of metaplastic transformation – Barrett's esophagus. When an epithelium undergoes malignant transformation, the keratin profile tends to remain stable; therefore, their expression patterns are commonly used as markers to identify different types of epithelial malignancies. However, keratins have been also found in non-epithelial cells, including fibroblasts (Traweek et al. 1993), endothelial cells and smooth muscle cells (Jahn et al. 1987), and cardiomyocytes (Huitfeldt and Brandtzaeg 1984). In our study, we have detected low level of keratins *in vitro* in fibroblasts and sometimes *in vivo* in smooth muscle fibers (Fig. 3.5).

Mucins

Mucins are glycoproteins produced and mostly secreted by epithelial cells to protect themselves against pathogens from outside. At least 19 isoforms have been found in human, some of which are transmembrane proteins, for example, Mucin-1. Mucin-1 is localized to the apical side of the epithelium and its cytoplasmic domain binds to the actin cytoskeleton, and therefore Mucin-1 is also used as an epithelial polarity marker. However, Mucin-1 is also expressed in non-epithelial cells such as myofibroblasts (Kamoshida and Tsutsumi 1998). We also reported a higher expression of Mucin-1 in some fibroblasts (Chai et al. 2010a).

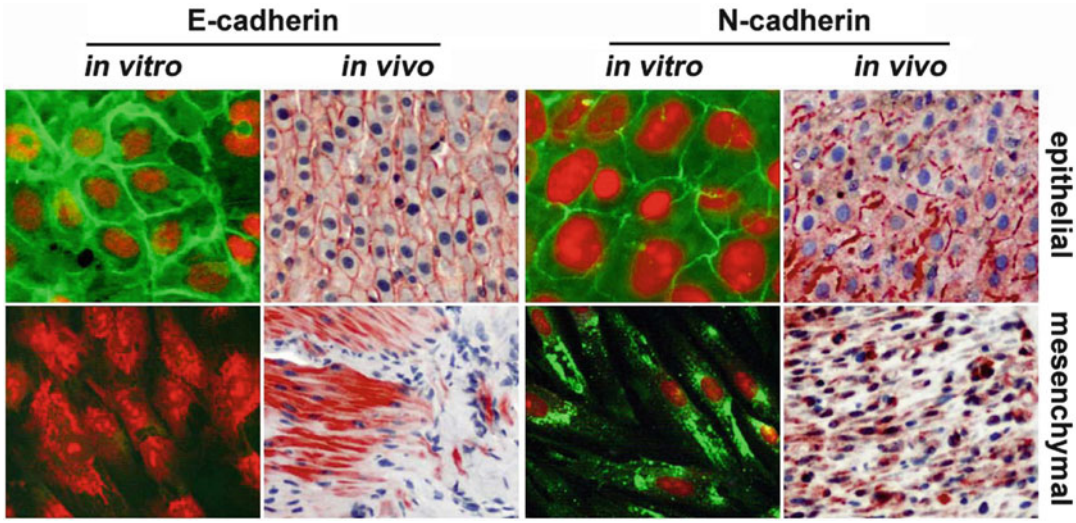

Fig. 3.6 Comparisons of E-cadherin and N-cadherin expressions in epithelial cells versus mesenchymal cells. *In vitro*, the target proteins are labeled with FITC and nuclei are counterstained with propidium iodide. *In vivo*, both E-cadherin and N-cadherin are identified in liver cells, and E-cadherin is also found in smooth muscle cells

Mesenchymal Markers

For mesenchymal cells, as Hay once said (Hay 2005), "There are, in fact, no specific biochemical markers by which we can define the mesenchyme". However, in contrast to epithelial cells, mesenchymal cells are known for having advanced cytoskeletal structure, high motility, and for producing more extracellular matrix proteins. Naturally, the main contributors to these features are also commonly used as mesenchymal markers in studies, including structural proteins (e.g. N-cadherin, Vimentin, etc.), cytosolic/secretive proteins (e.g. S100A4, Fibronectin, etc.), and extracellular proteins (e.g. fibroblast growth factors, collagens, periostin, metalloproteinases, etc.).

N-Cadherin

N-cadherin is normally found in neural tissue, retina, endothelial cells, fibroblasts, osteoblasts, mesothelium, myocytes, limb cartilage, oocytes, spermatids and Sertoli cells. Switch from E-cadherin to N-cadherin expression in epithelial cells is often considered a sign of EMT (Wheelock et al. 2008). While this switch is an integral part of several processes during normal development, aberrant expression of N-cadherin by cancer cells contributes to their invasiveness and metastasis in various tissues, including breast, thyroid, bladder, prostate and pancreas (Hazan et al. 2000), making this a widely used marker for EMT and cancer studies.

However, several types of epithelial cells express N-cadherin naturally (Fig. 3.6) and the E/N switch in cancer cell invasion is not always followed. For example, N-cadherin is required to maintain corneal limbal epithelial progenitor cells (Higa et al. 2009). Moreover, ovarian surface epithelial cells normally express N-cadherin, but switch to E-cadherin during progression to the neoplastic state (Patel et al. 2003), suggesting that an N/E switch, rather than E/N switch, might play a role in the initiation of ovarian carcinogenesis (Hazan et al. 2000). On the other hand, N-cadherin and E-cadherin are concurrently expressed in the epithelial cells of intra-hepatic bile ducts in normal liver (Mosnier et al. 2008).

Vimentin

Vimentin is an intermediate filament protein that has been widely used as a molecular marker for

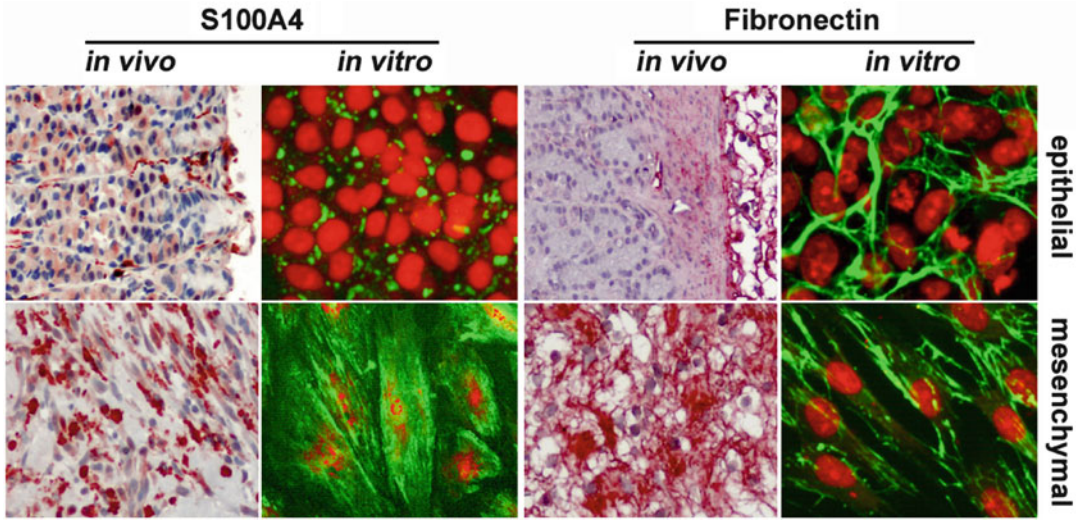

Fig. 3.7 Comparisons of S100A4 and Fibronectin expressions in epithelial cells versus fibroblasts. *In vitro*, the targets are labeled with FITC and nuclei are counterstained with propidium iodide. *In vivo*, gastric mucosa and submucosa are shown

mesenchymal cells, in opposite to keratins for epithelial cells (Fig. 3.5). However, vimentin expression has been found in ocular epithelial cells together along with cytokeratin (FitzGerald 2009). In addition, numerous studies have shown that vimentin can also be expressed in epithelial cells involved in physiological processes requiring epithelial cell migration, such as placentation and wound healing. As shown in our previous study (Chai et al. 2010b), during ulcer healing, some epithelial cells at the ulcer margin acquire a transit expression of vimentin, and once the wound is closed, vimentin signal in the epithelium disappears, suggesting a reversible EMT event.

S100A4

S100A4, also known as fibroblast-specific protein (FSP1), was once described as an absolute marker for fibroblasts (Strutz et al. 1995). Any cells that express S100A4 protein and/or show S100A4 promoter activity were classified as fibroblasts. However, more and more evidence shows that S100A4 is naturally expressed in various cell types, including blood cells, platelets, endothelial cells, smooth muscle cells, cardiomyocytes, astrocytes, and biliary epithelial cells (Schneider et al. 2008). Its elevation is usually associated with cell motility. Apparently, wherever cell migration is required, such as wound healing (Schneider et al. 2007), angiogenesis (Ambartsumianet al. 2001) and cancer metastasis (Boye and MÃ¦landsmo 2010), S100A4 is activated. As shown in Fig. 3.7, S100A4 expression can be detected in epithelial cells both *in vitro* and *in vivo* at considerable levels, although still lower than it in fibroblasts.

Fibronectin

Fibronectin exists in two forms, plasma fibronectin and cellular fibronectin. Plasma fibronectin is synthesized by hepatocytes and represents about 1% of serum protein, while cellular fibronectin is made by many different cell types, including fibroblasts, chondrocytes, endothelial cells, macrophages, as well as certain epithelial cells (Walia et al. 2004). The protein is deposited in the extracellular matrix as highly insoluble fibronectin filaments. As shown in Fig. 3.7, fibronectin exists in both epithelial and mesenchymal cells in culture, but in vivo, it is heavily accumulated in connective tissue and none in mucosal epithelium.

Extracellular Proteins

Mesenchymal cells are also known to produce more extracellular proteins such as collagens and metalloproteinases (MMPs), although epithelial cells also have this capability.

Collagens are the main component of connective tissue and also the main protein of the extracellular matrix that supports other tissues. They are primarily synthesized by fibroblasts. Among 29 types of collagens known so far, type I collagen is the most abundant one in the human body. As shown in our previous study (Chai et al. 2010a), this type of collagen was expressed drastically higher in mesenchymal cells than in any epithelial cells.

MMPs include over 25 proteases, either secreted or membrane-associated, that rely on metal ions for their catalytic activity. Collectively MMPs are capable of degrading any kinds of extracellular matrix components, therefore, their expression level is critical to embryonic development, tissue remodeling and cancer metastasis. Mesenchymal cells are the main source of MMPs, and for this reason, expression of MMPs in epithelial cells is often considered an indicator of EMT (Laffin et al. 2008). Most MMPs are tightly regulated at the transcriptional level. As shown in our previous study (Chai et al. 2010a), MMP2 (gelatinase A) was barely detectable in epithelial cells, but MMP9 (gelatinase B) was highly expressed in some epithelial cells.

Closing Remarks

First of all, EMT or MET is a continuous and dynamic process taking place in our system. Epithelial and mesenchymal represent two poles of this process, and there are numerous intermediate states or subtypes of cells existing in between. It is the cumulative effect of different expression of multiple genes that ultimately sets these two phenotypes apart. Therefore, it is almost impossible to draw an arbitrary solid line to separate epithelial from mesenchymal at any moment of life. For this reason, researchers are advised to examine as many markers as possible before claiming EMT or MET.

Second of all, those commonly used markers are all detectable in both epithelial and mesenchymal cells at certain point and, while some differences in expression and/or localization exist, nothing is consistent enough to be called a universal marker. However, that is not to say they should be abandoned from study. Although none of the markers exhibit exclusive epithelial or mesenchymal expression, based on the literature and our study, some of them are better than others. For epithelial cells, keratins appear to be the first choice, and E-cadherin can be the second. Since there are at least 20 different keratin isoforms and only a few of them are expressed in a given type of epithelial cells, researchers are advised to choose carefully. Catenins should not be used alone to identify epithelial cells. For mesenchymal cells, MMP2 would be the preferred marker, COL1A1 would be next. However, these are secreted molecules, and thus unsuitable for cell labeling. Fibronectin and S100A4 can be added to confirm the cell type. Vimentin and N-cadherin should not be used alone to define mesenchymal cell identity.

Finally, multiple molecules involved in EMT can be envisioned as targets of anti-EMT therapy to prevent or restrain invasion and metastasis of cancer cells. Given the complexity of the molecular and cellular pathways leading to EMT, a forced stimulation of MET can be a very neat approach to control EMT. In order to achieve this, however, it is imperative for us to gain a deeper understanding of this dynamic process through a detailed characterization of its different steps and components. Our study here provides ample data in that direction.

Acknowledgements This work was supported by the Department of Veterans Affairs of the United States.

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Mesenchymal Stem Cell Survival in Infarcted Myocardium: Adhesion and Anti-death Signals

4

Woochul Chang, Byeong-Wook Song, and Ki-Chul Hwang

Contents

Abstract

Mesenchymal stem cells (MSCs) possess the unique potential for use in cell-based therapy of heart diseases, especially in ischemic heart disease. The therapeutic ability of MSCs in myocardial regeneration can give rise to differentiate into cardiac tissue and to release the paracrine factors. However, advancement in MSC therapy is hindered by the poor viability of the transplanted cells due to harmful microenvironments like ischemia, inflammation and/or anoikis in the infarcted myocardium. Recently, many kinds of approaches have been developed in an effort to improve the survival of engrafted MSCs through *ex vivo* manipulation of MSCs, including genetic modification, pretreatment, preconditioning of MSCs. This chapter will discuss various approaches in MSCs for cardiac repair and summarize the current literature in the field.

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Introduction

Despite development of effective therapeutic strategies for heart failure, ischemic insult is still a main cause of myocardial death throughout the world. Myocardial infarction (MI), also called cardiac ischemia, leads to promote the associated death of functional cardiomyocytes and the inadequate situation of remaining cells. The resultant scar formation causes mechanical

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dysfunction, electrical uncoupling, loss of cardiac function, and ventricular remodelling (Segers and Lee 2008). Contemporary pharmacologic and interventional strategies commonly used for myocardial repair, whereas these treatments fail to improve function of heart with end-stage disease. The recent finding that a partial population of cardiac or skeletal muscle cells is possible to proliferate is proposed, however it is difficult to replace cardiac mass or myocardial scar area after MI (Anversa and Kajstura 1998). Unlike the above approaches, stem cell-based therapy has the therapeutic potential to improve cardiac function by proliferating the host cells and differentiating the injected cells because of their properties such as plasticity, the ability to transdifferentiate into multiple lineages, self-renewal and fusion with resident cardiomyocytes (Husnain and Ashraf 2005) (Table 4.1).

Among the different types of stem cells, mesenchymal stem cells (MSCs) are recognized as the best potential candidates for cell therapy for heart diseases. MSCs isolation from bone marrow was first described by Friedenstein and coworker in 1968 (Friedenstein et al. 1968). These cells have their beneficial properties, such as easy isolation, rapid expansion in vitro and rare formation of teratomas. Indeed, several studies have focused on inducing regeneration and protection in infarcted myocardium. However, some limitations exist that (i) transplanted MSCs are difficult to maintain and to generate in MI zone; (ii) The poor survival of the transplanted stem cells is another limitation to the therapeutic efficacy; (iii) The majority of implanted cells undergo apoptosis and/or necrosis within ischemic myocardium because of exposure to the poor environment. To overcome these limitations, various strategies, strengthening the cell adhesion and the anti-death signal, have been adopted to improve stem cell survival/number in the infarcted heart. While these approaches have shown promising results, more research is still necessary. This chapter will focus on the enhancing the MSC adhesion and survival after transplantation, and suggest the future directions to the therapeutic potential of modified MSCs for heart disease (Table 4.2).

Overview of Cell Therapy for Cardiac Repair

Stem cell-based therapy is a relatively new frontier against myocardial ischemia, cardiac dysfunction, or its combination. This therapy has the potential to improve cardiac function by enhancing angiogenesis, surviving the host cells, and protecting the myocardium (Fraser et al. 2004). More recently, the various cell types were used for cardiac repair. For example, bone marrow cells (BMCs), bone marrow-derived mononuclear cells (BMMNCs), hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs) and embryonic stem cells (ESCs) have been employed as a therapeutic source in ischemic heart disease (Fraser et al. 2004). Each cell type has its own strength and weakness for use in cell-based therapy.

ESCs are the prototypical cells in all three primary germ layers, ectoderm, mesoderm and endoderm, derived from the inner mass of the blastocyst. This cell has special property, including pluripotency, self-renewal capacity and high expansion of cell number. Many reports have demonstrated that ESCs can be differentiated into cardiomyocytes or cardiac precursor cells. However, there is no human trial using ESCs for cardiac repair because of teratoma formation, inmmunological incompatibility and considerable ethical concerns. Recently, inducible pluripotent stem (iPS) cells, which have autologous and ESC-like capacity, and are able to apply patient-specific therapy, have been minutely studied ever since their invention. However, this cell type also is restricted by unregulated tumor formation, inadequate conversion and persistent ectopic gene expression (Nelson et al. 2010).

Bone marrow (BM) is composed of heterogeneous populations, such as HSCs, MSCs, EPCs and other cell types. Among these BM-derived cells, BMCs, or BMMNCs, were vastly shown to replace cardiomyocytes in infarcted myocardium during preclinical trial. When BMCs or BMMNCs were transplanted directly or injected by intracoronary infusion, it was not only demonstrated improvement of left ventricular function, but also showed increase of cell

Table 4.1 Therapeutic approaches using manipulated MSCs for cardiac disease

Types	Target	Delivery	Cell number	Host	Efficacy	References
Gene overexpression	Akt	Retrovirus, Conditioned media	5×10^6	Rat	infarct↓, inflammation↓, collagen↓, cardiac hypertrophy↓, paracrine↑, heart function↑	Mangi et al. (2003), Gnecchi et al. (2006)
	Bcl-2	jetPEI transfection	6×10^6	Rat	cell survival↑, paracrine↑, engraftment↑, microvessel↑, scar size↓, heart function↑	Li et al. (2007)
	CXCR4	Adenovirus	2×10^6	Rat	migration and differentiation↑, capillary and vascular density↑, fibrosis↓, heart function↑	Zhang et al. (2008)
	ILK	Lentivirus	1×10^6	Rat	cell viability↑, survival protein↑, apoptotic signaling↓, infarct↓, fibrosis↓, apoptotic index↓ microvessel↑	Song et al. (2009)
	Hsp20	Adenovirus	1×10^6	Rat	cell viability↑, survival↑, fibrosis↓, vascular density↑, heart function↑, paracrine↑	Wang et al. (2009)
	PI3K-C2α	Lentivirus	1×10^6	Rat	viability↑, survival protein↑, apoptotic signaling↓, infarct↓, fibrosis↓, microvessel↑, heart function↑	Eun et al. (2010)
	tTG	Lipofectamine PLUS transfection	1×10^6	Rat	cell attachment, spreading and migration↑, ex vivo attachment↑, adhesion-related signaling↑, fibrosis↓, heart function↑, differentiation and integration potential↑	Song et al. (2007)
Pretreatment	FGF-2, IGF-1, BMP-2	Growth factor	1×10^6	Rat	cardiac marker and cytoprotection↑, infarct↓, heart function↑, arrythmia↓	Hahn et al. (2008)
	Hsp70	PTD	1×10^6	Rat	viability↑, apoptotic signaling↓, infarct↓, fibrosis↓, microvessel↑, heart function↑, differentiation and integration potential↑	Chang et al. (2009)
	hypoxia	Preconditioning	1×10^6	Rat	pro-survival and pro-angiogenic factor↑, cell death↓, heart function↑	Hu et al. (2008)
	SDF-1α	Preconditioning	5×10^5	Rat	cell apoptosis↓, proliferation↑, paracrine↑, homing↑, blood vessel↑, fibrosis↓, heart function↑	Pasha et al. (2008)

engraftment and cardiac differentiation. These efforts led to human clinical trials of autologous BMCs or BMMNCs. However, other investigation found some limitations including undefined functional fate and electrophysiology (Husnain and Ashraf 2005).

Similarly, EPCs, have CD34 positive antigen, are derived from BM or peripheral blood. These cells have been represented to mobilize into peripheral blood for improvement of cardiovascular disease. It is an advantage to induce neovasculogenesis through vessel regeneration

Table 4.2 Potential strategies for enhancing survival of MSCs

Effector	Related factors	Function	References
Anoxic preconditioning	Bcl-2 and Bax	Cell survival and angiogenesis for cardioprotective effect	Li et al. (2008)
Differentiation to a cardiac or an endothelial phenotype	TGF-β1, BMP, Wnt, and so on	Prosurvival signaling, proangiogenic effects, and cardiomyogenic differentiation	Chavakis et al. (2010)
Trophic activity	VEGF, HGF, IGF-2, and SDF-1	Non-invasive stem cell therapeutic approach, improvement of ventricular function and attenuation of apoptosis and fibrosis	Paul et al. (2009)
Anti-oxidant	HO-1, SOD, catalase, GPx, and NAC	Decreased apoptosis, increased cell viability, adhesion, and induction of survival pathway	Song et al. (2010a, b), Andreadou et al. (2009)
Paracrine factors	SFRP, HGF, IGF-1, VEGF, TGF-β1, SDF-1, FGF-2, MMP, IL-6, and so on	Cardiac repair, endogenous regeneration, cytoprotection and neovascularization	Gnecchi et al. (2008)

BMP bone morphogenetic protein, *HO-1* heme oxygenase-1, *SOD* superoxide dismutase, *GPx* glutathione peroxidase, *NAC* N-acetyl-cysteine, *SFRP* Secreted frizzled-related protein, *MMP* Matrix metalloproteinase

and remodeling, and enable small-scale clinical trials. However, EPCs also show their clinical boundaries such as rare population, heterogeneity and diminution with atherosclerosis and age.

MSCs represent a subset of stem cells that exist in the stroma of bone marrow, and peripheral and umbilical cord blood. MSCs possess plasticity, self-renewal, less immune rejection, and secretion of paracrine factors (Satija et al. 2009). Recent reports demonstrated that MSCs are able to differentiate into various cell types, including adipocyte, osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, and other mesenchymal phenotypes. These potentials enable various cell therapies to use MSCs.

Many studies reported that MSCs were used for gene delivery, cell implantation and tissue engineering. In preclinical studies using MSC therapy, post infarct animals demonstrated reduction of infarct size and improved regional and global left ventricular function. Furthermore, a clinical study of MSCs is successfully enforced in post-infarct patients. Difficulties of MSCs may arise, however, because of negative influences, i.e. anaerobic condition, increase of apoptotic factors, and inflammatory response resulting from oxidative stress, in ischemic myocardium (Segers and Lee 2008). There also remains significant heterogeneity of undifferentiated MSCs in MSC-transplanted myocardium. These causes resulted in therapeutic limitations, including poor viability and/or lower adhesion strength of cells. It has been reported that ~0.5% of transplanted human MSCs exist at 4 days after cell transplantation in ischemic murine heart (Toma et al. 2002). This section will be focused on the current knowledge of MSC therapy strengthening the specific function in the ischemic heart. And it will also be discussed the major challenges to MSC therapy toward heart regeneration.

Cause of Cell Death in the Infarcted Region

Three major reasons that lead to MSC death during cell transplantation are representative (Robey et al. 2008). Although pro-survival modification for successful therapy is currently studying in several studies, they do not solve the ultimate problems of poor adhesion of stem cells to the cell-matrix. The death of implanted MSCs in ischemic heart may begin when cells are detached from culture dish in in vitro. The first stress that is defined in cell biology as a

process of programmed cell death induced by loss of matrix attachment is produced during the engraftment process. This circumstance is called 'anoikis' (Michel 2003). At engrafted site, adhesion to structural glycoproteins of the extracellular matrix (ECM) by cross-linking is necessary for survival of adherent cells in the cardiovascular system. Adhesion of cells to the matrix, predominantly via integrin molecules, generates an endogenous tensile stress within the cells, called tensegrity. This integrity involves matrix, focal adhesion, integrin/cytoskeleton interaction, and folding of intracellular proteins associated with cytoskeleton (Michel 2003). Transplanted MSCs may undergo low tensegrity to adhere to myocardium via the loss of matrix anchorage, involving the effect of PI3K/Akt on focal adhesion and integrin-linked kinases (FAK, Shc, and ILK), obstruction of Raf-ERK signalling and jun N-Terminal kinases, bcl-2 repression, FAS ligand, and caspase activation.

The next potential initiator is ischemia (Segers and Lee 2008). Once MSCs are implanted to the infarcted zone, they meet severe cardiomyocytes exist in ischemic condition (e.g., the deprivation of nutrients and oxygen, and inflammation). This condition has an effect on engrafted MSCs in the ischemic area, following ATP depletion, activation of anaerobic glycolysis, and dysfunction of calcium and other ionic homeostasis (Robey et al. 2008). Ischemia also can lead to reactive oxygen species (ROS) formation, which is intensifying the anoikis signals in implanted MSCs (Song et al. 2010a). The ROS, such as superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), and others, are common by-products of many oxidative biochemical and physiological processes. When excessive ROS and oxidative stress are generated in ischemic myocardium, the inner mitochondrial membrane potential is damaged and this stress may induce apoptosis. In recent years, mitochondria have been recognized as the fate modulator via cell death as well as survival. In myocardial ischemia-reperfusion injury, intracellular Ca^{2+} and ROS are increased within the cytoplasm and mitochondria. Under these conditions of Ca^{2+} overload by oxidative stress and ATP depletion, mitochondria undergo a permeability transition that is associated with the formation of a non-specific permeability transition pore (PTP) in the mitochondrial membrane. During continued ischemic-reperfusion condition, the mitochondrial membrane depolarization induces further ROS production and ATP hydrolysis, and then cell death will occur by caspase activation via production of pro-apoptotic protein (Andreadou et al. 2009). ROS can induce an inflammatory response, and prevent cell adhesion, leading to cell death.

The last insult of injury is inflammatory response. Moderate inflammatory response may be essential for promoting angiogenesis and progenitor-cell recruitment, but excessive inflammation may also block the recruitment and survival of progenitor and/or implanted cells (Segers and Lee 2008). After MI, the necrotic cells generate strong inflammatory response to recruit neutrophil and monocytes/macrophage through chemokines and cytokines. These inflammatory cells produce oxygen-derived free radicals, inducing apoptosis and excessive fibrosis as well as inactivating cytoprotective nitric oxide (Chan et al. 2009). These inflammatory progresses are most severe in acute MI, and also have negative effects on implanting cells ratio independent of myocardial ischemia. However, MSCs have a beneficial effect in the allogeneic cell transplantation because of their immunomodulatory effects on inflammatory cells (Ryan et al. 2005).

Improving Therapeutic Potential of Transplanted MSCs

Many preclinical studies have demonstrated beneficial effects of MSC transplantation from small to large animal models of heart disease. Orlic et al. (2001) represented that $lin^-/c\text{-}kit^+$ bone marrow cells including MSCs was new myocardium occupied 68% of the infarcted zone for 9 days after stem cell transplantation. Although a limited number of MSCs was survived in injected site, engraft cells were expressed myogenic marker resembled the surrounding host

cardiomyocytes and left ventricular (LV) functions, LV end-diastolic pressure (LVEDP) and developed pressure (LVDP), were improved in transplantation group compared to sham-operated mice (Orlic et al. 2001). To find the injected MSCs, xenotransplantation using human mesenchymal stem cells labelled with *lacZ* was performed in CB17 SCID/*beige* adult mice. At 1 week after injection, a limited number of MSCs was survived in injected site, and engrafted cells were expressed myogenic marker resembled the surrounding host cardiomyocytes.

In fact, it is interesting now to modify combining functional genes or altering chemokine and growth factor in pre-clinical study with cell therapy approach to improve specific function, cell survival and/or adhesion. Modified cell therapy can be generalized into two approaches. First, MSCs can be engineered to overexpress a gene product or to activate specific signal, namely it modifies the biology of the injected cell, strengthening the effects of the cell itself. Second, MSCs can be used as a passive carrier, which introduced the specific gene, express and secrete cytokines and/or chemokines that beneficially alter the cell-injected myocardium (Penn and Mangi 2008). In the first approach, strategies of strengthening the MSC function can be categorized as follows: (i) pretreatment with growth factors or cytokines; (ii) preconditioning such as hypoxia; and (iii) genetic modifications to overexpress anti-death or adhesion signals (Song et al. 2010a, b). Chemokinetic pretreatment has been investigated successfully in MSCs and shows cytoprotective effects on cardiomyocytes and ischemic myocardium. The combination of growth factors including fibroblast growth factor (FGF)-2, insulin growth factor (IGF)-1 and bone morphogenic protein (BMP)-2, has recently been tested in an effort to improve therapeutic efficacy of MSC implantation (Hahn et al. 2008). Preconditioning of SDF-1, a member of the chemokine CXC subfamily, also suppresses MSC apoptosis, enhances their survival, engraftment, and vascular density, and improves myocardial function (Pasha et al. 2008). Heat-shock protein (Hsp) is molecular chaperone that it is triggered as a response to stress. Our study also demonstrated that transplantation of Hsp-70-pretreated MSCs using the Hph-1 protein transduction domain (PTD) leads to a decrease in area of both the fibrotic and apoptotic myocardium, but to improve LV function (Chang et al. 2009). The hypoxic- and anoxic preconditioning are stimulating the naive MSC. These effects were considered in the context of simulating hypoxia and anoxic exposition studies *in vitro* or the microenvironment *in vivo* in cardiovascular disease model system. When MSCs are exposed to 24 h of hypoxic preconditioning *in vitro*, the prosurvival, proangiogenic, and functional proteins are expressed (Hu et al. 2008). Moreover, anoxic preconditioning of MSCs improved cardiac function through the antiapoptotic effect and remodeling capacity in diabetic cardiomyopathy (Li et al. 2008).

Paracrine effect of MSCs has been investigated in many studies. It has been shown that MSCs produce and secrete a variety of cytokines, chemokines, and growth factors. Furthermore, hypoxic stress from ischemia increases generation of bioactive factors. Paracrine actions from MSCs include cardioprotection, neovascularization, and cardiomyogenesis (Gnecchi et al. 2008). During hypoxic preconditioning, MSCs can generate prosurvival, proangiogenic, and functional marker such as VEGF, phosphorylated Akt, connexin-43, and CD31 (Chacko et al. 2010). Under hypoxic condition, gene expression of secreted factors, VEGF, FGF-2, HGF, and IGF, increased in Akt-modified MSCs. When conditioned medium from hypoxic Akt-MSCs was also injected into ischemic heart, infarct size was decreased and LV function was improved (Gnecchi et al. 2006).

Now, it will be focused about the genetic alteration to enhance MSC function, which is cell survival and adhesion.

Genetic Modification to Enhance Anti-death Signals

General strategy to enhance anti-death signal of MSCs is targeting a specific molecular pathway. Many groups focus on expression of an anti-apoptotic protein or blocking a caspase. It

is known that PI3K and Akt are involved in promoting survival downstream of extracellular stimuli, and may mediate the anoikis-suppressing effects (Michel 2003). Dzau's group initially demonstrated the genetic modification of survival signalling in MSCs. They modulated survival of transplanted MSCs by overexpressing the prosurvival gene Akt1. MSC-Akt1-transplanted myocardium inhibited the cardiac remodeling, including inflammation, collagen deposition, and cadiac hypertrophy and improved the cardiac function (Mangi et al. 2003). Furthermore, Eun et al. recently reported that overexpression of PI3K-C2α is able to enhance the survival rate of MSCs under hypoxic condition. A decrease in PARP levels and an increase in the ratio Bcl2/Bax were significantly detected in MSCs overexpressing PI3K-C2α. It was also investigated that transplantation of PI3K-C2α-overexpressed MSCs in rats after MI resulted in a reduction of infarct size and area of fibrosis, with improved heart function (Eun et al. 2010).

Bcl-2, which is the prototypical member of a family of pro- or anti-apoptotic proteins, inhibits cell death by blocking cytochrome C release, and caspase activation. Genetically modified MSCs with Bcl-2 showed vascular endothelial growth factor (VEGF) secretion was improved under hypoxic condition in *in vitro*. A cellular survival of Bcl-2-MSC-transplanted heart was increased until 6 weeks after cell implantation. They changed infarct size and heart function remarkably (Li et al. 2007).

Heat shock proteins provide a defense mechanism against stress caused by high temperature, oxidative stress, pressure overload, and hypoxia/ischemia. Among these proteins, Hsp20 has a cardioprotective effect, interacting phosphorylated Akt. Wang et al. studied that overexpression of Hsp20 in MSCs protected against cell death *in vitro*. Hsp20-modified MSCs had an efficiency of reduced infarct size and improved the cardiac function after ischemia/reperfusion injury. Furthermore, Hsp20-overexpressed MSCs secreted various growth factors, VEGF, FGF-2, and IGF-1 by Akt activation (Wang et al. 2009).

SDF-1α and CXCR4, that is SDF-1α unique receptor, play an important role in stem cell homing, chemotaxis, and expression of adhesion. To enhance these effects, MSCs enhancing CXCR4 were transfected with adenovirus. In ligation model of left anterior descending (LAD) coronary artery, experimental group was set up, below: CXCR4/GFP, CXCR4/GFP + SDF-1α treatment (50 ng/μl), and microRNA targeting CXCR4 + SDF-1α treatment. CXCR4-expressing cells were increased in CXCR4/GFP and CXCR4/GFP + SDF-1α treatment group. And MSC engraftment was blocked in miRNA group. Finally, an increase of matrix metalloproteinases (MMPs) by CXCR4 tranfection improved cell engraftment in ischemic region (Zhang et al. 2008).

In the report of Shujia et al. (2008), they used double overexpression form of Akt and angiopoietin (Ang-1) under adenoviral vector in MSCs. When co-overexpressing MSCs were implanted to imtramyocardium, they formed many blood vessel, and development of vWFactor VIII and smooth muscle actin. Also, heart function by sonographic assessment was stable over a period of 3 months following Ang-1 and Akt co-overexpressed MSCs.

Genetic Modification to Enhance Adhesion

The adhesive strength is important to transplanted MSCs for cell engraftment and cardiac regeneration. Copland et al. represented how MSCs respond to a hypoxic and nutrient-poor condition environment. They found plasminogen activator inhibitor 1 (PAI-1) as a key regulator of MSC autograft survival *in vivo* by microarray and proteomic screens. Mechanistically, PAI-1 has directly an effect on the adhesive strength of MSCs to their surrounding matrices. Finally, they had postulated a negative effect that PAI-1 may have an anoikis via matrix detachment (Copland et al. 2009).

It is known that oxidative stress is potent pro-apoptotic agents. As discussed earlier, anoikis is potentially important to enhance transplanted

cell engraftment for cardiac repair. Focal adhesion kinase (FAK) activated by integrins may be able to suppress anoikis. To investigate the role of ROS on MSC adhesion, hydrogen peroxide was treated as a ROS generator. Under this condition, MSCs lost their adhesion-related molecules, including phosphorylation of FAK and Src, and αV and β1 integrin (Song et al. 2010a, b).

Previous reports demonstrated the effect of integrin-mediated genetic modification in MSCs. To enhance adhesion strength of cells to the matrix, there was initially used tissue transglutaminase (tTG) gene, acting as a coreceptor with ECM for fibronectin (Fn) in cell adhesion associated with integrin, was used. tTG overexpression in MSCs was led to increased survival of the implanted cells via an integrin-dependent mechanism, and strengthened cell attachment, spreading, and migration. tTG-overexpressed MSCs showed the enhanced adhesion of MSCs on cardiogel, 3D matrix adhesion, in comparison to 2D surfaces coated with Fn (Song et al. 2007).

Other studies have also demonstrated that ILK has positive effects to MSCs in hypoxic condition, including cell survival and adhesion, and improved engraftment efficiency of MSCs when compared with MSC only after cell transplantation. ILK has an interaction with β1 integrin, and plays a crucial role in integrin-mediated cell adhesion and signalling. Using lentiviral vector-integrated genetic modification of MSCs, ILK-transduced MSCs showed a decrease of pro-apoptotic signal, Bax and Caspase-3. ILK also enhances phosphorylation of PKB/Akt, which plays a critical role in the regulation of adhesion-mediated cell survival signals. Moreover, ILK-MSC-transplanted rats led to reduction of infarct size, apoptotic index, and fibrotic heart area and had an improvement of microvessel density in infracted myocardium (Song et al. 2009).

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Hepatogenic Differentiation: Comparison Between Adipose Tissue-Derived Stem Cells and Bone Marrow Mesenchymal Stem Cells

5

María José Gómez-Lechón and Laia Tolosa

Contents

Abstract

Mesenchymal stem cells (MSCs) are multipotent cells, able to differentiate into elements of the mesodermal lineage. Bone marrow and adipose tissue represent the main sources for MSCs isolation. In the last decade, several studies have reported the plasticity of MSCs toward a hepatocyte-like phenotype. Both types of cells have been cultured under similar pro-hepatogenic conditions by sequential exposure to cytokines, growth factors and hormones reflecting their temporal expression during *in vivo* hepatogenesis. The different capacities of hepatic differentiation of the two subsets *in vitro* have been investigated.

Abbreviations

ADSCs	adipose tissue-derived stem cells
AFP	alpha-Fetoprotein
ALB	Albumin
BMP 2 and 4	bone morphogenetic protein 2 and 4
BMSCs	bone marrow-derived stem cells
CYPs	cytochrome P450 enzymes
DEX	dexamethasone
FGF	fibroblastic growth factor
FGF1, 2 and 4	fibroblastic growth factor 1, 2 and 4
FGFR1 and 2	fibroblast growth factor receptor 1 and 2

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hESCs	human embryonic stem cells
HGF	hepatocyte growth factor
IGF-I	insulin growth factor
LDL	low-density lipoprotein
MSCs	mesenchymal stem cells
OSM	oncostatin
PLGA	poly lactic glycolic acid

Introduction

Mesenchymal stem cells (MSCs) are diversely distributed *in vivo* and, as a result, may occupy a ubiquitous stem cell niche. It has been reported that MSCs contribute to the regeneration of a variety of mesenchymal tissues and retain the ability to differentiate into cells of mesoderm lineage such as osteoblasts, chondrocytes, adipocytes, myoblasts and cardiomyocytes, and also into various types of tissue cells derived from other embryonic layers including neural and liver cells. The definition of MSCs requires minimal criteria including the expression of membrane antigens such as SH3, CD29, CD44, CD71, CD90, CD105, CD106, CD120a and CD124, the lack of expression of CD34, CD45 and CD14 as well as adherent properties (Ochiya et al. 2010). The immunosuppressive property of human MSC makes them an important candidate for cellular therapy in allogeneic settings. The use of allogeneic MSCs for the repair of large defects may prove to be an alternative to autologous and allogeneic tissue-grafting procedures. An allogeneic approach would enable MSCs to be isolated from any donor, expanded and cryopreserved, and would provide a readily available source of progenitors for cell replacement therapy (Ochiya et al. 2010).

The bone marrow stroma is heterogeneous in composition and a promising reservoir of several stem cell populations, such as MSCs and multi-potent adult progenitor stem cells. Of particular interest is the hepatogenic transdifferentiation potential of bone marrow-derived stem cells (BMSCs) that can be used for liver-directed stem cell therapy and transplantation. However, traditional bone marrow procurement procedures may be uncomfortable for the patient and may yield low numbers of MSCs upon processing. This has led recently to investigate alternative sources for MSCs outside the bone marrow microenvironment. It has been reported that MSCs can also be isolated from human umbilical cord vein, synovium, placenta, periosteum, skeletal muscle, and adipose tissue (Vemuri et al. 2011), suggesting that the MSC niche may not be restricted to just bone marrow.

Adult adipose tissue, like bone marrow, is derived from the embryonic mesenchyme, and a putative stem cell population within the adipose stromal compartment has been identified. Adipose tissue represents a rich source of MSCs, and provides an abundant and accessible source of adult stem cells with minimal patient discomfort. These cells have been termed adipose tissue-derived stem cells (ADSCs). This cell population can be isolated from human lipoaspirates and, like BMSCs, differentiate toward the osteogenic, adipogenic, neurogenic, myogenic and chondrogenic lineages (Zuk et al. 2002). In fact, some works have shown that human ADSCs have similar characteristics than BMSC *in vitro* and *in vivo* (Lee et al. 2004). Moreover, genome-wide transcriptome comparison of MSCs from bone marrow and adipose tissue was made by the group of Izadpanah who found that the differences between human bone marrow-MSCs versus human adipose tissue-MSCs were minimal (Izadpanah et al. 2008). Analysis of the transcriptome of the MSCs from early and late passages revealed that 8.8% of the genes were differentially expressed in human BMSCs versus ADSCs (Izadpanah et al. 2008). In addition, ADSCs compared with MSCs from other sources possessed the longest culture period and the highest proliferation capacity, and so adipose tissue may be an ideal source of high amounts of autologous stem cells attainable by a less invasive method than BMSCs. In recent years, several groups, included ours, have investigated the hepatic plasticity of BMSCs and ADSCs and hepatic integration *in vivo* with or without prior differentiation to hepatocyte-like cells *in vitro*.

In Vitro Differentiation of Progenitor Cells Towards a Hepatic Phenotype

Recent years have seen substantial progress in research on stem cell plasticity and regenerative medicine using stem-cell-derived cells, and technologies are now being developed to induce the differentiation of numerous cell types from embryonic stem cells and adult stem cells. In the natural milieu, the hepatic differentiation of stem cells involves multiple pathways. Great effort has been made in the last decade to identify the factors regulating the onset of hepatogenesis and embryonic liver development (Zhao et al. 2005). Figure 5.1 shows the external and transcription factors involved during liver embryogenesis. This *in vivo* process may be mimicked *in vitro* by using a combination of various factors and culturing conditions.

Based on the recent knowledge about the onset of hepatogenesis and liver development, different strategies have been attempted to induce hepatogenic trans-differentiation of BMSCs and ADSCs into functional hepatocyte-like cells. A first approach has been obtained using a cocktail of exogenous factors (Aurich et al. 2009; Seo et al. 2005) whereas other reports use multi-step trans-differentiation protocols by sequential exposure of MSCs to growth factors, cytokines and hormones, reflecting their temporal expression during liver embryogenesis *in vivo* (*commitment*, *differentiation* and *maturation* steps). Different protocols based on the use of pro-hepatogenic signals and mediators of liver development have been reported (Banas et al. 2008;

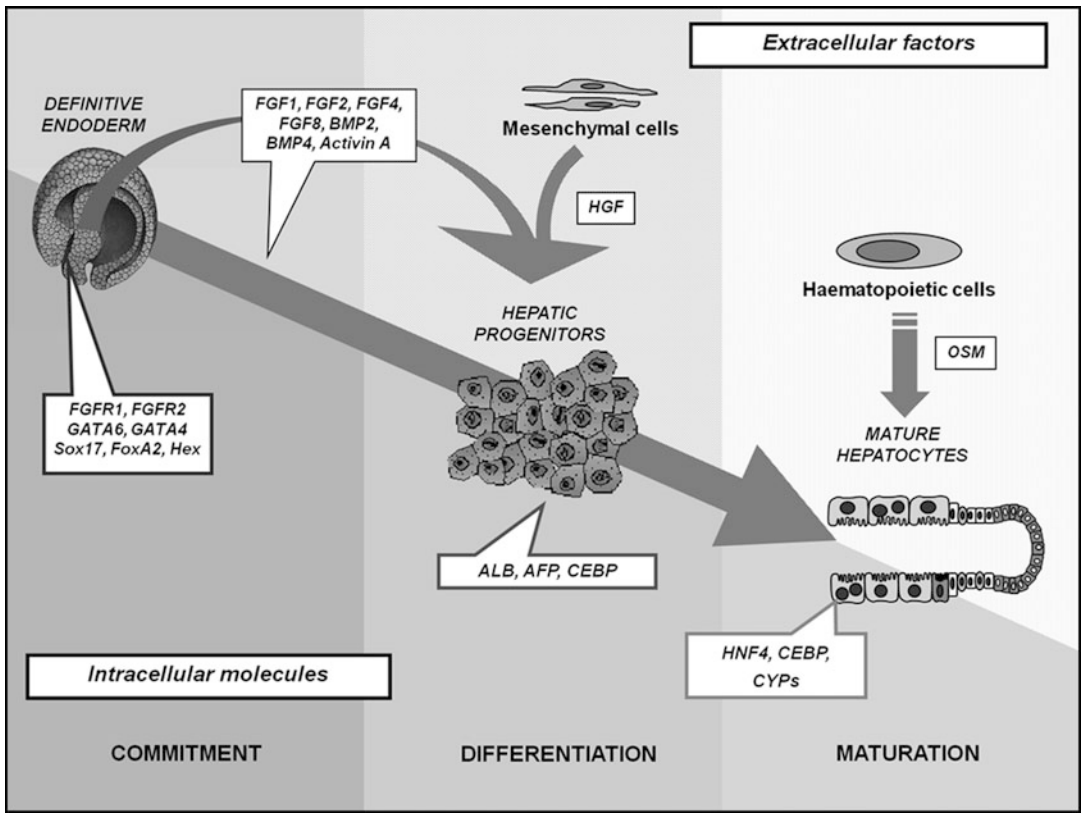

Fig. 5.1 Extracellular factors and intracellular molecules implicated in transdifferentiation events to hepatic phenotype during liver development. *AFP* alpha-Fetoprotein, *ALB* Albumin, *BMP 2 and 4* Bone morphogenetic protein 2 and 4, *CYPs* Cytochrome P450 enzymes, *FGF1, 2 and 4* Fibroblastic growth factor 1, 2 and 4, *FGFR1 and 2* Fibroblast growth factor receptor 1 and 2, *HGF* Hepatocyte growth factor, *OSM* Oncostatin

Bonora-Centelles et al. 2009; Lee et al. 2004; Talens-Visconti et al. 2006). All these reports have demonstrated the hepatogenic potentiality of MSCs by the detection of classical hepatic markers and biochemical functions.

Different fibroblastic growth factor (FGF) signals appear to initiate distinct liver development phases during mammalian organogenesis. The positive influence in hepatic differentiation *in vitro* of FGF2 (Lee et al. 2004) and, more recently FGF4, either alone or combined with epidermal growth factor (EGF) (Duret et al. 2007) or hepatocyte growth factor (HGF), have been reported (Banas et al. 2008; Snykers et al. 2006). FGF4 is one of the factors secreted by septum transversum mesenchyme and cardiogenic mesoderm at the early stages of endoderm formation in liver development *in vivo,* suggesting that related molecules probably function in a redundant capacity. In addition, exogenous FGF4 has been reported to increase the rate at which MSC proliferates, and has no significant effect on MSC pluripotency (Farre et al. 2007). Bone morphogenetic proteins (BMPs) are not only important for liver development, but also for hepatic specification of mouse and zebrafish embryonic stem cell-derived definitive endoderm. However, a lack of requirement for BMP4 addition was demonstrated in the generation of hepatocytes from human embryonic stem cells (Bonora-Centelles et al. 2009).

HGF has been reported to play an essential role in the early stages of hepatogenesis (Kinoshita and Miyajima 2002) and in liver regeneration after partial hepatectomy. Therefore, HGF and nicotinamide, which enhances the *in vitro* differentiation of fetal liver cells, have been included in several hepatogenic protocols. Some studies show induction of hepatocyte-like phenotype after exposure of MSC to HGF alone (Wang et al. 2004), or in cocktails combined with other factors (Snykers et al. 2006). However, many reports proposing multistep hepatogenic protocols point to a crucial role for HGF in the *commitment and differentiation* of MSCs, but it seems not to play a key role in further maturation stages of hepatocyte-like cells (Bonora-Centelles et al. 2009; Talens-Visconti et al. 2006).

A critical role for oncostatin (OSM) and dexamethasone (DEX) during the hepatic *maturation* step has also been demonstrated (Bonora-Centelles et al. 2009). This finding is consistent with the role reported for OSM to promote hepatoblasts differentiation to mature hepatocytes (Suzuki et al. 2003), and with the fact that OSM in combination with glucocorticoids is required to induce maturation of liver stem and progenitor cells, and that hormones, glucocorticoids and insulin are involved in the late *maturation* stage leading to increased liver-specific gene expressions.

Insulin growth factor (IGF-I) is a potent cytoprotective and anabolic hormone, synthesized mainly in the liver that regulate genes involved in cell survival, growth and differentiation of stem cells. Recently, Ayatollahi and collaborators have reported the use of IGF-I in combination with the growth factors HGF, OSM and DEX to achieve hepatic differentiation of human bone marrow derived MSCs to functional hepatocytes (Ayatollahi et al. 2011).

Some groups even include activin A at the *commitment* steps to induce differentiation from definitive endoderm. Another key point investigated has been the relevance of the extracellular matrix. In this sense, it has been reported that extracellular matrix components contribute to hepatoblast differentiation. Supplementing culture medium with matrigel induces a higher cellular proliferation rate and longer survival of MSC-derived hepatocyte-like cells (Bonora-Centelles et al. 2009; Talens-Visconti et al. 2006).

In summary, the conditions to obtain hepatocyte-like cells combine the EGF and FGF at the *commitment* step; FGF, nicotinamide and HGF at the *differentiation* step and, finally, OSM, DEX and insulin-transferrin-selenium at the *maturation* step (Banas et al. 2008; Bonora-Centelles et al. 2009; Lee et al. 2004; Snykers et al. 2006). Although differences can be found

between several hepatogenic differentiation protocols, basically all of them try to mimic the human liver development.

Cellular Reprogramming to Hepatocyte-Like Cells: Functionality Assessment

It seems to be no substantial qualitative or quantitative differences in the molecular or functional features of human undifferentiated MSCs either derived from adipose tissue or bone marrow for their capability to differentiate into hepatocyte-like cells *in vitro*. Several reports have shown a high degree of similarity in gene expression profiles and differentiation potential in BMSCs and ADSCs (Bonora-Centelles et al. 2009; Talens-Visconti et al. 2006). DNA microarray analysis of in vitro differentiated human BMSCs and ADSCs indicated upregulation of several liver-specific genes (Stock et al. 2008). Approximately 1,000 genes were upregulated in differentiated MSCs with a change greater than 3-fold. Among these, around 60% showed similar expression levels as adult liver, such as members of the serpin family or proteinase inhibitors or alcohol deshydrogenase isoenzymes. Approximately 1,400 genes were down-regulated in hepatocyte differentiated as compared to undifferentiated BMSCs, about 40% of which featured similar expression levels as the liver (Stock et al. 2008).

ADSC showed a similar behaviour to BMSCs (Bonora-Centelles et al. 2009). A whole genome expression analysis of ADSCs before and after differentiation treatment reveals a large readjustment in the expression profile of ADSCs during hepatogenic trans-differentiation with many genes related to development, proliferation and differentiation undergoing important expression adjustment, which suggests a profound reprogramming event. Clustering and principal component analyses demonstrated that the trans-differentiation process leads to a cellular population with a substantially different gene expression pattern from the same cell population before treatment. These results demonstrate that the hepatogenic differentiation protocol triggers the adjustment of multiple pathways involved in differentiation, morphogenesis, survival and hepatic-associated functions in MSCs.

Up to now, studies illustrating *in vitro* hepatic features of stem cells-derived hepatocyte-like cells were essentially confined at the phenotypic rather than the functional level. Acquisition of specific markers is a tool for evidencing a cell commitment while hepatocyte-like functionality is required to consider a cell for therapy. However, the functional characterization of stem cells-derived hepatocyte-like cells is principally achieved by commercially available assays (ELISA, colorimetric tests, PROD or EROD assays) whose handling are often poorly reproducible and quite difficult to interpret, or by other tests (such as lipoprotein uptake) with low specificity. Therefore, there is a crucial need for standardized and normalized techniques for characterizing hepatocyte-like functionality in differentiating cells. Regarding clinical application, one should provide the evidence of a specific functional activity after differentiation before considering stem cells for cell therapy. Different groups have studied the acquisition of hepatic functional characteristic such as the activity of several cytochrome P450 enzymes (CYPs) involved in drug metabolism, the capability to synthesize and store glycogen, urea secretion and the synthesis and secretion of plasma proteins to the culture medium (Aurich et al. 2009; Bonora-Centelles et al. 2009; Lee et al. 2004; Snykers et al. 2007). On the other hand, some groups have demonstrated the functionality of these cells by transplanting them into different experimental models (Tables 5.1 and 5.2).

It has also been reported that the expression of the adult MSCs surface marker Thy1 decreases during early fetal rat liver development, but is only expressed in oval cells in the adult liver, and not in mature hepatocytes (Isabel et al. 2006). The expression of Thy1 was elevated in undifferentiated MSCs, but a significant decrease was observed in the *maturation* step, suggesting glucocorticoids and/or OSM as likely mediators

Table 5.1 Hepatic differentiation strategies from human BMSCs

Reference	Protocol	mRNA markers	Protein markers	Functional assays	Cell transpl.	Overcome
Lee et al. (2004)	Multistep (EGF + bFGF) + (HGF + bFGF + NIC) + (OSM + DEX + ITS)	AFP, ALB, CK18, TAT, TDO2, G6P, HNF-4	ALB	UREA, CYP activity, LDL uptake		
Sato et al. (2005)		AFP, ALB	AFP, ALB, CK19, CK18, AGPR		Allylalcohol intoxicated rats	ALB secretion; no cell fusion
Talens-Visconti et al. (2006)	Multistep (EGF + FGF) + (HGF + NIC) + (OsM + DEX + ITS)	ALB, AFP, THY1, CK18, CK19, CYP3A4, CYP2E1, C/EBPβ, HNF4α	ALB, AFP			
Aurich et al. (2007)	Multistep (5'azacytidine) + (HGF + EGF)	AFP, CK19, CK7, CX43, CYP3A4, ALB, CPS, CK18, CX32, PCK1, TFN	CK18, CX32, HepPar1	UREA	Pfp/Rag2$^{-/-}$ mice	Engraftment, maintenance of hepatic qualities
Snykers et al. (2007)	Multistep (FGF-4) + (HGF) + (HGF + ITS + DEX) + (TSA)		AFP, ALB, CK18, HNF1α, HNF3β	ALB, UREA, CYP activity		
Kuo et al. (2008)	Multistep (EGF + FGF2) + (HGF + FGF2 + NIC) + (OSM + DEX)				NOD-SCID mice	Engraftment and differentiation. Improvement in liver function, ALB.
diBonzo et al. (2008)	Multistep (EGF + FGF2) + (HGF + FGF2 + NIC) + (OSM + DEX)	AFP, ALB, CK18, TAT, TDO2, G6P, HNF-4	HLA-I, GFAP, CK-7, Vimentin, αSMA, PDGFbR		Sublethally irradiated NOD/SCID mice exposed or not to acute liver injury	Low engraftment
Ayatollahi et al. (2011)	Multistep (IGF-1+ HGF + DEX) + (IGF-I + HGF + DEX + OSM)		ALB, AFP	UREA, GLYCOGEN		
Amer et al. (2011)	HGF		AFP	ALB	End-stage human liver failure	Significant improvement in liver functions
Wang et al. (2012)	PLGA scaffold + 1-step (HGF + FGF-4 + DEX)	AFP, ALB, CK18, CK19, CYP3A4	AFP, ALB	UREA, ALB		

Table 5.2 Hepatic differentiation strategies from human ADSCs

Reference	Protocol	mRNA markers	Protein markers	Functional assays	Cell transpl.	Overcome
Seo et al. (2005)	1-step DMSO + HGF + OSM	AFP, ALB, GADPH	ALB	LDL, UREA	NOD/SCID MICE CCl4 treatment	Engraftment, ALB expression
Taléns-Visconti et al. (2006)	Multistep (EGF + FGF) + (HGF + NIC) + (OSM + DEX + ITS)	ALB, AFP, THY1, KRT 18, KRT 19, CYP3A4, CYP2E1, C/EBPβ, HNF4α	ALB, AFP			
Sgodda et al. (2007)	1-step	GADPH, AFP, CK19, CK7, CX43, CYP1A1, CX32, ALB, CK18, PCK, CD26		UREA, GLYCOGEN	CD26 deficient rats	Engraftment
Banas et al. (2008)	Multistep (Activin A + FGF4) + (EGF + HGF + ascorbic acid + transferrin + FGF1 + FGF4 + OSM + Dex + ITS + NIC + DMSO)	ALB, FOXA2, TO	ALB		MICE CCl4	Improvement in liver functions ALT, AST, AMMONIA concentration
Aurich et al. (2009)	1-step HHMM + FCS + HGF + EGF	ALB, CD26, CK7, CX43		GLYCOGEN, UREA	Pfp/Rag2-/- mice 30% hepatectomy	Engraftment and expansion ALB, HepPar1, CX32
Bonora-Centelles et al. (2009)	Multistep (EGF + FGF2 + FGF4) + (HGF + FGF2 + FGF4 + NIC) + (OSM + DEX + ITS)	ALB, THY1, CYP3A4, CYP1A2, C/EBPβ, HNF4α, PGC1α, C/EBPα, Hex, GATA6, PEPCK, APOE, C3, AGTR1, ACSL1, ID3, α2M, SEPP1, ALDOB, APOCIII	APOE, C3, α2m	GLYCOGEN, CYP ACTIVITY		

(continued)

Table 5.2 (continued)

Reference	Protocol	mRNA markers	Protein markers	Functional assays	Cell transpl.	Overcome
Zemel et al (2009)	1-step DMSO + HGF + OSM	ALB, AFP A1AT	AFP, ALB	GLYCOGEN, UREA, uptake indocyanine green		
Ruiz et al (2010)	Multistep (EGF + FGFb) + (HGF + FGFb + NIC) + (OSM + Dex + ITS)	ALB, AFP, CK19, CYP3A4, TRF, DPP4, AAT1, AQP	ALB	CYP 450 ACTIVITY (1A2, 2C9)	CB.17 SCID MICE CCl4	Significant engraftment
Lue et al (2010)	Mult-step (FGF2 + EGF) + (FGFa + HGF + FGF4 + NIC + DMSO) + (OSM + Dex)	ALB, CYP7A1, AFP	ALB, CYP7A1, AFP	LDL		
Wang et al (2010)	3D scaffold				70% hepatectomized rats	Survival, ALB expression

of their repression during maturation of MSC-derived hepatic-like cells (Bonora-Centelles et al. 2009).

Several transcription factors involved in liver development have been also characterized, many of which were originally identified as regulators of liver-specific genes in the adult liver. GATA6 is the most critical player to regulate the differentiation of hepatoblasts (Zhao et al. 2005). Hex, a homeobox transcription factor, is the earliest identified marker for endoderm that will form liver (Bort et al. 2006). Both are essential factors in hepatic specification and the commitment to hepatogenic cell fate. However, medium supplementation with FGF4 and BMPs led to no noticeable differences in the induction of GATA6 and Hex, thus suggesting that FGF4 and BMPs do not likely play a critical role in the induction of GATA6 and Hex during MSCs hepatic trans-differentiation. Like GATA6 and Hex, CEBPα, CEBPβ and PGC1α expressions during hepatogenic induction of ADSCs revealed no significant response to FGF4 or BMPs (Bonora-Centelles et al. 2009). The expression of these five transcription regulators increased during the *commitment* and hepatic *differentiaion* steps, but none showed further increase during *maturation* step. In fact, C/EBP factors are master regulators of liver development, and the early expression of C/EBPα and C/EBPβ exclusively in the early liver bud has been reported (Westmacott et al. 2006). In addition, it has also been reported that HGF induces the expression of C/EBP factors (Suzuki et al. 2003). PGC1α is an important co-activator for the control of metabolic homeostasis, and developmental regulation of PGC1α co-activation can be an essential step to build up the appropriate regulatory machinery for liver energy metabolism. HNF4α is essential for hepatocyte differentiation during later development and crucial for metabolic regulation and liver function (Li et al. 2000). Some results are also consistent with previous observations, showing that GATA6 regulates HNF4α during development in a transcriptional cascade that controls the differentiation of the visceral endoderm (Bonora-Centelles et al. 2009). More recently, studies have shown that HNF4α is critical for hepatic morphogenesis. HNF4α deficiency in embryonic mouse liver results in abnormal tissue architecture and a lack of appropriate cell-cell contacts (Parviz et al. 2003). In this sense, the only transcription factor showing a significant up-regulation *in vitro* during the *maturation* step was HNF4α (Bonora-Centelles et al. 2009).

The term trans-differentiation refers to the phenotypic change of one differentiated cell type to another lineage. It has been suggested that pre-committed cells could change their phenotype during trans-differentiation by dedifferentiating into a primitive stem cell stage, perhaps through genome reprogramming. Induction of hepatogenic trans-differentiation *in vitro* of MSCs is a process that likely differs from embryonic liver development. Consequently, there are some concerns about *in vitro* differentiation protocols attempting to experimentally reproduce the embryonic development of the liver which may not adequately mimic the cross-lineage commitment between MSCs and hepatocytes during dedifferentiation-trans-differentiation processes. However, collectively experimental evidence demonstrates that sequential protocols not only induce a hepatic-specific expression profile, but also leads to the loss of the mesenchymal undifferentiated phenotype. Moreover, the results suggest the relevance of specific transcription factors (Hex, GATA6, C/EBPs, PGC1α and HNF4α) in promoting hepatocyte-derived MSCs and uncover new informative robust markers for the step-by-step trans-differentiation of MSCs into hepatocytes (Bonora-Centelles et al. 2009).

Hepatogenic Differentiation of Human Bone Marrow-Derived Stem Cells

Human bone marrow cells can be expanded *in vitro* and are expected to be a potential source for stem cell therapy without the risk of immune rejection. The bone marrow contains mesenchymal stem cells as well as hematopoietic stem cells. Accumulating evidence reveals that various bone marrow stem cells are capable of

differentiation into hepatocytes. Sato and cols. proved that both CD34 positive population and mesenchymal stem cell population differentiated into hepatocytes *in vivo* when directly inoculated in rat liver; with better results for mesenchymal cells (Sato et al. 2005). They showed that human BMSCs xenografted into rat liver treated with allyalcohol and differentiated into human hepatocytes, which express liver-specific markers, without cell fusion. These studies excluded spontaneous fusion between human MSCs and rat hepatocytes by identification of both human and rat chromosomes (Sato et al. 2005). Lee et al. (2004) demonstrated the evidence of hepatogenic induction of BMSCs using sequential treatment with factors. They showed different characteristics of hepatocyte-like cells, such as glycogen storage, albumin and urea secretion, low-density lipoprotein (LDL) uptake and CYP activity (Lee et al. 2004). Since then, several strategies have been proposed to differentiate mesenchymal cells derived from bone marrow into hepatocytes (Table 5.1). In general, they use a sequential approach to induce the hepatic differentiation (Ayatollahi et al. 2011; Kuo et al. 2008; Snykers et al. 2007; Talens-Visconti et al. 2006). All these reports have demonstrated the hepatogenic potential of BMSCs by the detection of classical hepatic markers and biochemical functions. Recently, other researchers have focused on the generation of a three-dimensional engineered tissue by culturing BMSCs and porous poly lactic glycolic acid (PLGA) scaffolds with an oscillatory perfusion system (Wang et al. 2012).

Additionally, several groups have studied the efficacy of BMSCs in animal models. DiBonzo et al. (2008) demonstrated the liver engraftment of cells of human origin under conditions of chronic injury; however, they described *in vivo* differentiation of intravenously transplanted MSCs into hepatocyte-like cells as a relatively rare and quantitatively unsatisfactory event (di Bonzo et al. 2008). However, Kuo et al. (2008) showed that BMSCs enhanced repopulation of necrotized tissue by endogenous cells, suggesting a role for paracrine effects in the rescue of fulminant hepatic failure. On the other hand, the transplantation of differentiated mesenchymal stem cells derived from bone marrow into livers of immunodeficient mice resulted in their engraftment in the periportal region and the maintenance of well-known qualities of hepatocytes such as glycogen storage or albumin expression after their regional integration, suggesting their importance to be used in cell therapy purposes (Aurich et al. 2007).

Recently, it has been described the short-term efficacy of autologous bone marrow-derived mesenchymal stem cell injection in patients with end-stage liver cell failure due to chronic hepatitis C (Amer et al. 2011). After cell transplantation, patients showed improved liver functions, especially serum albumin, Child-Pugh scores, Model for End Stage Liver Disease scores, fatigue impact scale and performance status (Amer et al. 2011). However, it should be further studied the possible risks associated with complex ex-vivo cell manipulation.

Hepatogenic Differentiation of Human Adipose-Derived Stem Cells

Adipose tissue contains a multipotent cell population (ADSCs) that can be isolated from human lipoaspirates and differentiated toward different lineages (Zuk et al. 2002), including hepatocytes (Aurich et al. 2009; Banas et al. 2008; Bonora-Centelles et al. 2009). The multipotent nature of ADSCs, together with their easy extraction and isolation, has made these cells an attractive alternative for autologous cell-based therapies. As in BMSCs, different strategies have been attempted to induce hepatogenic trans-differentiation of human ADSCs into functional hepatocyte-like cells (Table 5.2). In recent years, several groups have investigated the hepatic plasticity of ADSCs and, moreover, hepatic integration *in vivo* with or without prior differentiation to hepatocyte-like cells *in vitro*. A first approach was the use of a cocktail of exogenous factors (Seo et al. 2005), whereas other reports have based on the use of multistep sequential protocols (Banas et al. 2008; Bonora-Centelles et al. 2009; Lue et al. 2010;

Ruiz et al. 2010; Talens-Visconti et al. 2006). All these strategies have shown the hepatogenic potential of ADSCs by detecting specific biochemical functions and classical hepatic markers such as albumin or cytokeratin 18. Additionally, the use of these cells for tissue engineering purposes has been explored by culturing ADSCs in PLGA scaffolds (Wang et al. 2010). They showed that induced ADSCs on PLGA scaffold survived and maintained hepatic phenotype and function for at least 14 days after implantation in hepatectomized rats.

To date, studies on animal models have shown the efficacy of transplantation of ADSCs in promoting liver regeneration. Transplantation of ADSCs into SCID mice with acute liver failure revealed that these cells are able to engraft into the host liver and improve its function (Banas et al. 2008). It was demonstrated that undifferentiated ADSC *in vitro* produce different bioactive factors such as HGF; thus, their beneficial effects *in vivo* could be due to the secretion of these molecules that would contribute to hepatocyte proliferation and function (Banas et al. 2008). The results obtained reveal that differentiation of ADSC to hepatocyte-like cells prior to transplantation clearly improves long-term engraftment and functional hepatic repopulation *in vivo* compared to using undifferentiated ADSC (Aurich et al. 2009; Banas et al. 2008; Ruiz et al. 2010; Seo et al. 2005; Sgodda et al. 2007). In fact, Aurich et al. (2009) showed that human ADSCs are capable of extensive repopulation within the host liver similar to hepatocytes during liver regeneration. Additionally, they demonstrated a repopulation of 10% of the liver mass when ADSCs were transplanted whereas when BMSCs were used only 1% of repopulation was achieved (Aurich et al. 2007). Since it is generally accepted that a repopulation of 1–5% of the hepatic mass is sufficient to correct liver disorders in different injury models (Fox and Roy-Chowdhury 2004), the use of ADSCs would allow correcting these diseases. Additionally, it has been shown that MSCs isolated from human adipose tissue are immunocompatible and are easily isolated, which suggests that they may become an alternative source to hepatocyte regeneration or liver cell transplantation (Seo et al. 2005). Transplanted ADSCs have been found to incorporate into the host liver tissue and to form large cords of tissue within the parenchyma (Ruiz et al. 2010).

Future Perspectives

Stem cells are promising tools at the service of regenerative medicine for the treatment of inborn errors of metabolism and organ failure. Although BMSCs and ADSCs represent important sources for cell-based therapies, before clinical application, it is still required the scale-up production or "banking" of large numbers of the desired cell type. The current lack of large-scale GMP-grade cell culture methods presents a serious challenge to the progression of new therapies to clinical testing. Moreover, critical aspects such as the long-term safety, tolerability and efficacy of stem cell-based treatments should be further explored. Many issues must be addressed before these cells are safely applied; however, with appropriate validation of cell types, optimal performance and further characterization, they should yield a demonstrable benefit in cell therapy.

On the other hand, mesenchymal stem cells derived from both bone marrow and adipose tissue could represent a potential source to provide an unlimited number of human hepatocytes to be used for hepatotoxicity screening purposes, since they retain proliferation capacity *in vitro* and, under appropriate conditions, could differentiate into functional hepatocytes. The pharmaceutical industry is strongly interested in establishing screening models to early detect hepatotoxicity in the drug development process. Human hepatocytes are frequently used for assessment of metabolic activation and provide a valuable early screen. However, the use of human hepatocytes has been limited by the poor availability of human liver, the high cost, limited life and quality of frozen hepatocytes and the significant variability among different human hepatocyte preparations, making

this tool not applicable to a high-throughput screening in drug development. Therefore, it is necessary to determine if hepatocyte-like cells derived from both BMSCs and ADSCs could be used as an *in vitro* cell model to assess human hepatotoxicity.

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Fibrin for Encapsulation of Human Mesenchymal Stem Cells for Chondrogenic Differentiation

6

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Contents

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Abstract

Age-related wear and tear of cartilage (osteoarthritis) and traumatic cartilage damage are a leading cause of disability in developed nations. Articular (hyaline) cartilage covers the ends of the bones of synovial joints and is a complex, multilayered structure varying in composition with location in a joint, and in relation to load and shear forces at that specific site. When damaged, articular cartilage tissue does not have the ability to repair itself, but rather is usually replaced by fibrocartilage which does not have suitable compressive properties, leading to breakdown, pain and can ultimately require replacement by prosthetic joint. Thus, cartilage repair remains a clinical challenge and few current treatments yield satisfactory clinical results over the long term. Regenerative medicine, using tissue engineering-based constructs to enhance cartilage repair by mobilizing chondrogenic cells, is a promising approach for restoration of structure and function, and provides a scientific basis for integrating the proper cell populations, suitable cellular signals and appropriate scaffolds for optimum tissue development and organ replacement strategies. Fibrin has been used as both a delivery vehicle and as a scaffolding matrix for tissue engineering. The emergence of mesenchymal stem cells (MSCs) as an important tool in regenerative medicine is due to their capability to repopulate and differentiate into several tissue lineages,

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including both cartilage and bone. Human MSCs have been used in combination with a wide range of fibrin scaffolds including both autologous and allogeneic human fibrin glue either as a platelet-rich or normal formulation, in addition to commercially available bovine fibrin hydrogel precursors. This approach permits high density of cells to be implanted, wherein chemical manipulation of the fibrin scaffold modulates its stability, strength and complement of growth factors, while maintaining the promise of an autologous repair solution. This review focuses on recent advances in the application of the fibrinogen/fibrin system for tissue engineering of articular cartilage.

Introduction

Articular hyaline cartilage plays a key role as a covering in diarthrodial joints to provide a lubricating surface for frictionless movement (Ahmed and Hincke 2010). Articular cartilage mainly consists of a chondrocyte-secreted extracellular matrix (ECM) composed of type II collagen, aggrecan, chondroitin sulfate and other glycosaminoglycans, which are responsible for its specific biomechanical properties (Ahmed and Hincke 2010; Park et al. 2011a). The loss of cartilaginous tissues due to traumatic injury or pathological conditions is a major challenge to orthopaedic surgeons. A wide range of techniques involving the implantation of cells and tissue engineered constructs have been developed to enhance cartilage repair (Ahmed and Hincke 2010; Im et al. 2005). Tissue engineering is a promising strategy for restoration of cartilaginous tissue after injury (Baumgartner et al. 2010), and has the potential to improve the quality of life of millions of patients and delay future medical costs related to joint arthroplasty and associated procedures (Kessler and Grande 2008). Successful tissue engineering approaches depend largely on four pillars: functionally active cells, appropriate growth factors, a supporting 3D scaffold, and a maturation-promoting mechanical environment (Kessler and Grande 2008; Baumgartner et al. 2010).

A suitable cell source is an absolute requirement for a successful tissue engineering application. The most widely used cell sources for cartilaginous tissue engineering are chondrocytes and mesenchymal stem/stromal cells (MSCs). However, MSCs have advantages over chondrocytes, since they can be obtained in autologous form in a minimally invasive procedure and can be utilized for creating complex constructs such as the cartilage-bone interface (Ahmed et al. 2011). MSCs are multipotent cells which were first described as marrow stromal cells in the 1970s. They can be harvested in large quantities from a wide range of tissues in the human body and have emerged as an important tool in the field of regenerative medicine due to their easy-accessibility and abilities to self-renew, and to differentiate into several tissue lineages including cartilage, bone, and adipose tissues. MSCs are widely used in stem cell-based research and clinical trials, since they comply with key ethical issues, as compared to embryonic stem cells (Arita et al. 2011).

Tissue engineering generally requires an artificial extracellular matrix (ECM) (scaffold) in which the cells can proliferate and differentiate with subsequent new tissue generation. The scaffold must be biodegradable and facilitate reasonable cell adhesion. It should also provide sufficient mechanical support to withstand *in vivo* forces (Ahmed et al. 2008). A wide range of scaffolds have been evaluated for effective tissue engineering–mediated cartilage repair including protein based (collagen, fibrin, and gelatin), carbohydrate based (hyaluronan, agarose, alginate, and chitosan) (Kessler and Grande 2008), and synthetic polymers such as polyglycolic acids (PGA), polylactic acid (PLA), copolymers of glycolic and lactic acids (PLGA), and polyurethanes (Ahmed et al. 2010; Li et al. 2009, 2010b). Hydrogels are a class of biomaterials that have great scaffolding potential in many tissue engineering applications due to their tissue-like water content, high biocompatibility, efficient transport of nutrients and waste, powerful ability to uniformly encapsulate cells, and ability to be injected as a liquid that gels *in situ* (Ahmed et al. 2008). Although a range of synthetic to naturally-occurring polymers has been

investigated for cartilage regeneration, in this review we have focused on the application of fibrin, which can be isolated autologously from patients and fabricated into a hydrogel scaffold. Other features of fibrin scaffolds include biodegradability and biocompatibility. In addition, they can achieve high seeding efficiency and uniform cell distribution (Ahmed et al. 2007). Fibrin is a biopolymer of the monomer fibrinogen. Fibrin and fibrinogen have critical roles in blood clotting, fibrinolysis, cellular and matrix interactions, the inflammatory response, wound healing, and neoplasia. Fibrinogen/fibrin has been used clinically as a hemostatic agent in cardiac, liver, and spleen surgery. In addition, it can be used in surgery for patients with hemophilia. Further, fibrinogen is useful as a sealant in a variety of clinical applications, including procedures such as colonic anastomosis as well as in seroma prevention following soft tissue dissection. Moreover, it has been used to reduce suture vascular and intestinal anastomosis, to promote fistula closure, and in laparoscopic/endoscopic procedures (Ahmed et al. 2008). Because of the good manipulability of fibrinogen and its role in the natural healing process, the fibrinogen/fibrin system is a promising choice as both scaffolding and delivery agent for cells with regenerative capacity.

This review focuses on recent advances in the application of the fibrinogen/fibrin system for tissue engineering of articular cartilage (Table 6.1).

Fibrin Glue

Fresh fibrin (FG) produced by the CryoSeal® FS System, in combination with strongly CD90+ and CD105+ human bone marrow-derived mesenchymal stem cells (BM-hMSCs) under the effect of TGF-β_2 were evaluated in a recent *in vitro* study (Ahmed et al. 2011). Encapsulation of hMSCs into FG promoted enhanced aggrecan gene expression and increased accumulation of Alcian blue – positive extracellular matrix. In addition, enhanced collagen II gene expression was observed after encapsulation of hMSCs into FG which was consistent with the accumulation of collagen II protein as indicated by collagen II immunostaining and measurement of fluorescence intensity. Further, after encapsulation of hMSCs and induction of chondrogenesis, a majority of chondrocyte-like cells was observed in FG. In a related study, BM-MSCs was encapsulated into commercially available fibrin glue with TGF-β_1 and compared to constructs made of fibrin-alginate mixture (Ho et al. 2010). Hydrogel encapsulation influenced mesenchymal condensation which preceded chondrogenic differentiation. Early cell agglomeration was observed in fibrin as compared to fibrin/alginate composites. These fibrin encapsulated cells differentiated into chondrocytes which secreted aggrecan and collagen II. This study was completed by testing fibrin and fibrin/alginate constructs in the cartilage phase of the biphasic osteochondral constructs and only fibrin supported superior cartilage growth with higher cellularity, total glycosaminoglycan (GAG) and collagen II levels. According to this study, commercial fibrin glue in combination with BM-MSCs promoted chondrogenesis and cartilaginous growth in an osteochondral environment which can be utilized for cartilage repair (Ho et al. 2010). Finally, in a study conducted by Li et al. (2009), constructs of BM-hMSCs encapsulated into fibrin/polyurethane scaffold was evaluated as an optimized environment that promote chondrogenesis of MSCs *in vitro*. In this study, hMSCs at different densities were encapsulated into fibrin/polyurethane composites with TGF-β_1. The fibrin/polyurethane scaffolds promoted chondrogenesis of hMSCs comparable to that of MSC pellets, and chondrogenesis was dependent on the initial seeding density (Li et al. 2009).

Cartilage Fragments Promote Chondrogenic Differentiation

In a recent study conducted by Chen et al. (2012), cartilage fragments isolated from osteoarthritic knee were evaluated as a factor to promote chondrogenic differentiation of BM-hMSCs. In this study, intact non-injured parts of cartilage

Table 6.1 Summary of applications of different fibrin formulations in combination with hMSCs for chondrogenic differentiation

Cell source	Carrier/ scaffolding matrix	Bioactive factor	Inducing factor (s)	Outcome(s)	Reference
BM-hMSCs	FG or PR-FG (Cryoseal ® FS System)[a]	TGF-β_2	–	*Upregulation of aggrecan and collagen II gene expression, chondrocyte-like morphology*	Ahmed et al. (2011)
	FG or PR-FG (Cryoseal ® FS System)[a]	TGF-β_2/ HBDS	–	*Adverse effect on chondrogenesis*	Ahmed et al. (2011)
	FG or PR-FG (Cryoseal ® FS System)[a]	TGF-β_2	Tranexamic acid	*Stabilization without affecting cell viability*	Ahmed et al. (2011)
	PR-FG[b]	–	–	*Positive objective criteria, defect filling and surface congruity with native cartilage*	Haleem et al. (2010)
	Fibrin/ Polyurethane[a]	–	A combination of shear and dynamic compression	*Chondrogenic gene expression along with sulfated glycosaminoglycan and collagen II protein secretion*	Schatti et al. (2011)
	Fibrin/ Polyurethane[a]	TGF- β_1	Surface motion superimposed on cyclic compression	*Gene expression, protein synthesis, and chondrogenesis*	Li et al. (2010a)
	Fibrin/ Polyurethane[a]	TGF-β_1	Compression and surface rotation frequency and axial compression	*Chondrogenesis of hMSCs*	Li et al. (2010b)
	Fibrin/ Polyurethane[a]	TGF-β_1	–	*Aggrecan and collagen II gene expression, proteoglycans and collagen secretion*	Li et al. (2009)
	Fibrin hydrogel[a]	–	Dynamic mechanical compression	*ERK1/2 phosphorylation and chondrogenesis*	Pelaez et al. (2012)
	Fibrin hydrogel[a]	–	Cyclic compression	*Improved viability, proliferation and chondrogenic differentiation*	Pelaez et al. (2009)
	Fibrin hydrogel[c]	Heparinized NPs loaded with TGF-β_3	–	*Survival and proliferation, expression of extracellular matrix (ECM) genes and synthesis of their proteins*	Park et al. (2011a)
	Fibrin hydrogel[b]	Cartilage fragments	–	*Neocartilage-like structures, type II collagen gene expression*	Chen et al. (2012)
	Fibrin hydrogel[a]	TGF-β_3	–	*ERK1/2 phosphorylation and chondrogenesis*	Arita et al. (2011)
	Fibrin hydrogel[a]	TGF- β_1	–	*Improved cartilage markers and chondrogenesis*	Dickhut et al. (2010)

(continued)

Table 6.1 (continued)

Cell source	Carrier/ scaffolding matrix	Bioactive factor	Inducing factor (s)	Outcome(s)	Reference
	Fibrin hydrogel[a]	TGF-β_1	ε-aminocaproic acid	*Stabilization without affecting chondrogenesis*	Kupcsik et al. (2009)
	Commercial FG[c]	TGF-β_1	–	*Aggrecan and collagen II secretion, early cell agglomeration*	Ho et al. (2010)
				Cartilage growth, improved GAGs and collagen II levels	
	FG[a]	TGF-β_3	Hypoxic conditions (5% O_2)	*Stabilization under hypoxic conditions in the absence of any protease inhibitor*	Ahmed et al. (2010)
	FG (Tisseel/ Tissucol)[a]	TGF-β_1	–	*Retention of TGF- β1, chondrogenesis, deposition of proteoglycans and collagen-type I*	Diederichs et al. (2012)
	FG (Tisseel/ Tissucol)[a]	TGF-β	Hypoxic (5% O_2) and normoxic conditions (21% O_2)	*Rounded chondrocyte-like cell types and expression of collagen II*	Baumgartner et al. (2010)
	FG (Tisseel/ Tissucol)[a]	AAV-CMV-TGF-β_1	–	*Cartilage-specific gene expression, transduction, and chondrogenesis*	Lee et al. (2011)
BM-hMSCs or AT-hMSCs or amniotic fluid derived hMSCs	Fibrin hydrogel[c]	TGF-β_3	–	*Cartilage specific genes and proteins*	Park et al. (2011b)
AT-hMSCs	FG (Tisseel/ Tissucol)[b]	TGF-β		*Cell proliferation and cartilage formation*	Jung et al. (2010)
BM-hMSCs vs. AT-hMSCs	Fibrin hydrogel[a]	TGF-β_2 + IGF-I	–	*AT-hMSCs showed much weaker potential for chondrogenesis compared to BM-hMSCs*	Im et al. (2005)
BM-hMSCs and AT-hMSCs	Fibrin hydrogel[c]	–	SOX5, 6, and 9 genes (SOX Trio)	*Chondrogenic-specific gene expression and protein synthesis, differentiation of hMSCs into mature chondrocytes*	Yang et al. (2011)

[a] *in vitro*
[b] *in vivo*
[c] *in vitro and in vivo*

tissues were obtained during total knee arthroplasty (TKA) surgery. The minced fragments were mixed with immortalized BM-hMSCS and encapsulated into a fibrin gel, followed by subcutaneous implantation into nude mice. Histological analysis showed formation of neocartilage-like structures in the cartilage fragment – fibrin – MSC constructs, with elevated type II collagen gene expression after 4 weeks of implantation. In contrast, constructs of only BM-hMSCs in fibrin gel did not differentiate into a chondrogenic lineage. Thus, non-injured cartilage fragments from osteoarthritic knee can

promote chondrogenic differentiation of MSCs and might be a promising strategy for MSC chondrogenesis without the need for induction by exogenous growth factors (Chen et al. 2012).

Platelet-Rich Fibrin Glue (PR-FG)

Encapsulation of hMSCs in platelet-rich fibrin glue (PR-FG) led to initially increased expression of collagen II; however, no difference was observed between FG and PR-FG after long term *in vitro* culture. However, FG seemed to be more promising than PR-FG as a scaffold for chondrogenic differentiation of hMSCs, as indicated by morphology of the cells after encapsulation and expression of the known cartilage markers. The difference between FG and PR-FG might be due to the impact of various growth factors contained in the PR-FG hydrogels. A wide range of growth factors are released from platelets upon activation by thrombin during formation of PR-FG-based tissue engineering constructs, including platelet-derived epidermal growth factors, platelet-derived growth factor A + B (PDGF-AA, AB, BB), TGF-β_1, TGF-β_2, insulin-like growth factor 1 and 2, vascular endothelial growth factor, and basic fibroblast growth factor 2 (FGF-2). The TGF-β_1 superfamily is a potent inducer of chondrogenesis, whereas PDGF, insulin-like growth factor, and fibroblast growth factor mediate chondrocytic physiology rather than promoting chondrogenesis of MSCs. Vascular endothelial growth factor is a potent inducer of endothelial differentiation. Growth factors can either stimulate or inhibit cellular processes such as division, migration, differentiation, and gene expression, depending on the cells involved. For example, TGF-βs stimulate proliferation and migration of fibroblasts and promote differentiation of chondrocytes, while inhibiting proliferation of keratinocytes. Therefore, after encapsulation of hMSCs, the combination of growth factors in PR-FG is likely to have a complex effect on their cellular activity and patterns of gene expression, leading to altered accumulation of cartilage-specific markers (Ahmed et al. 2011).

In a related *in vivo* study, PR-FG prepared by the cell saver centrifuge system in combination with autologous BM-hMSCS was evaluated clinically in human subjects. Autologous BM-MSCs were culture expanded, placed on PR-FG intraoperatively, and then transplanted into full-thickness cartilage defects of femoral condyles under an autologous periosteal flap. All patients exhibited significant improvement by objective criteria over the follow-up period of 12 months. At this point, MRI examination revealed complete defect fill and complete surface congruity with native cartilage in three patients, whereas two patients showed incomplete congruity (Haleem et al. 2010).

Cell Source

Mesenchymal stem cells (MSCs) isolated from several types of tissues have the potential to differentiate into mesoderm cell lineages such as chondrocytes. In a recent study, several types of hMSCs, derived from bone marrow, adipose tissue, or amniotic fluid, were encapsulated in a commercial fibrin hydrogel containing TGF-β_3 and then evaluated for their capacity for differentiation *in vitro* and *in vivo*. The three different types of hMSCs mixed with TGF-β_3 encapsulated in fibrin hydrogels produced chondrocytes. However, it has been shown that hMSCs isolated from these different sources expressed high levels of cartilage specific genes and proteins when they were cultured *in vitro* and transplanted into nude mice after encapsulation with fibrin hydrogel in the presence of TGF-β_3, suggesting that these cells would be suitable for reconstruction of cartilage (Park et al. 2011b). Although scientists have focused their research on utilizing adult mesenchymal stem cells MSCs derived from the bone marrow (BM-MSCs) for chondrogenesis, isolation of MSCs from the bone marrow is accompanied by pain in the donor site and other possible various complications such as inflammation and bleeding. In addition, the number of cells that can be obtained from the bone marrow is limited. However, adipose tissue–derived MSCs

(AT-hMSCs) from adults pose no ethical problems and potentially could provide large number of cells (Jung et al. 2010). In this context, AT-hMSCs were differentiated into chondrogenic MSCs, and then combined with fibrin glue (Tisseel/Tissucol) for subcutaneous injection into nude mice to explore the feasibility of whether cartilage can be generated *in vivo*. These MSCs were found to proliferate and form new cartilage suggesting that formation of cartilaginous tissue from such a cell source *in vivo* can be achieved (Jung et al. 2010). In contrast, in a third study, AT-hMSCs were compared to BM-hMSCs for their cartilage forming potential. Both cell types were encapsulated in a commercial fibrin hydrogel in the presence of TGF-β_2 and insulin-like growth factor-I (IGF-I). AT-hMSCs showed much weaker potential for chondrogenesis compared to BM-hMSCs; these results weaken the value of adipose tissue as a source of MSCs (Im et al. 2005). In contrast, BM-hMSCs and AT-hMSCs transfected with SOX5, 6, and 9 genes (SOX Trio) and encapsulated in a fibrin hydrogel exhibited similar chondrogenic-specific gene expression and protein synthesis. Chondrogenic genes and proteins were more highly expressed in SOX Trio expressing cells than in untransfected cells. Both *in vitro* and *in vivo* analyses revealed that fibrin hydrogel–encapsulated cultured or transplanted cells transfected with the SOX Trio successfully differentiated into mature chondrocytes and could be used for the reconstruction of hyaline articular cartilage (Yang et al. 2011).

Mechanical Environment

The mechanical environment plays a crucial role during the normal development and homeostasis of cartilage, while excessive physical forces can lead to cartilage damage (Ahmed and Hincke 2010). Articular cartilage is subjected to a combination of compressive, tensile and shear stresses; consequently, one can assume that compression forces are not sufficient as a mechanical signal to generate a cartilage-like tissue *in vitro*. The effect of mechanical load on the differentiation of BM – hMSC (suspended into fibrin and seeded into biodegradable polyurethane scaffolds) under the effect of exogenous TGF-β_1 has been investigated (Li et al. 2010a). Mechanical load (surface motion superimposed on cyclic compression) stimulated chondrogenesis of hMSCs compared to the unloaded scaffolds, with a much stronger effect on gene expression at lower TGF-β_1 concentrations. In the absence of TGF-β_1, mechanical load stimulated gene expression and protein synthesis of TGF-β_1 and TGF-β_3. Thus mechanical load promotes chondrogenesis of hMSCs through the TGF-β_1 pathway by up-regulating TGF-β gene expression and protein synthesis (Li et al. 2010a). In another study (Schatti et al. 2011), a combination of shear and dynamic compression was applied to constructs made of fibrin/polyurethane composites in which human MSCs were encapsulated on the absence of any exogenous growth factor. The application of shear superimposed upon dynamic compression led to significant increases in chondrogenic gene expression. In addition, sulfated glycosaminoglycan and collagen II were only detected when compression forces were applied in combination with shear forces (Schatti et al. 2011). In a similar study, BM-hMSCs in combination with fibrin/polyurethane composites were subjected to various mechanical loads to determine the effect of compression and surface rotation frequency and axial compression magnitude on the induction of cartilage-specific gene expression and protein synthesis in the presence of TGF-β_1. Application of dynamic compression and surface shear (1 h/day for 1 week) led to enhanced chondrogenesis of hMSCs compared to no load controls. The load frequency and compression amplitude were positively correlated with the development of chondrogenic characteristics (Li et al. 2010b). In a fourth study, the capability of fibrin hydrogels to support chondrogenesis of BM-hMSCs under cyclic compression was evaluated. Different fibrin concentrations and stimulus frequencies were assessed for impact on viability, proliferation and chondrogenic differentiation of hMSCs,

demonstrating a threshold in these parameters for maintaining cellular viability within scaffolds. This study confirmed the suitability of fibrin hydrogel for supporting the cyclic compression-induced chondrogenesis of mesenchymal stem cells (Pelaez et al. 2009).

Heparin Delivery of Growth Factors

Heparin is a highly sulfated, anionic polysaccharide composed of repeating glucosamine and uronic acid residues, which binds a variety of growth factors and can provide localized release of heparin-binding growth factors. BM-hMSCs have been encapsulated in a fibrin hydrogel containing heparinized nanoparticles (NPs) loaded with TGF-β_3 or TGF-β_3 alone then subjected to growth factor release and denaturation tests (Park et al. 2011b). When transplanted into nude mice, hMSCs embedded in fibrin hydrogels survived and proliferated more readily in those samples containing TGF-β_3-loaded NPs, or TGF-β_3 alone, compared to those containing only NPs or the fibrin hydrogel alone. In addition, extracellular matrix (ECM) genes and their proteins were expressed at high levels by hMSCs embedded in hydrogels containing TGF-β_3-loaded NPs. The *in vitro* and *in vivo* results indicated that transplanted hMSCs together with TGF-β_3 may constitute a clinically efficient method for the regeneration of hyaline articular cartilage.

Another approach tested a heparin-based delivery system (HBDS) composed of heparin, heparin-binding growth factors, and a bifunctional peptide (linker) containing a heparin-binding sequence linked to a transglutaminase substrate sequence. During fibrin hydrogel formation, the bifunctional peptide becomes crosslinked to fibrin by transglutaminase activation, with concomitant immobilization of heparin and associated heparin-binding growth factors. HBDS was incorporated into fibrin glue and platelet-rich fibrin glue along with encapsulation of BM-hMSCs. Incorporation of HBDS into FG and PR-FG did not modify cell survival or promote chondrogenesis. Thus, although HBDS promoted central and peripheral nerve regeneration in chicken and rat models and represents a feasible method to enhance nerve generation in dorsal root ganglions, it exhibited adverse effects during cartilage regeneration (Ahmed et al. 2011).

Stabilization of Fibrin Hydrogels

Although the fibrin hydrogel combines some important characteristics as a scaffold for cartilage regeneration, fibrin constructs have been observed to degrade before the proper formation of cartilaginous tissue, which can be controlled by the addition of protease inhibitors such as aprotonin, galardin, and tranexamic acid to the culture medium (Ahmed et al. 2007, 2011). Encapsulation of BM-hMSCs into fibrin glue (FG) and platelet-rich fibrin glue (PR-FG) in the presence of tranexamic acid (1.5 mg/mL) resulted in stabilization of fibrin – hMSCs constructs without affecting the viability of cells (Ahmed et al. 2011). In a related study, the effect of ε-aminocaproic acid (EACA) on the TGF-β_1–induced chondrogenic differentiation of BM-hMSCs was assessed in a standard pellet culture system. EACA (5 mM) which was adequate to inhibit fibrin degradation did not affect chondrogenic differentiation (Kupcsik et al. 2009). In another study, BM-hMSCs were encapsulated into fibrin glue and cultured in chondrogenic media supplemented with TGF-β_3 under normoxic (21% O_2) versus hypoxic conditions (5% O_2). Fibrin-hMSCs hydrogels were stabilized under hypoxic conditions in the absence of any protease inhibitor, suggesting that hypoxic conditions might downregulate the expression of the enzymes responsible for fibrin gel breakdown after encapsulation of hMSCs (Ahmed et al. 2010).

Oxygen Tension

Cartilage is a hypoxic tissue; therefore it is pertinent to evaluate the effect of reduced oxygen tension on chondrogenic differentiation of MSCs. The fibrin sealant Tissucol has been combined with BM-hMSCs to compare normoxic *in vitro* conditions (21% O_2) to hypoxia (3% O_2)

for development of a cartilage substitute (Baumgartner et al. 2010). A highly porous hMSC-containing fibrin matrix was constructed that allowed hMSCs to survive throughout the period of culture (6 weeks). Only hypoxic conditions promoted the generation of rounded chondrocyte-like cell types and a chondral phenotype as indicated by mRNA expression of collagen II along with Alcian blue staining. While BM-hMSCs encapsulated into highly porous preparations of Tissucol maintained their stem cell character, culturing the cells in a chondrogenic medium under hypoxic condition promoted chondrogenic differentiation (Baumgartner et al. 2010).

Signalling During Chondrogenesis

In the field of cartilage and bone regeneration, the importance of the extracellular signal-regulated kinase (ERK) pathway is a matter of debate. Whether differentiation results from soluble chemical induction or a microenvironmental cue on the cells seems to have a determining effect on the role that this pathway plays in ultimate cell fate (Pelaez et al. 2012). The role of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway on chondrogenesis of mesenchymal stem cells (MSC) has been evaluated recently (Arita et al. 2011). BM-hMSCs were encapsulated into fibrin hydrogel scaffolds; TGF-β_3 significantly enhanced ERK1/2 phosphorylation and induced the cells down the chondrogenic pathway. Inhibition of ERK1/2 phosphorylation with a specific inhibitor PD98059 abrogated upregulation of chondrogenic-specific gene expression, suggesting that ERK1/2 is necessary for the TGF-β_3–mediated chondrogenic differentiation of MSC (Arita et al. 2011). In a related study, the role of the ERK1/2 pathway on the mechanical induction of chondrogenesis of BM-hMSCs was explored. BM–hMSCs were encapsulated in fibrin gel scaffolds and subjected to a dynamic mechanical compression stimulus previously demonstrated to induce chondrogenic differentiation of the cells, with and without the addition of PD98059. Inhibition of the ERK1/2 pathway completely abolished the chondrogenic differentiation otherwise induced by dynamic compression. These results indicate that the activation of ERK1/2 can be a key step in chondrogenic differentiation (Pelaez et al. 2012).

Fibrin Gel as a Delivery Vehicle

In a recent study, the release profile of TGF-β_1 from FG (Tissucol) alone or after encapsulation of BM-hMSCs was analysed for a week and chondrogenesis was assessed over 6 weeks. On the one hand, it has been shown that FG promoted retention of TGF-β_1. On The other hand, retention of TGF-β_1 after encapsulation of BM-hMSCs into FG increased by about 3-fold and almost stopped release beyond 24 h. In addition, TGF-β_1 remained bioactive and supported MSC chondrogenesis without impairing the deposition of proteoglycans and collagen-type II compared to standard scaffold-free MSCs pellets supplied with soluble TGF-β_1 (Diederichs et al. 2012). The effect of growth factor-augmented biphasic implants composed of different fibrin gels and a solid scaffold (collagen I/III) on chondrogenesis of BM-hMSCs has been studied *in vitro*. FG and collagen carriers induced strong upregulation of cartilage markers compared with biomaterial-free pellets. The biphasic carrier constructs showed a high biofunctionality with improved chondrogenesis and long-term local supply of bioactive TGF-β_1, which may be useful to enhance matrix-assisted repair strategies for damaged cartilage (Dickhut et al. 2010).

Gene Delivery from Fibrin Glue

Bioactivity of adeno-associated virus (AAV) – cytomegalovirus promoter (CMV) – TGF-β_1 (AAV-CMV-TGF-β_1) released from diluted (50%) and undiluted (100%) FG (Tisseel/Tissucol) was assessed by measuring induction of cartilage-specific gene expression in human

mesenchymal stem cells (hMSCs). AAV-TGF-β_1 released from diluted FG transduced hMSCs efficiently and subsequently higher concentrations of bioactive TGF-β_1 and greater upregulation of cartilage-specific gene expression were observed compared with hMSC from undiluted FG. This study demonstrated that diluted FG promoted enhanced release of bioactive AAV-TGF-β_1, efficient transduction, and improved chondrogenesis of BM-hMSC for potential tissue engineering applications (Lee et al. 2011).

In conclusion, the combination of hMSCs with the 3D carrier/scaffolding fibrin matrix is a continuously developing strategy to restore the function and structure of native cartilage after damage due to pathological or traumatic insults (Table 6.1). A wide range of effectors can influence the chondrogenic process of hMSCs including the nature of fibrin scaffold, source of the cells, bioactive agents (TGF-β growth factor superfamily and cartilage fragments), mechanical environment, delivery mode of the growth factors, fibrin-stabilizing agents, oxygen tension, ERK1/2 signalling, and finally genetic manipulation of the cell source.

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7 Differences Between Adipose Tissue-Derived Mesenchymal Stem Cells and Bone Marrow-Derived Mesenchymal Stem Cells as Regulators of the Immune Response

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Abstract

In general mesenchymal stem cells (MSCs) are considered to be promising agents for replacement of damaged tissues because of their potential to differentiate in a number of cell lineages. Moreover MSCs are potent regulators of the immune response and they are regarded as new and promising means for therapy of autoimmune diseases, graft versus host diseases etc. MSCs have been isolated by different tissues. The most studied are bone marrow derived MSCs (BM-MSCs), but the most easily accessible are the adipose tissue derived MSCs (AT-MSCs).

The object of this chapter is to discuss the basic data published as well as some data from our laboratory about immunoregulatory effects of human MSCs isolated either from bone marrow or adipose tissue. Direct effects of MSCs on different immune cell populations are discussed as well as some indirect effects mediated through immunomodulatory cells. The expression of some immunoregulatory molecules from MSCs is also the object of this chapter. In most cases tested so far, AT-MSCs express stronger immunosuppressive properties and this would raise the question of their preferred application in treatment of autoimmune diseases.

Introduction

Autoimmune diseases affect about 5% of human population; they have a chronic course and can lead to a permanent invalidization of the patients. Autoimmune diseases present serious problems both medical and social, which cannot be resolved successfully at the present status and achievements of the biomedicine science as one of the reason being that most of the applied therapeutics can cause multiple side effects. Investigations revealing some more details of the pathogenic mechanisms of the autoimmune process would create possibilities for searching of new therapeutic approaches. Obviously the most valuable therapeutics would be those which would correct the disregulation of the autoimmune response in autoimmune disorders.

Human mesenchymal stem cells (MSCs) are considered very important tools for use in autoimmune disorders because of their immunomodulatory activities. Most of the possible applications of human MSCs have been tested in animal models with good positive therapeutic results. Highly beneficial effects have been reported for children developing graft versus host disease (GVHD) after allogenic bone marrow transplantation and treated repeatedly with infusions of allogenic MSCs. Currently quite many clinical trials with MSCs are announced and officially registered at the web-site of the U.S. National Institute of Health. MSCs isolated from different sources and most often from BM-MSCs have been applied. A comparison between MSCs isolated from different sources is needed to select most suitable for clinical applications.

Characteristics of Human Mesenchymal Stem Cells

Mesenchymal Stem Cells – Isolation, Morphology, Clonogenicity, Immunophenotype, Differentiation Capacity

Mesenchymal stem cells were initially described as fibroblast-like elongated cells which can adhere to solid phase *in vitro* and could form cell colonies (Friedenstein et al. 1970). Over the last decade a huge number of papers have described different features of the MSCs concerning their phenotypes and plasticity which has lead to using different names for these cells such as marrow stromal cells, multipotent stromal cells, stromal precursor cells etc. The different names used to a define cell population with very similar characteristics but isolated from different organs or tissues caused some misunderstandings and this made the International Society for Cellular Therapy to publish the minimal requirements to be used for classifying a cell population as mesenchymal stem cells and these include adherence to plastic in culture, clonogenicity which means forming

cell colonies when seeded at low densities, capacity to differentiate into adipogenic, chondrogenic and osteogenic lineages, cell surface expression of CD90, CD73, CD105, but negative for CD45 and CD34 (Dominici et al. 2006). The precise phenotype of the MSCs is difficult to be specified mostly due to the lack of reliable/specific phenotypic marker for these cells *in vivo* or in suspension although a number of candidate molecules have been tested.

MSCs Share Similar Ways of Isolation and Morphology

Initially MSCs have been isolated from bone marrow aspirates (BM-MSCs) and it is generally accepted that these cells are components of the specific microenvironment which facilitates the process of hematopoiesis providing factors required for the development of the hematopoietic stem cells. These cells can adhere to solid phase and they proliferate forming colonies while at the same time they preserve their potential for self renewal and differentiation. In cell cultures the cells preserve their typical elongated fibroblast-like morphology although sometimes two distinctive types are described – small cells with high proliferative potential and larger cells which divide more slowly. It has been estimated that the MSCs comprise about 0.01–0.001% of the mononuclear bone marrow cells isolated by density gradient centrifugation. The BM-MSCs are a heterogeneous population but colonies originating from single cells can be cultured to form a monolayer of identical fibroblast-like cells which shows that these cells really possess high proliferative capacity.

Cells with the overall characteristics of BM-MSCs have been isolated from a number of different tissues such as adipose tissue (AT-MSCs), synovial membranes, muscles, skin, dental pulp, endometrium and decidua, fetal liver etc. which would suggest that MSCs or MSCs-like cells are widely distributed *in vivo* in human body. Both subcutaneous and visceral adipose tissue is a rich source of cells with the general characteristics of multipotent mesenchymal stem cells. The advantage of the subcutaneous adipose deposits as sources for AT-MSCs is that they are readily accessible; large volumes of fat can be aspirated without any risks for the donor. In most cases the stromal cells are isolated and cultured after treatment the lipoaspirates with collagenase following the laboratory protocol described by Zuk et al. (2002). It has been demonstrated by a number of studies that fibroblast-like cells isolated from subcutaneous adipose tissue can be induced to differentiate into numerous lineages: the adipocyte, chondrocyte, endothelial, epithelial, hematopoietic support, hepatocyte, neuronal, myogenic, osteoblast cells and support the undifferentiated growth of human embryonic cells.

Differences Between AT-MSCs and BM-MSCs

Comparisons between AT-MSCs and BM-MSCs have been performed early after the finding that adult stem cells are present in the adipose tissue. In case that AT-MSCs would have properties and potentials similar to that of initially described BM-MSCs, the former will have considerable advantages as regards to their putative clinical applications because they can be easily collected in high numbers. No differences between AT-MSCs and BM-MSCs are described regarding the morphology, the general growth characteristics; the most commonly used surface immunophenotype markers or the successful isolation rate. However, AT-MSCs are reported to have higher clonogenicity, higher proliferation rates and longer periods of culture as undifferentiated cells.

Both types of MSCs do not express CD45 and express the surface markers like CD73, CD90, CD105, and CD44. Results from detailed studies have revealed that there are some important differences and just to mention some of them AT-MSCs are positive for the expression of CD49d (4α integrin) but the BM-MSCs do not express this protein. Detection of low amount CD34 on subpopulation of AT-MSCs has been reported at early stages of cultures but its expression decreases with growing passages and

virtually disappears from the cells but CD34 is not detected at the surface of BM-MSCs at any time. Contrary to that CD106 (vascular cell adhesion molecule-1, VCAM-1) is lacking on the surface of AT-MSCs but is expressed by BM-MSCs.

Both AT-MSCs and BM-MSCs have similar adipogenic and osteogenic differentiation however it is considered that AT-MSCs have somewhat richer potential as they can differentiate into neural, glial or vascular endothelial cells. The profile of secreted paracrine factors analyzed at the levels of mRNAs shows that AT-MSCs express at higher levels of mRNAs for insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor-D (VEGF-D) and interleukin-8 (IL-8). Several studies have shown that AT-MSCs have greater secretion of VEGF (vascular endothelial growth factor) and HGF (hepatocyte growth factor), higher expression of MMP3 and MMP9. These facts would suggest that AT-MSCs might be more suitable in conditions when there is need to restore or to enhance the angiogenesis. Results from experimental animal studies prove the higher supportive effect in heart infarction, ischemic brain damages.

Systemic and qualitative studies using high informative methods of genomics and proteomics have compared AT-MSCs and BM-MSCs and found that in the greater percentage of cases the expressed genes were similar between the both cell types. Still, about 3.4% were expressed just in one cell type. Genes expressed in AT-MCSs only are predominantly related to the process of cell-to-cell communications (CCL3, FGF9, IL1R2, KDR) and control of gene transcription in the cell (PAX3, SP11, ZNF45) while genes expressed in BM-MSCs only are engaged in control of Wnt signaling and differentiation mechanisms (Noël et al. 2008). Taken together all data cited above show that AT-MSCs are easier for isolation, have higher rate of proliferation, higher clonogenicity, wider differentiation potential and better support of the vasculogenesis. All these would suggest that AT-MSCs have advantages for clinical usage in comparison to the BM-MSCs.

MSCs from Bone Marrow and Adipose Tissue Are Immunosuppressors

A crucial characteristic of the MSCs is their immunosuppressive activity which makes these cells important not only for use in regenerative medicine but makes them a better means for treatment of autoimmune diseases, clinical transplantation, graft versus host diseases, lymphoproliferative diseases etc. A lot of data have been reported that substantiate the notion that MSCs escape recognition as allogeneic cells or induce hyporeactivity in allogeneic designs. MSCs are proven to express HLA class I antigens but lack the expression of HLA II antigens as well as some co-stimulatory molecules. However, the expression or lack of HLA antigens by MSCs seems not to be the basic reason for their immune escape because it has been shown that even upregulation of MHC II expression by IFNγ do not induce immune response. This shows that MCSs are not just passive targets of immune recognition and response but they are active players in the interactions with the cells of the immune system (Rasmusson 2006). Multiple studies have shown that basically the immunosuppressive activities do not differ between AT-MSCs and BM-MSCs but still, there are some differences in their effects on the immune response.

Direct Influence of MSCs on Effector Immune Cells

Effects of AT-MSCs and BM-MSCs on T Lymphocyte Activities

A number of both *in vivo* and *in vitro* studies have proved that *ex vivo* cultured BM-MSCs are capable to suppress various activities of T lymphocytes which are considered to be the major factors in the cell mediated immune reactions (Bifari et al. 2010). It is known that BM-MSCs inhibit the T cell activation and proliferation both in model systems *in vitro* and in experimental animals *in vivo*, in mixed lymphocyte cultures or after

stimulation with non-specific mitogens and/or cognate antigens and this effect does not depend on the MHC compatibility. Even after being differentiated into osteocytes, adipocytes or chondrocytes the suppressive effect of BM-MSCs is preserved and their culture in the presence of IFNγ seems to enhance their suppressive effect (Le Blanc et al. 2003). Besides inhibiting the antigen specific proliferation of both naïve and memory T cells, the BM-MSCs suppress the expression of activation markers, the formation and functions of the cytotoxic lymphocytes as well as the production of cytokines such as IFNγ and IL-4 by Th-1 and Th-2 cells, correspondingly (Bifari et al. 2010). In regard to the CD8 + cells it is known that BM-MSCs inhibit their cytotoxicity only if they are present at the initial stages of the mixed lymphocyte culture (Rasmusson et al. 2003). The cell lyzing activity of the CTLs (cytotoxic T lymphocytes) was not affected if the BM-MSCs were added during the cytotoxic stage. It has been proposed that BM-MSCs are capable to suppress only the afferent phase of the alloreactivity by preventing the activation of cytotoxic CD8+ lymphocytes but the presence of BM-MSCs did not affected the function of already activated cytotoxic T lymphocytes.

In comparison to the numerous papers on the influence of BM-MSCs on the immune reactions and the significant progress achieved in this field the data about the immunomodulatory activities of the AT-MSCs are relatively scarce. Reviewing the published data in regard to the immunosuppressive properties and the immunogenicity of the AT-MSCs a general conclusion can be drawn and it is that BM-MSCs and AT-MSCs have quite similar immunosuppressive effect *in vitro* on the functions of the T lymphocytes (Puissant et al. 2005; Yanez et al. 2006). Alloreactive T cells are not activated by both BM-MSCs and/or AT-MSCs. Moreover the proliferation and the secretion of proinflammatory cytokines (TNFα, IFNγ and IL-2) by mitogen- or allogeneical activated T cells are suppressed by both BM-MSCs and AT-MSCs. Still, some studies have reported that AT-MSCs have higher inhibitory activity as compared to that of BM-MSCs. It has been established that AT-MSCs inhibit stronger the proliferation of pokeweed mitogen stimulated lymphocytes in comparison to the BM-MSCs (Puissant et al. 2005). However, another study on the comparison of the effects of the two types MSCs on the secretion of TNFα, IFNγ and IL-12 by T lymphocytes did not find statistically significant difference between the effects of the BM-MSCs and AT-MSCs (Yanez et al. 2006).

The experiments carried out in our laboratory assessed the changes of the secretion of IFNγ or IL-4 by mitogen activated T helper cells. Our data outlined the tendency of higher suppression of the T cell cytokine secretion by AT-MSCs as compared to the effect of the BM-MSCs (Bochev and Kyurkchiev 2009) and this will affect the whole chain of immune reactions regulated by IFNγ such as activation of macrophages, NK cells and dendritic cells, activation of cytotoxic T lymphocytes, up-regulation of the expression of MHC class I and II molecules, adhesion molecules and high-affinity Fc receptors. Similarly, the inhibition of the IL-4 secretion would affect the proliferation of B cells and their differentiation in antibody-secreting plasma cells as well as the activation of T lymphocytes. It seems that some data would suggest that the AT-MSCs seem to be a better immunosuppressor in regard to T cell activity with clearly defined advantages for therapeutic applications.

Effects of AT-MSCs and BM-MSCs on B Lymphocyte Activities

It has been established that human BM-MSCs have the capacity to inhibit the B cell proliferation, hemotaxis and differentiation in antibody-secreting plasma cells in *in vitro* experiments (Corcione et al. 2006; Tabera et al. 2008). Purified human CD19+ cells co-cultured with BM-MSCs and stimulated with combination of anti-Ig antibodies, recombinant CD40L, CpG 2006 oligonucleotides and cytokines have decreased proliferative potential and differentiation into

IgM-, IgA- and IgG-producing plasma cells. BM-MSCs inhibited the B cell proliferation not inducing apoptosis but caused a blockade of the lymphocyte development at G0/G1 phase of the cell cycle. Some experiments using cells separated by a semi-permeable membrane have shown that the inhibitory effect of the BM-MSCs on the B cell proliferation is preserved at the similar degree and this fact will suppose that still undefined soluble factors are responsible for that effect. BM-MSCs do not affect the antigen-presenting properties of the B cell since there were no changes detected in the expression of HLA-DR molecules as well as the co-stimulatory molecules CD80, CD86, CD40. However, the expression of chemokine receptors CXCR4, CXCR5 and CCR7 by the B cells was down-regulated during the co-culture with BM-MSCs which leads to some disturbances in their chemotaxis in response to the secretion of CXCL12 and CXCL13 chemokines.

In our laboratory it has been proved that AT-MSCs also exhibit considerable and even more powerful than BM-MSCs inhibitory effect on B-cell *in vitro* differentiation into antibody-producing plasma cells (Bochev et al 2008). We have demonstrated that in the presence of mesenchymal stem cells, regardless of their source, the immunoglobulin production by pokeweed mitogen stimulated human B cells was significantly inhibited as the suppressive effect of the AT-MSCs was 3-fold greater as compared to BM-MSCs. In regard to the possible molecular mechanism it is most reasonable to accept the soluble factors secreted by MSCs are responsible for the suppressor effect. However these putative suppressor factors have not been identified in these experiments. It is quite possible that factors such as TGFβ1, HGF, prostaglandin E2 (PGE) or indoleamine-2,3-dioxygenase (IDO) have a role in mediating the inhibitory effects of the MSCs on B-cell functions since it has been previously shown that all these factors are implicated in most MSC-mediated immunoregulatory activities (Bifari et al. 2010).

AT-MSCs seem to up-regulate the expression of CD69 by B lymphocytes while BM-MSCs, contrary to that, express inhibitory effect on the expression of this membrane receptor. However, the expression of CD69 by T lymphocytes is solely affected by BM-MSCs as the number of CD69+ expressing cells decreases after co-cultures. The effect on the expression of CD69 and its possible significance is discussed further in the text. These differences illustrate once again the various immunoregulatory potentials of MSCs from different tissue origins. The data presented above clearly show that both AT-MSCs and BM-MSCs have immunosuppressory activities regardless to the experimental design directly on different populations of lymphoid cells although there might be some differences in their effects. These differences might turn out to be quite useful in the putative clinical applications of the MSCs.

Indirect Immunomodulatory Effects of MSCs

MSCs exert their immunomodulatory activities either directly as described above or indirectly through influencing other cell populations with regulatory effect on the immune effector cells.

Effects of AT-MSCs and BM-MSCs on Peripheral Blood Monocytes

Monocytes are peripheral blood cells whose role in immune response is far more complicated than that of macrophages and monocyte-derived dendritic cells (mDCs) precursors as there are a lot of data about their engagement in the immune regulation processes. When co-cultured with AT-MSCs or BM-MSCs a significant up-regulation of the CD69 molecule expression on the monocyte surface is recorded and this effect is higher when AT-MSCs are used in co-culture. Classical ideas about the CD69 molecule are related to its role as an early activation marker expressed on all cells originating from the bone marrow with the exception of red blood cells. However, some new data reveal the role of CD69 as a molecule closely related to immunoregulation. It has been established that the CD69 expressed binds to its specific ligand CD69L

which located on the surface of the T lymphocytes and its binding triggers the secretion of the immunosuppressive factor TGFβ (Sancho et al. 2005). So, AT-MSCs and BM-MSCs activate the monocytes, up-regulate their CD69 expression and this molecule affects T lymphocytes to secrete TGFβ. The mechanism of this interaction is still unknown and the effect of AT-MSCs is higher than that of BM-MSCs.

Simultaneously with increased expression of CD69 from monocytes, the supernatants of PBMCs co-cultured with AT-MSCs or BM-MSCs contain significantly higher concentrations of IL-10 as the effect of AT-MSCs is stronger. It was not possible to determine the cellular source of the IL-10 under the experimental conditions used in our laboratory. However, in any case the MSCs exert an indirect immunosuppressive effect on the monocytes and/or lymphocytes by inducing the secretion of two cytokines with highly expressed immunosuppressive activity – TGFβ and IL-10 as the effect of the AT-MSCs is more potent than that of BM-MSCs. There are some data which complement the ideas about the role of the interaction between the monocytes and MSCs in the immunoregulation. It has been established that not only MSCs influence the activities of the monocytes in the direction of immunosuppression but *vice versa* as under the influence of the IL-1β, secreted by the monocytes, the MSCs produce TGFβ (Groh et al. 2005). Therefore it is clear that the blood monocytes and the MSCs are in complicated two-side interactions as partners in their immunosuppressive effects.

Effects of AT-MSCs and BM-MSCs on Monocyte-Derived Dendritic Cells

A basic characteristic of the peripheral blood monocytes is that they are precursors of the monocyte derived dendritic cells (mDCs). The classic model is that in *in vitro* cultures in the presence of IL-4 and GM-CSF (granulocyte-macrophage colony stimulating factor) the monocytes differentiate into immature dendritic cells which subsequently in the presence of LPS (lipopolysaccharide) proceed to mature dendritic cells. In addition to their major function as antigen presenting cells the mDCs are one of the most significant players in the modulation of the immune response. By direct interactions with their target cells and by secretion of various cytokines the mDCs can either stimulate the immune response (immunogenic mDCs) or can trigger induction of immune tolerance and apoptosis (tolerogenic mDCs) (Wallet et al. 2005).

Co-cultures of AT-MSCs or BM-MSCs with mDCs have different effects on the functions of the mDCs:

The Percentage of the Cells Expressing CD14+ Increased But the Cell Population Expressing CD83+ Significantly Decreased

It is well known that CD14 is a marker specific to the monocytes and these cells are supposed to lose its expression during their differentiation into dendritic cells under the control of IL-4 (Dilioglou et al. 2003), (Ivanova-Todorova et al. 2009a). On the other hand CD83 is a specific marker for the mature mDCs. Therefore, AT-MSCs and/or BM-MSCs induce a blockade of the process of differentiation of the monocytes into dendritic cells and the effect of the AT-MSCs is significantly higher as compared to that of BM-MSCs. It is supposed that the inhibition of mDCs differentiation is mediated via IL-6 secreted by the MSCs and which is present in high concentrations in conditioned medium samples of AT-MSCs or BM-MSCs (Djouad et al. 2007). It is not easy to interpret the described effect of both types of MSCs because of the very complicated and multilayered effects of the dendritic cells but most probably it is related to blocking of the major class of antigen-presenting cells and therefore blocking the afferent arm of the immune response. Another possible explanation although rather speculative might be that the blockade of the differentiation of dendritic cells from monocytes is related to the modulation mechanism of monocytic CD69 molecules as described above.

Decreased Expression of CD80 and CD86 Molecules by Dendritic Cells

The data of our experiments show that the basic effect of MSCs on the dendritic cells is the restriction of their antigen-presenting function which is mediated by inhibition of the expression of CD80 and CD86 induced by co-cultures of mDCs with AT-MSCs and BM-MSCs. Under these experimental conditions AT-MSCs have a higher suppressive effect (Ivanova-Todorova et al. 2009a). It is well known that these two molecules form the B7 complex which has a major role in the co-stimulation of lymphocytes in the process of the antigen presentation by mDCs. These molecules can be detected at the cell surface as early as the monocyte stage as the CD86 is the first to be expressed and around day 5 of the differentiation of the monocytes the CD80 molecules are detected. During the maturation of the mDCs induced by LPS the expression of these two molecules is contantly increased. The B7 complex is a key element in antigen presentation by binding with CD28 on the surface of the T lymphocytes and induces immune response. The same B7 complex binding to the CTLA-4 results in immune tolerance (Dilioglou et al. 2003). It has been described that the absence of B7 from the cell surface would induce anergy of the T lymphocytes. Comparing the both MSCs it can be established that the AT-MSCs have higher suppressive effect than the BM-MSCs on the expression of CD80 and CD86 which are the two major molecules needed for the effective presentation of antigens. Furthermore, their decreased expression is an immunoregulatory mechanism inducing an anergy in T lymphocytes.

Secretion of IL-10 by mDCs Is Increased During Co-cultures with MSCs

Co-culturing of mDCs with AT-MSCs or BM-MSCs causes a significant up-regulation of the secretion of IL-10 by mDCs and its concentration in mDCs/AT-MSCs conditioned medium is statistically higher than in mDCs/BM-MSCs conditioned medium (Ivanova-Todorova et al. 2009a). The secretion of this crucially important for the immunoregulation cytokine induced by the presence of MSCs has been observed in co-cultures with other populations of immunocompetent cells and most probably it is a common mechanism for immune suppression realized indirectly by the MSCs and will be discussed further in this text.

mDCs Co-cultured with MSCs Do Not Secrete CCL-3 and CCL-4

The chemokines CCL-3 and CCL-4 are not found in co-cultures of mDCs cultured with MSCs and this is one of the rare cases in our experiments when there are no qualitative differences between the immunosuppressive effects of the both MSCs types. Most probably this is due to the fact that their secretion by dendritic cells is completely blocked. These chemokines are secreted in high amounts by mDCs as their role is to recruit T cells, macrophages, NK cells and eosinophils. Their blocked secretion by mDCs is most probably an additional mechanism of MSCs to suppress the immune response by restricting the capacity of mDCs to recruit immunocompetent cells. In general, it should be underlined that under the influence of both AT-MSCs and BM-MSCs, the dendritic cells arrest their differentiation at the monocyte stage, restrict their capacity for antigen presentation and lose their potential to secrete some chemokines and the effect of AT-MSCs is significantly higher as compared to that of BM-MSCs.

AT-MSCs Conditioned Medium Increase the Percentage of Tregs

The higher immunosuppressive effects of AT-MSCs in comparison to the BM-MSCs in regards to other immunoregulatory cells prompt us to study in some more details these effects from different aspects. It is known that multiple T cell populations are engaged with

immunoregulatory functions as a very important role have the cells known as Tregs, distinguished by the expression of CD25 marker – CD4 + CD25 + FoxP3+ and the recently described and still unclear subpopulation CD4 + CD25- FoxP3. Both cell populations have immunosuppressive activity and the effect of CD25+ population is mediated via the secretion of TGFβ while the effect of CD25- subpopulation is believed to be mediated through the secretion of IL-10 (Curotto and Lafaille 2009).

In our experiments it was shown that AT-MSCs conditioned medium caused significant increase both subpopulations of Tregs present in homogeneous populations of CD4+ T helper cells purified by selective magnetic beads isolation. This fact suggests that AT-MSCs secrete active factors which induce specific increase of the expression of FoxP3 marker and the corresponding increase of the Tregs population. Our studies did not reveal any increase of the concentration of TGFβ in CD4+ T helpers conditioned medium but there was a significantly higher concentrations of the immunosuppressive IL-10. The fact that MSCs induce an increase of the numbers of CD4 + FoxP3+ cells as well secretion of IL-10 has been reported recently as the experiments have been carried out with BM-MSCs or UC-MSCs (umbilical cord-derived MSCs), as the cells are in direct cell-to-cell contacts with PBMCs. Our results reveal the same effect of AT-MSCs conditioned medium on T helpers isolated by specific magnetic beads. Several biologically active factors have been suspected to cause an increase of Tregs cells and the increased secretion of IL-10 and they include TGFβ, IDO, PGE2, HLA-G5, IL-6. We have recorded a rather high concentration of IL-6 and the meaning of this finding is discussed later in this chapter.

MSCs Induce the Immunoregulatory Cells to Secrete IL-10

The data about the effects of AT-MSCs and BM-MSCs on different immunoregulatory cells outline a general mechanism and that is the secretion of IL-10. In our experiments neither AT-MSCs nor BM-MSCs secrete themselves IL-10 but its secretion was elevated in co-cultures of MSCs and immunomodulatory cells (B lymphocytes, T helpers, mDCs). IL-10 is one of the major anti-inflammatory cytokines engaged in the induction of immune tolerance. Its major mechanism is to inhibit the expression of B7 by the antigen-presenting cells (mDCs) and CD28 by T lymphocytes and thus inducing T cell anergy (Yang et al. 2006). When secreted by the mDCs under the influence of MSCs it is most probably that IL-10 will have paracrine effect on CD28 expression as well as autocrine effect on the B7 molecules, expressed by the mDCs themselves. AT-MSCs and to lesser degree BM-MSCs induce secretion of IL-10 by various types of immunoregulatory cells and we believe that this may be one of the mechanisms by which mesenchymal stem cells indirectly induce immune suppression (Fig. 7.1).

Although we have not detected secretion of IL-10 in MSCs cultures the possibility cannot be ruled out that MSCs themselves are the source of this cytokine just in cases when they communicate with immunocompetent cells.

Immunomodulatory Molecules Expressed by MSCs

PIBF Expression by AT-MSCs and BM-MSCs

Progesterone Induced Blocking Factor (PIBF) has been identified initially by the group to Szekerez-Bartho as 34 kDa protein with immunomodulatory activity which is secreted by γδT lymphocytes triggered by progesterone during pregnancy. PIBF inhibits the NK cell and T cell cytotoxic activities and thus it is considered to be one of the major factors for the immunomodulation during pregnancy. Consequently data were piled up showing that PIBF seems to have much broader effects as inhibitor of immune reactions and directs the immune response to Th2 type with predominant secretion of IL-4, IL-6, IL-10. A lot of papers published describe

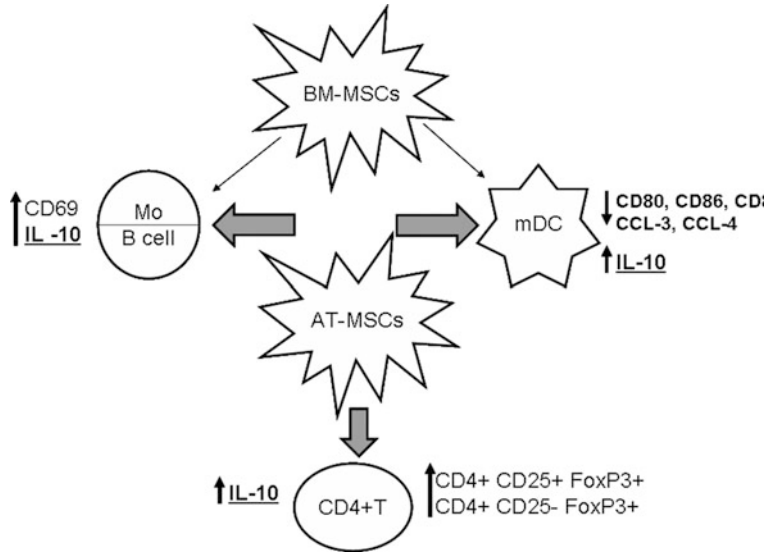

Fig. 7.1 MSCs lead to secretion of IL-10 by other immunoregulatory cells and AT-MSCs have stronger effect than BM-MSCs

the presence of mRNA or protein expression by rapidly proliferating cells such as human trophoblasts, tumor cell lines, endometrial or decidual mesenchymal stem cells (Check et al. 2009; Mikoa et al. 2011; Kyurkchiev et al. 2011). A hypothesis has been put forward that one of the possible mechanisms for the escape of tumor cells from the immune system is the secretion of PIBF which has immunosuppressive effect against NK cells and cytotoxic T lymphocytes (Check et al. 2009). All these data concerning the immunoregulatory role of PIBF and its relation to cell populations that don't have the characteristics of terminally differentiated cells prompted us to look for the PIBF expression by AT-MSCs and BM-MSCs. Using a monoclonal antibody produced in our laboratory PIBF could be detected to be expressed by both AT-MSCs and BM-MSCs. The specific cytoplasmic staining was localized perinuclear while no expression on the cell surface could be detected (unpublished data). The fact should be pointed out that AT-MSCs expressed PIBF in higher amount as compared to the BM-MSCs. These results could at least partially explain the fact that AT-MSCs have higher immunosuppressive potential while there are no significant differences in the profile of cytokines secreted by the both stem cell types. It can be speculated that the MSCs use PIBF secretion as a mechanism for survival at their niches because by secreting PIBF they would exert locally immunosuppression against the adjacent immune cells.

HLA-G Expression by AT-MSCs and BM-MSCs

MSCs have a diminished expression of MCH II but express HLA-G which is a MHC class Ib molecule which renders them non-immunogenic and it is known that MSCs do not provoke an alloreactive reactions. Non-classical MHC class Ib antigens (HLA-G, HLA-E and HLA-F) seem to induce immune tolerance acting as ligands of inhibitory receptors, expressed by the immune cells. Limited polymorphism is characteristics for HLA-G since there are four membrane isoforms (HLA-G1 to - G4) and three soluble splicing variants (HLA-G5 to -G7). Similarly to the classical MHC class Ia proteins, HLA-G is comprised of α chain with three domains which is no-covalently bound to β2-microglobulin. Peptides presented by HLA-G have usually nine aminoacids and have varying sequences. HLA-G is initial identified on trophoblasts and is

involved in building up the immune tolerance between the mother and the fetus during pregnancy. Later on it has been established that HLA-G is expressed by tumor cells, it can be detected in transplanted patients, in autoimmune diseases and viral infections. It should be pointed out that HLA-G mRNA can be identified in quite many different tissues but the expression of HLA-G protein is detected in a limited cell types and MSCs are among these cells. This fact proves that HLA-G expression is strictly regulated by the microenvironment at both transcriptional and post-transcriptional level.

The profile of inhibitory functions of HLA-G is quite broad and affects different types of immune cells. Its effects can be either directly exerted by cell-to-cell contacts by the membrane forms of HLA-G or can be indirect effect mediated via inducing secretion of cytokines by other cells or even by induction of immunoregulatory cells. The major effect of HLA-G is inhibition of the activities of T lymphocytes, NK cells and antigen-presenting cells. Studies *in vitro* have shown that this effect is mediated through interactions with inhibiting receptors such as immunoglobulin-like transcript-2 and -4 (ILT2, ILT4 also known as LILRB1 and LILRB2), and killer immunoglobulin-like receptor (KIR) -2DL4. Interactions with these receptors regulated the T cell proliferation, cytotoxicity of NK cells and maturation of dendritic cells. Even more, it has been demonstrated that HLA-G can induce the differentiation of various sub-populations of suppressive/regulatory immune cells. HLA-G expression by tumor cells protects the latter from the killing by NK cells. HLA-G1 expressed by the tumor cells inhibits the cytoskeletal re-organization of NK cells (Favier et al. 2010). The contact of NK cell with HLA-G negative target cells causes a rapid polarization of the lytic granules in the cytotoxic cells while in contact with HLA-G + target cells the lytic granules remain diffused in the cytoplasm. Another major mechanism of HLA-G effect is its engagement in the cytokine shift to Th-2 with increased secretion of IL-4, TGF-β1 and decreased secretion IFNγ and TNFα. A number of factors modulate the expression of HLA-G such as cytokines (IFNγ, TNFα, IL-10), hormones (progesterone), epidermal growth factor (EGF), viruses (herpes simplex virus type 1, rabies virus, HIV), ATP, IDO. IL-10 seems to be a key cytokine for the expression of HLA-G since it has been shown that it up-regulates both the expression and the secretion of HLA-G5 by CD14+ peripheral blood monocytes and MSCs. Later on it was reported that the same effect of IL-10 is seen in respect to the membrane isoforms of HLA-G (Rizzo et al. 2011). The effect of IL-10 is specific since it has been recorded that the classical MHC class I and MHC class II are down-regulated in monocytes following IL-10 treatment. A number of investigations have reported the expression of HLA-G by MSCs isolated from various tissue sources - chorionic-plate-derived mesenchymal stem cells (CP-MSCs) and Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs), fetal MSCs, decidual MSCs, BM-MSCs, and AT-MSCs. In our studies HLA-G expression by non-stimulated MSCs was detected at the level of mRNA, only while after stimulation with progesterone a high level of HLA-G protein expression was established (Ivanova-Todorova et al. 2009b). The higher increase of membrane HLA-G protein was detected in AT-MSCs compared with BM-MSCs and immunofluorescence results detected the higher cytoplasmic expression of HLA-G in AT-MSCs after treatment with progesterone. We could not detect secreted isoforms of HLA-G using commercially available ELISA kit for HLA-G5 although HLA-G5 mRNA could be detected in both progesterone treated and control cultures of MSCs. Briefly, our results show that the expression of HLA-G is higher in AT-MSCs as compared to BM-MSCs and this fact might be related to the higher immunosuppressive activity of the AT-MSCs. Some authors (Blanco et al. 2008) have detected low levels of HLA-G expression in decidual MSCs and showed that it could be up-regulated after treatment with IFNγ, progesterone and cAMP. Moreover, treatment of decidual MSCs with progesterone and cAMP caused a higher secretion of IL-10 by these cells. It was pointed out earlier in this chapter that when MSCs were in communication with PBMCs, CD4+

T cells or mDCs an increased concentration of the immunosuppressive IL-10 in the conditioned medium was found, while neither AT-MSCs nor BM-MSCs secreted IL-10.

It can be speculated that MSCs induce mDC, CD4+ T cells and other cell types to secrete IL-10, or even that MSC activated in the conditions of co-culture with immune cells secrete IL-10, which on its side would enhance the HLA-G expression by MSCs. The up-regulated HLA-G expression by MSCs would inhibit the activities of the immune cells. It is well known that "naive" MSCs have weaker immunosuppressive and other functions (suppressing the proliferation of tumor cells) in comparison to MSCs "activated" by cell-to-cell contacts with other cell types. These facts give us ground to suppose that when MSCs are in a particular microenvironment their immunosuppressive activities might be more powerful than under *in vitro* conditions due to cross-talk with other cells.

Generally it is assumed that HLA-G has a central role in the entire reshaping of the immune system for establishing a local specific immune tolerance. This assumption concerns not only physiological phenomena as pregnancy but also for some pathological states as neoplasms, transplantation, autoimmune diseases. MSCs are promising tools to be used in regenerative medicine and it is necessary to keep in mind the tendency of MSCs to migrate at site of early tumors development as well the suspected possibility of epithelial-mesenchymal transition and possible carcinogenesis. HLA-G expression by MSCs is a major caveat because of the phenomenon called togocytosis which is the capability of HLA-G molecule to be transferred from one cell to another when the cells are in close contact. This phenomenon has been described for tumor and immune cells. However in other cases such as transplantations and autoimmune diseases the HLA-G expression and its involvement in the immunosuppressive mechanisms are considered to be quite good for the patients. All these speculations necessitate detailed investigations of the factors controlling the HLA-G expression and the other participants in the cascade on immunotolerant events.

Comparison of Cytokine Profiles of BM-MSCs and AT-MSCs

Currently a number of investigations are focused on the secretory profile of mesenchymal stem cells isolated from different sources (Hwang et al. 2009; Jenhani et al. 2011). The majority of authors do not find significant differences and it is generally accepted that the MSCs secrete a broad range of predominantly chemokines, growth factors and comparatively small number of cytokines as in regard to immunomodulation IL-6 seems to be of major importance (Hwang et al. 2009; Jenhani et al. 2011). In our laboratory conditioned supernatants from AT-MSCs and BM-MSCs from different donors were compared using a standard cytokine panel for screening of 36 cytokines and chemokines (C5a, CD40L, G-CSF, GM-CSF, GRO, I-309, sICAM-1, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1ß, Serpin E1, RANTES, SDF-1, TNFα, TREM-1). When analyzing those cytokines and chemokines detected in every supernatant there were not any significant differences between the secretory profiles of AT-MSCs and BM-MSCs. Secretory profiles of AT-MSCs and BM-MSCs isolated from one donor and tested at the same cell culture passage were very similar or almost identical. This result seems to be very intriguing because of the known data for the better immunomodulatory effects of the AT-MSCs. One of the possible explanations might be that so far the key secretory factors related to the higher suppressory potential of AT-MSCs have not been identified. Another more probable speculation is that a combination of secretory products and direct cell-to-cell contact between the MSCs and immunocompetent cells which triggers a cascade of complex cellular interrelations is responsible. Both cell types secrete a number of chemokines such as IL-8, GROα, MCP-1, MIF, Serpin E1, RANTES, and SDF-1 which recruit immunocompetent cells. Having that in mind one can speculate the secretion of chemokines by AT-MSCs or

BM-MSCs is just the initial step in the complex process of immunomodulation exerted by MSCs. Recruitment of the suitable populations of immune cells guarantees the presence of target cells susceptible to the paracrine or direct cell-to-cell effects of the MSCs. SDF-1 (stromal derived factor-1) is a chemokine of particular interest because its specific receptors are expressed by the MSCs themselves which would suggest some autocrine control of the homing of MSCs (Hwang et al. 2009).

In regard to the secreted cytokines in our experiments we could not detect the presence of IL-10 in AT-MSCs or BM-MSCs conditioned medium which makes us believe that in our experiments the MSCs have induced the secretion of IL-10 by the immunocompetent cells present during the co-cultures. In regard of immunoregulatory cytokines in BM-MCSs conditioned medium as well as in AT-MCSs conditioned medium a high concentration of IL-6 was found. During the last years lot of data have been published which strongly suggest that the conception of IL-6 just as pro-inflammatory cytokine should be reassessed. It has been established that IL-6 triggers the differentiation of anti-inflammatory subtype of blood monocytes, inhibits the differentiation of mDCs when secreted by human MSCs (Djouad et al. 2007) and stimulates the differentiation of CD8 + FoxP3+ cells (Nakagawa et al. 2009). These data as well as our results suggest that IL-6 might be one of the immunoregulatory factors secreted by MSCs.

In conclusion, MSCs influence different subpopulations of immune cells and their effects are predominantly immunosuppressive. MCSs either directly suppress the functions of effector cells of the immune response or they exert indirect effects through altering the functions of other immunoregulatory cells. MSCs have constitutively or induced expression of immunoregulatory proteins such as PIBF and/or HLA-G. Still, it is not clear whether the immunosuppressive effect of MSCs is mediated through secreted factors only, and which are these mediators or direct cell-to-cell contacts are mandatory for the immunosuppressive effects. These are questions which need further research to reveal new approaches for manipulating the immune response via MSCs. MSCs derived from both bone marrow and adipose tissue manifest immunoregulatory activities. However the AT-MSCs have some advantages for clinical application because they are easily accessible and cultured and have higher immunosuppressive effect. Thus, AT-MSCs seem to be the better candidates for therapeutic agents applied in treatment of autoimmune diseases.

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8 Transforming Growth Factor-Beta Induced Chrondrogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells: Role of Smad Signaling Pathways

Peter M. van der Kraan

Contents

Abstract

Mesenchymal stem cells can be differentiated to a number of lineages, adipocytes, osteoblasts and also chondrocytes. In the differentiation process of chondrocytes, members of the TGF-β superfamily play a decisive role. These factors control not only lineage determination but also the subsequent steps of chondrocyte differentiation. The intracellular signaling molecules of this family, the Smads, are indispensable to regulate, in concerted action with Runx2, this process. Specific Smad subtypes play differing roles in the various stages of chondrocyte differentiation. This feature of the Smads can be used to control chondrocyte differentiation and to generate stable cartilage that can be used for repair purposes.

Introduction

In the 1980s of the last century it was shown that deminarilized bone powder could induce cartilage formation in muscle. From this source two cartilage-inducing (chondrogenic) factors could be isolated, Cartilage-Inducing Factor A and B (CIF-A and CIF-B), of which CIF-A was the most abundant in demineralized bone (Seyedin et al. 1985). In a later study, it was shown that CIF-A and TGF-β, isolated from human platelets, were both able to differentiate rat muscle mesenchymal cells to cells with a

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chondrocyte-like phenotype and that CIF-A and TGF-β were identical molecules (Seyedin et al. 1986). Several years later, Joyce et al. (1990) demonstrated that injection of TGF-β1 or β2 in the periosteum of new born rat femurs triggered chondrogenesis and subsequent endochondral ossification. In addition, studies in embryonic chicken limbs showed that TGF-β accelerated precartilage condensation formation and cartilage formation (Leonard et al. 1991). These early studies indicated the important role of TGF-β in the induction of chondrogenesis in mesenchymal stem cells. Since then a great deal has become clear about the role TGF-β in chondrogenesis and the intracellular pathways involved.

Chondrogenesis

Cartilage is a unique connective tissue with essential roles in skeletal growth and is present at sites that require flexibility in combination with robustness. Examples of the latter are the synovial joints, covered at their ends by articular cartilage, that make nearly frictionless movement possible. Cartilage consists of an abundant extracellular matrix that is maintained by the sparsely present cells, the chondrocytes. Many joint diseases result in destruction of articular cartilage. Unfortunately, once damaged articular cartilage is unable to repair itself. Therefore there is a great need for methods to repair destructed cartilage. Mimicking cartilage formation (chondrogenesis) as observed during development is an opportunity to regenerate damaged cartilage.

Chondrogenesis in the embryo is a strictly regulated process. Non-differentiated mesenchymal precursor cells condensate and initiate chondrogenesis. Differentiation of prechondrocytes into differentiated chondrocytes involves a chondroblast phase characterized by high cell proliferation and deposition of cartilage specific molecules, such as type II collagen and aggrecan. In the embryo, the phase of differentiated chondrocytes is in general followed by chondrocyte terminal differentiation and endochondral ossification. During terminal differentiation chondrocytes become hypertrophic, characterized by cell enlargement and expression of type X collagen. Hypertrophic chondrocytes finally die and are lastly replaced by bone. However, in the formation of articular cartilage the process of terminal differentiation is blocked which results in permanent cartilage residing at the end of the long bones. TGF-β plays an essential role in many stages of chondrocyte differentiation, stimulating the initiation of chondrogenesis and blocking chondrocyte terminal differentiation.

Signaling of TGF-β

TGF-β is produced as a pre-pro-peptide of which the signal peptide is removed in the endoplasmatic reticulum (ER). This is followed by further proteolytic cleavage and dimerisation. The cleaved N-terminal precursor peptide, called Latency Associated Protein (LAP), remains non-covalently bound to the mature TGF-β peptide, forming the small latent complex (SLC). The SLC is unable to bind the TGF-β receptors. The SLCs bind to large latent TGF-β-binding Protein (LTBP) forming large latent complexes (LLC). Dissociation of TGF-β from LLC and LAP is accomplished by integrins, shear force, thrombospondin-1 (TSP-1), plasmin, changes in pH, heat treatment, radiation and other agents.

TGF-β signals via heteromeric complexes of transmembrane serine/threonine type I and type II receptors . The type I receptors, called activin receptor-like kinases (ALKs), act downstream of type II receptors and determine receptor specificity. The canonical TGF-β signaling route is through the type I receptor ALK5. In endothelial cells, TGF-β not only signals via ALK5 but also via ALK1, a trait that recently has been found to be shared with other cells such as chondrocytes (Blaney Davidson et al. 2009; Finnson et al. 2008). The type I receptors phosphorylate the intracellular receptor Smads, ALK1, -2, -3 and -6 signal via Smad1, 5 and 8 (canonical BMP route) while ALK4, -5 and -7 signal via Smad 2 and 3 (canonical TGF-β route). Phosphorylated

R-Smads form heteromeric complexes with the common mediator (co)-Smad4. These complexes translocate to the nucleus where they, together with co-activators and repressors, control transcriptional responses. The two intracellular Smad pathways regulate gene expression differently, even antagonizing each other (Goumans et al. 2003). The Smad2/3 and Smad1/5/8 signalling routes both have a crucial role in control of chondrogenesis.

Role of TGF-β in Initiation and Progression of Chondrogenesis

The formation of aggregates of mesenchymal cells, condensations, is one of the initial steps in cartilage formation . During condensation, N-cadherin mediates cell-cell interactions and after that fibronectin production is augmented, contributing to cell-matrix interactions stimulating progression of chondrogenesis. As mentioned earlier, TGF-β accelerates and increases cellular condensation of mesenchymal cells in chick embryos (Leonard et al. 1991). In cultures of mouse embryonic limb mesenchymal cells, TGF-β stimulated chondrogenesis and enhanced the expression of N-cadherin, N-CAM, fibronectin and tenascin, suggesting that TGF-β plays a vital role in the formation of cell-cell and cell-extracellular matrix interactions during precartilage condensations in the embryonic stage (Chimal-Monroy and Diaz de Leon 1997).

Chondrogenesis is studied using primary mesenchymal precursor cells but also cell lines, such as ATDC5 cells and C3H10T1/2 cells, are used for this purpose. Also these cells show, when correctly cultured and stimulated, a sequential progression of chondrogenesis. Treatment of undifferentiated ATDC5 cells with anti-TGF-β 2 neutralizing antibodies or transfection of these cells with a dominant-negative mutant of mouse TGF-β type II receptor completely blocked cellular condensation. Moreover, administration of TGF-β2 enhanced expression of fibronectin and type II collagen mRNA but downregulated that of N-cadherin. These results indicate that TGF-beta stimulates chondrogenesis by stimulating the transition from an initial N-cadherin stage to a fibronectin stage during progression of chondrogenesis (Kawai et al. 1999). However, others have shown that TGF-β enhances the early stages of condensation, for instance in C3H10T1/2 cells (Song et al. 2007). Remarkably, more recently it was demonstrated that TGF-β3 inhibited condensation in chick leg bud mesenchymal cells through shedding of N-cadherin (Jin et al. 2010). The exact role of TGF-β in the initial stage of cellular condensation and the regulation of N-cadherin expression by TGF-β remains uncertain. However, it appears to be clear that in general TGF-β drives chondrogenesis in the circumstances that close cell-cell contact is established.

In this context it might be that species differences in responses to TGF-β exist or that TGF-β plays a different role in different culture conditions and various cell types, depending on the additional growth factors and transcription factors expressed by these cells. For instance, murine C3H10T1/2 cells might be incomparable in this respect to other precursor cells, like human bone marrow-derived mesenchymal stem cells. Human bone marrow-derived mesenchymal stem cells need forced cell-cell contact (pelletation or micromass cultures) to commence chondrogenesis while C3H10T1/2 can undergo chondrogenesis when cultured in (high-density) monolayer after TGF-β exposure.

In addition to TGF-β also other members of the TGF-β superfamily have been shown to stimulate chondrogenesis. Bone Morphogenetic Protein-2 (BMP-2), an activator of the Smad1/5/8 pathway, stimulates N-cadherin expression in C3H10T1/2 cells that undergo chondrogenic differentiation (Haas and Tuan 1999). Moreover, expression of a dominant negative N-cadherin mutant inhibited BMP-2-stimulated chondrogenesis. In a study by Roark and Greer (1994), it was shown that TGF-β3 and BMP-2 were capable of inducing chondrogenesis in chick limb mesenchymal cells cultured in micromass culture. However, they noted that TGF-β was most effective on cells which have not yet undergone cell condensation while

BMP-2 was most effective after cells have condensed. However, since they used micromass cultures forced cell-cell contact was present in their experiments. In an another study with chicken micromass cultures it was shown that BMP-4 stimulated chondrogenesis and this was accompanied by nuclear translocation and accumulation of Smad1 (Nonaka et al. 1999). However, using C3H10T1/2 cells, Ju et al. (2000) found that Smad1 contributed for the most part in osteogenic and not in chondrogenic differentiation. Very clearly it has been shown that blocking BMP signaling by lentiviral overexpressed noggin blocks aggregation of cells in prechondrogenic condensations (Pizette and Niswander 2000). In studies with human bone marrow-derived mesenchymal stem cells we have shown that inhibition of either the Smad2/3 or Smad1/5/8 pathway in an early stage fully blocked chondrogenesis (Hellingman et al. 2011). These findings suggest that both Smad2/3 and Smad1/5/8 pathways are involved in the early stages of chondrogenesis and that both pathways play an essential, although dissimilar, role in the initiation of cartilage formation by mesenchymal stem cells.

Besides the crucial Smad pathways also non-Smad pathways appear to be involved in early stages of chondrogenesis. Nakamura et al. (1999) showed that GDF-5 (a TGF-β superfamily member) was more efficient in inducing cellular condensation and chondrogenic differentiation of ATCD5 cells than BMP-2 or TGF-β1. Furthermore, they showed that P38 mitogen-activated protein kinase (MAPK) played an essential role in GDF-5-induced chondrogenesis in these cells. In another study, it was shown that TGF-β induced rapid expression of chondrocyte-specific aggrecan which was not only accompanied by fast and transient phosphorylation of Smad2, but also by activation of extracellular signal-activated kinase 1/2 (ERK1/2), and p38 MAPK (Watanabe et al. 2001). Inhibition of the Smad pathways using a dominant negative Smad4 construct blocked TGF-β-induced aggrecan expression, demonstrating that Smad signaling is essential for this response. What is more, inhibition of either the ERK1/2 pathway or the MAPK pathway also dimished TGF-β-induced aggrecan expression. This indicates that activation of several pathways is involved in TGF-β-induced aggrecan expression in ATDC5 cells.

With regard to Smad signaling it appears that canonical TGF-β signaling (Smad2/3) is more important in the initial stages of chondrogenesis than canonical BMP signaling (Smad1/5/8). In a study that compared multipotent mesenchymal C3H10T1/2 cells with chondroprogenitor MC615 cells it was shown that BMP-4 stimulated chondrogenic differentiation in MC615 cells via a Smad1/5/8-dependent mechanisms while chondrogenesis of C3H10T1/2 was not stimulated by BMP-4 (Hatakeyama et al. 2003). Also in vivo it appeared that BMP signaling mainly activates proliferation and matrix synthesis in cells already committed to the chondrocyte lineage while TGF-β is able to direct mesenchymal stem cells in the course of chondrogenesis.

The initial stage of chondrogenesis is followed by a phase of cell proliferation and extracellular matrix synthesis. Mesenchymal stem cells of different sources show that both TGF-β and BMPs stimulate synthesis of chondrocyte-specific molecules like type II collagen and aggrecan. Exposure of adipose tissue–derived mesenchymal stem cells to a combination of BMP-7 and TGF-β 2 showed enhanced cartilage formation compared to the factors alone (Kim and Im 2009). Cartilage formation by synovium-derived stem cells was efficiently stimulated by BMP-2 and TGF-β3 (Rui et al. 2010). We found that a combination of TGF-β1 and BMP-2 was more efficient in stimulation of chondrogenesis in human bone marrow-derived mesenchymal stem cells than TGF-β alone (Heldens et al. 2012). It appears that cartilage formation after the initial stage of condensation is clearly stimulated by members of the TGF-β superfamily and that a combination of factors is more efficient than separate growth factors. Although not proven, this suggests that during the stage of cell proliferation and matrix synthesis the Smad2/3 and Smad1/5/8 routes work cooperatively. This in contrast to the role of these pathways in the final phase of chondrocyte differentiation, know as terminal differentiation.

Control of Terminal Differentiation by TGF-β

The last stage of chondrocyte differentiation is terminal differentiation (TD), this is characterized by cell enlargement (hypertrophy), expression of type X collagen and synthesis of proteolytic enzymes, such as matrix metalloproteinase 13 (MMP13). Finally the hypertrophic chondrocytes die and are replaced by bone. TGF-β, BMPs and Smad signaling play a crucial role in TD. Already in 1993 it was shown that TGF-β1 inhibited TD of rat epiphyseal chondrocytes and that TGF-β1 stabilized the prehypertrophical phenotype (Ballock et al. 1993). A similar inhibiting effect by, in this case, TGF-β2 was observed on chick embryo sternum chondrocytes (Bohme et al. 1995). In cultures of primary mouse limb bud mesenchymal cells TGF beta inhibited TD (Zhang et al. 2004). Moreover, deficiency of Smad3 resulted in increased TD, implying that TGF-beta/Smad3 signals repress chondrocyte TD (Yang et al. 2001). In addition, in transgenic mice that overexpressed a truncated, kinase defective TGF-beta type II receptor in skeletal tissue, TD was enhanced (Serra et al. 1997). In contrast to TGF-β, canonical BMP signaling appears to stimulate TD. For instance, mice overexpressing the BMP signaling inhibitor Smad6 showed normal chondrocyte proliferation but inhibited chondrocyte TD (Horiki et al. 2004). Furthermore, TD was blocked in cartilage explants from Smad6 transgenic mice. In proliferating and maturating chondrocytes of rat growth plates, Smad1 and Smad5 are highly expressed. Retting et al. (2009) showed that cartilage-specific deletion of individual Smads (1, 5 and 8) resulted in viable and fertile mice while combined loss of Smads 1, 5 and 8 led to severe chondrodysplasia. Chondrocytes of mice deficient for Smad1 and Smad5 showed impaired TD.

Chondrocyte differentiation during endochondral bone formation appears to be controlled by Smads but also the transcription factor Runx2 plays an essential part in this process (Sato et al. 2008). Mice that lack Runx2 demonstrate total lack of TD and bone formation in these animals is absent (Hecht et al. 2007). It is shown that that BMP-related Smads cooperate with Runx2 to control chondrocyte TD and that Runx2 and Smad1 or 5 are indispensable for TD (Retting et al. 2009; Miyazono et al. 2004). Runx2 undergoes a physical interaction with Smads to render Runx2 functional in stimulating TD (Javed et al. 2009). Interaction of Runx2 with Smad1 or Smad5 has been shown to stimulate TD of pre-hypertrophic chondrocytes chondrocytes (Leboy 2006). Apparently Runx2 in combination with the Smad function as a hub to control TD, complex formation of Runx2 with Smad1 or 5 stimulates TD while combining Runx2 with the canonical TGF-β Smads blocks this.

In conclusion, members of the TGF-β superfamily, TGF-βs, BMPs, GFDs are essential molecules in all stages of chondrocyte differentiation of mesenchymal stem cells. These factors not only stimulate the initiation of chondrogenesis but also regulate the final step in this process, terminal differentiation. While in the initial stages of chondrogenesis most TGF-β superfamily members appear to enhance this process, during TD canonical TGF-β signaling is inhibitory while canonical BMP signaling is stimulatory. In the regulation of TD binding of Runx2 to the different Smad signaling members is crucial. Although Smad signaling is dominant in the regulation of chondrocyte differentiation, other signaling pathways play additional roles. Modulation of Smad signaling pathways can be an attractive option to regulate mesenchymal stem cell-derived chondrocyte differentiation and be applied to create stable articular cartilage for repair purposes.

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Part II

Induced Pluripotent Stem Cells

Drug Discovery Using Human iPSC Based Disease Models and Functional Hepatic Cells

9

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Contents

Abstract

The development of human induced pluripotent stem cell (iPSC) technology has generated enthusiasm about the therapeutic potential of these cells for treating a variety of diseases. During the past few years, iPSC generation and hepatic differentiation methods have been significantly improved. These will provide an unlimited source of functional hepatocytes not only for transplantation, but for efficient drug discovery via patient relevant modeling of liver diseases and of drug-induced hepatotoxicity. Here we discuss the near future applications (and challenges) of iPSC-based cellular models, with an emphasis on liver diseases, cancer and hepatocytes.

Introduction

The process of drug discovery is lengthy and costly with an average of 15 years and $1 billion spent before a single drug reaches the market with a high failure rate (approximately 95%) (DiMasi et al. 2003; Paul et al. 2010; Ledford 2011). The current drug development process includes the following steps: drug discovery (target identification, high throughput screening, and lead optimization), preclinical studies with animal models, and clinical trials. While it is a time and cost-consuming process about 85% of drug developments fail in early clinical trials because

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of effectiveness and safety (i.e. toxicity; Ledford 2011). Hepatotoxity is the most frequent among adverse drug reactions which cause over 100,000 deaths every year in the United States (Wilke et al. 2007) and thus a major contributing factor to high attrition associated with new drug development. Primary hepatocytes isolated from fresh human liver are the gold standard for drug metabolism and toxicity testing however the current inability to culture and expand primary hepatocytes which have unimpaired physiological functions represents a major challenge in the field.

The pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to self-renew indefinitely in culture with retaining potential to differentiate into specified cell types such as hepatocytes and cardiomyocytes.

The iPSC technology provide an alternative for generating functional, renewable and relevant cell sources for disease modeling and drug development (Sharkis et al. 2012; Choi et al. 2011a). Towards this goal, we and others have successfully differentiated human iPSCs into various cell types including functional hepatocytes that are most relevant for drug metabolism and toxicity studies (Liu et al. 2010, 2011; Sullivan et al. 2010; Rashid et al. 2010). In addition, the discovery of iPSCs along with the directed differentiation protocols have enabled disease modeling in a dish for multiple human diseases since the iPSCs can be generated from patient tissues harboring disease-related genetic and epigenetic changes in contrast to ESCs (Maherali and Hochedlinger 2008).

Integration of these iPSC based disease- or toxicity- models in early preclinical stages of drug development will significantly shorten the timeline and reduce the costs that are associated with lengthy clinical trials and the high failure rates. In this chapter, we discuss the potential and challenges of iPSC-based disease modeling and drug discovery, with an emphasis on liver diseases, cancer and differentiated hepatocytes.

The Importance of Disease-Specific iPSC Generation from Patient Liver Tissues

The greatest advantage of iPSC technology is that it allows for the generation of pluripotent cells from any individual in the context of his or her own particular genetic identity. Recently, a number of studies have reported the generation of patient-specific iPSC lines from individuals with different diseases and modeling of several human diseases has been indeed demonstrated (reviewed in Chun et al. 2010). So far the reports for the generation of iPSCs from patient tissues have been mostly limited to inherited (genetic) diseases including those shown with inherited liver disorders (Rashid et al. 2010; Ghodsizadeh et al. 2010; Choi et al. 2011a). Even though some of these non-hepatic sourced (e.g. skin fibroblasts- or blood cell-derived) iPSCs could be sufficient for modeling the inherited diseases, hepatic-sourced iPSCs will be required for disease modeling of acquired liver diseases such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. This requires the technology to generate human iPSCs from viable primary hepatocytes, which we have previously established (Liu et al. 2010); this technology provide a more amenable system to generate many of the acquired liver disease-specific iPSCs. Based upon this technology, we have indeed achieved reprogramming of primary hepatocytes from hepatocellular carcinoma (HCC) tissues and a few more liver diseases (Choi et al. 2011b). While these data suggest that non-genetic or acquired liver disease patient (i.e. liver cirrhosis and HCC) tissues can be reprogrammed into iPSCs, it remains to be determined whether these various liver disease patient-derived iPSCs still retain not only genetic but also epigenetic memory of the original disease and if these iPSCs can be utilized for disease modeling, in order to study the multifactorial pathogenesis and drug screening.

Several groups have recently reported generation of iPSCs from cancer cells and some of these

Table 9.1 Cancer cell-derived iPSC lines

Cancer types	Reprogramming factors	Characterization	Differentiated cell types	Malignancy phenotypes	Reference
Melanoma: Colo[a]	miRNA302	Expression of pluripotency markers Demethylation of Oct4 Teratoma-like tumor(+)	Neuron-like cells	Reduced migration ability (iPSCs) Reduced cell cycle-related gene expression in iPSCs	Lin et al. (2008)
Prostate cancer: PC-3[a]					
Colorectal cancer: DLD-1[a], HT-29[a]	Oct4, Sox2, Klf4, c-Myc	(The characterization is limited to DLD-1) Expression of pluripotency markers Demethylation of Nanog	Adipocytes	Acquired sensitivity to chemotherapy (EB cells) Reduced invasion and tumorigenicity of EB cells Higher expression of p16 and p53 of EB cells as compared to the parental cells	Miyoshi et al. (2010)
Esophageal cancer: TE-10[a]					
Gastric cancer: MKN45[a]					
Hepatocellular cancer: PLC[a]					
Pancreatic cancer: MIAPaCa-2[a], Panc-1[a]					
Cholangiocellular cancer: HuCC-T1[a]					
Chronic myeloid leukemia: KBM7[a]	Oct4, Sox2, Klf4, c-Myc	Expression of pluripotency markers Demethylation of Oct4 and Nanog Teratoma (+)	Hematopoietic cells, neuronal cells	Acquired insensitivity to imatinib (iPSCs) Loss of BCR-ABL dependency in iPSCs but restored after differentiation	Carette et al. (2010)
Chronic myeloid leukemia: bone marrow cell[b]	Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28, SV40 large T antigen	Expression of pluripotency markers Teratoma (+)	Hematopoietic cells	BCR-ABL fusion (iPSCs) Maintained patient-specific complex karyotype (iPSCs)	Hu et al. (2011)
Lung Cancer: A549[a]	Oct4, Sox2, Lin28, Nanog, HIF-1/2	Expression of pluripotency markers Demethylation of Oct4 Teratoma (-)	Endothelial-like cells	Increased tumorigenic properties of iPSCs in mice More aggressive and invasive (iPSCs)	Mathieu et al. (2011)

EB embryonic body
[a]Cell line
[b]Patient-derived primary cells

cancer cell-derived iPSCs have shown cancer phenotypes once differentiated into specified cell types (Table 9.1). Although still in its infancy, the cancer-specific iPSC disease model(s) can be highly useful for effective anti-cancer drug discovery by overcoming the weakness of conventional cancer research; the limitations with the conventional disease models (e.g. cancer cell

lines, murine syngenic models and subcutaneous/orthotopic xenograft models) for solid cancers including HCC are: (1) Although cell lines have been established from certain tumors, these cell lines are known to accumulate additional genomic abnormalities that may not be associated with the original tumor. (2) The primary tumor tissues are of limited availability which affects the reproducibility of the experiments. (3) Genomic information of the non-tumorous hepatic tissues from HCC patients or heterogeneous tumor clones of the same patient tissue may serve as indicators of risks for *de novo* tumor formation, metastasis, and drug resistance (Hoshida et al. 2008; Budhu et al. 2006). However, there is a lack of model systems to study these cell types. (4) Another disadvantage of current xenograft models is the impaired immune system of the animal host. Although it facilitates the engraftment of human cells, it prevents the study for the interactions of tumor and immune systems during cancer development and treatment.

The recent advances in the iPSC research may provide novel solutions to these problems. Human iPSCs resemble ESCs in the ability to be maintained in culture indefinitely without karyotype changes. It is possible to derive patient-iPSCs from both non-tumor and different portions of tumor tissues that contain genomic information representing different stages of HCC development. The pluripotency of iPSCs allows generation of transplantable hepatic cells as well as hematopoietic stem cells (the precursor for immune cells) from the same patient iPSCs, which provides unique advantage to eventually create humanized animal models for liver diseases with a compatible immune system. We and others have been able to successfully differentiate human iPSCs into either hematopoietic stem/progenitor cells or hepatic cells (Ye et al. 2009; Liu et al. 2010, 2011). This holds great promise as unlimited and renewable hepatocyte and immune cell sources which can be utilized not only for cell therapy but also for establishing a better humanized disease model system in the future.

The major limitations of the conventional drug development include the utilization of non-human cells or immortalized human cell lines that often have little similarity to primary cells. This adds a significant burden to the subsequent lengthy and much more costly animal testing and clinical trials. In addition, many potentially effective compounds can be missed using these less physiologically relevant cell lines. Therefore, it is of significant to generate iPSCs from relevant primary tissues (i.e. liver tissues from HCC patients) and to establish a more patient relevant disease models for effective drug screening.

Modelling of Liver Diseases Using Disease Specific iPSCs

One of the main advantages of the iPSC technology is that it provides an unlimited quantity of human disease-relevant cells. In addition, cell types of different liver commitment stages, such as definitive endoderm cells, hepatic progenitor cells and mature hepatocyte-like cells, can be generated from iPSCs (Liu et al. 2011). It is plausible that disease associated phenotypes presented on the patient specific iPSC-derived hepatic cells could be utilized for phenotype-based drug screening even before establishing detailed molecular mechanisms of the disease pathogenesis, as we have shown such feasibility in a cellular model of a metabolic liver disease (Choi et al. 2011a). During the past few years, along with the improvement of hepatocyte differentiation technologies, several independent laboratories have demonstrated iPSC based modeling of disease phenotypes *in vitro* for various liver diseases such as alpha-1 antitrypsin deficiency, glycogen storage disease type 1a, Wilson's disease and an infectious liver disease (hepatitis C) (Rashid et al. 2010; Zhang et al. 2011; Choi et al. 2011a, Schwartz et al. 2012). However, the disease phenotypes for most of the cases need to be established (Ghodsizadeh et al. 2010) in the near future (Table 9.2). Current challenges of using patient iPSC-derived hepatic cells to model liver diseases include; (1) Although significant progress has been made for hepatic differentiation of human

Table 9.2 Human iPSC-derived liver disease models

Disease	Molecular defect	Differentiated cell type/phenotype	Drug test	Gene correction	Reference
Alpha 1-Antitrypsin (AAT) deficiency	Homozygous mutation in the AAT gene	Hepatocyte/AAT polymerization	NA	ZFN (efficiency: one allele 54%, both alleles 4%)	Rashid et al. (2010), Ghodsizadeh et al. (2010), Yusa et al. (2011), Choi et al. (2011a)
Glycogen storage disease 1a	Defect in glucose-6-phosphate gene	Hepatocyte/ hyperaccumulation of glycogen	NA	NA	Rashid et al. (2010)
Glycogen storage disease 1b	Defect in glucose-6-phosphate gene	Hepatocyte/ hyperaccumulation of glycogen	NA	NA	Ghodsizadeh et al. (2010)
Familial hypercholesterolaemia	Mutation in LDLR gene	Hepatocyte/ impaired ability to incorporate LDL	NA	NA	Rashid et al. (2010), Cayo et al. (2012)
Crigler-Najjar syndrome	Deletion in UGT1A1	Hepatocyte/ND	NA	NA	Rashid et al. (2010), Ghodsizadeh et al. (2010)
Hereditary tyrosinaemia, type 1	Mutation in fumarylacetate hydrolase 1 gene	Hepatocyte/ND	NA	NA	Rashid et al. (2010), Ghodsizadeh et al. (2010)
Wilson's disease	Mutation of ATP7B	Hepatocyte/ mislocalization of ATP7B and defective copper transport	Curcumin	Lentivirus (efficiency: NA)	Zhang et al. (2011)
Hepatitis C	Hepatitis C virus (HCV) infection	Hepatocyte/ infection with HCV	NA	NA	Schwartz et al. (2012), Zhu et al. (2012)
Progressive familial cholestasis	Unknown	Hepatocyte/ND	NA	NA	Ghodsizadeh et al. (2010)
Cirrhosis	Unknown	Hepatocyte/ND	NA	NA	Choi et al. (2011b)
Hepatocellular carcinoma	Unknown	Hepatocyte/ND	NA	NA	Choi et al. (2011b)

ND Not determined; *NA* Not applicated; *LDLR* low density lipoprotein receptor; *UGT1A1* UDP glucuronosyltransferase 1 family, polypeptide A1; *ATP7B* ATPase, Cu++ transporting, beta polypeptide; *ZFN* Zinc Finger Nuclease

iPSCs during recent years, the hepatic differentiation efficiency as well as functionality of these hepatic cells can be variable depending upon genetic backgrounds of the donor cells, culture related epigenetic (perhaps genetic) changes and lot to lot variations of differentiating reagents. (2) The pathogenesis of many liver diseases involves interaction between hepatocytes and other cell populations such as hepatic stellate cells and Kupffer cells, which is currently limited by absence of specific differentiation protocols to generate other cell types. Modeling of certain liver diseases would require recapitulating subtle interactions to precisely reflecting their pathogenesis. (3) To establish a reliable disease modeling system for multifactorial diseases such as HCC and liver cirrhosis, it will be necessary to derive a representative set of disease-specific iPSCs from the same as well as different patients; as the phenotype and severity of many of these diseases can vary markedly within the population. (4) In the

case of diseases with unidentified genetic defects or disease phenotypes, establishing iPSC-based cellular models could be even more challenging; however, novel strategies taking advantage of up to date high throughput OMICS type technologies might provide the opportunity to help identify and recapitulate representative disease phenotypes.

Although derivation of human iPSCs from several gastrointestinal cancer cell lines has been reported (Miyoshi et al. 2010; Table 9.1), it is valuable to establish cancer patient-derived iPSC lines from primary tumor tissues, which could be far more patient relevant, accurate and informative by utilizing both non-tumor and tumor portion-derived iPSC lines (as well as their differentiated cell types). A good example for such cancers is HCC; even before the tumor onset, patients with chronic hepatitis accumulate genetic alterations in liver tissues that may contribute to progressive liver disease, toward development of cirrhosis and the subsequent risk for HCC (Thomas and Zhu 2005). Patient iPSC lines that represent genomic information of these disease stages may provide valuable insight to the pathogenesis of HCC and the development of a novel drug therapy. We have recently achieved reprogramming of cells from a chronic liver cirrhosis patient (Fig. 9.1) and multiple HCC patient liver tissues (Choi et al. 2011b). These cells were able to engraft the mouse liver after hepatic differentiation and transplantation, indicating the functionality of these hepatic cells and potential for in vivo disease modeling. It still remains to be determined whether these acquired liver disease patient-derived iPSC lines can be utilized for disease modeling and drug screening.

Applications of iPSC Based Disease Models and Hepatic Cells for Drug Discovery

The biggest challenges modern medicine faces are the long timeline, high failure rate and cost associated with developing a single new drug (DiMasi et al. 2003; Paul et al. 2010; Ledford 2011). In addition, drug-induced liver toxicity is a major cause of morbidity and mortality worldwide (Wilke et al. 2007; Ledford 2011). Lack of preclinical drug testing models to effectively predict candidate drugs' efficacy and toxicity, especially among individuals of diverse genetic backgrounds, contributes significantly to these challenges. The major drawback of traditional drug testing systems is that they rely mostly on transformed cell lines and animal models. Most cell lines do not express functional drug metabolizing enzymes that normally present in primary cell types, especially human hepatocytes. Animal models have high failure rate for predicting drug toxicity in humans, and more importantly, none of the existing systems has integrated the increasing knowledge from pharmacogenetic research which has demonstrated the important roles of genetic variations in differential drug response. These limitations have put significant burden on the lengthy and high cost of clinical trials.

Human hepatocytes are the most relevant cell type for drug evaluation since most drugs are metabolized in the liver, yet they are highly inaccessible. Having recognized the importance of hepatocytes in pharmacogenetic study and the significance of inter-individual genetic variations in predictive drug evaluation, several biocompanies have begun the effort to collect primary hepatocytes from diverse background individuals. These cells provide a valuable resource for the research community and pharmaceutical industry. However, unlike other types of primary cells such as fibroblasts, primary hepatocytes are very resistant to *in vitro* expansion (i.e. not renewable). The limited availability significantly hinders their broad and standardized application in drug testing. Clearly, new tools for pharmacological discovery are urgently needed.

The recent development in the iPSC technology (e.g. generation of patient specific iPSCs using virtually any type of cells in the body and improved hepatic differentiation methods) has brought even higher enthusiasm in the field. The use of diverse background iPSCs obtained from both healthy and diseased individuals to personalize drug development may prove to be a powerful

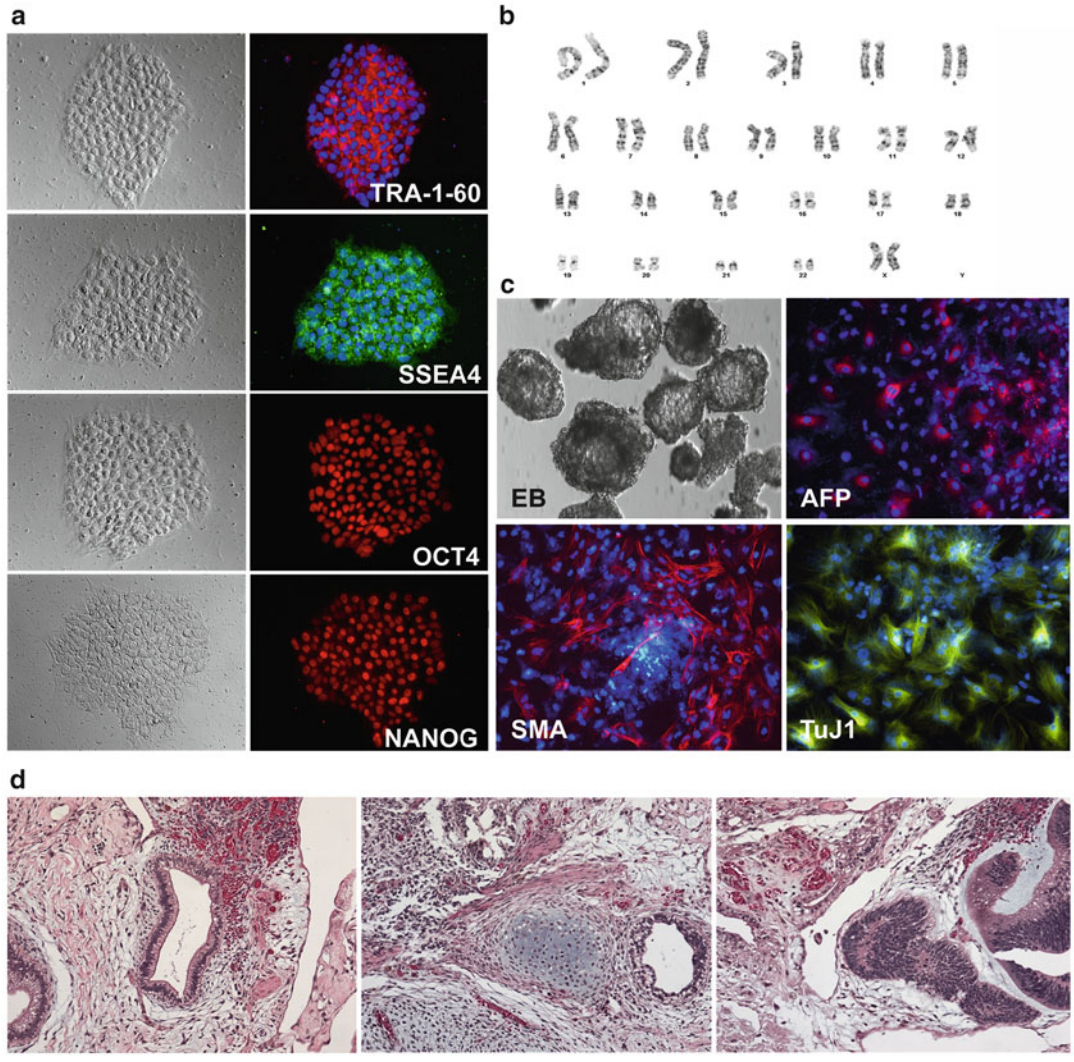

Fig. 9.1 Generation and characterization of liver cirrhosis-derived iPSCs. (**a**) Representative immunofluorescence analysis of liver cirrhosis patient-derived iPSC cells growing on Matrigel. Clear expression of the pluripotent stem cell surface antigens TRA-1-60 (*green*) and SSEA4 (*red*), the nuclear transcription factors OCT4 (*red*) and NANOG (*red*) are observed. *Blue* nuclear staining is DAPI (×200). (**b**) Karyotyping of the iPSCs. After 30 passages, these cells showed normal karyotypes. (**c**) *In vitro* differentiation of this iPSC line into all three primary germ cell layers. After generation of embryoid bodies the iPSC cells spontaneously differentiated into endoderm (AFP positive, *red*), mesoderm (SMA positive, *red*) and ectoderm (TuJ1-positive neuronal cells, *green*). *Blue* nuclear staining is DAPI (×100). (**d**) Spontaneous differentiation into all three germ layers is evident in teratoma. Endoderm (*left panel*), mesoderm (*middle panel*), ectoderm (*right panel*) (×200)

means of reducing drug toxicity, stratifying patient response, and reducing late-stage clinical failure. It is therefore of great significance to establish a bank of iPSCs from individuals with diversified backgrounds. However, enormous costs and time would be required for generating iPSC banks that have comprehensive coverage of human genetic variations. Therefore, as an alternative, an innovative and expedited iPSC-based approach which could provide novel tools for significantly advancing current pharmacological discovery and drug development processes, needs to be established.

Importantly, the ideal cellular models of liver diseases or compound induced hepatotoxicity (associated with drug candidates for all human diseases) will require high expression of cytochrome P450 (CYP) enzymes that are inducible and functional, since CYPs are critical for drug metabolism and generation of hepatotoxicants. As such, significant progress has been made for human iPSC based hepatic differentiation protocols during the recent several years; hepatocytes derived from human iPSCs not only have various CYP450 metabolic enzyme activities in vitro but also secret multiple hepatocyte specific functional proteins in vivo (Liu et al. 2010, 2011; Sullivan et al. 2010). However the levels of some of these drug metabolic enzymes and liver specific proteins within human iPSC-derived hepatocytes are significantly lower than human primary counterpart, which warrants further improvement of the current hepatic differentiation technology. Equally important is that the feasibility of using hepatotoxicity models for prediction of drug induced liver injury needs to be established before developing such novel tools for advancing pharmacological discovery and drug development processes.

Conclusion and Future Direction

In addition to the potential cell therapy, another area that iPSCs can have more imminent impact, but currently under-studied, is drug discovery. With the rapid advancement in both sequencing and iPSC technologies, it is now anticipated that research in these area will eventually lead to tailored therapy that is most effective for each individual by maximizing efficacy and limiting adverse drug effects. Recognizing the importance of personalizing drug selection and dosing, the FDA has started to label the approved drugs with pharmacogenomic information such as the major drug-metabolizing enzymes known to be associated with each drug. Integration of the pharmacogenetic knowledge to the aforementioned patient iPSC-based disease models in early stages of drug development will help to more accurately predict toxicity and therapeutic failure; thereby significantly shortening the timeline and reducing the costs associated with clinical trials and high failure rates. Given the rapid pace of developments within the iPSC field, it is likely that developing safe and effective technologies for using iPSC-derived hepatic cells in disease modeling as well as tailored drug- and cell- therapy will be achieved in the near future.

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10 Generation of Antigen-Specific T Lymphocytes from Induced Pluripotent Stem Cells for Adoptive Immunotherapy

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Contents

Abstract

Conquering cancer, one of the leading human health problems, is a major task for both basic and clinical scientists. With the understanding of recently developed cancer immunotherapy, in particular the cytotoxic T lymphocyte (CTL)-mediated immunotherapy, T cell development as well as the applications of stem cell technologies, an individualized cancer immunotherapy could be promisingly designed by avoiding several main obstacles in obtaining a large number of autologous and highly-reactive tumor-specific CTLs. Through induced pluripotent stem (iPS) cell technologies, tumor-specific CTLs could be generated from a piece of skin or blood from the individual cancer patient, following genetic modification as well as a directed differentiation. This novel approach could potentially further expand the weaponry to battle cancer.

Introduction

Cancer, a broad group of heterogeneous diseases which are characterized by unregulated cell growth, has become one of the major concerns in modern medicine. Because of the fact that both morbidity and mortality of cancer has increased sharply in recent years, an urgent need to find a cure to various types of cancers has become demanding. Cancer is the second leading killer in humans following the

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M.A. Hayat (ed.), *Stem Cells and Cancer Stem Cells, Volume 10*,
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cardiovascular diseases. In modern medicine, the treatment of cancer patients basically falls into three major categories as surgery, chemotherapy, and radiotherapy. Although individual or/and combined cancer therapy has achieved certain efficacies and prolonged the survivals of some cancer patients, there is no cure to most of the cancer patients, especially those patients with distant metastases. Under the guidance to further increase the prognosis of cancer patients in terms of extending survival time and enhancing the quality of life, a new anticancer regimen has been proposed and tested. Our current understanding is that in most of the cancer patients, their immune systems have been severely compromised due to the impacts of tumor or tumor-secreted molecules (Schreiber et al. 2011). To devise a better strategy to boost patients' immune system, currently anticancer immunity, or cancer immunotherapy has been under intense investigation at both laboratory and clinical levels. According to the intrinsic properties of the human immune system, it is generally divided into two major components, humoral and cellular immunity. Both components of the immune system have been shown to play important roles in fighting cancer; however, the cellular-based anticancer immunity has been shown in a pattern of direct killing, whereas humoral-based always executes its tumor-killing by indirect mechanisms. The direct killing mechanism is more efficient, relatively hassle-free, and it is the method of choice to enhance the antitumor immunity as compared to indirect killing that requires a collective action of multiple events. Supplement of tumor-reactive, direct killing cytotoxic T lymphocytes (CTLs) has been shown promising in the context of controlling tumor growth and maintaining tumor-free status in certain cancer patients.

Beyond the obtained success of this option for tumor management, there are also several problems that need to be addressed to achieve a better therapeutical efficacy. First is the source of tumor-reactive CTLs, the second is the persistence (or memory) of the tumor-reactive CTLs transfused into cancer patients, and the third is the avoidance of side-effects caused by graft-versus-host (GVH) diseases. A successful CTL-based immunotherapy requires to overcome the above problems and to meet those three criteria because of several intrinsic characters of CTLs: peripheral effector CTLs are generally short-lived; therefore, it is difficult to maintain their survival after infusion. CTLs isolated from cancer patients are usually modulated or suppressed by tumor cells; hence, they are functionally incapable of fighting tumors. Allogenic CTLs will cause autoimmunity and further complicating the situation. The introduction of stem cells provided researchers a better approach to generate large numbers of tumor-reactive, long-lived, and non-terminally differentiated CTLs through both *in vitro* and *in vivo* differentiation mechanisms. The biggest problem in any stem cell-based application is the source of stem cells. Embryonic stem cells (ESCs) have been shown to be the best source of stem cells based on its omnipotency and unlimited division. However, it is difficult to obtain ESCs from adult individuals due to the ethical issues and other technical problems. As an alternative, hematopoietic stem cells (HSCs) have been considered as the best source of stem cells in adults. However, there are several defects in terms of self-renewal and further differentiations have been reported. Allogenic stem tissue has been widely applied in the treatment of hematopoietic malignancies and the most-frequent side effect is the GVHD, which always requires the administration of immunosuppressant. As mentioned above, it seems that several technical difficulties have delayed the application of stem cell-based cancer immunotherapy. The debut of induced pluripotent stem cell (iPSC) technology gave people a new hope of generating tumor-reactive CTLs that meet those three criteria: a large number of persisting nonterminally differentiated T cells that arose from the self-tissue. In this chapter, we will discuss recent updates in generation of tumor-specific T lymphocytes from iPSCs for adoptive immunotherapy.

Cancer Immunotherapy

Besides the introduction of cytotoxic chemotherapy in the curative treatment of metastatic germline tumors and choriocarcinomas over 30 years ago, there was little progression in the development of a more effective cancer therapy. The only exception is the cancer immunotherapy. Following a comprehensive understanding of the immune system, it is realized that by harnessing certain components of the host immune system, it could help design a better strategy to battle cancer. There are two major categories of the host defense systems: innate and adaptive immune systems (Murphy et al. 2008). The innate immunity mediates the initial and first line immune responses towards microbial infections. It is also called the native or natural immunity, which has a fixed pattern of recognition that could only recognize certain particles or molecules expressed by the microbes. The members of innate immunity include epithelial barriers, neutrophils, complements, NK cells, and phagocytes. Because of the limited pattern recognition of innate immunity, it is difficult to engineer those components for attacking tumor cells, which merely express any molecules shared by an invading microbe.

The second line of defense is the adaptive immunity or also called the specific or acquired immunity. The adaptive immune system consists of lymphocytes and their products such as cytokines and antibodies. As innate immunity recognize certain structures shared by some classes of microorganisms, the lymphocytes in adaptive immunity specifically recognize different microbial substances as well as noninfectious molecules by their surface-expressed receptors, namely, B cell receptor (BCR) and T cell receptor (TCR). In general, the substances or molecules that could be recognized by lymphocytes are called antigens. The recognition patterns are, for instances, B cell recognizes B cell antigen through its BCR and T cell reacts with correspondent T cell antigen through its TCR. These recognition mechanisms could not only respond to invading foreign substances but also to some self-substances; for example, the autoimmune diseases are typically caused by overreacted adaptive immune system. The flip side of self-reaction is the foundation for designing a potential cancer immunotherapy because cancer cells are basically a group of uncontrollable self-tissues. The adaptive immunity could be further divided into two subcategories, humoral and cellular immunity. Humoral immunity is mediated by B cells and their secreted antibodies, and cellular immunity is mediated by T cells. Generally, humoral immunity plays a role in controlling extracellular microorganisms, and cellular immunity acts in eliminating intracellular microbes. Although the immune system has been categorized into two major types, they are not completely independent. Actually, the innate and adaptive immune systems are interconnected. Dendritic cells (DCs) and other antigen presenting cells (APCs) play a critical role in connecting both innate and adaptive immunity.

Theoretically, any functional components in the immune system could be modulated for conducting a cancer immunotherapy. However, their anti-tumor efficacies may vary depending on the roles they are playing in the immune network. As mentioned above, the innate immunity basically recognizes a fixed pattern of structures/substances that are found only on intruding microbes; this limits its application in the cancer immunotherapy, which focuses on targeting abnormal self-tissue. The versatile molecule/substance or antigen recognition properties of the adaptive immune system could serve as the basis of carrying out possible cancer immunotherapy in the awareness that, compared to the original, normal tissue tumor cells always express certain types of aberrant proteins or molecules. These tumor-specific proteins/molecules could be used as the antigens to educate adaptive immune system to be more tumor-specific. Through intensive research, several major components of the immune system have been identified, which could provide therapeutical effects in the cancer treatment, such as antibodies, cytokines, vaccines, and CTLs.

Antibody

Antibody is the major product of activated B cells. Upon stimulation by a certain antigen, B cells will be activated and produce the relevant antibody that could specifically recognize the antigen. This mechanism plays a significant role in eliminating extracellular pathogens. From the understanding of some autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE), antibody could also recognize and bind to self-tissue. This also suggests that an antibody could be used in cancer immunotherapy due to its binding property. Typically, an ideal tumor-specific antibody should acquire three major characters: specificity, affinity, and avidity. In search of a more potent antibody, the technique of monoclonal antibody (mAb) has surfaced. Literally, mAb is unanimously identical, which is selected, engineered, and expanded from a pool of antigen reactive antibodies based on those three selective criteria mentioned above. With an identified tumor-associated antigen, the intrinsic properties of antibodies render them as a good arsenal for tumor cells.

Currently, mAbs have had a significant impact on clinical oncology. The three top-selling anticancer drugs on market: rituximab, trastuzumab, and bevacizumab are all mAbs. Because of the limitations in both *in vitro* and *in vivo* testing systems, the exact mechanisms of mAbs' antitumor effects are still vague. However, strong data have supported that the immune responses play central roles in the killing of tumor cells, and, in particular, antibody-dependent cell-mediated cytotoxicity (ADCC) is the most important mechanism. In ADCC, after mAbs binding to the tumor cells with their Fab fragments, the Fc fragments on mAbs will bind to the Fc receptor (FcR) expressed on the NK cells, which execute the tumor lysis. This phenomenon has been well recorded in the clinical administration of rituximab (Weiner 2010). The other possible mechanisms as observed in various studies include a combined anticancer action initialed by mAb binding and followed by activation of other members of the immune system such as DCs as well as complement system which mediates the complement-mediated cytotoxicity (CMC) towards tumor cells.

To achieve a better therapeutical result in killing tumor cells, mAbs have been further engineered and modified to increase the antitumor efficacy. One example is the increase of Fc fragment affinity to FcR, and the other includes the generation of bifunctional mAbs which could bind to tumor associated antigen and, at the same time, either recruit T cells to exert a CTL-mediated cytotoxicity or deliver anti-tumor drugs with engineered components on mAbs. As described, antibody mediated cancer immunotherapy requires a combined action of several components of the immune system, any defects in the system such as NK cell anergy could possibly fail the regimen. Extensive work needs to be done to further elucidate the mechanism of action of mAbs mediated killing of tumor cells, which will significantly benefit the application of mAbs in clinical oncology.

A second category of antibodies have been applied in cancer immunotherapy are the immune-modulating antibodies. One important mechanism in modulating T cells' function is the surface expressed T cell costimulatory molecules. In the two steps T cell activation scheme, without the engagement of stimulating costimulatory molecules, T cells will not be activated even when TCR has already bond to major histocompatibility complex (MHC) loaded antigen.

There are two groups of costimulatory molecules, *i.e.*, stimulatory and inhibitory, in order to maintain the homeostasis of immune system. Stimulatory molecules activate the T cells, but inhibitory molecules shut down and/or inhibit the activation of T cells. In many cancer patients, it is well observed that circulating T cells have developed tolerance and/or anergy due to the stimulatory insufficiency or inhibitory prevalence. Hence, boost stimulatory and/or block inhibitory costimulatory molecules might break the tolerance. The immune-modulating mAbs either boost stimulatory or block inhibitory costimulation have been developed and clinical studies have

shown promising outcomes in treating advanced tumor patients. For example, mAbs specifically blocking CTLA-4 ligation, an inhibitory costimulatory molecule highly expressed in several cancer patients, has gained initial successes in treating patients with metastatic melanoma and renal cell carcinoma (Ribas 2010). Further studies on generating other costimulatory molecules-specific mAbs are still extensively undergoing.

Cytokine

Cytokines are a group of small molecules that are secreted by different types of cells. Usually, they play the role as signal transduction messengers between different cells. In immunology, cytokine is the pivotal element in connecting different groups of cells. Different types of cytokines have different mechanisms of action, some are immunoenhancing and others are immunosuppressing. For example, in the superfamily of interleukins (IL), IL-2 is an immunoenhancing cytokine, which boosts the expansion and activation of T lymphocytes. In recent clinical studies, it is shown that administration of IL-2 could significantly inhibit the growth of melanoma and renal cell carcinoma by reversing the tumor-induced T cell tolerance, although it always accompanies with global, massive side effects (Klapper et al. 2008). In the context of boosting host immune system, other cytokines such as Interferon-alfa (IFN-α) and Granulocyte-monocyte colony stimulating factor (GM-CSF) have also been used clinically in the treatment of melanoma and several hematologic malignancies (O'Donnell et al. 1995). In contrast, the other IL superfamily members such as IL-6 and IL-10, play the opposite function in that they tuning down the activated T cells. In the intratumor microenvironment, it is found that IL-6 and IL-10 along with transforming growth factor-beta (TGF-β) are highly expressed than in normal setting. Those cytokines are able to silence the tumor infiltrating lymphocytes (TIL); thus, providing a mechanism evading the normal immune surveillance. Supplement immunoenhancing and blocking immunosuppressive cytokines will possibly facilitate the achievement of a profound efficacy in a combined cancer immunotherapy.

Vaccine

The idea of cancer vaccine was proposed many years ago following the tremendous success of vaccination in the control of many infectious diseases; however, no major advance has been reported until recently. Although microbial components-derived vaccine could elicit strong protective adaptive immune responses due to the education and activation of the T cell responses, many clinical trials of cancer vaccine by using tumor-specific or tumor-derived antigens failed to arouse both $CD4^+$ and $CD8^+$ T cells to generate a noticeable anti-tumor responses. The successful cancer vaccine, Provenge, which has been approved by the FDA for treating advanced hormone-independent prostate cancer, is a DC-based cellular vaccine. Provenge has achieved a 4-month overall survival benefit in the past studies; however, it was not able to cure the patients with advanced prostate cancer (Kantoff et al. 2010). Therefore, the DC-based cancer vaccine could only serve as a supplement to other mainstream of cancer therapies in the notion of getting a cure. Besides cellular cancer vaccine, peptide-based cancer vaccine has also been shown acquired certain positive results. A combined vaccine approach by using gp100, peptide which is isolated from melanoma cells plus IL-2, has shown an improvement in patients with metastatic melanoma (Schwartzentruber et al. 2011). No cure in this approach has been documented. In summary, current knowledge in basic immunology is still inadequate to develop an ideal cancer vaccine to render patients with an established anti-cancer immunity as microbial vaccine has achieved. However, it is possible that following a better understanding of the immune system the development of a potent cancer vaccine such as polio vaccine will be feasible.

Adoptive T Cell Transfer

Above mentioned anti-cancer strategies are indirect approaches, all of them need executors in killing the tumor cells. The well-known executors in our immune system are NK cells and CTLs. NK cells are a part of the innate

immunity and their killing patterns are not antigen-restricted. However, CTLs have a more versatile and specific mechanism of killing that is TCR-mediated precise targeting and killing. This property is the basis of carrying out a directed, tumor-specific cancer therapy or the adoptive T cell transfer (ACT)-based immunotherapy. The idea of carrying out ACT-based immunotherapy is derived from the observation obtained in the treatment of hematologic malignancies by allogenic stem cell transplantation (alloSCT) (Horowitz et al. 1990). It was found that the donor T cells were able to target and kill leukemic cells by recognizing the aberrantly expressed leukemic antigens, which is described as the graft-versus-leukemia (GVL) effect in the literature. From this discovery, it is recognized that T cells are able to respond to the autoantigens that are abnormally expressed on the surface of malignant cells, and this paves the way to explore the further evidence of tumor-reactive T cells.

Another substantial discovery is the identification of tumor infiltrating T lymphocytes (TILs) in patients with melanoma (Dudley et al. 2002). Following surgical excision and processing of the melanoma tissue from patients, a group of T lymphocytes was isolated and expanded *ex vivo*. Because of their property of infiltrating into the tumor tissue, they are named as TILs. This heterogeneous population of T cells shows different profiles of phenotype, antigen-reactivity, and functionality; however, after infusing back into the tumor-bearing patients following *ex vivo* expansion and manipulation, they are able to control tumor growth in melanoma patients. By further combing with preconditioning lymphodepletion which aimed at depleting immunosuppressive regulatory T (Treg) cells and myeloid derived suppressor cells (MDSCs) as well as cytokine conjugation in terms of boosting infused TIL persistence, this TIL-based therapy can cure patients with advanced, metastatic melanoma. The existing problems for using TILs to conduct cancer immunotherapy are also quite obvious: first, TILs could only be identified in melanoma but not in other types of carcinomas; second, recovered effector-phenotyped TILs are always short-lived compared to naïve-phenotype T cells; third, the heterogeneous properties of TILs bear different efficacies in targeting tumor cells. Addressing those problems will help design a unanimous strategy that also suits other types of cancers.

One approach has been recently tested to meet those criteria is the genetic modification of T lymphocytes that will render T cells with a specific antigen-reactivity. In this approach, two major strategies that allow T cells with an identical and specific antigen-reactivity have been utilized: one is overexpressing a tumor-associated antigen specific TCR and the other is introducing a chimeric antigen receptor (CAR) that recognizes the tumor-specific antigen. Both of them have achieved substantial success in both artificial animal models and clinical trials in human patients. The mechanism of generating tumor-specific antigen reactive TCR modified T lymphocytes in targeting and killing the tumor is straightforward as in the screening of a high-efficacy mAb. The most antigen-reactive T cell clone will be isolated to amplify its recombined, full length TCR gene. This TCR gene bears both antigen-recognition and signal-transduction capabilities and introducing it into naïve T lymphocytes will instruct the latter to target and kill the antigen-bearing tumor cells through the classical cytotoxic effects (Morgan et al. 2006).

For CAR, the mechanism of tumor targeting and T cell activation is somewhat complicated. A functional CAR is a single chain structure that consists of several different components: an antigen-recognition domain, a transmembrane hinge domain and a T cell activation domain. Usually, a single chain variable fragment (scFv) of a tumor-antigen reactive mAb serves as the tumor/antigen recognition domain. The intracellular signaling domain of several different T cell activation molecules are recombined to exert function of T cell activation (Gross et al. 1989). In general, when CAR binds the antigen via the scFv region, it activates its modified T cells through the intracellular signaling domain to express Perforin and Granzyme B for tumor cell execution (Kalos et al. 2011). The great advantage of CAR is that it is MHC-independent. Because TCR-based antigen recognition is MHC-dependent, the CAR is able to give T cell an extended targeting capability.

The general protocol of generating genetically modified T cells is more or less the same in both scenarios. First step is the most important, that is the identification of tumor-specific antigens and correspondent TCRs as well as CARs. This is the limiting step for carrying out a successful ACT immunotherapy by using genetically modified T cells. The expression profiles of the tumor-associated antigen, along with the specificities and affinities of TCR and CAR are both the determining factors. The second step is the isolation and *ex vivo* expansion of autologous T lymphocytes from the cancer patients to obtain a large number of T cells for following genetic manipulation. These types of genetic manipulation provide T cells with antigen specificity and reactivity. In this step, either TCR or CAR is genetically introduced into the expanded T cells by a viral vector-mediated transduction. After *ex vivo* manipulation and characterization, TCR or CAR bearing T cells are reinfused back to patients. In the very beginning, TCR-engineered ACT was applied in the treatment of advanced melanoma that was shown to be effective. As documented in recent studies, this regimen has been broadened into other types of tumors, for example, neuroblastoma, synovial cell sarcoma, leukemia, and lymphoma. In the context of CAR-engineered ACT, the most important achievement is the management of B cell lymphoma by targeting CD19 molecule, a common B cell marker. With current promising data and in the light of advancement in tumor associated antigen identification in different types of cancers, additional therapeutical proposals of using TCRs and CARs are under intense investigation.

Although ACT with genetically modified autologous T lymphocytes has gained tremendous therapeutical efficacies and even cure in certain cancer patients, it is still far from perfect. The most important result from the use of autologous T cells is that most cells are terminally differentiated into antigen-experienced effector T cells. These cells are short-lived compared to naïve and memory T cells, and *ex vivo* expansion by cytokines and costimulation causes a sharp reduction in the of cell numbers after reinfusion. Second, terminally differentiated T cells have already expressed an original, functional TCR on their surfaces, overexpression of an additional TCR will possibly cause TCR mismatch, which could greatly reduce the antitumor potency of the engineered T cells. To find alternative sources of undifferentiated or less-differentiated T cells could help solve this problem because precursor cells do not have a mature TCR that would interfere with the introduced TCR, and more importantly undifferentiated or less-differentiated cells are usually self-renewal capable which could possibly provide a significant number of cells for therapeutic purposes. In our current understanding of the system biology, the best candidate that suits this scenario is stem cells.

Stem Cells in Immunology and Immunotherapy

In definition, stem cells bear the name because of their unlimited capabilities of division, differentiation, and self-renewal. In general, there are two categories of stem cells, the totipotent embryonic stem cells (ESCs) and somatic stem cells (SSCs) with different differentiation potencies from multipotency to oligopotency and unipotency following sequential progressions. ESCs are found in the inner cell mass of a blastocyst in the early embryo, and they are able to differentiate into all three germ layers: ectoderm, mesoderm, and endoderm, which could further sequentially differentiate into >200 types of cells in the human body. Because of their omnipotency in differentiation and indefinite ability of self-renewal, they are always the first choice in the application of regenerative medicine as well as tissue engineering. There are numerous reports available indicating that under different conditions ESCs are able to differentiate into different cells and tissue in both physiologic and artificial settings. However, the major problems in ESCs application are both technically and ethically related. In the former case, due to the short window of blastocyst stage obtaining ESCs is technically demanding and from their definition, it is not feasible to acquire any of them in adult humans. On the other hand, the isolation of human

ESCs will unavoidably destroy the embryo, which might raise the ethical issue. Third, in terms of clinical application, allogenic ESCs could possibly be immunogenic and may cause severe side-effects including host-versus graft diseases (HVGD).

For future therapeutical purposes, scientists started to use the more-differentiated, less-potent SSCs. As documented, additional SSC subpopulations have been identified, for example; hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), endothelial stem cells (endoSCs), and neural stem cells (NSCs). The major role of SSCs is to maintain the tissue homeostasis in the adult, and the activity of different SSCs depends on the turnover rate of related tissue and organ. For example, due to high turnover rates in the hematologic and digestive systems, intestinal SSCs and HSCs are relatively large in numbers and always more active in their differential and renewal potencies. This observation draws intense attention for possible research and treatment applications. HSCs, because they are technically easy to obtain, have been widely studied and further applied in the clinical settings. Physiologically, HSCs are multipotent stem cells in the hematologic system that give rise to a full spectrum of blood cells from both myeloid lineage (monocytes/macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, and DCs) and lymphoid lineage (T lymphocytes, B lymphocytes and NK cells) (Doulatov et al. 2012).

The presence of HSCs could be dated back in the blood island of yolk sac; however, in adult they mainly reside in the bone marrow (BM) and only a few are circulating in the peripheral blood. Although normally, HSCs are hiding deeply in the bone marrow niches, it is possible to force them to leave their habits and enter into circulation, which is described as HSC mobilization. Currently, by mobilizing HSCs with granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor (GM-CSF and G-CSF), it is easy to harvest a significant number of HSCs from peripheral blood for clinical applications such as alloSCT in the treatment of hematologic disorders and malignancies. Harvested HSCs could also be easily expanded *ex vivo* via certain cytokines such as stem cell factor (SCF), IL-3, IL-6, and thrombopoietin (TPO).

Because of its easiness in isolation and readiness in application, HSCs have been widely utilized in both laboratory and clinic. One of the most prominent applications of HSCs is the alloSCT-based treatment of leukemia, which aims at reconstituting the dysfunctioned hematopoietic system in the affected individuals (Gooley et al. 2010). After transplant, the donor-originated HSCs will engraft, expand and further differentiate into both lineages of blood cells, including T cells in the recipient, which is designated as bone marrow chimerism. In some patients, a significant graft-versus-leukemia phenomenon could also be observed. This observation not only helps foster the idea of conducting a T cell-based cancer immunotherapy but also intrigues scientists to find a fast and efficient mechanism to generate lymphocytes directly from HSCs. Under intensive research, the general lymphopoiesis is concluded as both B and T lymphoid progenitors that arise from HSCs. After stepwise differentiation from HSCs, B progenitors stay and get final mature in the BM; however, T progenitors will have to migrate into thymus to become matured, which indicates that there are significant regulatory events in the thymus that direct T lymphocytes development and maturation.

For the efficient generation of B cells from HSCs, it has been reported that in an *in vitro* BM stromal cell-based system, mature B-lymphocytes could be generated. In terms of T cell differentiation from HSCs; several models have been studied and one of the successful *in vitro* models is the fetal thymic organ culture (FTOC) system, which is based on the HSCs coculture with isolated fetal thymus. The microenviroment in fetal thymus helps the differentiation and maturation of progenitor T cells that existed in the HSCs pool (Lind et al. 2001).

Notch Signaling

In the search for the governing mechanism of T cell differentiation/development in the thymus, the Notch signaling pathway has been highlighted (MacDonald et al. 2001). The Notch signaling pathway was originally found and characterized in the wing-development of *D. melanogaster*. In following years, it has been proposed as a conserved, key regulatory mechanism in the development process of many organs and systems, including the hematopoietic system. In general, there are two types of Notch receptors, Notch1 and Notch2, and two major categories of Notch ligands: Jagged (Jagged-like 1 and 2) and Delta (Delta-like 1, 2 and 4). Notch1 has been shown to play critical role in the HSCs generation in the embryo however dispensable in the HSCs maintenance and homeostasis in adult (Bigas and Espinosa 2012). Notch signaling pathway's governing mechanisms in the hematopoietic system are largely unknown except for an identified role in T lymphocyte commitment. Previous studies have outlined the possible relationship between Notch signaling and T cell development based on the observation that HSCs express a low level of Notch receptors, and thymic stromal cells express a high number of Notch ligands, especially delta-like 1 (DLL1) and DLL4. Further studies have validated that Notch signaling pathway is the critical, indispensable component in T lymphocyte commitment from HSCs. As reported previously, BM stromal cells support the differentiation and maturation of B cells from HSCs, in an *in vitro* study (Kodama et al. 1994). By overexpressing Notch ligand DLL1 in a mouse BM stromal cell line (OP9-DL1), the switching of B lymphocyte commitment to T lymphocyte commitment becomes possible (Schmitt and Zuniga-Pflucker 2002). Subsequent studies further confirmed the function of DLL1 as well as of DLL4 in the commitment and development of T cells from HSCs such as TCR rearrangement and surface marker changes.

In summary, Notch signaling controls T lineage differentiation from HSCs and *in vitro* system of T lineage induction could greatly facilitate any clinical applications in which supplementation of T cells are required. However, the major problem in this *in vitro* system is the absence of, thymic microenvironment-regulated positive and negative selections. Therefore, *in vitro* generated T cells pool might contain T cells with autoreactive or futile TCRs, which could hinder their clinical applications. For example, in ACT-based immunotherapy, naïve T cells with a unanimous tumor-antigen specific TCR are the best choice because tumor antigen specific naïve T cells are long-lived compared to effector T cells and so could be able to further develop into central memory phenotype. Under this guidance, an animal study showed that by overexpressing an antigen-specific TCR into HSCs, TCR-bearing HSCs could be able to develop into antigen-specific T cells *in vivo*. HSC-derived *in vivo* developed T cells are able to control tumor growth in this animal model (Yang and Baltimore 2005). Another study using the *in vitro* T cell development system (OP9-DL1) as indicated above has further confirmed that HSCs could be induced into antigen-specific T cells *in vitro* by a synergistic reaction of both TCR and Notch signaling (Zhao et al. 2007). Based on the available information, HSCs could be able to serve as an outstanding source of unlimited, antigen-specific T cells for an ACT-based cancer immunotherapy.

Although ACT-based cancer immunotherapy has been reported the most promising single regimen in the recently developed immunotherapeutical approaches. Several combined/conjugated immunotherapies have shown further improved results, such as ACT-therapy combined with cytokine, mAbs, and DC-based vaccines. In addition to these observations, HSCs have been shown to develop into B cells, NK cell, DCs, and other blood cells *in vitro*.

In summary, HSCs could serve as a common source to conduct a combined, individualized cancer immunotherapeutical regimen with the options of HSC-derived T cells, NK cells, mAbs, and DC vaccines. On the other hand, ESCs, the predecessor of HSCs, have also been shown acquiring the same capabilities of *in vitro* differentiation into T cells, B cell, NK cells,

and DCs. These comprehensive understandings of stem cell-based immunocytes development will substantially advance cancer immunotherapy into a new era that also favors the current concept of a more-specific, more individualized medicine.

Because HSCs are somatic stem cells, their self-renewal capabilities are relatively down-regulated compared to ESCs, especially in some elder patients and patients who have hematologic problems. The second barrier for HSCs' clinical utilization is the human leukocyte antigen (HLA) matching which determines the success of allogenic transplantation. To overcome these major problems in HSCs utilization, significant efforts are still needed. In the context of ESCs, the problems remain: how to obtain ESCs without any ethical issue and how to avoid HLA matching?

The Concept of Induced Pluripotent Stem Cells and Its Update

In 2006, an exciting report from Takahashi and Yamanaka group for the first time showed that somatic cells could be reprogrammed into pluripotent stem cell status by only 4 transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) (Takahashi and Yamanaka 2006). Previous observation has indicated that nuclei transfer from stem cells to somatic cells converts somatic cells into stem cells; for instance, Dolly, the sheep was created by transferring nucleus substance into the adult mammary cells. It is emphasized that certain molecules expressed only in stem cells are not in somatic cells which govern the fates of stem cells. Through the endeavors of Takahashi and Yamanaka in understanding these mechanisms in maintaining the pluripotency and unlimited self-renewal of the stem cell, they narrowed a series of 24 transcription factors to 10 and finally to four key factors. By ectopic expression of a combination of these four factors via a retroviral mediated gene introduction method, mouse somatic fibroblasts could be able to switch back into their pluripotent status. The converted fibroblast cells are more similar to ESCs but not their prototype correspondents in both morphology and function, such as genetic profile, stem cell marker expression, embryogenesis, and teratoma formation. This novel type of genetically modified somatic cell is pluripotent stem cell-like but not quite related to any known stem tissues. Therefore, it is named the induced pluripotent stem cells (iPSCs). One year later, both Yamanaka (Takahashi et al. 2007) and Thompson group (Yu et al. 2007) reported the success of generating human iPS from human somatic fibroblasts.

Following these works, a wide variety of human somatic cells have been tested for their capability of generating iPSCs. So far, human mature B cells, stomach, liver, pancreatic β cells, skin-derived melanocytes and keratinocytes, adipose-derived stem cells, and neural stem cells have been reported, which are able to switch back into iPSCs. Also, in order to efficiently and safely generate iPSCs from somatic cells, many new improvements in iPSC generation have been reported. To increase the efficiency of iPSC generation, lentiviral-vector and inducible lentiviral-vector based gene introduction have been reported. However, with the first-generation retroviral-mediated gene introduction the major problem is the safety of gene introduction because both retroviral and lentiviral vector will integrate the target genes into the host genome, resulting in tumorigenesis.

To avoid the potential problems caused by gene integration, an excisable gene introduction approach such as transposons and *loxP*-flanked lentiviral vector have been introduced. Furthermore, non-integrating strategies such as adenoviral and plasmid vector based gene deliveries have been reported to be feasible. Recently, several studies of pluripotency induction further indicates that several non-DNA based approaches are also able in generating iPSCs from human fibroblasts; for example, Sendai (RNA) virus-mediated gene delivery, direct protein delivery of transcription factors, delivery of modified mRNA that encoding transcription factors, and microRNA based conversion (Warren et al. 2010). The advancement of gene delivery techniques eases the safety issues aroused from the oncogenic-prone gene

integration method. The second concern in the safety of using iPSCs in clinic is the introduction of potential oncogenes such as c-Myc. To overcome this, different combinations of transcription factors have been studied. In the previous mentioned trials, direct delivery of Oct3/4 and Sox2 proteins into somatic fibroblasts could generate iPSCs, although the efficiency is very low (Zhou et al. 2009). Another experimental approach to convert fibroblasts into iPSCs is by several microRNAs, which is relatively safe in the context of avoidance both gene integration and oncogene introduction (Miyoshi et al. 2011).

Taken together, this information further confirmed the concept of iPSC and offered new hope and opportunity for modeling human diseases and designing a personalized regenerative medicine. The discovery of iPSCs could also avoid the constraints from using ESCs. For the substitution of iPSCs to ESCs in laboratories and clinics, the most important issue is the similarity of iPSCs to ESCs. Although iPSCs have manifested broad similarities to ESCs, there are still inconsistencies in gene and protein expression and functionalities. Furthermore, generated iPSCs still bear more or less the markers of their prototype cells. To better determine the readiness of iPSCs in replacing ESCs in research and clinical application, it needs careful comparison between iPSCs and ESCs. The first key property needs to be evaluated is the pluripotency. Usually cell colony morphology is the first index to look into. Somatic cells and ESCs have significant difference in colony formation; therefore, the first step to characterize and isolate a potential iPSC colony after different approaches of reprogramming is based on the similarity of the colony to ESCs. However, similarity in morphology does not guarantee iPSCs' identification of gene expression profiles, and functionalities. Hence further markers in molecular level will be favored to accurately evaluate the reprogrammed iPSCs.

Fully reprogrammed iPSCs express a series of pluripotency genes in comparable with ESCs such as Oct4, Sox2, and Nanog. Another hallmark of entering the pluripotent stage is the reactivation of telomerase genes in iPSCs, which have been silenced in somatic cells. Other molecular markers paired with activated pluripotent genes in ESCs include SSEA1, SSEA3, TRA-1-60, TRA-1-81, DNMT3β, and REX1. Other than these documented genetic changes in pluripotency, epigenetic changes are also crucial in interpreting the reprogramming of iPSCs, i.e., the methylation status of certain gene promoters is a fair indicator after reprogramming. More importantly, in epigenetic turnovers, the reactivation of X chromosome silence has been shown to be another critical step in the conversion of somatic cells into iPSCs. Based on current comprehension, when reprogrammed cells acquire all these features, they could be designated as iPSCs and are supposed to behave like ESCs. But questions about their pluripotent function are still remaining because molecular similarities do not necessarily guarantee functionalities.

Well-described methods to evaluate the pluripotency functions of ESCs are also suitable in determining iPSCs (Maherali and Hochedlinger 2008). *In vitro* ability of differentiation into embryoid bodies and all three germ layers of cells is the first checkpoint to evaluate the pluripotency. In mouse, the *in vivo* development of embryo chimerism by injection of iPSCs into the developing blastocyst could help check the capability of iPSCs in the context of differentiation into adult tissue and cells. Apparently, the embryo chimerism is not suitable in testing human iPSCs. Therefore, a teratoma formation assay has been developed and currently serves as a gold standard in evaluating the pluripotency of reprogrammed human cells. In this system, any potential human iPSCs will be introduced into an immunedeficient mouse model (SCID mouse or nude mouse) either subcutaneously or intramuscularly.

True iPSCs will form a well-differentiated tumor mass consisted of cells from all three germ layers. Although it is called the gold standard, the problem is still remaining. iPSCs could be able to develop into three germ lines, but this does not guarantee that iPSCs will differentiate into all cell types found in human body. Because of the relatively young-aged concept of iPSCs, it is understandable that there are still debates

among scientists with both pro and con thoughts. It is believed that these debates will further promote the maturation of iPS technology in the future. Accordingly, considerable information is still needed to establish more consistent and effective standards to thoroughly evaluate the generated iPSCs, which could significantly benefit the worldwide application of iPSCs in both research and treatment.

The ultimate goal of studying iPSCs is to find a cure to many human diseases, including cancer. Generally, there are two approaches of using iPSCs in finding a cure: one is the remodeling of human diseases with patient-specific or disease-specific iPSCs, so that therapeutical strategy could be developed. The other approach is to repair or supplement defective cells or tissue with patient-iPSCs derived ones. Although only a few years have passed since the debut of iPS technology many applications of iPS have already been reported in the literatures. In terms of remodeling human diseases and drug screening with iPS cells, the first study is in patients with familial dysautonomia, a genetic disorder of the peripheral nervous system, which characterized as extensive autonomic nervous system defects and small-fiber sensory neuron dysfunctions (Lee et al. 2009). The etiology has been traced to the point mutation in the gene IKBKAP, and there are several *in vitro* models to evaluate the disease prognosis and drug intervention. However, none of those studies uses disease-specific or affected tissue. Therefore, it lacks specificity to accurately examine the problem. With the successful remodeling with affected tissue-derived iPSCs, for the first time, the cellular and molecular properties of this disease have been carefully evaluated and following a screen for pharmaceutical management, a plant hormone called kinetin has been identified as being effective. This pioneering research unveiled the new stage of iPSC-based research in human diseases. So far, iPSCs have been applied in major systems, including cardiologic, hematologic, metabolic, musculoskeletal, and neurological. Approximately, nearly 50 different types of diseases have been reported by iPSC remodeling, and some of them show satisfied results; for instance, familiar dysautonomia, Rett's syndrome, schizophrenia, type 2 long QT syndrome and retinitis pigmentosa. With the uses of iPS technology together with further unveiling of disease mechanisms, additional iPS-derived models will be setup to benefit the exploration of effective therapeutical managements.

The other iPSC-derived treatment option is the regenerative medicine. We have waited for a long time to find an optimal substituting mechanism to replace failed human cells, tissue, and organs. Although allogenic transplantation has been successfully carried out in past decades because of the improvement in modern medicine and introduction of more-effective immunosuppressive agents, the critical problem is still the shortage of the available tissue for transplantation. In the pre-iPS time, scientists even came up with the idea of raising genetically engineered pigs to provide xenogenic organs for the organ failure patients in the long waiting list for transplantation. The other concern involves the post-transplant rejection or side-effects following immunosuppressant administration that aims at suppressing or reversing the tissue rejection. In clinics, physicians have been trying hard to balancing these two events in post-transplant patients. Probably, difficulties in finding suitable organs and battling rejections will be seen only in history books thanks to the development of iPS technology.

It is impossible to summaries all the advanced developments of iPS science here. To date, cardiomyocytes, chondrogenic cells, endothelial progenitor cells, hepatocytes, male germ cells, neural progenitor cells, osteocyte progenitors, pancreatic β cells, retinal pigment cells, steroid-secreting adrenocytes, and various types of cells in the hematologic system including T cells, B cells, NK cells, DCs, megakaryocytes/platelets, and red blood cells are derived from different types of iPSCs. More specifically, certain subtypes of T cells could also be generated from iPSCs; for example, regulatory T cells (or Tregs) could be generated from iPSCs with both intrinsic and extrinsic stimuli (Haque et al. 2012). The continuous advancement in comprehending stem cell biology will keep expanding the pool of differentiated

cells from both natural and induced pluripotent cells for tissue engineering. There is an intense research in the area of blood cell differentiation from iPSCs, which will attract the attention by immunologists: will iPSCs be able to be applied in cancer immunotherapy?

Generation of Tumor-Specific T Lymphocytes from iPSCs for Adoptive Immunotherapy

As introduced earlier here, a successful cancer immunotherapy relies on highly specific tumor-antigen reactivity by T cells, mAbs, and other components. Supplementing tumor-reactive T cells in ACT therapy has shown promising results. So, the important question is whether iPSCs can be used to generate tumor-antigen specific T cells for potential ACT-based cancer immunotherapy. With the information that both HSCs and ESCs are able to differentiate into T cells in an *in vitro* culture system, we have advanced the idea to test whether iPSCs could be able to follow the same trend (Lei et al. 2009). In that study, we cocultured iPSCs with Notch ligand expressing bone marrow stromal cell line (OP9-DL1) for a certain number of days to induce the T lineage differentiation. At the different time points under differentiation, iPSC-derived cells were harvested for evaluation in different aspects including morphology, cell marker change, and functionality assessment. It is found that under the course of coculture, iPSC-derived cells are changing from stem like cells to more T like cells in terms of cell or colony morphology, and acquired T cell marker and T cell function. In the morphology, stem cell dome-like colonies disappeared; instead, mesodermal-like colonies and grape-like colonies were formed sequentially. Before the differentiation, iPSCs expressed a high level of CD117, a stem cell surface marker and Nanog gene. However, after 12 days, coculture induced differentiation, both CD117 and Nanog were gone, and T cell markers like CD4, CD8 had developed. *In vitro* differentiated iPSC-derived cells were also able to function in the context of secreting IL-2 and IFN-γ upon the stimulation with anti-CD3 and anti-CD28 antibodies.

Due to the absence of three-dimensional nature of thymic selection, in an *in vitro* culture system, iPSC-derived T cells were still considered as immature progenitor cells and the majority of them were stalled in the double-positive (DP) stage, which featured as bearing both CD4 and CD8 markers. After adoptive transfer of those differentiated progenitor cells into *Rag*-deficient mice, the DP progenitor cells were further differentiated into final matured either CD4 or CD8 single positive (SP) groups in 3 weeks time and reconstituted the T cell pools in animals. This initial report for the first time supported the possibility of using iPSCs in T cell differentiation, which paves the way to use iPSCs in cancer immunotherapy.

The next step to further explore the potentials of iPSCs in cancer immunotherapy is to test whether iPSC-derived T cells could acquire antigen reactivity and control tumor growth. In this study, iPSCs were genetically modified with MHC-I restricted, antigen-specific TCR by retrovirus-mediated gene integration. Previous reports have suggested that the TCR incorporated stem cells are able to direct stem cell differentiation towards T cells *in vivo* under the signal activation by the TCR. Following this information, TCR-bearing iPSCs were transferred into conventional C57BL/6 mice through the tail vein to evaluate their capabilities of differentiation, development, and further persistence. It was found that after 6 weeks of *in vivo* differentiation and development, iPSC-derived, $CD8^+$ T cells were detected in the lymphoid organs based on the expression of their unique TCR compared to endogenous T cell populations. Furthermore, in the tumor-bearing animals, iPSC-derived T cells were persisting longer than conventional T cells that were isolated from transgenic mice. These findings suggested that TCR could direct iPSCs to develop into T cells *in vivo,* and as hypothesized previously, iPSC-derived antigen-specific T cells are able to develop into central memory-like T cells which were able to persist longer than both naïve and effector T cells.

Subsequently, we tried to investigate whether iPSC-derived $CD8^+$ T cells are functional. In this test, after the *in vivo* development, $CD8^+$ T cell pools were isolated from mice and stimulated with antigen. Subsequently, T cell activation as IL-2 and IFN-γ secretions were monitored by intracellular staining. It was observed that upon antigen stimulation, iPSC-derived $CD8^+$ T cells were activated and secreted cytokines. A specially designed *in vivo* killing assay further supported that iPSC-derived T cells were cytotoxically functional.

In summary, iPSCs are able to give rise to antigen-specific T cells based on the observation that after adoptive transfer, iPSC-derived T cells are found morphologically and functionally similar to conventional antigen-specific T cells isolated from transgenic mice. However, in cancer immunotherapy, the most important part of T cells is the control of tumor growth and prolong the survival of affected individuals. To evaluate this key property of iPSC-derived T cells, after *in vivo* induction, iPSC bearing mice were challenged with tumor cell by intraperitoneal injection. In the following days, tumor growth and animal survival were closely monitored. Tumor cell growth was found to be significantly inhibited in TCR-iPSCs group as interpreted by tumor cell counting from peritoneal lavage. At the end of tumor protection experiment, the mouse group that received TCR-iPSCs, survived tumor challenge. Taken together, it is emphasized that iPSCs might possibly fit the requirements in cancer immunotherapy as the source of a large number of antigen-reactive T cells for an ACT-based therapy. In this initial study, to simplify the condition, we tested only the antigen-reactivity with an artificial antigen/TCR system. Therefore, the real tumor antigen/TCR will still be tested to further prove the concept of using iPSCs in ACT-based cancer immunotherapy. Following this information, considerable effort is till needed to modulate this approach as well as to minimize the potential side-effects for achieving an optimal and effective cancer immunotherapy (Lei et al. 2011).

In summary, the general approach to design an iPSC-based cancer/patient-specific ACT therapy could be divided into two major parts: one is the identification of tumor antigen-reactive TCR and the other is the preparation of patient-derived iPSCs. In the first part, TCR will be isolated and characterized from TILs obtained from surgical procedures or directly constructed from tumor antigen library. Through general protocol of antigen-specific TCR cloning, tumor-antigen reactive T cells could be identified and clonally expanded upon tumor antigen stimulation, and tumor antigen-specific TCR will be cloned in these clonally amplified T cells which have the strongest response towards antigen stimulation. The characterized TCR will be genetically processed and subcloned into a viral vector for upcoming gene transduction. For the second part of this approach, generation of iPSCs is relatively easy because of the great stride in iPS technologies. With the help of commercial iPSC induction kit, iPSCs could be easily generated and characterized from a small sample of blood or a small chunk of tissue. At this point, TCR will be introduced into iPSCs via well-documented viral transduction. To increase the T lineage differentiation efficacy and reduce the spontaneous full spectrum differentiation of stem cells, TCR bearing iPSCs could be briefly stimulated by Notch ligand *in vitro*. After finishing all *in vitro* steps, TCR-bearing iPSCs will be infused via patients peripheral veins as normal transfusion. This is further explained in Fig. 10.1.

In recent years, along with our study in iPSCs' application in T cell area, other groups have also made considerable efforts in understanding iPSCs function in hematopoietic development. Dendritic cells, NK cells, and B cells have been shown to be successfully induced from iPSCs. Thus, by using patient-derived iPSCs, tumor antigen-presenting DCs, tumor reactive T, and NK cells, could be generated on purpose to treat cancer patients. At the same time, iPSCs-derived B cells could be further engineered to express both antagonist and agonist mAbs to against cancer. In completing these studies, it would greatly fortify the concept of using iPSCs in the treatment of cancer from a combined network of immune cells/factors.

Also, recently developed a combined, targeted cancer therapy involving both immunotherapy

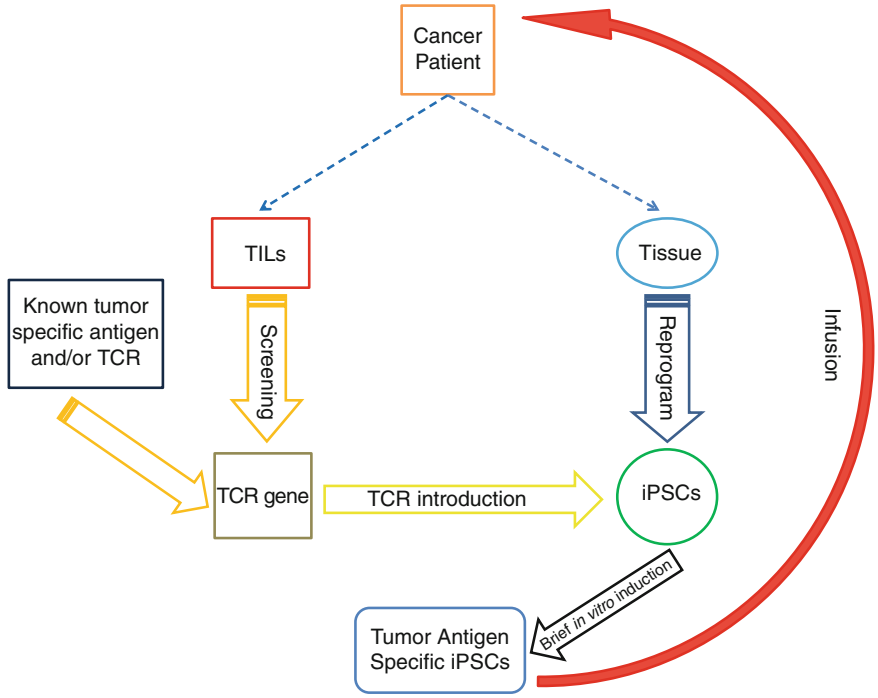

Fig. 10.1 The general approach to design an iPSC-based cancer/patient-specific ACT therapy could be divided into two major parts: one is the identification of tumor antigen-reactive TCR and the other is the preparation of patient-derived iPSCs. In the first part, TCR will either be isolated as well as characterized from TILs obtained from surgical procedures or directly constructed from tumor antigen library. Through general protocol of antigen-specific TCR cloning, tumor-antigen reactive T cells could be identified and clonally expanded upon tumor antigen stimulation, and tumor antigen-specific TCR will be cloned in these clonally amplified T cells that have the strongest response towards antigen stimulation. The characterized TCR will be genetically processed and subcloned into a viral vector for upcoming gene transduction. For the second part of this approach, generation of iPSCs is relatively easy because of the great stride in iPS technologies. With the help of commercial iPSC induction kit, iPSCs could be easily generated and characterized from a tube of blood or a small chunk of tissue. At this point, TCR will be introduced into iPSCs via well-documented viral transduction. To increase the T lineage differentiation efficacy and reduce the spontaneous full spectrum differentiation of stem cells, TCR bearing iPSCs could be briefly stimulated by Notch ligand *in vitro*. After finishing all *in vitro* steps, TCR-bearing iPSCs will be infused via patient's peripheral veins as normal transfusion

and chemotherapy could also be applied in iPSC-based cancer immunotherapy in the near future. Targeted tumor therapy intends to block the growth and survival of tumor cells through drug reactions on designated spots on regulating pathways of tumor cells. Presently, any drug that could suppress tumor growth and enhance immune response, especially T cell response, could be considered to combine with an iPSC-based cancer therapy (Vanneman and Dranoff 2012). For example, in blocking tumor growth, drugs (Sunitinib, Imatinib, Vemurafenib) and mAbs (Trastuzumab, Becacizumab, Cetuximab) could be supplemented in iPSC-based immunotherapy to enhance the tumor control. Other than targeting tumor cells, substantial enhancement of iPSC-derived immune cell responses are also needed. More specifically, for directly boosting iPSC-derived T cell actions, mAbs of both stimulating positive costimulatory signals (CD80, CD86, CD40) and inhibiting negative costimulatory signals (CTLA4, PD1) could be coadministrated. Indirect T cell function enhancement includes diminishing tumor-induced immunosuppression. To achieve this goal, blocking immunosuppressive cytokines by mAbs, and bleaching MDSCs and Tregs via chemocytotoxicity are possible options.

Cancer immunotherapy is a relatively newly developed approach in comparison with classical cancer managements. In this broad area, stem cell (especially iPSC)-based regimens are still in the cradle stage; therefore, current studies are still under development. In order to advance this idea into reality, both intensive and extensive inputs from all aspects are desired. Although it will be a winding road in front, we are optimistic regarding finding a cure of cancer provided significant comprehension of both cancer biology and immunology becomes available.

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Part III

Neural Cells and Neural Stem Cells

Genetic Identification of Human Embryonic Stem Cell-Derived Neural Cell Types Using Bacterial Artificial Chromosomes

11

Zeynep Tokcaer-Keskin and Dimitris G. Placantonakis

Contents

Abstract

Human embryonic stem cells represent a renewable source of pluripotent cells which can give rise to endodermal, mesodermal and ectodermal lineages. Not surprisingly, they have received enormous attention in the context of regenerative medicine. However, harnessing their tremendous potential requires a thorough understanding of their biology and the mechanisms that govern their pluripotency. Directed *in vitro* differentiation of human embryonic stem cells is typically based on recapitulating biological processes that take place during normal mammalian development. Unfortunately, differentiation of human embryonic stem cells to specific cell types is plagued by low yields and heterogeneous cell populations. This chapter will describe how the differentiation of human embryonic stem cells can be tracked using recombinant bacterial artificial chromosomes expressing fluorescent reporters driven by developmentally regulated promoters. As an example, we will describe how this technology can be applied within the neural lineage to track differentiation to neural stem cells and spinal motor neurons.

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Introduction

Neural Differentiation of Human Embryonic Stem Cells

Human embryonic stem cell (hESC) lines were first generated by Thomson et al. (1998) from blastocyst stage embryos that had been generated by *in vitro* fertilization. They have indefinite capacity to self-renew *in vitro* and remain pluripotent, a property that allows them to give rise to cell types derived from all three germ layers: endoderm, mesoderm and ectoderm. These properties make hESCs an ideal model system for understanding human developmental biology and pathogenesis. Modeling the development and diseases of the human central nervous system, in particular, has been an area of unprecedented success with the use of hESCs (Peljto and Wichterle 2011; Vazin and Freed 2010; Goldman 2005). Furthermore, hESCs represent a unique opportunity for developing cell replacement therapies for diseases where drug-based treatments do not exist or are inefficient.

Directed differentiation of hESCs into neural lineages requires a thorough understanding of human developmental biology. *In vitro* combinations of morphogens and small molecules, which either inhibit or activate critical signaling pathways, are commonly used to direct hESCs to specific neural fates by recreating developmental processes known to take place *in vivo*. The *in vitro* production of mature terminally differentiated neural cells from hESCs, such as neurons or glia, requires two distinct steps. First, hESCs are directed to acquire a primitive neuroectodermal stem cell phenotype. Second, such neutrally restricted stem cells are directed to differentiate to specific mature neural cell types (Zhang et al. 2001). Here, we will focus on the specification of spinal motor neurons (MNs) from rosette-type neural stem cells (NSCs) as an example of hESC directed differentiation.

The development of the central nervous system starts with the formation of the neural tube. The neuroepithelial cells of the neural tube generate progenitor cells, which in turn give rise to several types of neurons and glia. The first step in obtaining neural lineages from hESCs involves the conversion of hESCs to neural stem cells (NSCs), a process known as neural induction. To facilitate neural induction and maximize its yield, *in vitro* protocols utilize morphogens and small molecules that suppress cues for mesodermal and endodermal differentiation. In our laboratory, we promote hESC neuralization by using several factors: noggin (reviewed in Sasai and De Robertis 1997, Chambers et al. 2009), LDN-193189 (Yu et al. 2008) and SB431542 (Smith et al. 2008, Chambers et al. 2009). These agents act to suppress signaling through the BMP and TGFβ cascades, thereby inhibiting mesendoderm and ectoderm formation and promoting neuroectodermal formation. The neuroepithelial cells obtained through this process are organized radially in structures termed neural rosettes, which resemble the neural plate in terms of marker expression and multipotency (Zhang et al. 2001; Elkabetz et al. 2008). Neural rosette NSCs can be patterned toward virtually any neural fate within the central nervous system (Zhang et al. 2001; Elkabetz et al. 2008). In particular, they can be ventralized and caudalized, by treating with sonic hedgehog (Shh) and retinoic acid (RA) respectively, to differentiate into spinal motor neurons (MNs) (Lee et al. 2007; Placantonakis et al. 2009; Hu and Zhang 2009).

The two steps of hESC-derived spinal motor neurogenesis, neural induction and MN patterning with Shh and RA, can be tracked *in vitro* using the Notch pathway and transcription factor HB9 as respective markers. The maintenance of multipotency in rosette NSCs has been shown to be highly dependent on the Notch pathway (Elkabetz et al. 2008). The effect of the Notch pathway in maintaining NSCs has been well investigated. Notch is thought to support NSC maintenance via expression of the *Hes1* and *Hes5* genes, which suppress neurogenesis (reviewed in Ables et al. 2011; Kageyama et al. 2008). The development of MNs depends on the expression of several homeodomain transcription factors, including HB9, Islet1 and Lim3 (Shirasaki and Pfaff 2002). The homeobox gene *HB9* is a much more specific marker for MNs as its

expression during CNS development is restricted to MNs (reviewed in Shirasaki and Pfaff 2002; Arber et al. 1999). For this reason, the *Hes5* and *HB9* promoters are good candidates to drive the expression of reporter genes that identify hESC-derived NSC and MN lineages, respectively. These promoters have been successfully used for such purposes in mouse ESC and hESC-derived neural lineages in several studies to date (Placantonakis et al. 2009; Tomishima et al. 2007; Roy et al. 2004; Wichterle et al. 2002).

Bacterial Artificial Chromosomes and Their Modification

The neural differentiation of hESCs yields heterogeneous cell populations comprised of several cell types. Due to this inherent limitation using current differentiation protocols, developing efficient methods to discriminate between these cell types is very important. Transgenic reporter systems provide a live detection method to differentiate between these various cell types by harboring a reporter gene (such as a fluorescent protein) expressed under the control of cell-specific developmentally regulated promoters. The fidelity of such reporters is highly dependent on the promoter constructs used to drive reporter expression. We propose that bacterial artificial chromosomes (BACs) can produce high-fidelity reporter systems, by virtue of their ability to retain the numerous regulatory elements that tightly control endogenous gene expression by way of their large size.

Bacterial artificial chromosomes (BACs) were first generated by Shizuya et al. (1992), from the F plasmids of *E.coli*. The genes in the F plasmid backbone control the unidirectional replication and copy number of the BACs. In the initial generation of BACs, the chloramphenicol (cm) resistance gene (*cmR*) and *loxP* sites were included in the backbone to aid in engineering BACs toward desired cell culture applications (Fig. 11.1). Although BACs have the ability to carry up to 1,000 kb of DNA inserts, their usual sizes are between 100 and 250 kb. Because they can carry large inserts, they are the preferred vehicle for the construction of genomic libraries for many species. An advantage associated with the large insert size is that most and potentially all regulatory elements associated with transcriptional regulation of a gene may be present in BAC constructs (Yang and Gong 2005). It follows that reporter genes cloned into BAC constructs were shown to be expressed more faithfully providing a better tracking of cells (Yang et al. 1997; Lee et al. 2001). Moreover, the GENSAT BAC transgenic project showed that recombinant BACs can be generated very efficiently and rapidly (Gong et al. 2003).

Two common methods in BAC engineering take advantage of bacterial recombinases to replace sequences within the BAC with exogenous sequences: allelic exchange and recombineering. Here we will focus on the allelic exchange method, which is also described in detail in the methods section. This technique takes advantage of bacterial *recA* gene expression in a controlled manner (Yang et al. 1997). It employs a long 500 bp homology arm to serve as template for homologous recombination. The homology arm is ligated to the reporter gene carried by a shuttle vector (pLD53.SC2). The expression of this shuttle vector highly depends on the presence of the *pir* gene (encodes the π protein), which controls the vector's *R6Kγ* origin of replication. This explains why cloning of the homology arm is performed in *E. coli* strains that carry the *pir* gene. In this system, expression of the *recA* gene is introduced by another plasmid (pSV1.RecA). Expression of *recA* is regulated by a temperature-sensitive origin of replication, which is active at 30°C but not at higher temperatures. For the homologous recombination to take place, these two vectors are introduced into the *E.coli* strain DH10B which carries the BAC of interest and lacks the π protein and incubated overnight 30°C. At the end of this step, *recA* gene mediates the recombination of the reporter gene into the BAC backbone. Since DH10B bacteria lack π protein, the pLD53.SC2 plasmid is lost. During the subsequent step, which is incubation of the culture at 43°C, the temperature-sensitive origin of replication of *recA* plasmid is affected. At the

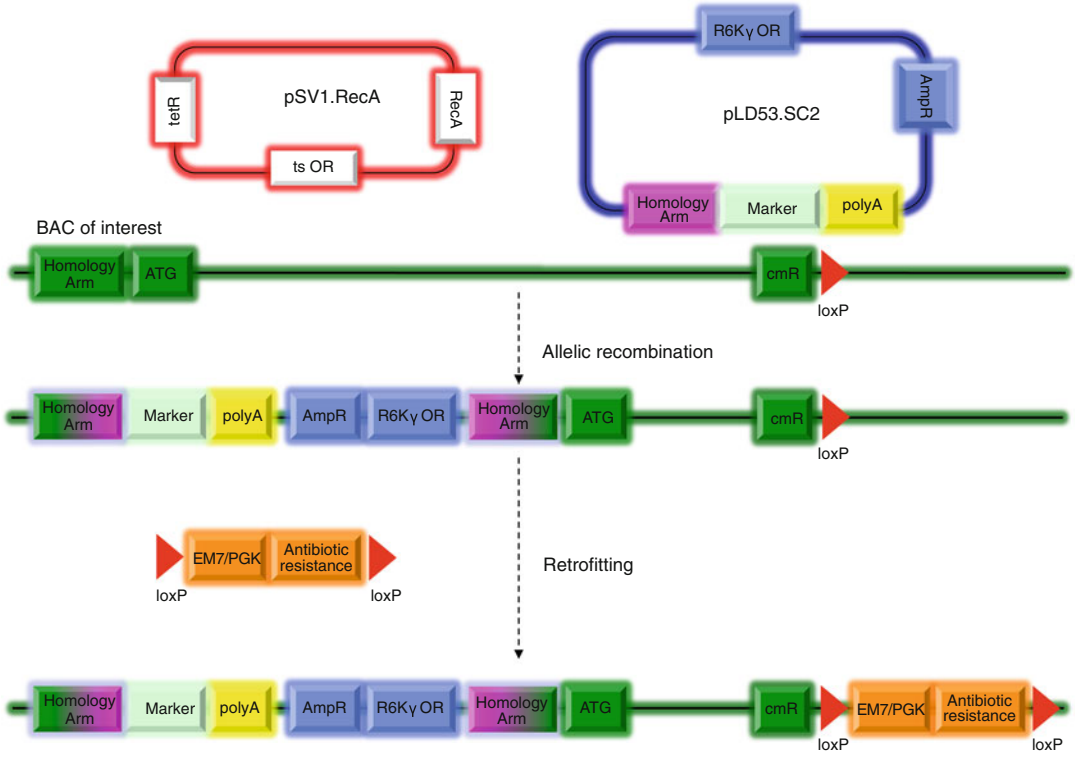

Fig. 11.1 Schematic presentation of a bacterial artificial chromosome. *tetR* tetracycline resistance, *tsOR* temperature-sensitive origin of replication, *R6Kγ OR*, *R6Kγ* origin of replication, *polyA* polyadenylation signal, *ampR* ampicillin resistance, *ATG* initiator ATG of target gene, *cmR* chloramphenicol resistance, *EM7*/*PGK* tandem of prokaryotic (EM7) and eukaryotic (PGK) promoters

end of this step, the bacterial culture should contain bacteria with the recombinant BAC but not the accessory plasmids (Gong et al. 2002, 2010; Fu and Maye 2011).

The chloramphenicol resistance gene present on the backbone of the BAC allows for prokaryotic selection in bacteria. However, for *in vitro* use of recombinant BACs with cultured cells, such as hESCs, BACs are retrofitted with eukaryotic antibiotic resistance markers (Tomishima et al. 2007). For this purpose a mammalian selection marker is introduced into the *loxP* site of the BAC backbone in Cre recombinase-expressing bacterial strains (such as EL350) (Wang et al. 2001, 2004).

Here, we will describe the steps (Fig. 11.2) towards the generation and use of BACs expressing fluorescent reporters driven by *Hes5* and *HB9* promoters to track the differentiation of hESC-derived rosette NSCs and spinal MNs, respectively.

Materials

Bacterial Cultures and Modification of Bacterial Artificial Chromosome (BAC)

LB medium, SOC medium, LB plates with or without antibiotics at final concentrations of: 12.5 μg/ml chloramphenicol (cm) (Sigma), 10 μg/ml tetracycline (tet) (Sigma), 50 μg/ml ampicillin (amp) (Sigma), 50 μg/ml kanamycin (Sigma), 50, 20 and 10% sterile glycerol solutions, Qiagen miniprep solutions P1, P2 and N3, isopropanol, 70% ethanol, sterile ddH_2O, EcoRI (NEB), restriction enzymes (RE) to digest plasmids of interest, agarose gel electrophoresis, Qiagen midi prep kit, *E.coli* strains PIR1, DH5α, DH10B and EL350, Qiagen Qia-quick gel extraction kit, T4 ligase (NEB), *Taq* polymerase

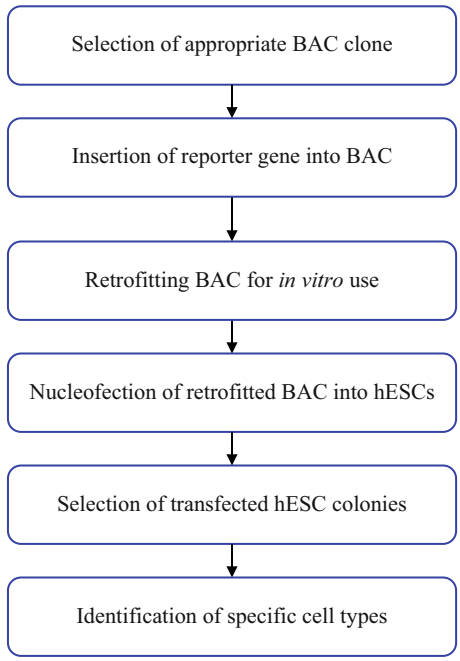

Fig. 11.2 Flow chart of bacterial artificial chromosome engineering and its *in vitro* use

(Qiagen), High fidelity *Taq* polymerase (Qiagen), 50 mM $CaCl_2$, 10% L-arabinose in ddH_2O (filter sterilized), Princeton Separations BAC midiprep.

Eukaryotic Cells Required for Tissue Culture

Human Embryonic Stem Cells (hESCs), mouse embryonic fibroblasts (CF6Neo MEF 4M Mito-C, GlobalStem), multidrug-resistant MEFs (DR4 MEF 2 M IRR, GlobalStem). DR4 cells are resistant to: neomycin, hygromycin, puromycin and 6-thioguanine.

Reagents

0.1% gelatin in sterile water (ES-006-B, Millipore), matrigel basement membrane matrix (BD), FGF2 (233-FB-025/CF, R&D), sonic hedgehog (464-SH-025, R&D), BDNF (248-BD, R&D), ascorbic acid (A4034, Sigma), Y27632 (1254/10MG S, Tocris Biosciences), retinoic acid (R2625, Sigma), noggin (719-NG-050, R&D), SB431542 (1614/10MG S, Tocris Biosciences), LDN193189 (04-0074, Stemgent), laminin (L2020-1MG, Sigma), 0.01% poly-ornithine (P4957-50ML, Sigma), Amaxa nucleofector kit (VPH-5022, Lonza), G418 (10131035, Life Technologies), accutase (AT104, innovative cell technologies), DMSO (D8418, Sigma), sterile 1 × PBS, sterile ddH_2O.

Media and Preparation

DMEM with 10% Fetal Bovine Serum (FBS): Mix 450 ml DMEM (11995073, Life Technologies) with 50 ml FBS (16000044, Life Technologies) and filter sterilize.

hESC medium: 400 ml DMEM/F12 (11330057, Life Technologies), 100 ml Knockout Serum Replacement (KSR; 10828028, Life Technologies), 2.5 ml L-glutamine (25030081, Life Technologies), 5 ml non-essential aminoacids (NEAA; 11140050, NEAA Life Technologies), 0.5 ml 2-mercaptoethanol (21985023, Life Technologies), 2.5 ml penicillin/streptomycin (15070063, Life Technologies). Filter-sterilize all the ingredients and then add FGF2 to a final concentration of 5 ng/ml.

Dispase solution: Dissolve 10 mg dispase (LS02100, Worthington) in 100 ml hESC medium and filter-sterilize.

KSR medium: 82 ml KO DMEM (10829018, Life Technologies), 15 ml KSR, 1 ml L-glutamine, 1 ml NEAA, 0.1 ml 2-mercaptoethanol, 0.5 ml penicillin-streptomycin. Filter-sterilize all the ingredients.

N2 medium: 0.155 g D-Glucose (Sigma), 0.2 g NaHCO3 (Sigma), 2.5 mg bovine insulin (Sigma) dissolved in 1 ml of 5 mM NaOH (Sigma), 10 mg transferrin (BD), 30 nM sodium selenite (Sigma), 100 μM putrescine (Sigma) and 20 nM progesterone (Sigma) in ethanol. Complete to 100 ml with DMEM/F12. Filter-sterilize all the ingredients.

Motor Neuron (MN) medium: 96 ml Neurobasal medium (21103-049, Life Technologies), 1 ml 100× N2 stock solution (17502-048, Life

Technologies), 2 ml 50× B27 (12587-010, Life Technologies), 1 ml NEAA. Filter-sterilize all the ingredients.

Methods

Amplification and Storage of Bacterial Strains

1. Choose the BAC DNA of interest. BAC libraries could be screened by using publically available browsers. We routinely use the UCSC Genome Browser for such purposes (http://genome.ucsc.edu). We typically choose BACs which contain ample amounts of sequences upstream and downstream of the coding portion of the gene of interest. BACs can be ordered from BACPAC Resource Center (BPRC) located at the Children's Hospital Oakland Research Institute in Oakland, California, USA (http://bacpac.chori.org/) or Invitrogen.
2. When the bacterial stock arrives in an agar slab, usually with DH10B *E.coli* containing the BAC of interest (DH10B/BAC), start an overnight (o/n) liquid culture in LB/cm or streak an LBagar/cm plate.
3. Make 20% glycerol stocks by mixing 400 µl 50% glycerol and 600 µl bacterial culture and store in −80°C for future use.

Miniprep of BAC

1. Initiate an o/n 5 ml liquid culture from DH10B/BAC in LB/cm. The final concentration of cm should be 12.5 µg/ml.
2. In a 1.5 ml Eppendorf tube, spin down 3 ml from the o/n culture at 13,000 rpm in a tabletop centrifuge for 30 s and aspirate the medium.
3. The following steps must be performed extremely gently.
4. Resuspend in 250 µl P1 solution of Qiagen miniprep kit.
5. Lyse with 250 µl P2 solution of Qiagen miniprep kit and invert a few times gently to mix.
6. Neutralize with 300 µl N3 solution of Qiagen miniprep kit and invert a few times gently to mix.
7. Centrifuge at 13,000 rpm in a tabletop centrifuge for 4 min.
8. Carefully pipette the supernatant to a new tube avoiding the cell debris.
9. Centrifuge at 13,000 rpm in a tabletop centrifuge for 4 min.
10. Mix supernatant with 750 µl isopropanol in a separate tube by inverting a few times and incubate at room temperature (RT) for 10 min.
11. Centrifuge at 13,000 rpm in a tabletop centrifuge for 10 min.
12. Carefully aspirate the supernatant and add 1 ml 70% ethanol without disturbing the pellet.
13. Centrifuge at 13,000 rpm in a tabletop centrifuge for 3 min.
14. Carefully aspirate the supernatant and air dry the pellet by inverting the tube for about 5 min.
15. Resuspend the pellet in 16 µl of ddH_2O and keep at 4°C. We recommend not freezing the purified BAC suspension, because repeated freeze-thawing can damage the integrity of the BAC. If the next step in the protocol is electroporation into bacteria, we recommend not storing the BAC for long periods of time, because its integrity may be compromised.
16. Digest with EcoRI to establish the restriction fragment length polymorphism (RFLP) pattern of the unmodified BAC.

Amplification of Plasmids Required for Allelic Exchange

1. Amplify pLD53.SC2 (Gong et al. 2010) in PIR1 at 37°C in LBamp media. Plasmid pLD53.SC2 carries the reporter gene, GFP.

2. Amplify pSV1.RecA (Yang et al. 1997) in DH5a at 30°C in LBtet media, which carries the *recA* gene.
3. Midiprep by using Qiagen midiprep kit.

Modification of BAC

1. Choose the 500 bp homology arm for homologous recombination that is 500 bp upstream of the start codon of the gene of interest.
2. Design primers to PCR-amplify the homology arm that have overhanging restriction enzyme (RE) sequences for RE digestion and cloning into pLD53.SC2. It is important to choose REs that have single cut sites at the vector backbone just in front of the marker gene and digest the homology arm and vector with the same enzymes to generate sticky ends for the ligation.
3. PCR-Amplify the homology arm by using BAC of interest as a template.
4. Optimize the PCR with regular *Taq* polymerase.
5. After optimization, PCR-amplify the homology arm with high fidelity *Taq* polymerase (HotStar Taq DNA polymerase, 203203, Qiagen). Run on a 1% agarose gel and isolate from the gel by using Qiagen Qia-quick gel extraction kit.
6. Digest homology arm and pLD53.SC2 with the appropriate restriction enzymes.
7. Run in agarose gel and isolate from the gel.
8. Ligate homology arm and vector with T4 ligase (NEB) at 16°C o/n.
9. Thaw chemically competent PIR1 cells (C101010, Invitrogen) on ice.
10. Mix 5 μl of ligation mix with bacterial cells by tapping gently.
11. Incubate on ice for 30 min and heat shock at 42°C water bath for 30 s.
12. Incubate on ice for 2 min and recover in 250 μl SOC medium for 1 h. Spread on LBamp plates.
13. Select transformed colonies on LBamp plates in 37°C bacterial incubator o/n.
14. Pick several colonies and amplify in LBamp o/n by vigorous shaking at 37°C.
15. Miniprep and diagnose the ligation with RE digestion and PCR for homology arm.
16. Expand bacterial cells that contain the BAC of interest in 50 ml LBcm by shaking vigorously at 37°C.
17. Pellet the bacteria at 2,500*g* for 10 min at 4°C and discard the supernatant.
18. Make them chemically competent by resuspending in 50 mM $CaCl_2$ on ice for 15 min.
19. Pellet the bacteria at 2,500*g* for 10 min at 4°C and discard the supernatant.
20. Resuspend the pellet in 180 μl 50 mM $CaCl_2$ and 120 μl 50% glycerol solution.
21. Transform 100 μl of chemically competent BAC-containing bacteria with pSV1.RecA as described before (but in this case increase the duration of heat shock to 50 s).
22. Select on LBtet/cm plates at 30°C.
23. Expand colonies from this plate o/n by vigorous shaking at 30°C in LBtet/cm.
24. Inoculate 1 ml from this culture to 50 ml LBtet/cm and continue culturing until the $O.D._{600}$ is 0.6–0.8 (approximately 3 h) with vigorous shaking at 30°C.
25. Centrifuge the bacterial culture at 2,500*g* for 10 min at 4°C and discard the supernatant.
26. Make the cells electrocompetent by washing with 25 ml ice cold water and 25 ml ice cold 20% glycerol and pellet at 2,500*g* for 10 min at 4°C. Discard the supernatant and repeat the wash one more time.
27. Resuspend the cells in 200 μl of ice cold 10% glycerol solution.
28. Electroporate 50 μl of these cells (which carry the BAC and pSV1.RecA) with the pLD53.SC2 reporter construct containing the homology arm using the following parameters: 1.75 kV, 25 μF, 200 Ω.
29. Recover in 1 ml of SOC media at 30°C for 2 h.
30. Transfer to LBtet/cm/amp and incubate at 30°C o/n
31. Plate on LBcm/amp plates and incubate at 43°C o/n.

32. Select colonies and perform BAC miniprep
33. Design primers to 3′ and 5′ sequences of the reporter construct.
34. Diagnose recombinant BAC colonies by PCR with primers amplifying the reporter from the junction of recombination.
35. Check the integrity and RFLP pattern of the BAC by digesting with EcoRI for 2 h at 37°C and running the reaction on a 0.8% agarose gel.
36. Make glycerol stocks from the colonies that contain the modified BAC.

Retrofitting the BAC

1. Use the minipreps from the colonies that have the intact and correct BAC to electroporate into electrocompetent EL350 *E.coli* (prepared as described above and electroporated under the same conditions: 1.75 kV, 25 μF, 200 Ω) and recover at 30°C for 2 h.
2. Select on LBcm/amp plates o/n at 30°C.
3. Perform a BAC miniprep and check the integrity and RFLP pattern with EcoRI digestion. Make glycerol stocks.
4. Using RE digestion of appropriate vectors, such as plasmid pL452 (http://web.ncifcrf.gov/research/brb/productDataSheets/recombineering/plasmid.aspx#PL452), retrieve the antibiotic resistance cassette (which could be the neomycin/kanamycin resistance gene or the hygromycin resistance gene) under the transcriptional control of both prokaryotic and eukaryotic selection promoters (EM7 and PGK, respectively). This cassette should be flanked by *loxP* sites.
5. Run the digestion reaction on a 1% agarose gel, gel-purify the antibiotic resistance cassette and determine its concentration.
6. Start an o/n culture of EL350 cells with the recombinant BAC at 30°C.
7. Inoculate 2 ml from the o/n culture into an 18 ml LBcm/amp culture and grow the cells until $O.D._{600}$ is 0.5 at 30°C.
8. Separate culture equally into two flasks.
9. Add 100 μl 10% arabinose solution to one of them and incubate for 1 h at 30°C. The Cre recombinase in EL350 cells is under the control of an arabinose-inducible promoter. The second flask serves as negative control.
10. Cool the flasks on ice by swirling and transfer to cold falcon tubes and cool 5 min more.
11. Pellet the cells in a tabletop microcentrifuge at 6,000 rpm for 5 min at 4°C. Aspirate the supernatant.
12. Resuspend in 1 ml ice cold ddH_2O and centrifuge in a tabletop microcentrifuge at 13,000 rpm for 30 s. Aspirate the supernatant. Repeat this step three times.
13. Resuspend the cells in 50 μl ice cold ddH_2O. Mix with 100 ng antibiotic resistance cassette. Electroporate as above (1.75 kV, 25 μF, 200 Ω).
14. Recover in 1 ml SOC medium for 2 h at 30°C.
15. Plate on LBcm/amp/kan plates o/n and incubate at 30°C.
16. Select colonies, miniprep the BAC and check for the presence of the resistance cassette by PCR. Confirm the insertion of the cassette with RFLP analysis. After obtaining molecular confirmation, make glycerol stocks from the colonies that are retrofitted.
17. Electroporate electrocompetent DH10B bacteria with the retrofitted BAC as above (1.75 kV, 25 μF, 200 Ω) and select on LBcm/amp/kan.
18. Pick colonies, miniprep and perform RFLP analysis, as above. Make glycerol stocks. The recombinant retrofitted BAC glycerol stocks should be in DH10B cells to prevent recombination events and unwanted BAC changes.

BAC Midiprep

1. Start an o/n culture from the frozen stock that contains the recombinant retrofitted BAC of interest for a midiprep.

2. Perform BAC midiprep with the Princeton Separations BAC midiprep kit according to the manufacturer's instructions and determine the concentration.

NOTE: BAC should be freshly prepared on the day of hESC nucleofection to increase the efficiency of nucleofection. BAC freezing should be avoided to keep the quality of the BAC high.

Preparation of Conditioned Medium

1. Plate ~10×10^6 mouse embryonic fibroblasts (MEFs) pretreated with mitomycin C or irradiated on a 175 cm^2 tissue culture flask with DMEM 10% FBS (day 0).
2. On day 1, aspirate the medium, wash the cells with 1× PBS and add hESC medium.
3. On day 2, pipette up the MEF-conditioned medium (CM) and replace with fresh hESC medium.
4. Aliquot CM in falcon tubes at desired volumes and store at −20°C.
5. Repeat the two steps above everyday until the required amount of CM is stored.
6. MEFs in the flask can be used for this procedure for at most 1 month.
7. Before using the CM, thaw required amount of aliquots and filter-sterilize.
8. hESCs cultured on matrigel in MEF-conditioned media require 10 ng/ml FGF2.

Culture of Human Embryonic Stem Cells

1. Coat 10 cm tissue culture dishes with 0.1% gelatin in sterile water for 15 min.
2. Plate mouse embryonic fibroblasts (MEFs) that were treated with mitomycin C or irradiated at 70% confluency (i.e. 1.5×10^6 cells/10 cm dish) in DMEM 10% FBS.
3. Before plating human embryonic stem cells (hESCs), aspirate the medium, wash once with 1× PBS, and replace the medium with 6 ml of hESC medium.
4. Take a frozen stock of hESCs and quickly thaw in water bath at 37°C.
5. Centrifuge at 150*g* for 5 min in 10 ml of hESC medium and in the presence of 10 μM Y27632 (Li et al. 2008).
6. Aspirate the supernatant and dissolve the pellet that contains the hESC colonies in 2 ml of hESC medium and add the whole content to the plate that has MEFs.
7. Add Y27632 at a final concentration of 10 μM.
8. Grow and expand hESCs on MEF-coated 10 cm tissue culture dishes. No additional Y27632 is needed after the first day of culture.

Passaging of hESCs

1. Discard differentiating colonies by scraping with the tip of a P1000 before each passage.
2. To passage, aspirate the hESC medium and add 4 ml 100 μg/ml dispase in hESC medium.
3. Incubate in the incubator until the colonies fold (approximately 15 min).
4. Dilute dispase with 6 ml hESC medium and collect as many colonies as possible by washing the surface of the plate. Transfer to a 15 ml Falcon tube.
5. Centrifuge at 150*g* for 5 min and aspirate the supernatant.
6. Add 10 ml of hESC medium to the Falcon tube and centrifuge again.
7. Aspirate the supernatant and resuspend the colonies in hESC medium. In this step the cells can be partitioned into separate tubes for differentiation or freezing if required. It is imperative to perform two washes when dispase is used.
8. Centrifuge for 3 min at 150*g* and plate.

Preparation of hESCs for Nucleofection

1. At least four or five 10 cm hESC culture plates with high numbers of colonies are required for nucleofection.
2. 3 days before the nucleofection remove the hESCs from MEF-coated plates by treating with dispase as described before.

3. Prepare 1:20 matrigel diluted in DMEM/F12 on ice using cold pipettes and falcon tubes.
4. Add diluted matrigel on 15 cm tissue culture plates at RT. Use just enough matrigel to cover the surface and wait for 45 min to gelatinize at RT.
5. After 45 min, wash the plates with DMEM/F12 and aspirate.
6. Plate hESCs on matrigel-coated 15 cm tissue culture plates with CM and add 10 ng/ml FGF2.

Nucleofection of hESCs with Retrofitted BAC

1. Grow hESCs on matrigel (you need 5×10^6 cells per nucleofection and 5 μg fresh BAC per nucleofection).
2. Warm solution V of Lonza Amaxa nucleofection kit to RT.
3. Aspirate the medium and dissociate the colonies with warm accutase at 37°C.
4. Collect the cells in a 50 ml falcon tube in hESC medium with 10 μM Y27632. Y27632 is a Rho kinase inhibitor and dramatically increases the survival of dissociated hESCs (Watanabe et al. 2007).
5. Centrifuge at 200*g* for 5 min.
6. Aspirate the supernatant and resuspend the cells in hESC medium with 10 μM Y27632.
7. Obtain cell count with trypan blue staining.
8. Centrifuge at 200*g* for 5 min.
9. Resuspend 5×10^6 cells in 100 μl solution V.
10. Add 5 μg BAC and transfer to nucleofection tubes.
11. Nucleofect using protocol B-16 of Amaxa nucleofector.
12. Immediately add 500 μl CM with 10 ng/ml FGF2 and 10 μM Y27632
13. Plate in equal amounts on two 15 cm dishes with MEFs in hESC medium with 10 μM Y27632 (day 0). Repeat the same protocol for negative control plate nucleofected with ddH2O and plate the whole content to one 10 cm dish coated with MEFs.
14. Change the media next day (day 1) and add again 10 μM Y27632.
15. On day 2 and 3 change the media.
16. On day 4, change the media and add hESC medium with 12.5 μg/ml G418.
17. On day 11, increase G418 concentration to 25 μg/ml.
18. Continue selection for approximately 3 weeks total or till all the colonies on the negative control plate die.
19. At that point, mechanically pick and expand individual colonies.

NOTE: All the MEFs used during the drug selection must be neomycin resistant if a neomycin selection cassette is used for BAC retrofitting. Alternatively, if a hygromycin cassette is used, DR4 multidrug-resistant MEFs should be used (These cells can be obtained from GlobalStem).

Neural Induction and Motor Neuron Differentiation

1. Select several colonies to start neural induction as serum free embryoid bodies (SFEBs) (Zhang et al. 2001)
2. On day 0, resuspend the colonies as a suspension culture in Petri dishes with KSR medium. Add 125 ng/ml noggin, 10 μM SB431542 and 200 nM LDN193189 to induce neural differentiation and 10 μM Y27632 to increase viability of the SFEBs.
3. On day 2, centrifuge SFEBs at 150*g* for 3 min, resuspend in fresh KSR medium and add 125 ng/ml noggin, 10 μM SB431542 and 200 nM LDN193189.
4. On day 5, centrifuge SFEBs at 150*g* for 3 min, resuspend in N2 medium and add 125 ng/ml noggin, 10 μM SB431542 and 200 nM LDN193189 (Neural Differentiation Medium – NDM).
5. On day 6, dilute sterile 0.01% poly-ornithine (po) at 1:3 ratio in sterile 1× PBS and coat 6-well tissue culture plates o/n.
6. On day 7, wash the plates with sterile 1× PBS three times and add sterile 10 μg/ml

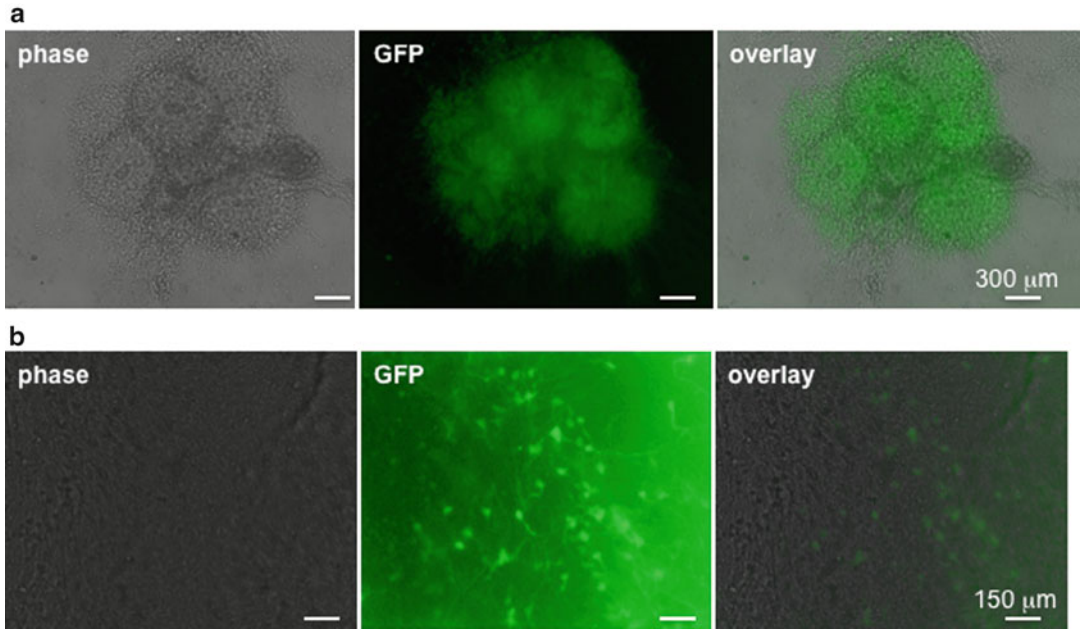

Fig. 11.3 (**a**) Neural rosettes differentiated from *Hes5::GFP* BAC transgenic hESCs. Live imaging of neural rosettes was performed under phase contrast and with fluorescence microscopy. (**b**) Live imaging of motor neurons differentiated from *HB9::GFP*-transgenic hESCs under phase contrast and with fluorescence microscopy

laminin. Wait at least 2 h either at RT or 37°C. Then aspirate the liquid before use.

7. Centrifuge SFEBs at 150*g* for 3 min and resuspend in NDM. Plate on po/laminin-coated plates.
8. Keep the cells in NDM for 7 days by changing the medium every 2 days.
9. If you are using the *Hes5::GFP*-BAC transgenic line for this experiment, check for the expression of the *Hes5::GFP* transgene in rosette NSCs (Placantonakis et al. 2009) (Fig. 11.3a).
10. On day 13, dilute sterile 0.01% poly-ornithine (po) at 1:3 ratio in sterile 1×PBS and coat 6-well tissue culture plates o/n.
11. On day 14, wash the plates with sterile 1×PBS three times and add sterile 50 μg/ml laminin. Wait at least 2 h either at RT or 37°C. Then aspirate the liquid before use.
12. On day 14, mechanically select the rosettes under the dissecting microscope with the help of a P1000 pipette tip.
13. Pool all the rosettes in a falcon tube and centrifuge at 150*g* for 3 min.
14. Resuspend the rosettes in Motor Neuron (MN) medium and add 1 μM RA, 50 ng/ml Shh, 20 ng/ml BDNF and 200 μM ascorbic acid (MN Differentiation Medium – MNDM).
15. Plate on po/laminin-coated plates.
16. Keep the cells in MNDM for 14 days, changing the medium every 2 days. If you are using the *HB9::GFP* BAC-transgenic hESC line, check for the expression of the *GFP* transgene in MNs starting from day 11 of MN differentiation (Placantonakis et al. 2009) (Fig. 11.3b).

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Moderate Low Temperature Preserves the Stemness of Neural Stem Cells (Methods)

12

Kosuke Saito, Noboru Fukuda, and Nariyuki Hayashi

Contents

Abstract

Moderate hypothermia has been known to protect the brain from inflammation and free radicals after brain injury. However, the cellular mechanisms underlying the amelioration of brain injury by moderate therapeutic hypothermia have not been established. It is considered that neural stem cells reside in the subventricular zone (SVZ) and subgranular zone (SGZ) of the adult mammalian brain and act to regenerate damaged brain tissues. We investigated the effects of moderate low temperature and the contribution of a cold-inducible molecule towards the stemness of neural stem cells. The MEB5 mouse neural stem cell line was cultured in the presence or absence of EGF, and apoptosis, mRNA expression, and immunocytochemistry of the differentiation markers, nestin and GFAP, were evaluated at 37 or 32 °C. We investigated the contribution of the cold-inducible RNA binding protein (CIRP) on apoptosis and differentiation of MEB5 cells at 32 °C. EGF deprivation increased the number of apoptotic cells, decreased expression of nestin, and increased expression of GFAP. Moderate low temperature prevented apoptosis and decreases in expression of GFAP in MEB5 associated with EGF deprivation. The moderate low temperature significantly increased expression of CIRP. siRNA against CIRP significantly increased the apoptotic cell population of MEB5 cells via EGF deprivation at 32 °C. These findings suggest that moderate

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low temperature preserved the stemness of neural stem cells and prevented cell apoptosis via the stimulation of CIRP. One of the mechanisms of rescue from brain injury via moderate hypothermia is associated with the preservation of neural stem cells.

Introduction

General cellular functions including gene transcription and translation in eukaryotic and prokaryotic cells are regulated around 37 °C. If the temperature environment is below or over 37 °C, maintenance of survival activity and intracellular metabolism will be difficult. Over the course of evolution, life forms have undergone cellular adaptations to adjust to such changes in environmental temperature.

Recently, the stabilization of metabolic reactions and protection of neurons with low temperatures via brain hypothermia therapy have been applied to treat patients with serious brain injury and trauma. Although a practical low temperature therapeutic strategy has been established for severe brain injury, the detail of the mechanisms that enable such protection have not been clarified. Thus, it is important to accumulate basic data on the effects of low temperature on cells and tissues *in vivo* and *in vitro*.

In this chapter we will describe new perspectives on the protective effects of low temperature treatments on neurogenesis.

Stem Cells in Adult Tissue

Stem cells are critical for organogenesis during the fetal stage of development. Stem cells are defined as cells having self-renewing capabilities and the capacity to differentiate into multiple cell lineages (Thomson et al. 1998; Weissman 2000a, b). Therefore, stem cells hold great promise for tissue repair and regenerative medicine. Adult stem cells are tissue-specific cells in the postnatal organism showing a characteristic pattern of gene expression that enhances resistance to stress through increased expression of DNA repair and detoxification system genes (Ivanova et al. 2002). Previously, stem cells had not been thought to be present in postmitotic organs such as heart and kidney. However, in recent times the presence of cardiac stem cells in heart and renal stem cell in kidney has been reported (Beltrami et al. 2003; Torella et al. 2004). The study of stem cells is an exciting field that holds great promise for the development of regenerative medicines.

Stem cells have a self-renewal capacity as well as the capacity to differentiate into single or multiple lineages. The self-renewality and differentiation of stem cells are regulated by niche cells and various signals. Hematopoietic stem cells survive and grow, when provided with the trophic factors to maintain their viability, while facilitating an appropriate balanced output of mature progeny for the lifetime of an organism (Baksh et al. 2004). Understanding the relationships of stem cells to their niches should lead to development of new strategies directed toward regeneration.

The increased resistance against stress could exemplify a rather general stem cell feature beyond the protection against apoptosis. Transcriptional profiling of mouse embryonic as well as adult neural and hematopoietic stem cells revealed a characteristic pattern of stemness genes, the expression of which is typical for these cells and distinguishes them from mature cells (Ivanova et al. 2002). Series of stress response genes involved in the redox balance were identified to be a characteristic feature of stem cells, proposing that one essential attribute of the stemness includes the high resistance to stress (Ramalho-Santos et al. 2002). Observations in neural progenitor cells have shown that factors promoting self-renewal cause these cells to enter a more reduced redox state, whereas exposure to signaling molecules that promote differentiation leads to an excess of oxidation in these progenitors (Noble et al. 2003).

Adult Neural Stem Cells

Neural stem cells localized in the mammalian adult brain have a role in neurogenesis, and generate novel neural and glial cells continuously. The neurogenesis that occurs in the mammalian adult brain is produced by neural stem cells in the subventricular zone (SVZ) and subgranular zone (SGZ). For example, neural stem cells in the SVZ and SGZ of the rodent adult brain have been shown to undergo neurogenesis (Zhang et al. 2008). The generated neural cells from neural stem cells present in the SVZ transfer to the olfactory bulb during division via the rostral migratory stream. Finally, they differentiate into granular cells or juxtaglomerular cells. In the SGZ the neural cells generated from neural stem cells move immediately to the granular layer of the cortex, and are involved in the development of the functional neural network. During nerve degeneration in ischemic brain tissue, neural stem cells in the SVZ and SGZ proliferate, differentiate and migrate to the injured tissue site to differentiate into the astrocytes that facie the ventricle. The SVZ and SGZ act a microenvironment niches for the neural stem cells (Alvarez-Buylla and Lim 2004). The maintenance of neural stem cells by sending them into hibernation is regulated by unknown intracerebroventricular signal molecules that promote regeneration of nerve cells lost by brain damage.

Moderate and Mild Hypothermia

Alterations in temperature is a common environmental factor experienced by biological tissues in almost all species from bacteria to plants and mammals, which have been focused to adapt surgical management and to keep the cultured cells and organs in the medical field. In particular, low temperatures have been employed in clinical treatment as an antipyretic to reduce inflammatory responses. At first, hypothermia as a surgical management was exploited to protect the brain during cardiac surgery. However, therapeutic hypothermia below 30 °C after brain injury and cardiopulmonary resuscitation cannot exert beneficial curative effects since the strong low temperatures used (25–30 °C) can themselves induce damage to tissues and increase complications (Clifton 1995).

It was first reported that mild hypothermia (34–35 °C) efficiently protects brain from ischemia and damage in vivo, where a sufficient protection effect has been achieved in injured brain tissue without the need for cooling below 30 °C (Busto et al. 1987). Thus moderate hypothermia (32–33 °C) has been reported to enable efficient neurological recovery from severe brain injury and a shorter recovery period from injury than in patients with normothermia (Marion et al. 1997). In practice, a mild and moderate hypothermia achieved by keeping cerebral blood temperature at 32–34 °C using a water cooling blanket after the administration of a general anesthetic and muscle relaxant drugs to patients with severe brain injury or ischemic insult (Hayashi 2009). Mechanisms of amelioration of brain injury using brain hypothermia have been reported to prevent metabolic responses in brain tissue through baneful stress, which leads to abnormalities in the metabolism of neurotransmitters, depletion of energy metabolism, increase in reactive oxygen species (ROS), and neuronal cell death (Xu et al. 2002; Tasdemiroglu 1996). It has been considered that mild and moderate hypothermia protects neuronal cells by generation of specific proteins that suppress the metabolic responses of ischemic neuronal tissue. Therefore, it is important to clarify the mechanisms of this process to enable further improvement of brain hypothermia therapy.

Moderate Low Temperature Induces Protection and Suppresses Differentiation of Neural Stem Cells

Materials and Methods

To investigate the protective effects of moderate hypothermia on the injured brain we examined neurogenesis and activation of neural stem cells. Experiments using cultured neural stem cells are one of the most useful methods to analyze the

self-renewal and multipotency by which cells can differentiate to neuronal and glial cells. We investigated the effects of moderate low temperature on proliferation and self-renewal in neural stem cells.

We employed MEB5 cells, which are a multipotent neural stem cell line derived from mouse embryonic forebrain (Nakagaito et al. 1998) (Health Science Research Resources Bank, Osaka, Japan). The cells were cultured routinely at 37 °C in a 95% air/5% CO_2 humidified atmosphere using a CO_2 multi gas incubator. To achieve EGF deprivation, EGF was omitted from the medium. Cells with a floating neurosphere epithelium-like morphology were used for all assays and were dissociated and plated in MEB5 culture medium on 24-well plates or 10-cm dishes coated with poly-L-lysine, laminin, and fibronectin. For moderate low temperature culture conditions, cells cultured at 37 °C were transferred to 32 and 28 °C in MEB5 culture medium in a 95% air/5% CO2 humidified atmosphere using a CO_2 multi gas incubator.

Results and Discussion

In comparison to the 37 °C culture, the number of neurospheres formed by MEB5 cells cultured under 32 °C conditions was decreased by 59%, although the growth and form of typical neurospheres was preserved. However, at 28 °C, neurospheres were not formed (Fig. 12.1). In addition, moderate low temperature prevented apoptosis of MEB5 cells cultured under EGF deprivation (Fig. 12.2).

Acute brain damage following cerebral ischemia promotes an increase in the neural stem cells that are inherent in the adult brain, and this process is accompanied by exogenous and endogenous modulators, such as growth factors, that stimulate neurogenesis (Dietrich and Kempermann 2006). The aim of hypothermia is to avoid a rapid fall in temperature and to protect cells in the body by causing a gradual suppression of the effects of harmful metabolic reactions from low temperature. Additionally, hypothermia induces cell protection systems that are not associated with the inhibition of metabolic the reaction pathway triggered by excessive cold. Busto et al. (1987) reported in a rat model of forebrain ischemia that lowering the temperature in the brain by only 2 °C during ischemia dramatically reduced ischemic neuronal cell death in the hippocampus CA1 region, and immediately after ischemia, energy metabolism in the brain cells was not affected by the temperature change. In addition, in a rat focal cerebral ischemia model, hypothermia suppressed activity of δPKC, which induces apoptosis in one of the subtypes of protein kinase C (PKC), and maintained εPKC in one of the subtypes of PKC activity with its anti-apoptotic effect (Shimohata et al. 2007a, b). Zhao et al. (2005) reported that hypothermia reduced neural cell apoptosis via the phosphoinositide 3-kinase /Akt pathway, phosphorylating substrates that have apoptotic effects such as forkhead transcription factor and glycogen synthase kinase 3. Thus it is possible that mild hypothermia ameliorates the injured brain by activation of neural stem cells, accompanied by increases in cell protection molecules.

Cold chock proteins (CSPs) are factors whose expression is significantly altered in the cell at low temperature (Ermolenko and Makhatadze 2002). The main functions of CSPs are to protect and stabilize important processes that determine the fate of the replicating cell, such as transcription and translation of DNA and association with the ribosome (Fujita 1999). Cold-inducible RNA binding protein (CIRP) was the first mammalian CSP to be identified, and it binds to target RNA (Fujita 1999). CIRP has been shown to bind to heterogeneous nuclear ribonucleoprotein (hnRNP), and the reported CIRP proteins are synthesized cells exposed to low oxygen (Wellmann et al. 2004) and UV irradiation (Sheikh et al. 1997), in addition to moderate low temperature. Xue et al. (1999) reported the expression of CIRP in a transient ischemic rat brain model, with of CIRP mRNA level being decreased in the hippocampus of ischemic rats after reperfusion. Sakurai et al. (2006) reported that CIRP prevents apoptosis by induction of tumor necrosis factor alpha (TNFα) through the activation of an extracellular signal-regulated kinase (ERK) known to regulate the proliferative capacity of cells. Artero-Castro et al. (2009) recently demonstrated that CIRP is involved in the activation of ERK 1/2 signaling pathways,

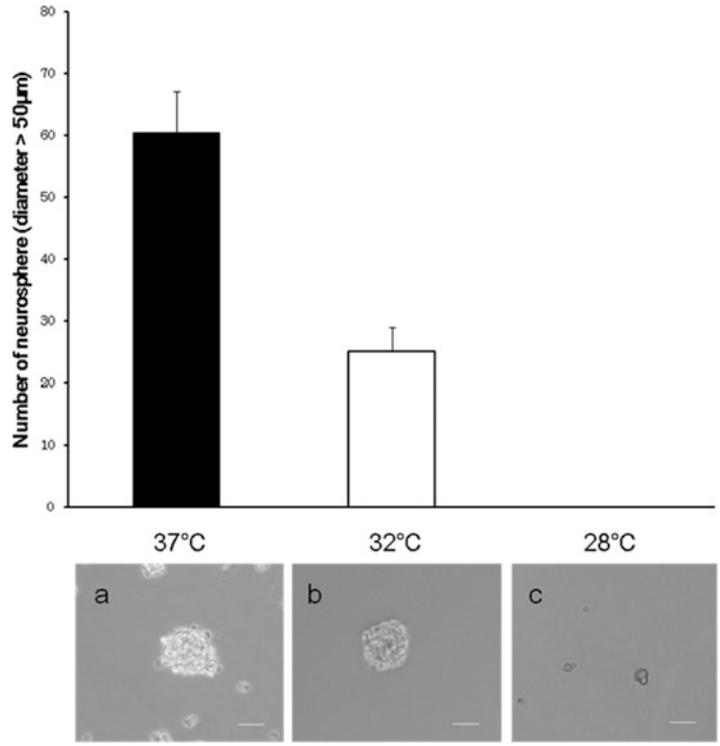

Fig. 12.1 Effects of different temperatures on the neurosphere formation of MEB5 cells. MEB5 cells were cultured at 37 °C (**a**), 32 °C (**b**), and 28 °C (**c**). Data are mean ± SEM (n = 3). *Scale bars* represent 50 μm

which is related to the capacity for self-renewal of embryonic stem cells. Schmitt et al. (2007) suggested that moderate low temperature effects suppress the inflammatory response in microglia, which play an important role in immune responses in the central nervous system via the ERK signaling pathway. More recently, we demonstrated that CIRP is a pivotal factor to protect neural stem cells from apoptosis *in vitro* (Saito et al. 2010). Thus, it has been suggested that moderate hypothermia has a protective effect in neural stem cells via CIRP. Neurogenesis is associated with the proliferation of neural stem cells in the SVZ and SGZ regions of the adult brain (Li et al. 2010). Protection for stemness, and the survival and growth of endogenous neural stem cells are involved in the maintenance of neurogenesis in the injured brain.

In our study, MEB5 cells in the absence of EGF showed increased gene and protein expression of GFAP, and decreased expression of Nestin (Fig. 12.3). The absence of EGF is known to suppresses the ability of neural stem cells to differentiate into glial cells. However, when stem cells were cultured under EGF depletion and the moderate low temperature of 32 °C, expression of nestin was maintained and expression of GFAP was suppressed when compared to culture at 37 °C. It has been reported that regeneration of neuron and glial cells from neural progenitor cells in GFAP knockout mice was enhanced compared to wild type mice (Widestrand et al. 2007). We observed the moderate low temperature prevented apoptosis and decreases in expression of GFAP in MEB5 cells under EGF deprivation. These findings suggest that moderate low temperature preserved stemness of neural stem cells and prevented cell apoptosis via the stimulation of CIRP, and one of the mechanisms of rescue from brain injury by moderate hypothermia is associated with preservation of neural stem cells.

One way to preserve the function of brain tissue is to remove the immediate consequences of brain injury such as ischemia and metabolic abnormalities. Currently, a combination of regenerative medicine techniques has been considered as an attractive treatment for brain injury. It has been demonstrated that even in the mammalian adult brain, neurons are generated sustainably by activation of neural stem cells in the

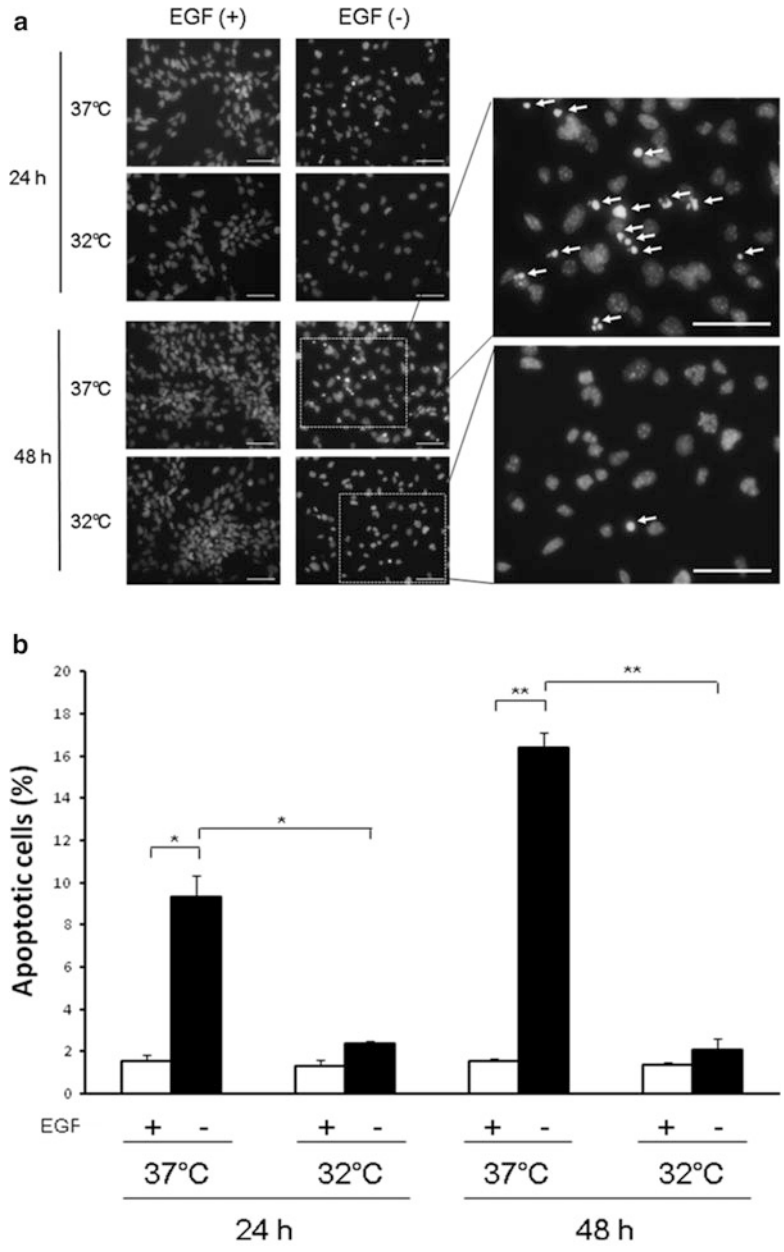

Fig. 12.2 Effects of different temperatures on proliferation and apoptosis of MEB5 cells. (**a**) The apoptotic cell (*arrows*) population was evaluated by staining with Hoechst 33342. (**b**) MEB5 cells were incubated for 24 and 48 h in the absence or presence of EGF at 37 ° C or 32 °C. Data are mean ± SEM (n = 3). *P < 0.05, **P < 0.01, between the indicated bars. *Scale bars* represent 50 μm

SVZ and SGZ areas (Doetsch et al. 1999; Alvarez-Buylla and Lim 2004). Moreover, such activated neurogenesis has been reported after a variety of stresses such as ischemia (Nakatomi et al. 2002). However, it is difficult to repair injury sites via neurogenesis with endogenous neural stem cells alone. To overcome such problems, it has been considered that the process of differentiation and migration due to proliferation of neural stem cells could be promoted with hematopoietic cytokines and neurotrophic factors.

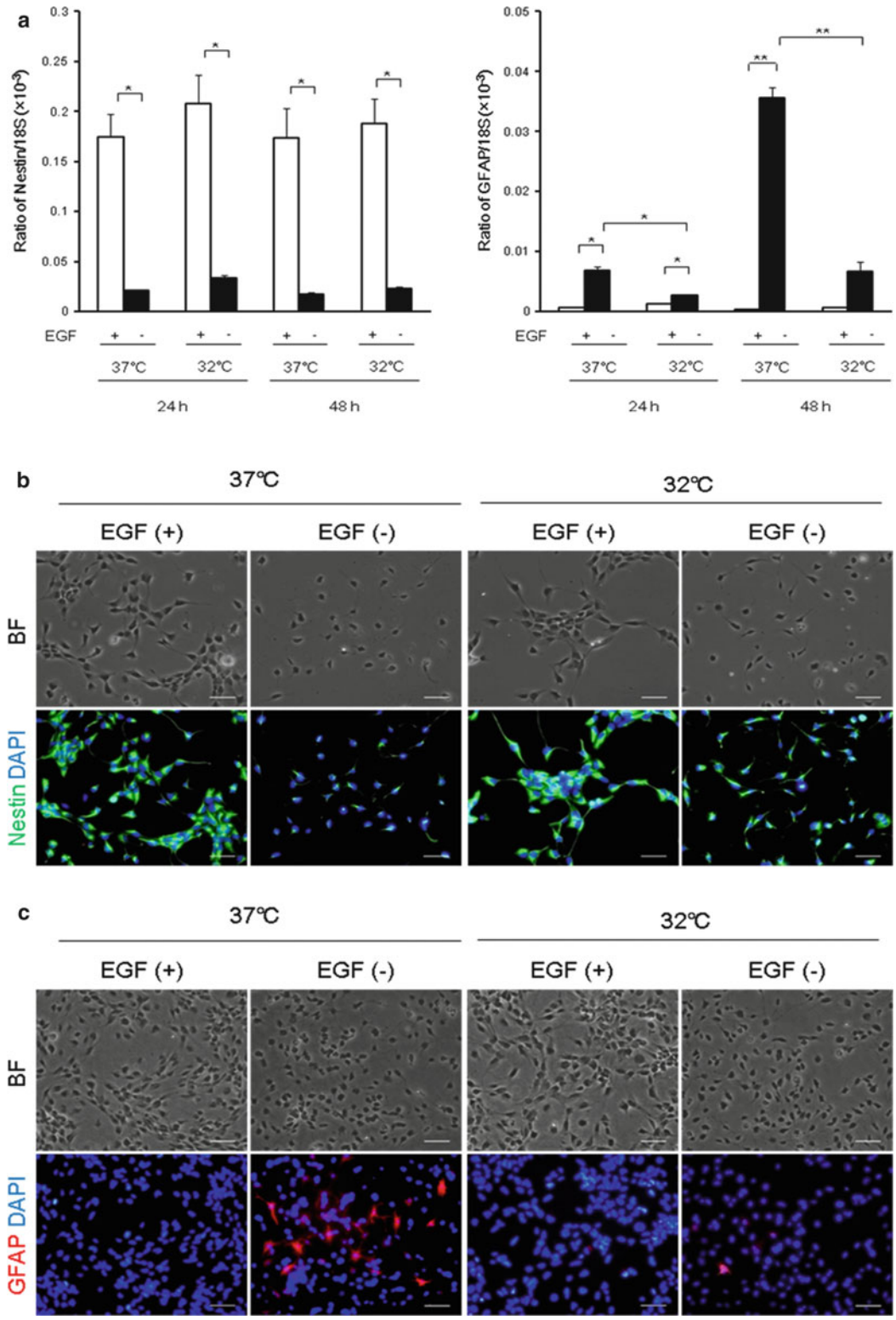

Fig. 12.3 Effects of moderate low temperature on differentiation of MEB5 cells under EGF deprivation. Expression of nestin and GFAP mRNAs in cultured MEB5 cells at 32 or 37 °C in the presence or absence of EGF for 24 or 48 h (**a**). The abundance of all mRNAs was determined by real-time PCR. Relative gene expression was analyzed by the comparative Ct method with 18S. Immunocytochemistry for nestin (**b**) and GFAP (**c**) in MEB5 cells at 32 or 37 °C for 48 h. Cells were stained with nestin and GFAP antibodies. Nuclei were stained with DAPI. BF: bright field. Merged views of fluorescence images are shown. Data are mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$, between indicated bars. *Scale bars* represent 50 μm

Some patients with severe brain damage cannot be rescued with brain hypothermia therapy, which has been considered to be associated with a management for brain injury treatments including the contents and procedures. In particular, brain hypothermia therapy has disadvantages associated with the invasive brain temperature. To protect endogenous neural stem cells from injury, moderate and mild hypothermia may induce efficient neurogenesis, it may be one of the findings of fill the part of the unknown mechanism of brain hypothermia.

In conclusion, findings from our study indicate that moderate low temperature preserves the stemness of neural stem cells, and suppresses apoptosis via CIRP activation. These findings suggest that one of the mechanisms of rescue from brain injury by moderate hypothermia is associated with preservation of neural stem cells.

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Part IV

Role of Stem Cells in Disease

13 High-Dose Chemotherapy with Autologous Stem Cell Support in the Treatment of Transformed B-Cell Non-Hodgkin's Lymphomas

Marianne Brodtkorb Eide and Harald Holte

Contents

Abstract

Malignant lymphomas are a heterogeneous group of lymphoid neoplasias of which B-cell lymphomas constitute approximately 75%. The most frequent types are follicular lymphomas and diffuse large B-cell lymphomas, both originating from lymphoid germinal centers. While follicular lymphomas most often have an indolent clinical course, diffuse large B-cell lymphomas are more aggressive and require immediate therapy. Follicular lymphomas and other indolent B-cell lymphomas transform to diffuse large – and other aggressive B-cell lymphomas at a yearly rate of 3%. B-cell lymphomas are most often treated with combined immuno-chemotherapy: anti-CD20 antibody therapy combined with cytostatic drugs. The majority of patients respond to therapy with long-lasting remissions. While the follicular lymphomas tend to relapse, diffuse large B-cell lymphomas are often cured.

Patients with histological transformation, especially in a relapsed setting, have a much more dismal prognosis with a median overall survival from transformation of 7 months to 1.7 years. As there is a dose-response relationship in curative lymphoma treatment, high dose therapy (HDT) with autologous stem cell support has been investigated for these lymphomas during the last 20 years. No prospective randomized phase III studies have been

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performed, but a number of phase II studies give support for the use of HDT in this setting. In this chapter, the principles of HDT and its effect in malignant lymphomas in general are shortly reviewed while the documentation of effect and morbidity in transformed B-cell lymphomas are described in some more detail.

Introduction

Malignant Lymphomas

Malignant lymphoma is a heterogeneous group of lymphoid neoplasias, constituting approximately 4% of all malignancies. The two main entities are the non-Hodgkin lymphomas (NHL, 85% of the cases), and Hodgkin lymphomas. NHL is further subdivided into B- (85%) and T/NK cell lymphomas (15%), based on the cell of origin of the malignant lymphoid cells. The various entities are since the first edition of the "WHO classification of tumors of Haematopoietic and Lymphoid Tissues" in 1999 classified according to histopathological morphology, immunophenotype, molecular characteristics and clinical information. Now more than 60 entities exist, the most prevalent being the diffuse large B-cell lymphomas (more than 30% of all NHL) and the follicular lymphomas (20–25%). The clinical course varies tremendously, from cases which do not need treatment for years to those with a fatal course within days. Even within the same entities with similar clinical risk profile and given the same treatment, the course may be highly different between patients. Most patients with disseminated malignant lymphomas are treated with chemotherapy; for B-cell lymphomas in combination with immunotherapy using an anti-CD20 antibody. For localized disease, the treatment of choice for indolent NHL types, i.e. follicular lymphomas (FL) is radiotherapy while radiotherapy most often is preceded by (immuno)chemotherapy in aggressive lymphomas. High-dose chemotherapy followed by auotologous stem cell support (HDT) is used in various settings in several of the NHL-entities. The focus of this review is the usage of HDT in transformed NHL (t-NHL) and mainly in transformed Follicular Lymphoma (t-FL).

Follicular Lymphoma, Transformed B-Cell Lymphomas and Diffuse Large B-Cell Lymphomas; Histopathological Features, Grading and Molecular Characteristics

In follicular lymphoma (FL) the malignant B-cells are organized in a follicular growth pattern resembling the secondary follicle of a reactive germinal centre FL cells are positive for B-cell associated antigens such as CD19, CD20, CD22 and CD79a as well as for germinal centre B-cell markers such as CD10 and Bcl-6. CD5 and CD43 immunostaining is negative, as opposed to chronic lymphatic leukaemia. In contrast to B-cells of a reactive secondary follicle, FL cells are positive for Bcl-2 in most cases (Swerdlow et al. 2008). There is a close relationship between FL-cells and the meshwork formed by the follicular dendritic cells as visualized by CD23 and CD21 immunostaining. In addition, the neoplastic follicles contain follicular T helper cells positive for CD4 and CD57. The inter-follicular areas are composed of variable elements of T-cells, macrophages, micro vessels and fibroblasts.

FLs are graded from 1 to 3a or 3b according to the content of centroblasts relative to centrocytes as determined by counting of 10 high power fields (40× objective and 18 mm field of view). Although this method shows poor reproducibility, it has clinical relevance as the higher grades of FL shows a more aggressive clinical behaviour with increased risk of progression to diffuse large B-cell lymphoma (DLBCL). FL grade 1–2 are suggested to be grouped together in the latest revision of The WHO classification and is dominated by centrocytes, containing up to 15 centroblasts/hpf. FL grade 3 is subdivided into 3A and 3B, the former containing >15 centroblasts/hpf and the latter in addition showing solid sheets of centroblasts. Grade 3B shows similarities to DLBCL with less frequent BCL2

translocation and more frequent chromosomal breaks at 3q27 and at 8q24, rearranging BCL6 and MYC respectively.

The hallmark of FL is the t(14;18)(q32;q21) detected in 90%. The translocation confers over expression of Bcl-2 protein as the BCL-2 gene expression is regulated by the Ig heavy chain enhancer. In a minority of cases, the BCL-2 gene becomes regulated by the light chain κ or λ light chain enhancer by the variant t(2;18) and t(18;22) translocations. Recurrent secondary genomic aberrations in FL are deletions of 1p and 6q, gains of 1q, 7, 12, 18, X occur in 20–30% of cases (Cheung et al. 2008; Eide et al. 2010). Target genes have not been identified.

Upon transformation the follicular growth pattern is lost and larger, blast-like cells growing in diffuse areas take over, commonly showing the histological features of DLBCL. Areas of FL may be retained adjacent to the diffuse areas in a composite lymphoma. Transformation to an aggressive and often treatment resistant phenotype occurs in various proportions of patients in several of the mature B-cell neoplasms and is generally followed by short survival. In FL, transformation has been reported to occur in 10–70% of patients, depending on the diagnostic method (histological examination, clinical criteria or detected at autopsy), the observation time and the frequency of biopsies taken during the course of disease (Montoto et al. 2007; Al-Tourah et al. 2008; Conconi et al. 2012). The probability of transformation seems to be constant at least during the first 5–10 years of observation, and after 10 years of observation approximately 30% of patients have experienced transformation. Whether or not the transformation incidence declines in patients remaining at risk thereafter is not clear and the existing data are conflicting (Montoto et al. 2007; Al-Tourah et al. 2008). Neither the timing of treatment onset (immediately following diagnosis of FL versus "watch and wait"), nor the intensity of the primary treatment regimen seem to influence the incidence of transformation (Al-Tourah et al. 2008; Conconi et al. 2012).

The diagnosis of transformation is confirmed by a biopsy showing DLBCL, or more rarely a Burkitt-like lymphoma harboring both the t(14;18) and a rearrangement of the MYC oncogene. Higher grade transformation of CLL to DLBCL occurs in 5–10% of cases (termed Richter's transformation); marginal zone lymphomas (MALT) rarely transform. Until recently, and before the WHO 2008 classification discriminated between follicular lymphoma grade 3A and – grade 3B, grade 3 was considered more aggressive than grade 1 and 2, and was treated like DLBCL at many centers. Thus, follicular lymphomas with increasing grade from 1 and 2 to grade 3 were by many considered as a transformation. Today, the important distinction is between grade 3A and grade 3B as 3A has the better prognosis, even with rituximab monotherapy (Wahlin et al. 2012). Obtaining a representative biopsy is sometimes difficult as the site of transformation may be surgically unavailable or clinically and radiologically occult. The appearance of a least one of the following clinical characteristics in a patient with a history of FL, is associated with the same outcome as for patients with histologically verified transformation; rapid, discordant tumor growth, involvement of unusual extranodal site, new B symptoms, hypercalcemia or sudden rise in LDH-level (Al-Tourah et al. 2008). The diagnosis of transformed lymphoma should in such cases be based on these clinical findings and treatment planned accordingly.

Diffuse Large B-cell Lymphomas (DLBCL) is morphologically and clinically a more heterogeneous disease than FL, and there are certain subgroups of DLBCL with distinct phenotypic features and different outcome (i.e T-cell/histiocyte-rich large B-cell lymphoma, primary diffuse large B-cell lymphoma of the CNS, primary cutaneous DLBCL leg type, EBV positive diffuse large B-cell lymphoma of the elderly). Histologically DLBCL is characterized by large lymphoid cells completely effacing the architecture of the lymph node. Three main morphological variants can be distinguished; the centroblastic variant, the immunoblastic variant and the anaplastic variant. There is no clear correlation between different morphological subtypes and patient outcome (Swerdlow et al. 2008).

In recent years, gene expression studies defined three molecularly distinct subgroups which are largely indistinguishable at a morphological level, but show different outcome with conventional treatment; The Germinal Centre B-cell (GCB), Activated B- Cell (ABC) and Primary Mediastinal B- Cell (PMBC) Lymphoma subtypes. The majority of GCB can be cured by R-CHOP-like regimens, while 50% of ABCs die of lymphoma (Lenz et al. 2008). GCB and ABC show gene expression patterns resembling germinal centre B-cells and B-cells on the way to plasma cells, respectively. In GC subtype there is over expression of Bcl-6 and translocations or mutations of the BCL6 gene are seen more frequently in the GC subtype. Forty-five percent of GC subtype lymphomas harbour t(14;18) and over express of Bcl-2. Thirty percent have inactivation of PTEN by different mechanisms leading to activation of PI3K/AKT signalling. In the ABC subtype the NF-kappa B pathway is constitutively activated by various somatic mutations. The ABC subtype lymphomas express plasma cell differentiation markers like Blimp-1. However, inactivation of PRDM1 blocks the terminal differentiation into plasma cells. The PMBLs share similarities with HL nodular sclerosis both in terms of clinical characteristics (young female patients, mediastinal mass) and molecular features (overlapping gene expression profiles, similar genomic aberrations such as amplification of 9p24 associated with JAK2 over-expression and activation of NF-kB pathway).

Clinical Features and Treatment

The staging of lymphomas is based on clinical examination, lymphoma tissue- and bone marrow biopsy and CT-scan including neck, thorax, abdomen and pelvis. Malignant lymphomas are staged according to the modified Ann Arbor classification (Table 13.1).

Most patients with follicular lymphoma present with moderately enlarged peripheral nodes, although some present with symptoms from enlarged, mostly infra-diaphragmatic, nodes which may threaten normal organ function (hydronephrosis, intestinal obstruction, compression of vena cava inferior, cholestasis). Primary mediastinal or spleen involvement is rare. General performance status is commonly preserved, and only 20% of patients have B-symptoms. Bone marrow involvement is common (50–60%). A large multicentre retrospective analysis identified five risk factors (see below). A minority of patients present with primary extra nodal involvement like in the gastrointestinal tract, salivary or thyroid gland, skin etc. The Follicular Lymphoma Prognostic Index (FLIPI) is based on a set of clinical criteria, including stage (Solal-Celigny et al. 2004):

1. Age $\geq$60 years
2. Ann Arbor stage III–IV
3. Hemoglobin level $<$12 g/L
4. Involved lymph node regions $>$4
5. Serum LDH $\geq$ upper reference value

Based on these criteria, patients can be allocated to low (0–1 criterion), intermediate (2 criteria) and high risk (3–5 criteria) groups with significantly different survival. There is considerable overlap between the clinical factors associated with inferior survival of FL and factors associated with transformation risk, reflecting the poor treatment response and short survival time following transformation.

Treatment strategies in first line and relapse of FL have been comprehensively reviewed by others (Hiddemann et al. 2007; van Oers and Kersten 2011). Patients with a low FLIPI-score who are in a good condition with diminutive symptoms can be observed untreated ("watch and wait"). Eventually, disease progression with declining general condition, uncomfortable lymph node enlargement, signs of bone marrow failure, B-symptoms or organ complications enforces treatment. In younger patients and patients presenting with bulky tumour or intermediate to high FLIPI-score at diagnosis, treatment is initiated early.

Standard first line therapy for most patients consists of a combination of immunotherapy with the anti-CD20 antibody rituximab and chemotherapy: cyclophosphamide, vincristine, prednisolone

Table 13.1 Modified Ann Arbor classification

Stage	Description	
I	Involvement of one lymph node region (spleen, thymus, Waldeyer's ring is considered as separate regions)	
II	Involvement of two lymph node regions on the same side of the diaphragm	
II_1	Involvement of two lymph node regions which can be included in the same radiation field	
III	Involvement of lymph node regions on both sides of the diaphragm	
IV	Involvement of the bone marrow (40% of patients)	
E	Extra nodal tumour	
X	Bulky disease; tumour $\geq$ 10 cm in diameter	
A/B	A = No general symptoms	
	B = One or more general symptoms (B-symptoms)	Fever $\geq$ 38 °C (repeating or persisting during the last month)
		Weight loss $\geq$10% (during the last 6 months)
		Night sweats repeatedly during the last month

(CVP) or cyclophosphamide, doxorubicine, vincristine, prednisolone (CHOP). An alternative for patients who wish to postpone chemotherapy is immune- monotherapy. Maintenance rituximab treatment has been shown to confer an improved progression free survival. Chlorambucile, which also may be combined with immunotherapy, is an alternative for elderly patients, but may be detrimental for the bone marrow function and increase the risk for inadequate bone marrow harvest yield and secondary myeloid neoplasia for patients eligible for autologous bone marrow transplantation at a later stage. Treatment response is usually very good and in the majority of patients CR or a good PR is achieved after three to six courses (3–6 months of treatment). At relapse of follicular lymphoma, different treatment options can be chosen depending on the patient's general condition and the severity of symptoms. If a good and long-lasting response to rituximab (R) or R-CVP was obtained in first line, the same or the alternative regimen may be chosen. Maintenance rituximab immunotherapy is recommended if not given up front. For patients relapsing after a shorter period or who do not respond, high dose therapy with autologous stem cell support (HDT) may be the treatment of choice. At relapse with transformation to higher grade lymphoma the patient's general condition may be poor and call for prompt treatment initiation. Various chemotherapy regimens in combination with rituximab can be used to induce remission depending on the patient's age and previous treatment. Patients with chemosensitive disease should be considered for HDT (Bernstein and Burack 2009).

Patients with DLBCL experience a more rapid clinical deterioration and 40% have B-symptoms at diagnosis. Primary extra nodal manifestation is seen in 40% of the cases, the most frequent being the gastrointestinal tract. Bone marrow infiltration is seen in only 10% of the cases and may show small-cell monoclonal B-cell infiltrates, suggestive for a transformation in the diagnostic biopsy. Standard treatment today consists of CHOP-like chemotherapy plus rituximab, either with two (with G-CSF support) or 3 weeks interval. The majority of patients (>80%) achieve a complete remission, but some experience a relapse so that approximately 60% of the patients will be cured. For DLBCL, a clinical risk score was published in 1993 with the following factors being of equal importance:

> All patient age groups: stage III–IV, elevated LDH, WHO performance status $\geq$ 2, age > 60 years, >1 extranodal site.
>
> Patients less than 60 years: Stage III–IV, elevated LDH, WHO performance status $\geq$2.

Relapsing patients with DLBCL histology aged less than 65 years will normally be eligible for HDT if they are still chemosensitive (Philip et al. 1995). If rituximab has been part of first line therapy, only 50% will respond while more than 80% will respond if patients are rituximab naïve at relapse. Accordingly, 3 year event free survival after relapse for patients less than 65 years

have decreased from 47 to 21% (Gisselbrecht et al. 2010).

Traditionally, patients with transformed non-Hodgkin's lymphoma (t-NHL) have received salvage treatment with combination chemotherapy regimens like CHOP, alternatively dexamethasone, cytarabine and cisplatin (DHAP), ifosfamide, carboplatin and etoposide (ICE) or etoposide, ifosfamide and methotrexate (VIM) in cases where doxorubicin has to be avoided (elderly, patients with heart disease or patients that already received doxorubicin-containing regimens for their FL). Despite CR close to 40%, the median overall survival obtained with this conventional chemotherapy was as low as 7 months to 1.7 years (Yuen et al. 1995; Al-Tourah et al. 2008). However, a subgroup of patients in CR after salvage treatment obtained long-term survival (Yuen et al. 1995). Studies prior to 1999 had shown longer survival in patients with localized transformation, no previous chemotherapy, no B symptoms, a normal LDH-level as well as in patients attaining CR after salvage chemotherapy (Yuen et al. 1995). Post-transformation survival was not dependent on the interval from primary diagnosis to transformation (Yuen et al. 1995).

High Dose Therapy (HDT) with Autologous Stem Cell Support

In high dose therapy with autologous stem cell support (HDT) chemotherapy and/or total body radiation (TBI) is administrated in doses exceeding the tolerance of the haematopoietic stem cells with the aim to completely eradicate the neoplastic lymphoid cells. Chemotherapeutic agents with suppression of bone marrow function as the dose limiting toxicity are chosen. In advance, the patient's own haematopoietic stem cells are harvested either from bone marrow or from peripheral blood. The autologous stem cells are re-infused intravenously following HDT to re-populate the bone marrow and restore haematopoiesis. Only patients with disease response to immuno-chemotherapy tested in induction or salvage regimens will benefit from HDT. Thus, patients achieving at least a PR following two to three courses of chemotherapy are deemed eligible for HDT.

Bone marrow harvest was previously performed by repetitive aspirations from the posterior and anterior iliac crests, but is today largely abandoned as haematopoietic stem cells can be efficiently harvested by aphaeresis of peripheral blood. The time to restoration of bone marrow function after HDT is approximately 1 week shorter following reinfusion of stem cells harvested by aphaeresis of peripheral blood. Haematopoietic CD34+ stem cells are efficiently mobilized from the bone marrow to peripheral blood by combining chemotherapy and G-CSF. Doses of cyclophosphamide in the range of 3–5 g or 2–3 g of cytarabine are recognized as agents with a good ability to mobilize stem cells. In our experience, the combination of mitoguazon, ifosfamid, MTX, etoposide (MIME) and G-CSF is efficient in mobilizing CD34+ cells in pre-treated lymphoma patients. When the blood cell levels again increase following chemotherapy induced aplasia, the fraction and number of CD34+ stem cells in peripheral blood are increased 20–100-fold, and a sufficient number of stem cells may be collected by the use of aphaeresis one to 2 days in a row. Normally, harvest is performed when the CD34+ count in blood is above 1.0×10^9/mL. A total yield of $>2.0 \times 10^6$ CD34+ cells/kg are by most centers demanded for HDT. From the majority (90%) of patients, the required amount of CD34+ cells are collected by 1 or more days of aphaeresis. A high number ($>5 \times 10^6$ CD34+ cells/kg) of re-infused stem cells is associated with faster haematological recovery, less bleeding- and infectious complications and improved outcome following HDT. G-CSF monotherapy may also be used in mobilizing CD34+ cells. Although the harvested number of CD34+ cells is reduced, no impact on first mobilization failure rates, engraftment or overall survival has been shown for mobilisation with G-CSF alone compared to G-CSF and chemotherapy (Vose et al. 2009). A proportion of patients show poor mobilization of stem cells to peripheral blood. High age and especially several previous chemotherapy cycles are risk factors for poor mobilization (Vose et al. 2009). For some of these patients re-mobilization or harvesting of stem cells from the bone marrow may be successful.

More recently, the additional use of the chemokine plerixafor (Mozobil®, Genzyme) with the ability to disrupt the adhesion of stem cells to the bone marrow stromal cells seems to improve the success rate of stem cell harvest from peripheral blood.

Contamination with Malignant Cells in the Autologous Graft; Purging of Stem Cells Before Re-infusion

From a theoretical point of view, voiding the autologous graft of viable tumor cells was considered important in order to reduce the risk of re-infusing tumor cells that could cause lymphoma relapse. Different methods for graft purging were thus applied, mostly anti-B cell antibody and complement-mediated lysis of tumor cells or mono-dispersed magnetic micro beads coated with monoclonal anti-B cell antibodies. For the latter method, positive (removing tumour cells) or negative (removing all non-tumour cells) selection or a combination has been applied with high purging efficacy. However, the clinical benefit of the procedure was difficult to prove and was the subject of only one randomized study (Schouten et al. 2003), which was closed prematurely due to slow patient inclusion. The study could not show an improved survival following graft purging compared to patients receiving un-purged grafts. Other studies have shown increased risk of severe infections following the use of purged grafts compared to unmanipulated autologous grafts. By the introduction of anti-CD20 antibody treatment (rituximab), in vivo purging became an efficient procedure for mature B-cell lymphomas. Administration of rituximab prior to stem cell mobilisation, results in nearly total eradication of CD20 positive normal and neoplastic cells in the harvest product.

High-Dose Therapy

The term "conditioning regimen" is used for the high dose treatment before re-infusion of stem cells, originating from its use in the allogeneic stem cell transplantation setting, where it reduces the host immune function so that the re-infused allogeneic bone marrow cells are not rejected by the patient. In HDT the purpose of the conditioning regimen is complete eradication of the malignant cells. In animal models, there is a linear-logarithmic relationship between drug dose and the killing of tumour cells. The drugs used show a steep dose-response relationship between chemotherapy-dose and the killing of tumour cells, have short half-life and myelotoxicity as the major (only) dose-limiting side effect.

The most widely used regimens for lymphoid malignancies are either a combination of fractionated total body radiation (TBI), 10–14 Gy followed by cyclophosphamide (1.2 g/m^2 2 days in a row) or carmustine (BCNU), etoposide, cytarabin, melphalan (BEAM). Melphalan may be substituted with cyclophosphamide in the BEAC regimen. The procedure related mortality rate has dropped substantially from the mid 1980s (10–15%) till today (approximately 1%). This drop is most probably due to shorter cytopenia duration after stem cell harvest from peripheral blood, to better patient selection and to improved supportive care during aplasia. In an unpublished series of all 711 Norwegian lymphoma patients undergoing HDT since 1987 until 2008, 10 year overall survival is 55%, ranging from 45 to 75% for the various entities. For many of the entities, there are few deaths beyond 5 years after HDT, indicating that a high percentage of these patients are cured from their disease.

Documentation for Effect of HDT in Lymphoid Malignancies

Randomized studies comparing HDT with continued conventional (immuno)-chemotherapy have been performed in the following lymphoma entities and settings:

Diffuse large B-cell lymphomas, first line (Greb et al. 2008; Strehl et al. 2003).

Diffuse large B-cell lymphomas, second line or later (Philip et al. 1995).

Table 13.2 Commonly accepted standard indications for HDT in lymphoma types

Lymphoma entity	Time of transplant	References
Hodgkin lymphoma	Primary resistant or in second- or later remission	Schmitz et al. (2002)
Diffuse large B-cell lymphoma	Second- or later remission	Philip et al. (1995)
Mantle cell lymphoma	First remission	Dreyling et al. (2005) and Geisler et al. (2008)
T-lymphoblastic lymphoma	First remission	Sweetenham et al. (2001)
Follicular lymphoma	Second- or later remission	Schouten et al. (2003)
Peripheral T-cell lymphoma	First remission	d'Amore et al. (2012)

Mantle cell lymphomas, first line (Dreyling et al. 2005).

Lymphoblastic lymphomas (Sweetenham et al. 2001).

Follicular lymphomas, first line (Schaaf et al. 2012).

Follicular lymphoma, second line or later (Schouten et al. 2003).

Hodgkin lymphoma, primary resistant, second line or later (Schmitz et al. 2002).

Based on these studies as well as promising results from phase II studies in T-cell lymphomas, first line, most centres would accept the following indications for HDT as standard treatment (Table 13.2):

Effect of HDT in Transformed B-Cell Lymphomas

Relatively small retrospective phase II analyses of HDT in t-NHL indicate that a subgroup of patients with chemo-sensitive disease obtains long-term survival following HDT. No randomized trial has been performed for transformed B-cell lymphomas. The reviewed 11 studies below are ordered chronologically, and those including more than 20 patients with t-FL are shown in Table 13.3. The first publication in 1989 from Nebraska, Omaha (Schouten et al. 1989) describes 18 patients with relapsed disseminated follicular lymphoma, of whom 10 patients showed histological transformation HT. The results of this study showed a statistically inferior survival for the transformed group, and the authors recommend that if HDT is considered, it should be performed before transformation.

Bastion et al. (1995) report on feasibility and therapeutic efficacy of HDT in 60 patients with poor-prognosis follicular lymphoma, 16 of the patients had t-NHL. 12 patients were in first PR, 34 in second CR/PR and 14 were in subsequent remission. Patients with t-NHL tended to have a worse failure free survival, but there was no OS difference. Patients in a later than second remission had a high treatment related mortality rate and a dismal survival. Heavily pre-treated patients are at high risk when undergoing HDT.

St Bartholomew's Hospital, London treated 27 t-NHL patients with HDT (Foran et al. 1998). All received high dose cyclophosphamide and total body irradiation (TBI) as conditioning, 24 of the patients were harvested from bone marrow (BM), only 3 from blood. In vitro purging with anti-B-cell antibodies and complement was performed in the 24 BM harvested patients. Nineteen of the patients were treated in their first remission following transformation. Death due to myelodysplasia was seen in two patients. The results are encouraging with median survival at 8.5 years. They recommend considering HDT for young patients with transformed FL (t-FL) in remission.

The Dana Farber experience covering 21 patients with t-NHL (from FL or CLL) all with BM harvest and in vitro anti-B-cell monoclonal antibody - and complement mediated purging, was also encouraging (Friedberg et al. 1999). All patients received cyclophosphamide plus TBI as

Table 13.3 Previously published retrospective analyses of high-dose chemotherapy with autologous stem cell support to transformed follicular lymphoma

Study	No. of patients	Histological subtypes	Median follow-up	HDT PFS	HDT OS	Early TRM	Late TRM	Secondary malignancies	Conditioning and graft manipulation
Hamadani et al. (2008)	24	FL to DLBCL	3.2 years	3 years: 40% 5 years: 33%	3 years: 52% 5 years: 52%	8%	16%	4% (MDS)	Cond. with chemotherapy. Non-purged PBSC grafts
Sabloff et al. (2007)	23	Histological criteria for transformation not specified	7.6 years	5 years: 25%	5 years: 56%	0	4%	18% (9% MDS, 9% Adenocarcinoma)	TBI Non-purged BM and PBSC grafts
Andreadis et al. (2005)	22	FL to FL 3 or DLBCL	5.5 years	Median: 1.4 years	Median: 4.6 years	2%	2%	2% (MDS)	TBI to 50% *In vitro* purged BM grafts and non-purged PBSC grafts
Williams et al. (2001)	50	FL to FL diffuse, DLBCL or any high-grade lymphoma	4.9 years	5 years: 30%	2 years: 64% 5 years: 51%	8%	10%	6%	TBI to 70% Non-purged BM and PBSC grafts
Chen et al. (2001)	35	FL to FL3 or DLBCL, initial DLBCL with FL relapse	4.3 years	5 years: 36%	5 years: 37%	20%		8% (MDS)	TBI to 91% Non-purged BM and PBSC grafts
Friedberg et al. (1999)	21	FL to DLBCL	36 months	5 years: 46%	5 years: 58%	0	14%	19% (MDS and AML)	TBI *In vitro* purged BM grafts
Foran et al. (1998)	27	Histological criteria for transformation not specified	2.4 years	N/A	Median: 8.5 years	7%	7%	7% (MDS)	TBI *In vitro* purged BM grafts

Only studies including more than 20 patients are shown in the table
FL follicular lymphoma, *DLBCL* Diffuse large B-cell lymphoma, *PFS* Progression free survival, *OS* Overall survival, *TRM* Treatment related mortality, *MDS* Myelodysplastic syndrome, *TBI* Total body irradiation, *BM* Bone marrow, *PBSC* Peripheral blood stem cells

conditioning. There were no treatment related deaths; disease free and overall survival at 5 years was 46 and 58%, respectively. Twelve of the patients were alive and in remission at a median of 36 months. Those with an early transformation had the better survival.

Berglund et al. (2000) report the experience from Uppsala, Sweden. Eleven of the 22 patients had t-FL, 9 patients received a purged graft. The conditioning regimen varied with time, those with TBI had higher toxicity (including three cases with MDS) and worse outcome. After a long median follow up of 74 months, they had an impressive DFS and OS of 72 and 81%, respectively.

The Stanford experience (Cao et al. 2001) with 92 primary induction failures or relapsed FL, were treated with either high dose chemotherapy alone or in combination with fractionated TBI. In this study, there was a trend for better survival with TBI containing regimen and with early transplantation. The 17 patients with t-FL had a survival (OS at 4 years 50%) comparable to FL without transformation.

Chen et al. (2001), Toronto, Canada report results from 35 t-FL patients treated with HDT. Patients were harvested from BM or blood, no purging was performed. All received high dose chemotherapy plus TBI. They experienced a high treatment related mortality (20%) and in addition three deaths (8%) from myelodysplasia. Five year PFS and OS was 37 and 36%, respectively. High age was the only significant risk factor.

The European Bone Marrow Transplant Registry receives and registers allogeneic and autologous transplant procedures and - results from a number of European centres. The results from 50 patients undergoing HDT for t-FL were matched with 200 patients similarly treated for either FL or intermediate-grade NHL (mostly DLBCL) (Williams et al. 2001). The procedure related deaths among the t-FL patients was high (18%) while the OS and PFS at 3 years were 51 and 31%, respectively. The authors found no difference in survival compared to the other two groups, and conclude that HDT should be seriously considered for t-FL, taken that the disease is still chemo sensitive.

Andreadis et al. (2005) report results from HDT in 49 FL patients; 22 of the patients had t-NHL. Nine of the patients received a purged graft, in 10 patients were harvested from blood. The conditioning regimen was TBI in 50% of the cases. Only high age and non-CR after HDT were statistically significant factors for short survival in multivariate analysis (PR versus CR before HDT had no impact). Thus, there was no difference between patients with or without transformation before HDT.

In another series of 138 patients treated with HDT, 23 showed t-FL (Sabloff et al. 2007). They report a better 5-year PFS for non-transformed compared to transformed cases ($p = 0.007$), but there was no difference in OS. Also these authors conclude that HDT alters the course of the disease positively for t-FL.

Finally, Hamadani et al. (2008) report their results with this treatment modality in 24 patients. The treatment related non-relapse mortality was high (25%), but 3 year PFS and OS was satisfactory (40 and 52%, respectively).

The only prospective study in this patient group was performed as a Norwegian multicentre study during the time period 1999–2004, with participation from all centres performing HDT in Norway (Eide et al. 2011). Patients were included at start of salvage chemotherapy for t-FL so that the prognosis for the whole cohort of patients was available. Inclusion criteria were: age 18–65 years, previously treated with chemotherapy, transformation to DLBCL from indolent B-cell lymphomas (CLL excluded), adequate bone marrow function and solid organ functions. The study was performed before rituximab was routinely used. One of the centres performed stem cell in vitro purging. Forty-seven patients from five Norwegian centres were included of whom the majority responded to induction chemotherapy and 30 (63%) were treated by HDT. Eighteen (60%) achieved CR, 7 (23%) PR and 5 (10%) had progressive disease following HDT. Median follow-up for patients alive was 75 months. PFS rates at 2 and 5 years were 50% (95% CI 0.32–0.68) and 32% (95% CI 0.18–0.46), respectively, while OS rates at 2 and 5 years were 73% (95% CI 0.57–0.89) and 47%

(95% CI 0.29–0.65), respectively. Median OS was only 10 months for the 17 patients not eligible for HDT. Patients receiving CD34+ enriched/B-cell depleted grafts had inferior PFS and a trend for inferior OS compared to patients receiving non-purged grafts (Log Rank 0.025 and 0.151, respectively).

In conclusion, results from 11 retrospective phase II studies and one prospective phase II study indicate that HDT should be considered for patients with t-NHL and chemo sensitive disease. Treatment results are similar to those seen in relapsed untransformed FL and in relapsed de novo DLBCL. Further, the procedure should not be performed late after a high number of previous treatments; treatment related mortality is reduced to well below 10% during recent years, but elderly patients are at increased risk; a partial remission on salvage chemotherapy is sufficient to proceed to HDT; the use of TBI may be associated with higher treatment related morbidity and mortality, including myelodysplasia; and there is no demonstrated benefit of *in vitro* purging of stem cell graft on patient survival.

HDT for Transformed Lymphomas in the Rituximab Era

The anti-CD20 monoclonal antibody rituximab is the first drug that definitely has changed survival prospects for FL patients. There is no indication that a "watch and wait" policy or first line treatment with single agent rituximab increases the rate of transformation; an Italian retrospective study (Conconi et al. 2012) indicates that upfront rituximab treatment may rather reduce the risk. This may, however, be due to selection of low risk patients for rituximab monotherapy. Retrospective analyses indicate both that patients who have received rituximab at the time of t-FL as well as patients who receive rituximab as part of therapy after t-FL have a better prognosis (Montoto and Fitzgibbon 2011). On the other hand, patients with de novo DLBCL relapsing after rituximab – containing therapy have a considerably worse prognosis than patients who have not received rituximab previously (Gisselbrecht et al. 2010). Thus, we anticipate in the near future a better prognosis for patients with t-NHL who have not received rituximab previously than those who have because the former group will have the better response to salvage chemotherapy before HDT. As far as we know, there is no study on HDT to t-FL patients in the rituximab era, but we are presently examining this topic.

Rituximab maintenance therapy improves PFS in patients in PR or CR after first line immunochemoptherapy (Salles et al. 2011) and both PFS and OS after immuno-chemotherapy for relapse (van Oers et al. 2006). Whether maintenance therapy improves survival after HDT for t-FL has not been investigated properly.

Treatment for Relapse After HDT

Patients who relapse after HDT for FL or t-FL have a variable course. The prognosis may be excellent for patients with low-grade localized relapse with FL histology. Also for patients with widespread FL relapse, the prognosis may be quite good, even with rituximab single agent therapy (unpublished results). In cases with an aggressive clinical relapse and/or t-FL histology, the prognosis is dismal. Allogeneic stem cell transplantation with reduced conditioning (RIC-allo) may be an option, but results are clearly inferior to RIC allo than for FL without transformation (Montoto and Fitzgibbon 2011). We are not aware of studies using radio-immunotherapy in relapse after HDT. Bone marrow toxicity may be a concern in this setting.

Future Perspectives

Patients with t-NHL are frequently excluded from studies on new drug development. Data on the potential effectiveness of drugs like novel antibodies to CD20 and other B-cell line specific antigens, immunoconjugates (i.e. anti-CD19-maytansine), tyrosine kinase inhibitors, NFκB inhibitors like bortezomib and immunomodulatory drugs like lenalidomide are therefore

limited. However, there are some promising data on the use of lenalidomide . Further, promising data are obtained with the radioimmunoconjugate iodine-131 tositumomab with response rates around 60% and response duration of approximately 1 year (Montoto and Fitzgibbon 2011). Ongoing research on redirecting allogenic T-cells to lymphoma cells are promising, and may be of benefit also to t-NHL-patients in the future. Soluble monoclonal T-cell receptors capable of activating T-cells to lyse tumor cells are under development in pre-clinical studies. Further improvements in the therapy of FL may also reduce the relapse rate and reduce the incidence of t-NHL.

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The Wnt/β-Catenin Pathway as a Potential Target for Drug Resistant Leukemic Stem Cells

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Contents

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Abstract

Components of the canonical Wnt/β-catenin signaling pathway are frequently mutated in cancer, and their deregulation has also been recently associated with the development of cancer stem cells (CSCs). Elevation of β-catenin activity is critical for blast transformation and drug resistant property of the stem cells in both chronic and acute myeloid leukemia, although the underlying mechanisms are still largely unknown. In this chapter, we will focus on the role of Wnt/β-catenin pathway in normal hematopoietic and leukemic stem cells. We will also review currently available small molecule inhibitors that target the canonical Wnt signaling, and propose their potential applications in combination with chemotherapy for cancer treatment.

Introduction

Cancer Stem Cells in Acute Myeloid Leukemia

Acute myeloid leukemia (AML), is characterized by an accumulation of immature white blood cells with myeloblast features in the bone marrow (BM), can be initiated by different genetic mutations originating from hematopoietic stem cells (HSC) or early myeloid progenitors. Cancer stem cells (CSC) have been functionally

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identified in all major subtypes of AML by xeno-transplantation models, suggesting that the diseases are sustained by a relatively small pool of functionally distinctive leukemic stem cells (LSC) with unlimited self-renewal and potential drug resistant properties (Hope et al. 2003). Combination chemotherapy using daunorubicin and cytarabine targets rapidly proliferating cells by interfering with DNA synthesis, and results in a complete remission in about 70% AML cases. However, the disease frequently relapses and only 30% of patients can achieve a long-term remission. This leads to the hypothesis that chemo-resistant LSC could evade the toxicity of chemotherapy and cause subsequent relapse. Allogeneic hematopoietic stem cell transplantation is the mainstay of treatment for high-risk AML but leukemia relapse occurs in 30–40% patients. As a result, the treatment outcome has remained dismal and the development of specific cancer therapeutics targeting drug-resistant LSC is critical to eradicate the disease.

The Canonical and Non-canonical Wnt Pathways

The Wnt signaling pathway governs a wide range of biological processes, from early developmental events during embryogenesis to tissue homeostasis, by regulating a specific set of genes that are key for various biological functions such as cell adhesion, polarity, proliferation, differentiation and self-renewal (Barker and Clevers 2006). The Wnt signaling can be classified into the canonical and non-canonical pathways. Both can be activated by the binding of specific Wnt ligands to their corresponding Frizzled receptors (Fz), however the effector proteins downstream of Wnt/Fz are different. The central mediator of the canonical pathway is β-catenin, which translocates into nucleus to form transcriptional complexes to modulate gene expression. In contrast, the non-canonical pathway can be mediated by various downstream messengers like small Rho GTPases, c-Jun N-terminal kinase and calmodulin kinases. Over the past decade, the canonical Wnt/β-catenin signaling pathway has been shown to regulate stem cell function in various tissues, and its deregulation is frequently associated with different types of cancers including colon, liver, lung, breast, ovarian and leukemia, making it an attractive target for cancer therapeutics. In this chapter, we will focus on two aspects: firstly, the implication of aberrant Wnt/β-catenin activity in LSC, particularly in myeloid leukemia, and secondly, the potential therapeutic intervention via the targeting of the Wnt/β-catenin pathway using small molecule inhibitors.

Molecular Regulation of the Canonical Wnt Pathway and Its Deregulation in Cancer

At the basal state when Wnt ligand is absent, β-catenin is mostly excluded from the nucleus and is maintained at low levels through continuous protein destruction (Fig. 14.1). β-catenin interacts with its proteolytic machinery called destruction complex containing Axin, adenomatous polyposis coli (Apc), glycogen synthase kinase 3 β (Gsk3β) and casein kinase 1 α (Ck1α). Axin serves as a scaffold for serine/threonine kinases, whereas Gsk3β and Ck1α phosphorylate serine/theronine residues (Ser33, 37 and 41 by Gsk3β and Thr45 by Ck1α) of β-catenin. Phospho-serines in β-catenin provide recognition sites for E3 ubiquitin ligase Skp1-cullin-F-box (SCF)-β-TrCP complex, which promotes poly-ubiquitination mediated degradation by 26S proteosome. When Wnt ligands bind to Fz, a seven-span transmembrane receptor and induce its dimerization with low-density lipoprotein (LDL) receptor related protein (Lrp), this results in the recruitment of Disheveled (Dvl) and Axin to the cell membrane, which in turn leads to the dissociation of the destruction complex. β-catenin protein that has escaped proteolysis can accumulate and translocate into the nucleus via a mechanism that has not been fully elucidated. β-catenin then forms transcriptional complexes with other transcription factors (*e.g.*, T-cell factor (Tcf) and lymphoid enhancer binding factor (Lef)) and histone modification enzymes (*e.g.*, creb-binding protein (Cbp), p300

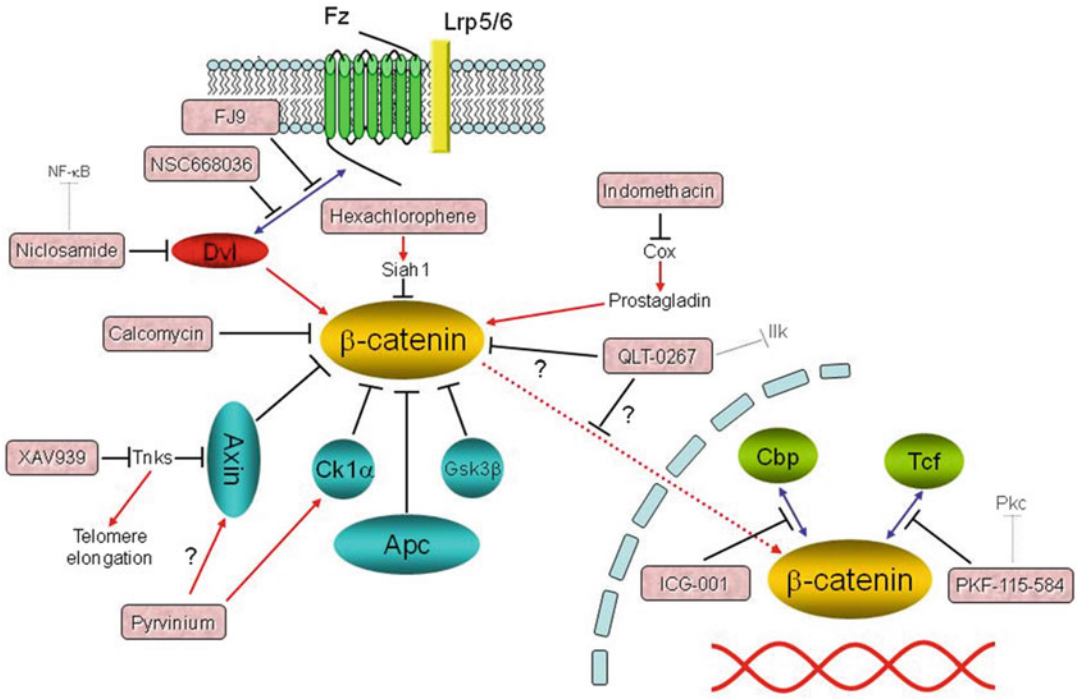

Fig. 14.1 The canonical Wnt signaling pathways and its inhibition by small molecule inhibitors. *Red arrows* and the blunt *black arrows* represent activation and inhibition actions respectively. The *red dashed arrow* represents translocation of β-catenin from the cytoplasm to the nucleus; whereas the *blue double arrow-headed line* indicate protein-protein interaction. *Pink rectangles* represent small molecule inhibitors of the pathway; *gray blunt arrows* indicate an inhibition of non-specific targets; "*?*"indicates an unknown mechanism. For clarity sake, the different extracellular Wnt regulators are not shown in this diagram

and mixed lineage leukemia (Mll)) to activate a specific set of genes containing a unique DNA sequence called Tcf/Lef transcriptional response element (TRE) that usually associates with cell proliferation like cyclin D1 and c-Myc or anti-apoptosis like survivin (Fig. 14.1). On the other hand, the Wnt signaling activity can also be modulated by extracellular Wnt antagonists: soluble Fz-related proteins (SFRP) and Wnt inhibitory factor-1 (WIF-1) that segregate Wnt ligand and prevent it from interacting with Fz. Dickkopf (Dkk) binds to the LRP5/6 co-receptor, and inhibits its interaction with Wnt ligands.

In cancer, various key components of the canonical Wnt pathway are frequently mutated or deregulated, which results in the aberrant activation of β-catenin (Barker and Clevers 2006). Among those, mutations in *APC* are probably the most well studied examples, since they occur in 80–90% of sporadic colon cancers. Mice that bear a nonsense mutation called *multiple intestinal neoplasia (min)* on the *Apc* locus (Apc_{Min}) develop intestinal malignancy in a manner that is similar to that observed in patients with familial adenomatous polyposis (FAP). Also about 25% of colorectal cancer harbor *AXIN2* mutations associated with defective DNA mismatch repair. On the other hand, *β-catenin* can also be directly mutated in liver cancer, with somatic mutation in exon 3 affecting the serine/threonine residues in position 33, 41, 45 or the region surrounding these phospho-residues. These β-catenin mutants can no longer be phosphorylated by GSK3β or CK1α kinases, thus avoiding poly-ubiquitination by the E3 ubiquitin ligase that is responsible for proteolysis. Similarly, activating mutations of β-catenin have been found in gastric cancer, and relatively rare, in colon cancer. Both the over-expression of Wnt ligands and the reduced expression of Wnt antagonists like SFRP, WIF-1 or DKK have also

been reported in several human cancers, including leukemia. Down-modulation of Wnt antagonists is usually associated with DNA methylation on their CpG islands, which leads to the hyper-activation of Fz and the stabilization of β-catenin.

The Wnt Signaling in Normal Hematopoiesis

The Canonical Wnt Pathway Activates HSC Functions

Enhanced canonical Wnt activity through over-expression of the constitutively active form of β-catenin expands murine HSC pool and increases the repopulation activity (Reya et al. 2003). *In vivo* activation of β-catenin by inhibition of GSK3β enhances long-term repopulation of human and murine HSCs, and improves survival of lethally irradiated recipients (Trowbridge et al. 2006). Interestingly, in spite of an increased number of Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) population in the transgenic mice carrying a conditional knock-in allele of the constitutively active form of β-catenin, their HSCs have impaired repopulation potentials, leading to multiple hematopoietic defects in both myeloid and lymphoid lineages (Kirstetter et al. 2006). These results indicate that constitutive activation of β-catenin can in fact impair intrinsic HSC function and a precise dose of β-catenin activity is crucial for normal hematopoiesis. Consistently, a recent study using various *Apc* mutant mice with different nuclear β-catenin activity demonstrates that only the intermediate dose of β-catenin promotes HSC functions (Luis et al. 2011).

β-Catenin Is Largely Dispensable for Normal Hematopoiesis

Despite the fact that β-catenin plays an important role in HSC functions, its loss does not compromise normal hematopoiesis and lymphoid development (Cobas et al. 2004). One possible explanation for the relatively mild phenotype would be a compensatory effect by γ-catenin. However, the double knockout of β-catenin and γ-catenin does not exhibit any major impact on normal hematopoiesis, and HSCs are still capable of multi-lineage reconstitution of the recipient (Jeannet et al. 2008). It is noteworthy that an unexpected expression of truncated β-catenin upon Mx-1-Cre mediated deletion and residual β-catenin/Tcf transcription activity observed in LSK cells may complicate the conclusions. In a separate study where β-catenin was inactivated by Vav-Cre in hematopoietic lineages, depletion of β-catenin hampered long-term repopulation of HSC in recipient mice upon serial transplantations (Zhao et al. 2007). Together, these results suggest that although β-catenin can positively regulate HSC functions, it is largely dispensable for normal hematopoiesis, making it as an ideal target for cancer therapeutics.

The Wnt Signaling in Leukemic Stem Cells

The Canonical Wnt Signaling in CML Stem Cells

While the Wnt/β-catenin signaling has long been speculated to play a pivotal role in the development of CSC, the first and most well established evidence comes from leukemia studies. In 2004, Jamieson et al. fractionated BM cells from chronic myeloid leukemia (CML) patients in different disease phases and found that β-catenin was highly expressed in granulocyte-macrophage progenitors (GMP) in blast crisis or imatinib-resistant CML (Jamieson et al. 2004). Unlike its normal counterpart, GMP from CML with activated β-catenin activity acquired self-renewal capacity. Down-regulating β-catenin by lentiviral transduction of Wnt inhibitor Axin reduced *in vitro* replating efficiency, while enforced expression of β-catenin conferred replating ability to normal GMP. Activation of β-catenin during CML blast transformation may be mediated by aberrant in-frame splicing of GSK3β resulting in inactivation of the kinase activity (Abrahamsson et al. 2009). The crucial role of β-catenin in LSC function has been

further demonstrated using β-catenin deficient cells transformed with BCR-ABL, which failed to induce CML in recipient mice (Zhao et al. 2007).

The Canonical Wnt Signaling in AML Stem Cells

In AML, elevated β-catenin expression has been linked to adverse prognosis with poor relapse-free and overall survival (Ysebaert et al. 2006). The evidence for a critical function of β-catenin in LSC was recently demonstrated in AML driven by MLL fusions (Wang et al. 2010a; Yeung et al. 2010). β-catenin level increased in MLL-fusion-transformed murine GMP compared with normal GMP (Wang et al. 2010a), and further elevated during the transition from pre-LSC to LSC (Yeung et al. 2010). While naïve GMP cells cannot be transformed by Hoxa9 and Meis1, enforced expression of stable-form of β-catenin resurrects the transformation ability. Moreover, suppression of β-catenin by Cre-mediated gene deletion prolonged disease latency and survival of MLL/AML in both studies. On the other hand, β-catenin expression in human AML blasts enhanced the replating efficiency (Ysebaert et al. 2006), whereas its silencing in HL-60 AML cell line by short hairpin RNA diminished the engraftment potential although the effect of β-catenin depletion in primary human AML varied significantly (Gandillet et al. 2011), suggesting that β-catenin is possibly dispensable for certain AML subtypes. While further studies are required to define its underlying mechanisms and a complete array of AML subtypes that are sensitive to β-catenin inhibition, these results strongly suggest that β-catenin is a potential target for suppression of AML stem cells.

The Canonical Wnt Signaling in Drug Resistant LSC

In addition to its role in the maintenance of LSC, β-catenin has also been shown to contribute to drug resistance. Although imatinib treatment can successfully inhibit BCR-ABL kinase activity and prolonged the overall survival of CML mice carrying BCR-ABL, the mice eventually died due to the selection of LSC clones with elevated expression of β-catenin (Hu et al. 2009). The mechanisms that lead to enhanced β-catenin expression in imatinib resistant LSC remain however unknown. The most striking example for β-catenin's involvement in drug resistance is documented in MLL leukemia, which is in general dependent of GSK3 activity. The phosphorylation of CREB by GSK3 is an essential step for HOX-mediated transcription to promote oncogenesis by MLL fusions (Wang et al. 2010b). Inhibition of GSK3 by small molecule inhibitors or gene inactivation of GSK3α and/or GSK3β suppressed MLL development in a murine model. However, activation of β-catenin contributes drug resistance to GSK3 inhibitor in MLL LSC (Yeung et al. 2010). While MLL pre-LSC is sensitive to various GSK3 inhibitors, MLL LSC with elevated levels of β-catenin are resistant to the inhibitors. Strikingly, full-blown MLL leukemia can be sensitized to lithium carbonate treatment resulting in a prolonged disease-free survival by specific knock down of β-catenin expression (Yeung et al. 2010). Although the mechanisms by which activated β-catenin promotes GSK3 inhibitor resistance in LSC are still unclear, the observation that the suppression of β-catenin activity restores drug sensitivity in MLL leukemia unveils a novel therapeutic avenue by targeting both β-catenin and GSK3 in certain leukemia.

Small Molecule Inhibitors Targeting the Wnt Signaling Pathway

Over the past years, a number of potential small molecule inhibitors that target canonical Wnt signaling at different levels by either directly or indirectly acting on the key components of the pathway have been identified by several high-throughput (HTP) chemical screens, and cell-based or enzyme-linked immunosorbent assays (ELISA). In addition, the availability of high-resolution protein structure of β-catenin transcription complex provides invaluable

information for designing inhibitors disrupting essential protein-protein interaction among different transcriptional components. Here, we will review these small molecule inhibitors according to their mode of action along the Wnt signaling cascade (Fig. 14.1).

Inhibitors Acting on Disheveled and Frizzled Receptors

NSC668036 is a small organic molecule derived from the United States National Cancer Institute small molecule library that can bind Dvl PDZ domain, thus attenuating the Wnt signaling by disrupting the interaction between Dvl and Fz. It induces a double body axis formation in Xenopus, an effect that can be overcome by forced expression of β-catenin but not by Wnt3a ligand, indicating that the inhibitor acts upstream of β-catenin (Shan et al. 2005).

FJ9 is a compound of non-electrophilic indole-2-carbinol–based chemical scaffold derived from a structure-directed design. It binds to the PDZ domain of Dvl, and disrupts Dvl binding to Fz7, leading to the down-regulation of the canonical Wnt signaling by decreasing β-catenin protein levels. This inhibitor has been tested in a colon cancer cell line, where it induces apoptosis coupled with a reducted expression of β-catenin downstream targets such as cyclin D1, c-Myc and survivin. Administration of FJ9 *in vivo* can reduce tumor size in a xenograft nude mouse model (Fujii et al. 2007).

The antihelminth compound, Niclosamide, down-regulates the Wnt/β-catenin signaling specifically through disruption of Dvl2 expression, which in turn diminishes β-catenin. This compound suppresses proliferation of colon cancer cell line as well as primary colorectal tumor explanted *in vitro* and reduces tumor growth in a xenotransplant NOD/SCID mouse model (Osada et al. 2011). However, there are reports suggesting that niclosamide inhibits the NF-κB pathway in leukemia cell lines (Jin et al. 2010). The specificity of this inhibitor has to be considered, especially when treating leukemia.

Inhibitors Inducing β-Catenin Degradation

Non-steroidal anti-inflammatory drug indomethacin is a COX inhibitor that diminishes the canonical Wnt signaling by degrading β-catenin protein. Recent findings suggest that COX activity can induce prostaglandin level which in turn suppressing β-catenin proteolysis (Castellone et al. 2005). This inhibitor has been shown to suppress murine MLL-leukemia through β-catenin degradation (Wang et al. 2010a).

XAV939, identified by a HTP chemical genetic screen using a TOP/FLASH reporter, targets poly(ADP-ribose) polymerases PARP1, PARP2, Tankyrase (TNKS) 1 and 2 (Huang et al. 2009). XAV939 inhibits TNKS polyADP-ribosylation of Axin, enhancing protein stability. The stabilization of Axin facilitates β-catenin degradation, hence inhibits β-catenin mediated transcription. XAV939 specifically reduces the colony forming ability in a colon cancer cell line that is dependent on β-catenin activity (Huang et al. 2009). Since TNKS1 is also involved in telomere elongation, XAV939 may suppress tumor cell growth via telomere shortening mechanisms.

Pyrvinium is a Foods and Drugs Agency (FDA)-approved drug that was identified due to its ability to stabilize Axin in a chemical screen. It also binds to CK1α and potentiates its activity. Both properties favor degradation of β-catenin. In addition, pyrvinium promotes the proteolysis of pygopus, a key component required for nuclear localization of β-catenin. Functionally, pyrvinium induces axis duplication in Xenopus embryos and exhibits a cytotoxic effect on colon cancer cells (Thorne et al. 2010). Calcimycin was identified in a HTP screen for candidates that suppress the expression of S100A4, a downstream target of β-catenin. Through an unknown mechanism, this molecule reduces β-catenin expression at both the mRNA and the protein level. It down-regulates a panel of β-catenin target genes and prevents colon cancer growth both *in vitro* and *in vivo* (Sack et al. 2011).

QLT-0267 attenuates the Wnt/β-catenin signaling activity by suppressing Wnt3a-mediated β-catenin nuclear translocation and enhancing its

degradation. However, QLT-0267 was firstly identified as a potent integrin-linked kinase (Ilk) inhibitor that inhibited protein kinase B (PKB) phosphorylation on serine 473 and suppressed proliferation of breast cancer cells (Troussard et al. 2006). The effect of this inhibitor has also been tested in human primary AML cells. While it inhibited long-term culture of primary AML and reduced their engraftment potential in NOD/SCID mice, its effect on β-catenin in AML LSC has not been investigated (Muranyi et al. 2010).

Hexachlorophene is a relatively less specific inhibitor that mediates proteolysis of β-catenin through activation of E3 ubiquitin ligase Siah-1. This compound has been tested in Epstein Barr virus (EBV)-infected B-cell lymphoma and efficiently suppressed tumor growth (Park et al. 2006).

Inhibitors Disrupting the Interaction Between β-Catenin and Its Co-factors

ICG-001 was identified in a HTP screen for the inhibition of β-catenin/Tcf mediated transcription. This compound disrupts the interaction between β-catenin and its transactivator Cbp. By binding to the N-terminal region of Cbp, ICG-001 decreases the expression of β-catenin downstream targets such as survivin. ICG-001 inhibits colon cancer growth both *in vitro* and *in vivo* by suppression of survivin expression and inducing caspase 3-mediated apoptosis (Ma et al. 2005). Moreover, ICG-001 can also block γ-catenin/Cbp interaction. In CML, it reduces colony formation of imatinib-resistant CD34-enriched progenitors (Kim et al. 2011).

PKF-115-584 is one of the inhibitors identified from an ELISA screen for compounds that disrupt the binding of β-catenin to Tcf (Lepourcelet et al. 2004). This compound inhibits survivin and cyclin D1, and exhibits tumor suppressor activity on colon and prostate cancer cells. Notably, PKF-115-584 is also known as calphostin C and is a very potent protein kinase C (PKC) inhibitor. PKF-115-584 inhibits PKC at a nano-molar range, whereas its action on β-catenin/Tcf disruption requires micro-molar dose. Thus, the specificity has to be considered when applying this inhibitor for cancer therapeutics.

Prospective

There are increasing bodies of evidence indicating that β-catenin plays a pivotal role in cancer stem cells, especially in myeloid leukemia. Given that *β-catenin* knockout does not grossly affect normal hematopoiesis, it further highlights the Wnt/ β-catenin signaling as an attractive target for elimination of myeloid leukemia without severely impacting on vital normal hematopoiesis. Whereas a number of the canonical Wnt/β-catenin inhibitors have been developed, several important issues still remain to be resolved. The first is drug specificity. Compounds that intercept the components upstream of β-catenin (like Disheveled and Fz receptors) will likely interfere with the non-canonical Wnt signaling pathway. Certain canonical Wnt inhibitors, such as QLT-0267 and PKF-115-584, may target other pathways such as Ilk for the former and PKC for the latter. Ideally, drugs directly act on β-catenin should provide better specificity although it has been proven to be very difficult to effectively target transcription factor. The second concern is the impact of β-catenin inhibition on other normal tissues. The Wnt/β-catenin pathway is widely used by various tissue specific stem cells to support diverse important physiological functions. Even in hematopoiesis, β-catenin is required by osteoblasts for the maintenance of primitive hematopoietic progenitors. Thus a specific drug delivery strategy is required to avoid targeting vital organs that heavily rely on β-catenin. The clonal selection/evolution of LSC is the other issue. The functional dependency on β-catenin may be overcome by leukemic clones carrying additional genetic/epigenetic events that either pre-exist or evolve during the treatment. Thus, an appropriate design of combination therapy targeting multiple pathways is critical to

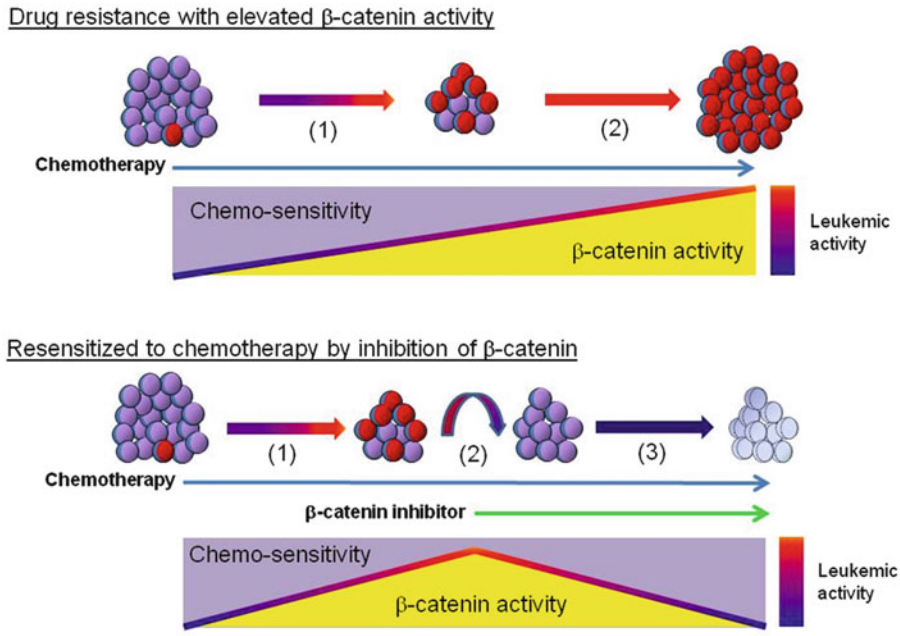

Fig. 14.2 β-catenin inhibitor resensitizes drug-resistant leukemia to chemotherapy. *Upper panel*: During chemotherapy, leukemic clones develop drug-resistance properties, conconmitantly with increased β-catenin activity: chemo-sensitive clones (*purple cells*) get eliminated and chemo-resistant clones (*red cells*) emerge (*1*). Therefore, continuous chemotherapy is not effective to eradicate all leukemic clones and results in relapse (*2*). *Lower panel*: Alternatively, suppressing β-catenin activity by small molecule inhibitors in chemo-resistant clones with an activated canonical Wnt pathway (*1*), renders chemo-resistant clones sensitive to chemotherapy (*2*). Combined chemotherapy with β-catenin inhibitor thus would potentially achieve a total remission (*faint cells*) (*3*)

eradicate the LSC. Chemotherapy has proven effective in inducing remission in a large number of patients, however relapses are common and remain to be a major clinical problem. Given the link between β-catenin and drug resistance in CML and MLL/AML, combining chemotherapy with small molecule inhibitors that suppresses β-catenin activity can be an attractive therapeutic approach (Fig. 14.2). Future research into related areas will be useful in facilitating rational design of more effective cancer therapies for targeting CSC in not just leukemia but also other solid tumors.

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Bone Marrow Stem Cell Therapies for Diabetes Mellitus and Its Complications 15

Ming Li and Susumu Ikehara

Contents

Abstract

Diabetes mellitus (DM) will affect the lives of 380 million people by 2025. Curative therapies mainly include diet, insulin, and oral hypoglycemic agents. However, these therapies fail to maintain blood glucose levels in the normal range all the time. Pancreas or islet-cell transplantation achieves better glucose control and substantially improves the quality of life. In this chapter, we summarize stem cell therapies for type 1 and type 2 DM and diabetic complications.

Introduction

Diabetes mellitus (DM) is characterized by hyperglycemia resulting from defects in insulin secretion and insulin action. The chronic hyperglycemia induces failure of the eyes, kidneys, nerves, heart, and blood vessels. DM therapies mainly include diet, insulin, and oral hypoglycemic agents, and pancreas or islet-cell transplantation. However, because of the shortage of donor organs, research has focused on stem cells as a means to generate β cells for DM treatment (Hussain and Theise 2004). Stem cells, which can be classified into embryonic stem cells (ESCs) and tissue stem cells (TSCs), are potential sources for β cell replacement. TSCs include SCs derived from the bone marrow, liver and pancreas. This chapter provides an

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overview of stem cells therapies for DM and of diabetic complications.

Classification and Criteria of DM

According to the WHO (World Health Organization), DM will affect the lives of 380 million people by 2025. Etiologic classification of DM (2004) includes two main types: Type 1 DM (T1DM), in which pancreatic β cells are destroyed by the immune system, and Type 2 DM (T2DM), which results from decreased insulin sensitivity and inadequate insulin production by β cells. Other specific types of diabetes include, (1) genetic defects in β cell function (maturity onset diabetes of young (MODY)), (2) genetic defects in insulin action, (3) disease of the exocrine pancreas, (4) endocrinopathies, (5) drug-chemical-induced DM, (6) infections, (7) uncommon forms of immune-mediated diabetes, and (8) other genetic syndromes sometimes associated with diabetes. There is also what is known as gestational DM. The criteria for the diagnosis (2004) of DM are either symptoms of diabetes (classically polyuria, polydipsia, and unexplained weight loss) plus a casual plasma glucose concentration of $\geq$200 mg/dl, or fasting plasma glucose concentration of $\geq$126 mg/dl, or a 2 h postload glucose concentration of $\geq$200 mg/dl, during oral glucose tolerance test.

Current Therapies for Diabetes Mellitus

DM therapies mainly include diet, insulin, oral hypoglycemic agents, and pancreas or islet-cell transplantation. Exogenous insulin replacement has been the primary therapy for controlling blood glucose levels because T1DM is insulin-dependent. However, most patients with T1DM are unable to maintain their blood glucose levels in the normal range at all times. Pancreas and islet transplantation achieves better glucose control and allows a substantially improved quality of life (Ryan et al. 2004). However, although these various studies are underway, there remain limitations to allogenic organ transplantation. Recent research has focused on the use of stem cells in the treatment of DM.

Stem Cell Therapies for Type 1 Diabetes Mellitus

Insulin injections are the standard therapy for T1DM because of the absolute insulin deficiency. Unfortunately, insulin secretion is exquisitely sensitive to the minute-to-minute changes in blood glucose, and glycemia-stimulated insulin secretion cannot be mimicked by exogenous insulin injections. Thus, the ability to generate new β cells is an important approach in the treatment of T1DM. Bone marrow-, liver- and pancreas-derived stem cells are potential sources for β cell replacement. Induced pluripotent stem (iPS) cells and ESCs also can differentiate into insulin-positive cells.

The non-obese diabetic (NOD) mouse is a spontaneous model of T1DM, with genetic and pathophysiological roots comparable to the human disease (Anderson and Bluestone 2005). NOD mice reconstituted with BALB/c nu/nu bone marrow cells displayed normal T- and B-cell functions, and newly developed T cells in the allogeneic bone marrow recipients were tolerant to cells with both donor- and host-type major histocompatibility complex determinants. These results suggest that allogenic bone marrow transplantation (BMT) might contribute to the prevention of islet destruction, and to the restoration of self-tolerance. BMT may thus ultimately be developed as a component of a strategy to be employed for the treatment of T1DM in humans (Ikehara et al. 1985).

The bone marrow is an invaluable source of adult, pluripotent stem cells, including hematopoietic stem cells (HSC), endothelial progenitor cells (EPCs), and mesenchymal stem cells (MSCs). Bone marrow cells have the ability to differentiate *in vivo* into functionally competent β cells (Ianus et al. 2003). Currently, there are eight clinical trials underway, including phases I to IV, on the use of bone marrow stem

cells for the treatment of DM patients (Fotino et al. 2010).

The liver and pancreas are both differentiated from endoderm during development, and pancreatic duodenum homeobox protein-1 (Pdx-1) is a homeodomain protein of 283 amino acids whose expression is absolutely required for the pancreas to develop. Liver stem cells expressing Pdx1 long-term exhibited similar profiles for the expression of genes related to pancreatic development and β cell function, and reversed hyperglycemia in diabetic mice (Yang 2006).

The pancreas mainly consists of exocrine tissue, which is composed of acinar cells that produce digestive enzymes, and duct cells that form the afferent system to the duodenum. There is a report that fully differentiated exocrine cells can be directly reprogrammed into cells that closely resemble β cells in adult animals by a combination of just three transcription factors Ngn3, Pdx1 and Mafa (Zhou et al. 2008). Another report demonstrates convincingly that the adult mouse pancreas contains islet cell progenitors and that the expansion of the β cell mass following partial duct ligation depends partly on the activation of Ngn3 gene expression and the ensuing differentiation of endogenous progenitor cells in a cell-autonomous, fusion-independent manner, providing the first direct evidence for the existence of endogenous endocrine islet cell progenitors in the adult mouse pancreas. This cell population is similar to the one that gives rise to the islets during embryonic development and represents an obvious target for therapeutic regeneration of β cells in DM (Xu et al. 2008).

iPS cell were able to derive β-like cells similar to the endogenous insulin-secreting cells in mice. These β-like cells secreted insulin in response to glucose and corrected a hyperglycemic phenotype in two mouse models of T1DM and T2DM via an iPS cell transplantation (Alipio et al. 2010). ESCs are pluripotent stem cells derived from the inner cell mass of the blastocyst, and can generate insulin-positive cells *in vitro* (Segev et al. 2004). However, although it can produce endocrine pancreatic cells from ESCs, *in vitro* differentiation can not be controlled (Brolen et al. 2005).

Stem Cell Therapies for Type 2 Diabetes Mellitus

T2DM shows relative insulin deficiency usually in the presence of insulin resistance. Genetic and environmental factors are involved in the development of T2DM. Inflammation is considered to have a pivotal role in the development of metabolic diseases, including obesity and T2DM (Hotamisligil 2006). Some youths with a clinical diagnosis of T2DM show evidence of islet-cell autoimmunity, with autoantibodies present in 10–75% of patients; islet-cell antibodies (ICA) in 5–8%, glutamic acid decarboxylase (GAD) in 8–30%, islet-autoantibodies (IA)-2 in 8–42% and insulin antibodies in 5–35% (Hathout et al. 2001; Gilliam et al. 2005). Obese youths with a clinical diagnosis of T2D may show evidence of islet autoimmunity contributing to insulin deficiency (Klingensmith et al. 2010). Recently, it has been shown that obese adipose tissue activates CD8 T cells, promoting the recruitment and activation of macrophages in the adipose tissue (Nishimura et al. 2009). Adipocytes regulate and mediate inflammatory cytokines such as tumor necrosis factor-α (TNFα), IL-6, matrixmetalloproteinases (MMPs), peroxisome proliferation activated receptor-r (PPAR-r) and fatty acid-binding protein-4. These cytokines inhibit or enhance each other, and their activities contribute to insulin resistance (Suganami et al. 2005). As such, both an autoinflammatory as well as an autoimmune response are involved in the pathogenesis of T2DM.

We previously showed that allogenic BMT could be used to treat insulin-independent DM in KK-Ay mice. KK-Ay mice, a T2DM model reconstituted with KK-Ay bone marrow cells showed glycosuria, hyperinsulinemia, and hyperlipidemia. However, KK-Ay mice that had been lethally irradiated (9.0 Gy) and then reconstituted with T cell-depleted bone marrow cells from normal BALB/c mice showed negative urine sugar with decreases in serum insulin and lipid levels 4 months after BMT (Than et al. 1992). A previous report suggested that the transplantation of bone marrow MSCs via intra bone

marrow-BMT (IBM-BMT) in conjunction with the induction of HO-1 could eradicate T2DM. The beneficial effect of HO-1 induction further suggests that the abnormality in endothelial progenitor cells is due to MSC-stromal cell disorder exacerbated by oxidative stress and decreases in adiponectin. Thus, the transplantation of bone marrow MSCs using the IBM-BMT strategy in conjunction with HO-1 induction offers a novel approach for the treatment of T2DM (Abraham et al. 2008).

We have also reported that increased insulin sensitivity and decreased blood glucose levels result from normalizing the imbalance of lymphocyte subsets by IBM-BMT + thymus transplantation (TT) in db/db mice. The novel effects of IBM-BMT + TT are that this combination induces adiponectin secretion, followed by enhanced pLKB1-AKT-AMPK crosstalk, signaling pathway, insulin phosphorylation, and also HO-1. We think that IBM-BMT + TT is thus a potential therapeutic intervention for metabolic disorders such as T2DM, insulin-resistant diabetes and metabolic syndrome (Li et al. 2010).

Autologous bone marrow-derived rat MSCs (i) promote PDX-1 and insulin expression in the islets, (ii) alter T cell cytokine patterns, (iii) preserve regulatory T cells in the PB, and (iv) induce sustained normoglycemia (Boumaza et al. 2009). Combined therapy of intrapancreatic autologous stem cell infusion and hyperbaric oxygen treatment can improve metabolic control and reduce insulin requirements in patients with T2DM (Estrada et al. 2008). Bone marrow derived stem cells have been used to treat T2DM in phases I to IV of clinical trials (Fotino et al. 2010).

Stem Cell Therapies for Diabetic Complications

Diabetic complications mainly include diabetic retinopathy, nephropathy, neuropathy, and compromised wound healing. Vision loss associated with ischemic diseases such as retinopathy of prematurity or diabetic retinopathy are often due to retinal neovascularization. A previous report suggesting that BM-derived progenitors could exert effects on both developing and degenerating retinal vessels prompted our study of their possible role in ischemic retinopathies using the oxygen-induced retinopathy (OIR) mouse model. In the report, the researchers show that transplanted BM-derived progenitors dramatically accelerated retinal vascular repair of OIR with no observed long-term toxicity, increasing the rate of physiological intraretinal revascularization while at the same time markedly reducing the formation of abnormal, preretinal neovascularization. The report demonstrated that the transplanted myeloid progenitors differentiated into cells with characteristics of microglia following their injection into the vitreous humor of mice. Autologous adult BM-derived progenitor cells can be used to rebuild and stabilize functional vasculature in hypoxic retinal tissue, rather than to eliminate the problematic vessels that result (Ritter et al. 2006).

Diabetic nephropathy is the most common cause of end-stage renal failure in the world. MSCs have been shown to have the ability to slow the progression of DN independently of glycemic control (Ezquer et al. 2009). Bone marrow cells have the capacity to differentiate into mesangial cells and to transdifferentiate into podocytes (Wong et al. 2008). This is accompanied by the re-expression of the defective collagen chains and improved renal histology and function (Sugimoto et al. 2006). MSCs have the capacity to repair renal injury, accelerate tubular proliferation and improve renal function (Morigi et al. 2004). Nephrons are of mesenchymal origin and stroma cells are of crucial importance for signaling, leading to the differentiation of both nephrons and collecting ducts (Anglani et al. 2004). Bone marrow-derived mesangial cell progenitors may play a crucial role in the development and progression of extracellular matrix accumulation and mesangial cell proliferation in the db/db mouse.

Diabetic neuropathy, which is the most frequent and earliest diabetes complication, decreases the quality of life and increases the morbidity of diabetic patients. It occurs because of the impairment

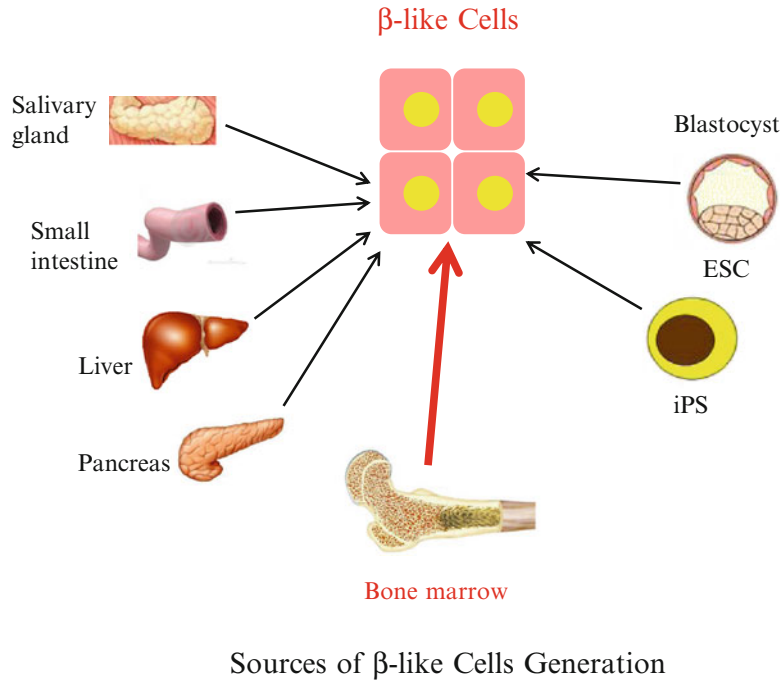

Fig. 15.1 Sources of β-like cell generation

of nerve blood flow and metabolic imbalances in the neural compartment of the peripheral nerve. The epidemiology and natural course of diabetic neuropathy is clouded with uncertainly, largely due to confusion regarding the definition and measurement of this disorder (Vinik et al. 2000). EPCs can be isolated from the bone marrow, cord blood, and peripheral blood. One report has demonstrated that therapeutic neovascularization using human umbilical cord blood-derived EPCs thus reverses diabetic neuropathy (Naruse et al. 2005). Bone marrow-derived EPCs could reverse various manifestations of diabetic neuropathy. These therapeutic effects were mediated by direct augmentation of neovascularization in peripheral nerves through long-term and preferential engraftment of EPCs in nerves and particularly vasa nervorum and their paracrine effects. These findings suggest that EPC transplantation could be an innovative therapeutic option for treating diabetic neuropathy (Jeong et al. 2009).

EPC transplantation accelerates cutaneous wound repair in a dermal excisional wound model. The transplantation of EPCs was directly involved in the formation of new capillaries in the granulation tissue. Furthermore, the improved wound healing might have been mediated through abundant monocyte/macrophage recruitment, as well as by increased neovascularization (Suh et al. 2005). Bone marrow MSCs have been shown to enhance wound healing in non-diabetic and diabetic mice by promoting re-epithelialization, cell infiltration and angiogenesis (Wu et al. 2007), and bone marrow-derived stem cells have been used in phases I and II of clinical trials for the treatment of diabetes complications (Fotino et al. 2010).

In conclusion, the use of ESCs is fraught with potential complications, including a tendency to form teratomas and the ethical debate revolving around their derivation. Induced pluripotent stem cells (iPSC) are derived from skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state that enables the development of an unlimited source of any type of human cell needed for therapeutic purposes. However, the benefits of the use of iPSCs needs to be proven in clinical trials. In contrast, bone marrow stem cells are an invaluable source of adult pluripotent stem cells (Fig. 15.1) and have

already been used in the Phases I to IV of clinical trails for the treatment of diabetes and its complications.

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Thyroid Cancer Stem Cells – Strategies for Therapeutic Targeting

16

Reigh-Yi Lin, William Sewell, Kyle Spradling, Ashley N. Reeb, and Wen Li

Contents

Abstract

Thyroid cancer stem cells are capable of sustaining neoplastic growth. Because they are often resistant to chemotherapy and radiation therapy, they present a major obstacle to effective thyroid cancer treatment. This review discusses the recent discovery of cancer stem cells in many subtypes of thyroid cancer, including papillary, follicular, medullary and anaplastic. It emphasizes potential new therapeutic strategies targeting multidrug resistance, DNA repair and oncogenic signaling pathways activated in the stem cells during cancer progression. These new, targeted therapies, if realized, may improve the efficacy of current therapeutic treatments against advanced thyroid cancer, prevent disease relapse and enhance patient survival.

Introduction

Over the last two decades, the discovery that hierarchically organized, self-renewing subsets of cells with stem-cell-like properties are responsible for the initiation, progression, metastasis and even the relapse of many types of tumors has changed remarkably the way we think about and treat cancer. These cancer stem cells are characterized by an ability to undergo both symmetrical and asymmetrical division, as well as to differentiate into a multitude of tumor cell types

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that both initiate and support tumor growth (Lin 2011). The cells are largely quiescent, which allows them to evade standard cancer therapies aimed at rapidly dividing cells (Boman and Wicha 2008). They also express efflux transporters that effectively pump chemotherapeutic agents out of the cell (Moitra et al. 2011) and may repair radiation-damaged DNA more efficiently than do other tumor cells. Their resilience explains why many advanced cancers recur despite an initial response to treatment. Understanding the mechanisms underlying this resistance to chemotherapy and radiotherapy is crucial to the development of new, more-effective therapies. Here, we summarize the current knowledge of cancer stem cells and discuss the utility and limitations of targeting a cancer stem cell pathway to eliminate thyroid cancer, which affects more people than all other endocrine cancers combined.

Cancer Stem Cells in Thyroid Carcinoma

Papillary, follicular, medullary, and anaplastic thyroid cancers all harbor a small subset of cancer stem cells. In particular, thyrospheres with properties resembling those of stem or progenitor cells have been isolated from human papillary thyroid cancer cell lines (Malaguarnera et al. 2011). Medullary thyroid cancer, which accounts for 3–4% of all thyroid cancers, arises through the transformation of parafollicular C cells within the thyroid gland. It is frequently caused by a mutation in *RET*, which encodes a receptor tyrosine kinase. Although a cell-surface marker for medullary thyroid cancer stem cells has not yet been identified, a self-renewing CD133-positive population plays a crucial role for *RET* proto-oncogene activity (Zhu et al. 2010).

Papillary thyroid cancer is far more prevalent than follicular thyroid cancer (accounting for about 80–85% *versus* about 10–15% of all thyroid cancers, respectively). They are both well-differentiated thyroid cancers that still display some or all of the characteristics of mature thyroid tissue. When detected early, both have an excellent prognosis (nearly 100% of patients survive 5 years). In contrast, anaplastic thyroid cancer is the rarest and most aggressive subtype of thyroid carcinoma. It comprises only 1–2% of thyroid malignancies but accounts for more than half of all thyroid cancer-related deaths (Gimm 2001). Compared to other subtypes of thyroid cancer, anaplastic thyroid cancer is undifferentiated and has a higher tendency to invade neighboring tissues and metastasize to distant organs. It is resistant to all types of therapy, and anaplastic thyroid cancer patients usually have a poor prognosis.

It has been reported by Todaro et al. (2010) that, although cancer stem cells make up only 2 and 1.2% of the cells in papillary and follicular thyroid cancer, respectively, up to 3.5% of anaplastic thyroid tumor cells may be cancer stem cells. These cancer stem cells exhibited high levels of aldehyde dehydrogenase activity, grew as thyrospheres in suspension, and divided both symmetrically and asymmetrically. When injected orthotopically into the thyroid glands of immunodeficient mice, anaplastic thyrospheres generated more-aggressive tumors with advanced distant metastasis than did thyrospheres derived from follicular and papillary thyroid cancers (Todaro et al. 2010). Although we lack a comprehensive understanding of the mechanisms of thyroid cancer, therapies targeting the cancer stem cell population may be the key to halting tumorigenesis and overcoming drug resistance in advanced thyroid cancer.

Targeting Multiple Drug Resistance

Many malignant tumors demonstrate a chemoresistant phenotype, and researchers continue to identify genes (collectively known as multi-drug resistance (MDR) genes) that alter the susceptibility of thyroid carcinoma cells to chemotherapeutic agents. Studies have shown that tumor cells gain resistance to synthetic and natural toxins by modulating four basic processes: drug efflux, apoptosis, cell cycle progression, and DNA repair. In particular, the drug-resistant phenotype of anaplastic thyroid cancer cells is

due in part to their expression of ATP-binding cassette transporters (ABC transporters) (Sugawara et al. 1994; Yamashita et al. 1994). Side population (SP) cells from the anaplastic thyroid cancer cell line HTh74 enriched for stem and progenitor cells express substantially higher levels of the ABC transporter genes ABCG2 and MDR1 than do non-SP cells (Zheng et al. 2010), and long-term culture of the cell line with doxorubicin resulted in the establishment of a doxorubicin-resistant line with a flourishing SP fraction. The fact that combination treatment of doxorubicin-resistant cells with doxorubicin and the L-type calcium channel blocker verapamil eliminated the SP phenotype and ended resistance, however, highlights the necessity of a therapy that deliberately targets cancer stem cells.

Apotosis inhibitors have also been linked to chemotherapeutic resistance in thyroid cancer cells. Expression of the anti-apoptotic genes Bcl-2 and Bcl-xL is elevated in papillary, follicular and anaplastic thyroid cancer cells (Stassi et al. 2003). Additionally, exogenous expression of these anti-apoptotic genes is sufficient to bestow cytotoxic protection to normal thyrocytes. Further scrutiny of various histological thyroid cancer specimens and purified thyroid cancer cells uncovered a previously unknown aspect of the tumor microenvironment and an autocrine component to its pathology in the form of intense immunoreactivity to both interleukin-4 (IL-4) and interleukin-10 (IL-10). Treatment of normal thyrocytes with IL-4 and IL-10 increased Bcl-2 and Bcl-xL levels in the cells and protected them from apoptosis in the presence of cisplatin, doxorubicin, and taxol. On the other hand, IL-4- and IL-10-neutralizing antibodies down-regulated Bcl-2 and Bcl-xL expression in thyroid cancer cells, substantially increased the proportion of cells undergoing apoptosis, and sensitized the cells to chemotherapeutic drugs (Stassi et al. 2003).

Elevated levels of several other apoptosis inhibitors, including survivin, c-IAP1, c-IAP2, and XIAP, have also been found in many anaplastic thyroid cancer cell lines (Ito et al. 2003; Tirro et al. 2006). Expression of these apoptotic inhibitors and their antagonist, Smac, was examined in thyroid cancer cell lines after cisplatin, doxorubicin, or taxol treatment (Tirro et al. 2006). Although the different cell lines displayed variable levels of both inhibitors and Smac in response to each chemotherapeutic compound, c-IAP1 and survivin levels were generally increased, while Smac levels were reduced. Several studies show that targeting IAPs and survivin (Tirro et al. 2006), as well as NF-kB (which up-regulates anti-apoptotic gene expression) (Meng et al. 2008; Smallridge et al. 2009; Starenki et al. 2004), restores the sensitivity of thyroid cancer cells to cytotoxic agents.

In 2006, a study demonstrated higher pathologic complete response rates in diabetic patients who were receiving both metformin (an oral anti-diabetes drug) and neoadjuvant chemotherapy for breast cancer (Jiralerspong et al. 2009). Metformin operates as an anti-mitogenic agent by activating AMP-activated protein kinase pathway (Zakikhani et al. 2006). Differentiated human thyroid cells and thyroid carcinoma cells treated with metformin exhibited appreciable cell-cycle arrest and enhanced apoptosis, and metformin potentiated the anti-mitogenic effect of doxorubicin and cisplatin in anaplastic thyroid cancer cells (Chen et al. 2012). In addition to inhibiting the growth of thyroid cancer cell lines, metformin also antagonizes the proliferative effect of insulin and suppresses the self-renewal of thyroid cancer stem cells. Clonal colony and thyrosphere formation were dramatically inhibited by metformin in the anaplastic thyroid cancer cell lines HTh74 and HTh74R. Hyperinsulinemia and insulin resistance are major contributors to the increased risk of thyroid cancer experienced by people with type 2 diabetes mellitus. These studies suggest that metformin may be a potentially effective adjuvant treatment for type 2 diabetic patients with thyroid cancer.

Targeting DNA Repair

Radiation therapy or chemotherapy with alkylating agents are designed to damage a cancer cell's DNA beyond repair. Anaplastic thyroid

cancer cells, however, are highly resistant to these types of DNA damaging therapies. Many studies of thyroid carcinomas have revealed mutations in or the misregulation of a number of DNA repair pathway genes linked to genomic instability and, ultimately, to tumor development, progression and/or metastasis (Gatzidou et al. 2010).

The Gadd45 (growth arrest and DNA-damage-inducible gene) protein family is associated with DNA replication and repair (Smith et al. 1994, 2000), G2/M checkpoint regulation (Vairapandi et al. 1996, 2000), and apoptosis (Takekawa and Saito 1998). Although transcript levels of Gadd45 family members increase following treatment with DNA-damaging agents, Gadd45γ is present at appreciably lower levels in anaplastic thyroid cancer cells than in cultured primary thyrocytes (Chung et al. 2003). Adenovirus-mediated Gadd45γ expression in anaplastic thyroid cancer cells dramatically inhibited proliferation *in vitro* and tumor growth *in vivo*. Although this effect has been attributed to increased levels of apoptosis, the precise mechanism of Gadd45γ action in the cells is still unknown. However, because the growth suppression of anaplastic thyroid cancer cells by Gadd45γ is similar to the effect of the tumor suppressor p53, Gadd45γ may be a potential target for gene therapy in anaplastic thyroid cancer.

Anaplastic thyroid cells exposed to ionizing radiation activate c-Jun NH2-terminal kinase (JNK), and selective inhibition of JNK with the pharmaceutical agent SP600125 clearly suppresses anaplastic thyroid cancer cell growth (Bulgin et al. 2006). Treating anaplastic thyroid cancer cells with both a JNK inhibitor and ionizing radiation considerably compromised DNA damage repair in the cells and promoted a greater decrease in clonogenic survival than did either treatment alone. Interestingly, this effect was due to the induction of a senescence-like terminal growth arrest. Thus, JNK inhibition coupled with ionizing radiation may be a promising strategy for the treatment of anaplastic thyroid cancer.

The L1 cell adhesion molecule (L1CAM) has recently been shown to regulate the activation of cell-cycle checkpoint proteins in glioblastomas in response to DNA damage. Clinical observations establishing an association between L1CAM expression and tumor progression and metastasis in both ovarian (Stoeck et al. 2006) and colorectal carcinomas (Kaifi et al. 2007) has led researchers to also examine L1CAM expression in thyroid cancer. A study by Kim et al. (2012) showed that L1CAM expression was universal in all anaplastic thyroid tumor samples studied, but was undetectable in normal thyroid follicular epithelial cells or in papillary thyroid carcinomas. Knockdown of L1CAM in human anaplastic thyroid cancer cell lines by short hairpin RNAs (shRNA) significantly decreased the proliferation, migration, and invasiveness of the cells and also enhanced their sensitivity to both gemcitabine and paclitaxel. L1CAM depletion also markedly reduced tumor burden in immunodeficient xenograft model mice and prolonged their survival (Kim et al. 2012), and L1CAM targeting sensitized glioblastoma stem cells to radiation (Cheng et al. 2011). These results suggest that anti-L1CAM therapy may synergize with traditional therapeutics to overcome the radioresistance of cancer stem cells.

Targeting Developmental Signaling Pathways

The Wnt signaling pathway plays many important roles in embryogenesis, cell differentiation and carcinogenesis. It contributes to the functional and structural maintenance of the tissues of the body by controlling proliferation, stem cell activation, and self-renewal. Signaling by Wnt protein, as well as that of other stem-cell regulatory pathways such as Hedgehog and Notch, is dysregulated in thyroid malignancies.

β-catenin, a key oncogenic protein in the Wnt pathway, is hyperactive in poorly differentiated and undifferentiated thyroid cancer. Targeting this pathway with an adenovirus-based therapy significantly inhibited anaplastic tumor growth *in vivo* and prolonged the survival of mice with the condition (Abbosh et al. 2007). The therapy relied on a synthetic promoter with TCF/β-catenin

response elements driving adenovirus E1A and E1B expression, which allowed the adenovirus to replicate and kill only those cells in which the β-catenin pathway was hyperactive. This approach shows it is possible to kill cells by specifically targeting the Wnt signaling pathway, and suggests that a similar approach may be useful in the treatment of thyroid cancer.

Current cancer therapies with tyrosine kinase inhibitors such as imatinib or vandetanib work in part by inhibiting the Wnt/β-catenin pathway. Imatinib treatment decreased the proliferation and invasiveness of anaplastic thyroid cancer cells positive for c-abl tyrosine kinase significantly by reducing nuclear β-catenin and increasing β-catenin/E-cadherin binding to the plasma membrane. This redistribution in turn reduced expression of the β-catenin target gene cyclin D1 and led to cell growth arrest (Rao et al. 2006). Similarly, vandetanib treatment of papillary TPC1 cells carrying a RET/PTC rearrangement stabilized β-catenin in the plasma membrane, reducing the expression of c-myc and cyclin D1 and decreasing cell proliferation and migration (Tartari et al. 2011). Together, these results highlight the importance of Wnt signaling pathway activation in thyroid cancer progression. Sulindac, a non-steroidal anti-inflammatory drug, also reduces β-catenin expression in human colon cancer cells (Rice et al. 2003). In human papillary thyroid cancer cell lines overexpressing $BRAF^{V600E}$, sulindac targets the Wnt/β-catenin pathway and reverses the aberrant expression and localization of β-catenin and is accompanied by a decrease in cell growth (Cho et al. 2010). Further studies should investigate the potential for β-catenin-directed therapy for patients with advanced thyroid cancers.

Recent findings demonstrate a considerable upregulation of β-catenin, cMet (the hepatocyte growth factor receptor), and Akt (a serine/threonine protein kinase) in thyrospheres derived from undifferentiated thyroid cancer as compared to those from papillary and follicular thyroid cancers (Todaro et al. 2010). The upregulation of cMet and Akt correlates with the migration capabilities of a small population of cancer stem cells whose expression is correlated with malignancy. Silencing either cMet or Akt expression with short hairpin RNAs completely abrogated the metastatic activity of thyroid cancer stem cells *in vivo* (Todaro et al. 2010). These studies suggest that both cMet and Akt play a critical role in the growth of cancer stem cells and the metastasis of anaplastic thyroid cancer.

Finally, a study by Malaguamera et al. (2011) described the use of the thyrosphere culture technique to isolate thyroid cancer stem cells from patients who had undergone surgery for papillary thyroid cancer. These thyrospheres expressed high levels of stem cell markers, including Oct-4, SOX-2, NANOG and CD133, and low or absent expression of thyroid differentiation markers such as thyroglobulin, thyroperoxidase and the TSH receptor – characteristics consistent with more-aggressive forms of papillary thyroid cancer. The thyrospheres also expressed several signaling molecules from the insulin-like growth factor (IGF) pathway, including IGF-1, IGF-II, and receptors for IGF-I and insulin, and the insulin receptor to IGF-I receptor ratio of the thyrospheres from papillary cancers exceeded that of thyrospheres derived from normal thyroid tissue. Treatment with insulin and/or IGF-1 increased the volume of cancer thyrospheres and the growth of cancer stem cells, and treatment with IGF increased the expression and activity of Oct-4 and NANOG. Together, these findings suggest a role for insulin and IGF-1 in the development of thyroid cancer stem cells and imply a possible future differentiation-based therapy in thyroid cancer.

In conclusion, the studies reviewed here highlight recent progress in the identification of cancer stem cells in thyroid cancer, as well as current and future challenges in thyroid cancer therapy. It is clear that much work is needed to identify the molecular machinery that drives the regulation of cancer stem cells and the resistance of thyroid cancer stem cells to chemotherapy and radiation. Such studies are likely to increase our understanding of the aggressive nature of advanced thyroid cancer and the development of effective therapies for affected patients.

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Role of Cancer Stem Cell in Mammary Carcinogenesis and Its Clinical Implication

17

Yajing Liu and Suling Liu

Contents

Abstract

Accumulating evidence shows the presence of a subpopulation of cancer stem cells in many cancers including breast cancer. The breast cancer stem cells are resistant to treatments and able to initiate tumorigenesis, these features suggest that they may contribute to therapy resistance and relapse and thus making them a promising target for improving outcome of treatments. The expression of specific markers in breast cancer stem cells and development of mouse model has facilitated the study and several intrinsic and extrinsic pathways maintaining breast cancer stem cell population have been exploited. The discovery of different states of breast cancer stem cells provides more insights in how cancer stem cells are maintained and regulated. Compounds targeting pathways maintaining cancer stem cells are under development and several have already entered clinical trials. Breast cancer stem cell studies deliver a potential and effective regimen for improving outcomes of breast cancer patients.

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Introduction

The female breast functions primarily as a lactating gland; it mainly consists of lobules, lactiferous ducts, and connective tissues. It undergoes massive morphological changes during puberty

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and pregnancy including accumulation of fat tissue and development of lactating glands and ducts. In fact, the epithelium that lines lobules and ducts undergoes continuous change via apoptosis and proliferation throughout life because of oscillations in hormone levels during the menstrual cycle. Therefore, abnormalities are not surprisingly seen in breast tissue due to the frequent proliferation/involution cellular events. Breast cancer is defined as a malignant tumour arising from uncontrolled proliferating breast epithelial cells. Breast cancer is the second most common cancer among women, it is estimated that ~190,000 women are diagnosed with breast cancer each year and 40,000 women will die from this. Breast cancer is highly heterogeneous, which is why a single treatment is not equally effective for all patients. Thus identifying different types of breast cancer becomes essential for a more effective therapy and thus a better outcome. The categorization into different types based on histopathology, the grade and stage of the tumor and expression of proteins helps clinicians to choose the most effective therapies. For example, estrogen and progesterone receptors (ER & PR) are important prognostic indicators in breast cancer; tumors with these two receptors tend to be well differentiated and respond to hormone-therapies, and such patients tend to have a prolonged disease-free survival rate. Tamoxifen that inhibits cell proliferation by competing with endogenous estrogens on the ER site is the most frequent prescription medicine for ER positive breast cancer patients. Although early detection and development in adjuvant systemic chemotherapy and radiotherapy increase relapse-free and overall survival rates, development of therapy-resistance, metastasis and disease recurrence remain problematic. These drawback features are often found associated with the cancer stem cell (CSC), which is a subpopulation of cancer cells that display stem cell properties. Therefore, understanding the biology of CSCs becomes essential for targeting this population in order to improve patients' survival.

Normal Breast Stem Cells

Stem cells are defined by their ability to self-renew and differentiate into multiple lineages. They are important for embryonic development and tissue regeneration in adults. In adult tissues, stem cells sit in specific niches, which are important in stem cell regulation and maintenance by responding to intrinsic and extrinsic signals. In human mammary gland, the presence of breast epithelial stem cells were initially described by their ability to form colonies, which morphology resemble myoepithelial or luminal phenotypes or express markers exclusive for these two populations (Stingl et al. 1998). Mammary epithelial stem cells exhibit the following properties:

- Expression of distinct proteins has been used to identify stem cell populations for a long time, although the functions of these markers are not always well understood. In mammary epithelial cells, MUC-1 glycoprotein (MUC-1)+/common acute lymphoblastic leukaemia antigen (CALLA)-/epithelial-specific antigen (ESA)+ and the MUC-1- to +/−/CALLA +/− to +/ESA + were suggested as the progenitor markers for ductal and alveolar cells by the group that first identified human breast epithelial progenitors (Stingl et al. 1998).
- The ability to eject fluorescent dyes such as Hoechst due to high activity of several transmembrane transporters leads to formation of a side population (SP). Research on normal mammary epithelium found limited SP cells, but these cells were able to differentiate into ductal and lobular cells both *in vitro* and *in vivo* (Alvi et al. 2003), indicating their stem/progenitor property.
- The ability to display aldehyde dehydrogenase activity, which can be assessed by the ALDEFLUOR assay via flow cytometry. *In vivo* study showed only Aldefluor + ve cells of normal mammary epithelium were able to repopulate fat pad in mouse but not the Aldefluor-ve population. And the structures

formed by Aldefluor + ve mammary epithelial cells resembled the human mammary duct phenotype as well as expressed same pattern of cytokeratins (Ginestier et al. 2007).

- The potential to survive and proliferate when grown in anchorage-independent environment with the presence of growth factors in the form of spheroids. These floating 3D structures, termed mammospheres were capable to differentiate into both epithelial and myoepithelial lineages. More importantly, this culture technique maintained the self-renewal and multilineage potential of the mammary epithelial stem/progenitor populations (Dontu et al. 2003). Taken together, the establishment of biomarkers, *in vitro* and *in vivo* models from studies of normal mammary stem cells has facilitated the isolation and characterization of such cells in malignant breasts.

Breast Cancer Stem Cells

Although the concept of cancer stem cells was implied decades ago (Furth and Kahn 1937), identification of the cell population only became feasible more recently (Bonnet and Dick 1997). Our laboratory first isolated putative breast cancer stem cells that express epithelial specific antigen (ESA) and CD44 but not CD24 (Al-Hajj et al. 2003). As few as 200 $ESA^+CD44^+CD24^-$ cells were capable to generate tumor *in vivo*, whereas a 100-fold more cells without these markers isolated from the same tumors were non-tumorigenic. In addition, the secondary tumors resemble the phenotype (morphology and ESA/CD44/CD24 expression profile) of the initial tumor and the tumorigenic ESA^+-$CD44^+CD24^-$ tumor cells could be serially passaged at least four passages *in vivo*. Subsequent studies employed several methodologies adapted from stem cell research to also isolate or study breast CSC; these included side population (SP), ALDEFLUOR assay, anchorage-independent growth conditions. The SP assay is based on the ability of stem cells to exclude DNA dye such as Hoechst 33342 by membrane transporters, and the SP has been shown to contain the most tumorigenic population within breast cancer cell line when injected *in vivo* (Dontu et al. 2003). The Aldeflour assay represents a group of enzymes catalysing the oxidation of aldehydes. In malignant mammary epithelium, cells with high ALDH activity were associated with the greatest self-renewal and differentiation ability when grown *in vitro* or as xenotransplants, and positive ALDH immunostaining in breast carcinomas correlated with poor prognosis (Ginestier et al. 2007). Mammary stem/progenitor cells are able to survive in serum-free and anchorage-independent conditions in the form of spheroids (Dontu et al. 2003). Breast CSCs were also enriched when grown as non-adherent spheroids *in vitro* (Ponti et al. 2005). Interestingly, these markers (ALDH+ and $ESA^+CD44^+CD24^-$) with greater tumorigenicity than the rest of the population only showed limited overlapping (Ginestier et al. 2007). Similar finding was also proposed by the other group demonstrating that different breast cancers may contain tumor initiating cells that display different cell surface markers (Wright et al. 2008). Despite the heterogeneity between breast CSC markers mentioned above these cells are usually associated with development of therapy resistance and relapse, the two main drawbacks of cancer treatment. Therefore, understanding the biology of CSC population will help the development of new treatment targeting them and lead to more effective therapies and ultimate cure for cancer.

Breast Cancer Stem Cell Regulation and Maintenance

The similarity shared between normal stem cells and CSCs has drawn great attention on their intrinsic properties. By definition, stem cells are capable of self-renewal and differentiation as well as maintaining a delicate balance between the two states. Several key signalling pathways such as Hedgehog, Wnt and Notch that function in normal mammary stem cell maintenance and regulation during development and tissue homeostasis. Notch is required for self-renewal of mammary stem cells as shown by our lab that

activation of the pathway increased secondary mammosphere formation by 10-fold (Dontu et al. 2004). Elevated level of Hedgehog and Wnt signalling is found associated with mammospheres and differentiation is induced when the pathways are inhibited (Liu et al. 2006; Korkaya et al. 2009). More importantly, the authors also found activation of these pathways are also found in breast CSCs and blocking these pathways decreased the breast CSC population.

Stem cells are thought to protect themselves from intrinsic and extrinsic insults through a variety of different mechanisms including avoid and/or repair the damage more efficiently. Our preliminary data show that the CSCs are association between Rad51, which is involved in homologous recombinant repair pathway of DNA damage and PARP inhibitor resistance in triple negative breast cancer cell lines, and Rad51 expression is highly expressed in $ALDH^+$ population. Contradictory results showed that either fewer or similar DNA damage was induced in CSCs in the first place or stem cells repaired the DNA damage more efficiently than the rest of population to become resistant to ionizing radiation (Phillips et al. 2006; Li et al. 2008). Moreover, dysfunctional Notch, Wnt and Hedgehog signaling pathways are often disassociated with therapy-resistance.

Adult stem cells interact with a local microenvironment termed the stem cell niche and this interaction is crucial for their function and the breast tumor microenvironment may exhibit similar roles towards CSCs. In tumors, this niche contains a variety of cellular elements including inflammatory cells, fibroblasts, endothelial cells and mesenchymal stem cells. Interactions can exist between CSCs, their progenies and surrounding cells, some of these interactions involve signaling pathways described previously, including Wnt, Notch and Hedgehog. Such interaction was seen in the study of carcinoma-associated fibroblasts isolated from patients: they promoted tumor cell growth as well as angiogenesis in mouse model (Orimo et al. 2005). Tumor microenvironment also involves inflammatory cells, and it has been shown by us that bone marrow-derived mesenchymal stem cells (MSC) interact with breast CSCs through a cytokine network including CXCL7 and IL-6 and the attraction of MSC to the CSC to the primary tumor site was seen in patients' samples (Liu et al. 2011). Subsequent study demonstrated that the IL-6 inflammatory loop was activated in acquired and de novo trastuzumab resistance to expand CSC population (Korkaya et al. 2012). In addition, the inflammation cytokine TNFα was found to stabilize EMT through NF-κB, which in turn promote metastasis (Wu et al. 2009).

Growing evidence suggests that CSCs are also regulated by microRNAs, a group of small non-coding, single-stranded RNAs. Increasing expression of microRNAs were often found in cancers (Liu et al. 2012a), in mouse mammary stem/progenitor cells isolated by ALDH activity, a negative association with expression level of microRNA93 was found (Ibarra et al. 2007), indicating the higher microRNA levels are linked with non-stem bulk tumor populations. Our recent results confirmed the negative association between microRNA93 and $ALDH^+$ population, tumorigenecity and metastasis, and it does so by interfering cell proliferation as well as genes involved in breast CSC regulation. Interestingly, this reverse association between microRNA93 and breast CSCs was only restricted in less differentiated basal and $claudin^{low}$ breast cancer models, in luminal breast cancer cells, overexpression of microRNA93 actually increase CSC population and promote tumorigenesis, indicating distinct roles of this molecule at different differentiation stages (Liu et al. 2012b). A summary of signalling pathways and molecules involved in regulating breast CSCs is listed in Fig. 17.1.

Breast Cancer Stem Cell Plasticity

In order to travel to other organs, cancer cells need to loss cell-cell contact and undergo a series of cytoskeletal remodeling to acquire a mesenchymal phenotype which can migrate easily to

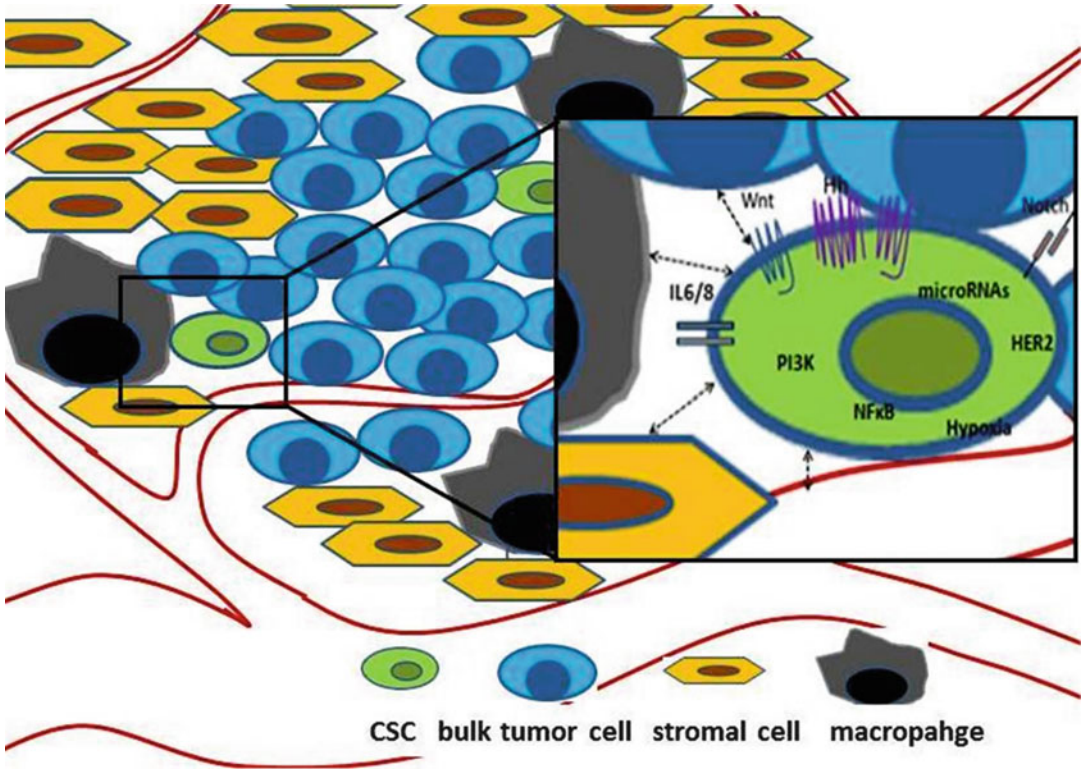

Fig. 17.1 CSCs and their niche. Cancer stem cells reside in a habitable community composed of immune cells, fibroblasts, myoepithelial, endothelial and bulk tumor cells, all of which nourish, regulate as well as are being regulated by CSCs. Signalling pathways such as Wnt, Hh, Notch, PI3K, NFκB and Her2 within the CSCs and the cytokine loops with IL-6 and IL8 that act as messengers between CSCs and their neighbours function together to retain the harmony. The newly discovered member microRNAs and the acquired hypoxia condition within the tumour are also actively involved maintaining the CSC population

enter the circulations system. And this morphological change is called epithelial to mesenchymal transition (EMT), which is involved in many biological processes including embryonic development, wound healing and cancer progression. Importantly, circulating cancer cells are also capable to undergo a mesenchymal to epithelial transition (MET) for them to leave the blood stream and settle at a new site to regenerate the tumor for metastasis. It was found that both inflammatory immune response and hypoxia that are often linked with tumor microenvironment induce EMT (Yang et al. 2008; Wu et al. 2009) and EMT plays an important role in the metastasis of breast cancer (Gjerdrum et al. 2010). Recently it was found that both EMT and non-EMT population contribute to metastasis, indicating other states of metastasizing tumor cells (Tsuji et al. 2008).

Because CSCs are competent to differentiate and recapitulate a new tumour resembling all the features at the original site, CSC and tumour metastasis have been linked conceptually. As described previously by our lab, $CD44^{+}CD24^{-}$ and $ALDH^{+}$ identify overlapping, but not identical cell populations, our preliminary data studying expression molecules associated with MET as well as EMT showed that $ALDH^{+}$ cells are more enriched in MET cells, whereas $CD44^{+}CD24^{-}$ cells are mainly contained in EMT cells. Previous studies plus our current work suggest a model where highly proliferative MET-like cells expressing ALDH count for majority of the CSC populations within the

mass tumor while $CD44^+CD24^-$ CSCs with EMT status are predominantly found at the front edge of invasion and metastasis. Our hypothesis is supported by studies demonstrated that elevated expression of EMT markers: Vimentin, Slug and Twist were found in malignant $CD44^+CD24^-$ stem cells (Mani et al. 2008), and invasiveness was directly associated with $CD44^+CD24^-$ phenotype (Sheridan et al. 2006). In addition, there is limited evidence linking homing and proliferation at the metastasis site, indicating that MET is required for EMT-like invasive cells to settle at the secondary site.

In normal mammary gland, two distinct progenitor populations were recognized by $EpCAM^-CD49f^+$ and $EpCAM^+CD49f^+$ phenotypes and both of which could form complete mammary gland (Keller et al. 2012). Our preliminary data showed that MET-like ALDH+ cells express a $EpCAM^+CD49f^+$ phenotype whereas $CD44^+CD24^-$ enriched EMT-like population is associated with $EpCAM^-CD49f^+$ phenotype, suggesting our model of CSC plasticity: EMT and MET may also apply to normal mammary stem cells.

Targeting Breast Cancer Stem Cells and Their Niche

The relative resistance of breast cancer stem cells to radiation and cytotoxic chemotherapy highlights the need to develop agents able to target this cell population. Self-renewal is one important feature of CSCs and it is been related to metastasis, several signaling pathways mentioned above have been shown to regulate this mechanism therefore targeting these pathways is likely to bring a promising outcome. LPR, which is the coreceptor for Wnt was demonstrated as a promising target for this signalling pathway and both antibodies and compound specific for the blocking the protein showed inhibitory effect in Wnt-driven tumor growth in mouse model (Ettenberg et al. 2010). The most studied compound targeting Notch signalling pathway are γ secretase inhibitors (GSIs), they prevent the releasing of Notch receptor of intracellular domains, which activation of the pathway is dependent on. In vitro breast cancer studies demonstrated efficient induction of apoptosis and prevention of mammosphere formation by GSIs; although severe gastrointestinal toxicity causes termination in majority of leukemia patients, co-treatment with glucocorticoids may overcome the side effects (Seveno et al. 2012). Hedgehog signaling pathway is activated by binding of the extracellular hedgehog protein to patched homologue 1 and thus prevents its inhibitory effect on smoothened homologue (SMO) that activates the downstream molecules. Phase I clinical trial found that the anti-tumor effect of SMO inhibitor in basal-cell carcinomas (Von Hoff et al. 2009).

Increased inflammatory cytokines were observed in serum of patients with high risk of metastasis (Sansone et al. 2007) and poor outcome and the association between inflammatory cytokines and breast cancer stem cells suggest that developing strategies to interfere with these loops may provide a novel strategy to target cancer stem cell populations. The most predominant nonsteriodal anti-inflammatory drug aspirin has been shown reduce the risk of breast and several other cancers (Algra and Rothwell 2012). Repertaxin, a CXCR1/2 antibody was developed to prevent graft rejection and has been reported to be relatively non-toxic in phase I clinical trials. It was found to reduced $ALDH^+$ population in *in vitro* and *in vivo* breast cancer model as well as systemic metastasis (Ginestier et al. 2010). IL-6 receptor also provides a promising anti-cancer target for its role in regulating breast CSCs, our group showed that blocking this receptor overcome the development of therapy resistance and reduce CSC population and tumorigenesis (Korkaya et al. 2012). This suggests that agents designed to target inflammatory cytokines in the breast cancer stem cell niche will be available for clinical testing. The monoclonal antibodies targeting IL-6 or its receptor are currently being evaluated in clinical trials for multiple myeloma (Kastritis et al. 2009).

Vasculature is essential for normal tissue and organs as well as for tumors, and thus it was

postulated as a potential therapeutic intervention for treating cancer four decades ago (Folkman 1971), however anti-angiogenic therapy in breast cancer patients rarely bring long-lasting benefit. Based on the CSC model, this population is likely to survive the treatment and our group demonstrated that anti-angiogenic agents indeed increased the CSC population despite the inhibition effect on tumor growth. More importantly, we demonstrated hypoxia, as a result of anti-angiogenic treatment activated Akt/Wnt/β-catenin pathway that regulates breast CSCs *in vitro*, and the CSCs were located near the hypoxia area of the tumor where β-catenin was also condensed (Conley et al. 2012), suggesting a potential combination treatment combining anti-angiogenesis and Wnt signaling pathway.

Clinical Implications of Breast Cancer Stem Cells

The development of majority of the anti-cancer agents has been focused on their ability to target fast-proliferating cells and the efficacy of these agents in clinical trials has been evaluated based on the so-called "RECIST" (response evaluation criteria in solid tumors) criteria which reflect tumor size as ascertained by direct measurement or through radiographic imaging. However, the non-CSCs or the bulk cell populations determine the tumor size thus this may explain why tumor regression observed in many solid tumors including breast cancer does not always bring better patient survival rate. Since these treatments only target the bulk population rather than the rare CSCs that are able to initiate tumor growth. The breast CSC biomarkers described by us provide the possibility to assess total effect of new drugs in both CSC and non-CSC populations, more importantly it opens the opportunity to design targeted therapy for CSCs.

The percentage of $ALDH^+$, $CD24^-CD44^+$ populations or mammospheres can serve either as the indicators of efficacy of the treatment or cells sorted based on these markers can be given the agent directly to test its CSC-specific effect. Targeting the signaling pathways regulating CSC population such as Notch/Wnt/Hh seems the most effective and straightforward therapeutic intervention. Clinical trials utilizing Hedgehog inhibitor and/or Notch inhibitors have been in Phase I or Phase II to treat breast cancer patients at University of Michigan Hospital. Induction of differentiation towards luminal type is also a potential strategy since these tumors are less aggressive and relatively easy to manage, most importantly we have developed effective hormone and biological therapies against this type of breast cancer. Metastasis remains the major cause of death for incurable cancers and our and others observations suggest that the CSCs are at the frontline of each single step of the process by shifting between different states: EMT and MET. Thus the design and assessment of new compounds need to include all CSC states in order to target not only the CSCs within the tumor at the original site but also those that migrate to local or distant sites and the ones in the circulating system.

The development of breast cancer is not an isolated event happening in mammary epithelium; interactions with local microenvironment are also vital for supporting disease progression. Therefore, the niche is equally important as the breast CSCs and hormones and growth factors secreted from the niche may provide potential therapeutic targets for CSC population. IL-6 and IL-8 for example are secreted by many cell types within the tumor microenvironment and their important role as a messenger between breast CSCs and niche put them under the spotlight of therapeutic intervention. Compounds targeting the receptors of above cytokines are now under clinical evaluation as a combination therapy with cheom-drugs at University of Michigan Hospital. The recent discovery of the rules of microRNAs in breast CSC by our lab also provides another biomarker for tumor diagnosis, prognosis and prediction of therapeutic responses as well as potential anti-cancer target.

The identification and isolation of breast CSCs facilitate us to study the biology of these unique populations and the scientists have made astonishing progress in the field. Many targets have been proposed and several are under

clinical trials aiming to eliminate the CSC populations. However, this does not imply that the elimination of bulk tumor populations is not of therapeutic benefit. Indeed, in metastatic disease, symptoms are largely caused by these bulk tumor populations. Thus the combination of cancer stem cell targeting agents with cytotoxic chemotherapy and/or radiation therapy may provide a means of targeting both cell populations. If the CSC populations are the answer for the hurdle in our fight against cancer, we are now witnessing the beacon of hope that penetrates the cloud in the battlefield.

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Critical Analysis of Parkinson's Disease Models and Cell-Based Therapy

18

Amit K. Jaiswal and Asok Mukhopadhayay

Contents

Abstract

The degeneration of neurons because of genetic mutations and other factors in aging populations causes serious complications in normal brain function, which can result in a variety of progressive dysfunctions. Parkinson's disease (PD) is one type of these dysfunctions that constitutes approximately 0.3% of the total population and 1% of the total old-age population (over 60 years). Because of the severity of this disease, it has drawn attention from medical and scientific communities around the world. The existing treatments, such as L-DOPA administration and/or deep brain surgery (DBS), have been found to further complicate the case of the patient. Therefore, alternate therapies have been investigated. Currently, cell-based therapy is a potential alternative to the previously mentioned treatments because of the promising results obtained from animal experiments and limited clinical trials. The experimental Parkinson's disease models have significantly increased our knowledge on the progression of this disease and the scope of cell therapy. At the same time, the data from these models also raise some questions concerning the relevance of these models in a clinical setting and the efficacy of the treatment.

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Introduction

The degeneration of neurons is one of the primary causes of central nervous system (CNS) damage. Several diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis/low Gehrig's disease (ALS) and Creutzfeldt-Jakob disease, are caused by neuro-degeneration. All of these diseases are characterized by the apoptosis of neurons and the disruption of neuronal networks that have specific physiological importance. Most of the neurodegenerative diseases develop in people who are older. In some cases, however, these diseases develop in younger patients. In such cases, the damage is not restricted to a specific cell type responsible for a specialized task, such as motor function, but affects many different types of neurons, such as nigrostriatal dopaminergic, noradrenalin, 5-hydroxytryptamin and acetylcholine neurons (Chaudhuri et al. 2006). Parkinson's disease is one such neurodegenerative disease that is characterized by tremors, slowness of movement, rigidity, bradykinesia and problems in maintaining balance. Treating patients with L-dopa can alleviate these associated problems, but there is still the possibility the condition of the patient will further deteriorate. Currently, cell-based therapy is considered a promising alternative form of treatment. Many different cell types have been proposed for this type of therapy, and each one of them has specific advantages and disadvantages over the other. Therefore, the search for the most suitable candidate for this cell-based therapy is of great interest, and it is important to increase our knowledge on how this disease progresses and use this knowledge to search for candidate cell types for this type of therapy.

Parkinson's Disease and Its Associated Complications

PD is one type of serious neurodegenerative diseases, which is characterized by the progressive loss of dopaminergic neurons that are specifically involved in regulating various motor and non-motor bodily functions. This disease affects the body asymmetrically. The affected areas are usually responsive to treatments with dopamine or its agonist because these treatments have shown that dopamine depletion is the major cause of the symptoms for this disease. The three principal signs of Parkinsonian syndrome are rigidity, tremors and bradykinesia. Other symptoms associated with this disease are dementia and akinesia. The onset of this disease generally occurs between 21 and 40 years of age, and it becomes especially apparent as the individual ages. In juvenile cases, the onset of this disease can be earlier than 20 years of age (Muthane et al. 1994). People from all ethnic backgrounds, especially males, are prone to this disease. The diagnosis of PD is very difficult in the early phase of onset. It is generally only diagnosed by expert clinicians using specific psychiatric approaches. A tissue biopsy is the only test that absolutely confirms PD detection. Aging and familial are two general forms of PD. The aging form of PD involves changes in cell physiology and accumulative responses because of mutations in genes that have mitochondrial functions. The familial form of PD is genetically transmitted to the offspring and can have an autosomal dominant or autosomal recessive pattern (Samii et al. 2004). This form of PD constitutes approximately 10–15% of all PD cases.

PD is a sporadic type of disease in which several specific pathological changes occur inside the cell, including excessive reactive oxygen species (ROS) production, mitochondrial dysfunction, defective proteasomal degradation of misfolded proteins and other factors that may cause neuroinflammation resulting in cell death. PD is multicentric in nature. This pathological characteristic is most evident in terms of the observed morphological abnormalities, which follow a retrograde trend that starts at the dorsal motor nucleus and ends in substantia nigra pars compacta (SNc). Among all of the neuronal cell types, the dopaminergic neurons in the SNc are the first to degenerate in PD, followed by the degeneration of nondopaminergic neurons. Specific inflammatory changes are considered a common feature of the PD pathology. The biochemical analysis of postmortem brains has

established that mitochondrial dysfunction, i.e., a deficiency in the mitochondrial respiratory chain protein complex I activity in SNc resulting oxidative stress, is another common feature of the PD pathology. Proteasomal inhibition has also been implicated in progressing PD. Proteasome-mediated cleavage leads to the clearance of defective or misfolded proteins. A decrease in cell activity and a reduction in protein subunits in the SNc indicate that there are abnormalities in the ubiquitin proteasomal system (McNaught et al. 2003). It has been reported that the systemic administration of a proteasomal inhibitor in rodents induces the loss of nigral dopaminergic neurons. A recent finding suggests that adult nigral dopaminergic neurons are especially dependent on voltage-dependent calcium channels that play an important role in the interaction between the mitochondria and free radicals. An elevated intracellular calcium flux results in an increase in mitochondrial-derived free radicals, especially within the dendrites, and these events could contribute to a neuron's predisposition to "dying back" neuronal degeneration (Chan et al. 2007). A mutant α-synuclein protein binds with high affinity to lysosomal receptors on the membrane and prevents translocation. This prevention appears to inhibit the chaperone-mediated autophagy (CMA) pathway. Defects in eliminating this mutant α-synuclein protein contribute to the formation of oligomers and aggresomes, which cause PD pathogenesis (Anglade et al. 1997). In the brains of patients with PD, the number of autophagy vacuoles increases over time.

Clinical Complications

PD is not fatal, but it reduces the life expectancy of the patient. It can seriously impair the quality of life for patients in any age group. In general, the disease progresses more quickly in older patients and may severely disable a patient within 10–20 years of onset. Older patients experience difficulty in performing the activities that are commonly conducted in daily routine and occasional restrictions in movement. As the disease progresses, the social life of the patient becomes significantly impacted, and patients become increasingly dependent on family members.

In approximately 70% of all cases, resting tremors with a frequency of 3–5 Hz, which are associated with stiffness in the limbs, is the first sign of PD onset. Eventually, tremors can also occur in the head, lips, hand and feet. Symptoms can develop in one or both sides of the body. These symptoms may worsen with anxiety and lead to the acquisition of contralateral mobility. Approximately a quarter of PD patients do not develop tremors. If PD develops without the symptom of tremors, then the disease will most likely be more severe. Rigidity involves stiffness in flexor and extensor muscle groups that are responsible for general body movements. Muscle stiffness occurs because of a defect in the relaxation mechanism. Postural instability generally occurs once the reflexes are affected, which is during a late stage of disease progression, and results in a difficulty in maintaining balance. Abnormalities in gait, such as reduced arm swing and flexion of trunk in the affected areas, are also observed in Parkinson's patients. These abnormalities are the result of a shortening in the stride length and flexed knees. Bradykinesia begins at the onset of Parkinson's disease, and patients find it more difficult to perform fine motor tasks, such as writing and performing hand movements while walking. PD may also cause non-motor related symptoms, such as depression and anxiety, among the patients. Dementia or confusion can also develop during the later stages of PD. Some patients experience a decreased sense of smell.

Limitation in Present Therapy

Currently, there is no effective treatment for PD. Certain chemotherapeutic agents, such as L-DOPA, which is an analog of dopamine, are used to reduce the symptoms of the disease. The drug L-DOPA is given with inhibitors of aromatic L-amino-acid decarboxylase (AADC).

Carbidopa is used as an inhibitor to prevent the degradation of L-DOPA in the gut, which is associated AADC. The brain striatum also contains AADC. Fresh dopamine synthesized from L-DOPA is stored in the nerve terminals and is slowly released. It stimulates the postsynaptic dopamine receptors and mediates antiparkinsonian activities (Koller and Rueda 1998). The administration of exogenous L-DOPA may result in serious complications, and the severity of the complications varies from patients to patients. In the early stages of PD, L-DOPA treatments can alleviate the symptoms over an extended period of time, but as the disease progresses the longevity of its therapeutic effect decreases (Hauser et al. 2000). Fluctuations in motor responses and involuntary movements are two of the most common complications observed in these patients. Approximately 40% of the patients develop psychiatric complications, including nocturnal confusion, hallucination, vivid dreams, depression, anxiety and delirium (Fenelon et al. 2000). In extreme cases, subthalamic nucleus deep brain surgery (DBS) is performed. An electrode is implanted in the subthalamic nucleus, and a low voltage electrical impulse is generated that improves motor function. Although this treatment is considered safe, complications such as a decline in intelligible speech, postoperative seizures with hemorrhage, edema, and ischemia, may occur. Furthermore, there is a risk of infection, lead migration, vasovagal attack, confusion and lead replacement with this procedure (Doshi 2011). Generally, DBS is beneficial for symptom relief, but it does not alter the progression of PD. Therefore, different treatment strategies are being explored that will provide a definitive solution to PD. These treatments include gene therapy and cell based therapy.

Model Organisms and Clinical Relevance

Animal models not only provide us with insight into the pathophysiology of this disease, but they also enrich our knowledge on the mechanisms underlying the progression of this disease. Currently, many animal models are available that allow us to study various diseases, including neurodegenerative disorders. Generally, the animal models preferred for PD studies are toxin-induced models. Toxins are delivered locally or systemically into rodents or nonhuman primates and manifest pathological and symptomatic behaviors that are similar to those symptoms observed in human cases. Additionally, there are a few genetic models available that allow us to study the progression and pathological behavior of this disease. These genetic models have been generated in rodents and nonhuman primates either by introducing a point mutation in one of the mitochondrial genes or by introducing heterozygous allelic pairs that resemble the monogenic familial form of this disease (Alberio et al. 2012). There are, however, disadvantages to each of these models. Most of the genetic- and toxin-based animal models have problems associated with the reproducibility of results and lack an exact match to the pathology observed in patients. In genetic models, collecting homozygous animals is a rigorous process, and the phenotype is often not uniform within the population. Furthermore, it takes approximately 6–12 months for the animals to develop PD symptoms. Toxin-based models are considered superior for transplantation and engraftment studies, as well as for restoring normal behavior after the neurotoxin challenge. However, the pathophysiology caused by the natural progression of this disease in humans, such as the formation of lewy bodies or α-synuclein protein aggregates, are not seen in these models. In these cases, critical aspects of PD, such as disease progression, pathology and symptomatic changes in behavior, generally observed in patients as the disease progresses must be disregarded. For a better understanding, these types of models are explained in greater detail.

Drug Induced Models of Parkinson's Disease

The toxins that are commonly used in these models are broadly classified into two groups based on the nature of the damage. The first group of neurotoxins, which includes reserpine,

causes reversible damage. The second group of neurotoxins, which includes 6-OHDA, MPTP, paraquat and rotenone, causes irreversible damage to the dopaminergic neurons.

Reserpine

When injected into animals, reserpine depletes monoamines and dopamine, which produce conditions that mimic PD. The symptoms observed after reserpine administration include slowness of movement, rigidity, a hunched posture and akinesia. The drug is administered subcutaneously at a concentration of 3–5 mg/kg body weight, and symptoms are detectable within 48 h of injection. Reserpine acts by increasing the oxidative stress at the striatum. It prevents the storage of dopamine in the transporter vesicles at nerve termini and alters vesicular monoamine transporter (VMAT) function. These events result in an increase in the oxidative breakdown of cytosolic DA by monoamine oxidase, a mitochondrial enzyme. The depletion of DA, as well as the formation of acidic metabolites, cellular oxidants and hydrogen peroxide occur simultaneously. These conditions partially mimic the increased turnover rate of dopamine in the unaffected neuron termini that is observed in PD patients. These symptoms generated by reserpine can be alleviated by administering L-DOPA. Because the fundamental goal is to study the mechanisms regulating disease progression and to test the efficacy of potential therapeutic agents, the reserpine-induced animal model is not suitable.

6-OHDA

6-Hydroxydopamine (6-OHDA) is a derivative of dopamine and is the most widely used neurotoxin for developing a PD model. Approximately 50 years ago, it was first used to create nigrostriatal lesion in rats (Ungerstedt 1968). 6-OHDA administration results in the retrograde degeneration of tyrosine hydroxylase (TH^+) neurons from the striatum to the cell bodies sitting in the substantia nigra pars compacta (SNpc), which precisely mimics the degeneration that occur in patients with PD. 6-OHDA is always used with desipramine to prevent noradrenergic neurons from damage. Although 6-OHDA mimics some of the symptoms of PD, such as the depletion of dopaminergic neurons from the SNpc, memory loss and motor deficit, it does not affect other neurons, which is observed in PD patients. Lewy body formation has been reported in mice treated with 6-OHDA, but these bodies react with α- synuclein monomers, which is not observed in PD patients (Blandini and Armentero 2012). Generally, 6-OHDA is locally injected into the brain striatum, mid-forebrain bundle (MFB) and substantia nigra region. The site of injection can be unilateral or bilateral; however, a bilateral injection can increase the chance of mortality of the animals. Unilateral damage is preferred for behavioral studies and the testing of therapeutics. The drug dosage will generally vary from 3.5 to 7.5 μg per animal. 6-OHDA administration will damage approximately 70–90% neurons of the injection site. The severity of this lesion depends on the number of injections, the drug dose and the site of injection. It has been reported that multiple injections produce more lesions when compared with single injections. 6-OHDA injected in the MFB and substantia nigra produces more severe lesions and results in more animal deaths when compared with injections into the striatum (Blandini and Armentero 2012). This neurotoxin damages catecholaminergic neurons by producing ROS and other derivatives, such as quinones. When 6-OHDA is exposed to oxygen and alkaline condition, it is readily oxidized producing H_2O_2 and para-quinones (Fig. 18.1).

6-OHDA is the most suitable neurotoxin for generating a PD model that can be used in cell-based therapy studies. 6-OHDA selectively affects DA neurons, the ablation of DA neurons by 6-OHDA is reproducible, and it is a derivative of the dopamine metabolic pathway.

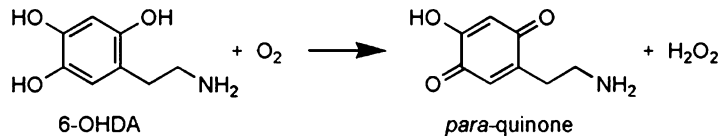

Fig. 18.1 Reaction of 6-OHDA in the physiological conditions

MPTP

1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is a derivative of MPPP (1-methyl-4-phenyl-4-propionpiperidine). MPTP was accidently discovered in 1982 by a graduate student in the United States, when he was trying to synthesize MPPP and was exposed to MPTP. He developed Parkinson-like symptoms, including severe bradykinesia (Fahn 1996). MPTP causes irreversible damage to dopaminergic neurons and the effects of this drug are instantaneous. Most of the dopaminergic neurons are depleted within a few days of administration, and the remaining neurons may take years. Apart from 6-OHDA, use of MPTP has now become popular because of its ability to generate irreversible Parkinsonian symptoms in the mouse, monkey, and other species. Notably, rats are resistant to MPTP-based neurodegeneration because of some unidentified reasons. This drug is administered by either subcutaneous or intraperitoneal injection. The local administration of this drug in the brain has a high mortality rate in mice. The intranasal administration of MPTP is also effective in depleting the expected amount of DA neurons. The severity of lesion formation depends on the dosage; the most effective dose is 20 mg/kg of body weight. Frequently, 4 injections per day will deplete 90% of striatal dopaminergic neuron and 70% of SNpc dopaminergic neurons (Jackson-Lewis et al. 2012). It forms the active compound MPP^+ through a complex oxidative process. Pathological changes that take place in mice include neuroinflammation and increased ROS production. The oxidative stress and reduced energy generated by the mitochondria impair the functioning of the mitochondrial protein complex I of the electron transport system (Singer and Ramsay 1990). MPTP is highly lipophilic and readily crosses the blood-brain-barrier (BBB). Once inside the brain, astrocytes take up MPTP,

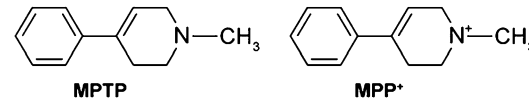

Fig. 18.2 Structure of MPTP and MPP^+

and monoamine oxidase-B (MAO-B) cleaves it, generating MPP^+ (the active compound) as shown in Fig. 18.2.

This active metabolite is released in the extracellular space through the OCT3 transporter (Cui et al. 2009). The DA neurons internalize MPP^+ through DAT (dopamine transporter) and store it in vesicles through vesicular monoamine transporter-2 (VMAT2). This active metabolite impairs the ETS protein complex I, which results in the excessive generation of ROS, and decreases in energy production by the cells. In non-human primates, such as the rhesus macaque and squirrel monkey, the administration of MPTP generates symptoms that are comparable to the symptoms observed in PD patients. Studies using MPTP provide insight into the molecular crosstalk involved in the neuronal degeneration characteristic of PD and the complex events involved in mitochondrial dysfunction. This model is also beneficial for drug research studies that aim to prevent neuronal loss due to mitochondrial dysfunction, which is another symptom of PD pathogenesis. Major limitations of MPTP studies are the variety of neurotoxic responses observed in different animal strains and the reproducibility of the lesions.

Rotenone

Rotenone is the most potent neurotoxin of the rotenoid family. It is used as a herbicide and pesticide. Rotenone is a flavonoid that is frequently found in the roots and stems of leguminous plants from tropical environments. It is

sensitive to light with a half-life of 3–5 days, depending upon the exposure to light. Similar to MPTP, rotenone is highly lipophilic in nature and can easily cross the BBB. It accumulates in the CNS and reaches its full concentration in just 15 min (Talpade et al. 2000). It is administered by either subcutaneous or intraperitoneal injection. In some cases, however, the oral administration is also found to be effective. To increase the percentage of lesions, the neurotoxin is injected using an osmotic pump. This drug produces nigrostriatal damage, but the results are not consistent. The cellular mechanisms induced by rotenone are similar to the mechanisms induced by MPTP. It inhibits the mitochondrial electron transport system. It binds with the PSST subunit of mitochondrial protein complex I and thereby inhibits NADH–ubiquinone reductase activity. It also inhibits microtubule formation, which arrests cell division. Some reports suggest that rotenone inhibits the proteasomes and leads to the excessive accumulation of aggregated proteins in the cell and increased production of ROS. Characteristic pathological changes produced by this neurotoxin include the formation of α-synuclein protein aggregates and lewy bodies. The behavioral symptoms observed in rodents include motor function deficits, such as reduced mortality, flexed posture and rigidity. The rotenone-based PD model is used to study the mechanisms of disease progression caused by mitochondrial dysfunction, α-synuclein protein aggregate accumulation and disease pathogenesis. Disadvantages of using the rotenone-based model are that the lesions appear to be less severe when compared with other neurotoxins and that lesion severity may vary from animal to animal and strain to strain and are often not reproducible.

Paraquat

Paraquat generates oxidative stress by inducing the redox cycling of nitric oxide synthase, which leads to the production of ROS. The mechanisms induced by Paraquat are similar to the mechanisms induced by MPP^+, and it has been found to be more effective when used in combination with maneb. Paraquat is hydrophilic by nature. It is not able to cross the BBB and must be transported using a neutral amino acid transporter. This toxin is frequently administered subcutaneously. In many reports, the reproducibility of the results and efficacy of treatment were reported to vary depending on the animal strain, the weight and age of the mice, and the duration of toxin administration. Older mice appear to be more sensitive to paraquat toxicity. Paraquat activates mitochondrial Cytochrome c and induces apoptosis. This model is ideal for studying the mechanisms and pathways that result in DA neuronal death.

Genetic Models of Parkinson's Disease

In addition to aging and environmental factors, familial forms of Parkinson's disease have also been observed in 10–15% of all patients. The familial forms of PD occur because of mutations in genes that are essential for normal cell function. Because a mutation in a specific gene can result in PD, scientists have been compelled to develop animal models using genetic mutations. Currently, many different genetic models are available for studying the progression and pathology of this disease. These genetic models have been generated by introducing one or more mutations in specific genes that have been found to be responsible for developing Parkinsonism in humans.

Alpha-Synuclein Deficient

To investigate the functional role the *SNCA* gene plays in the progression and pathology of PD, a model based on an α-Synuclein protein deficient was generated. To generate this model, three types of point mutations, i.e., A30P, A53T and E46K were introduced in the α-synuclein gene. This model is important for studying disease progression, the aggregation of defective α-synuclein and the formation of lewy bodies. As the animals age, they develop a nigrostriatal loss

of dopamine content as well as reduced motor and non-motor dysfunction. Slowness of movement, gastrointestinal alterations and olfactory deficits are also been reported in these mutants. However, this model fails to replicate the neurodegeneration observed in the nigrostriatal region of PD patients.

LRRK2

Leucine-rich repeat ser/threonine kinase 2 (LRKK2) mutations are more prevalent in the autosomal form of Parkinson's disease and cause a dominant form of PD (Blandini and Armentero 2012). There are six different forms of this disease caused by mutations in this gene. Genetic models generated by introducing G2019S and R1441G mutations in the LRRK2 protein demonstrate an age-dependent reduction in DA content, where the uptake and release of DA from the striatal region is affected, motor deficit and akinesia is also noticed. Mutations in LRRK2 promote the aggregation of α-synuclein and the phosphorylation of α-synuclein at Ser129, which is frequently observed in lewy bodies. This mouse model, however, fails to result in DA neuron death.

Parkin

Parkin is an E3 ubiquitin ligase and plays an important role in proteasome-mediated degradation. A large number of mutations in parkin may cause PD pathogenesis. One of these mutations is Q311X, which selectively affects dopaminergic neurons and causes nigrostriatal degeneration, followed by a hypokinetic motor defect and a resistance of α-synuclein to proteinase K activity. It has been reported that parkin mouse models are more susceptible to neurotoxins and other inflammatory signals. This model is frequently used to study the neurodegeneration that occurs during PD progression (Lu et al. 2009).

In addition to genetic mouse and rat models, various other model organisms have also been used, such as *C. elegans, Zebra fish* and *Drosophila melanogaster*, because of their more simplistic body architecture, their neuronal circuitry and their similarity to human neurophysiology. Animal models other than mammalian models may further enrich our understanding of how this disease progresses and may be beneficial in testing various pharmacological drugs in their efficacy against PD progression.

Current Cell Therapies in Practice

Because there is no effective treatment for PD, there is an urgent need to find a new and efficient therapy for Parkinson's disease, and recent findings indicate that gene- and cell-based therapies are the most promising types of therapy for this disease. To be an effective cell-based therapy the following criteria must be met: (1) it should be safe and non tumorigenic, (2) it should be readily engrafted into the host system and should not cause any side effects, such as graft-induced dyskinesia, (3) therapeutically effective cells should readily proliferate in culture to meet the number requirement for a clinical transplantation, and (4) the graft should support the system and completely alleviate motor and non-motor behavioral defects. Many candidate cells have been tested in animal models, and used in therapeutic applications in patients.

Embryonic Stem (ES) Cells

ES cells have the capacity to differentiate into a vast number of cell lineages from all three germ layers. In a previously reported study DA neurons that had been generated by over-expressing Nurr1 in ES cells were transplanted into mice (Kim et al. 2011). These mice showed partial recovery in terms of function, morphology and electrophysiology (Kim et al. 2002). In another study, the survival and/or phenotypic stability of ES derived DA neurons was found to be poor and tumors were observed among the intrastriatal transplanted cells. Newer techniques for this type of therapy have been used to

increase the engraftment potential and propensity for neuronal differentiation as well as to minimize the occurrence of tumors. ES cells were first differentiated into neuroblasts that were then differentiated into specific cell types, such as glial cells and DA neurons, using a cocktail of cytokines. When these *in vitro* differentiated neurons were transplanted into a mouse and monkey PD model, they produced a better engraftment and formed more TH^+ neurons. Furthermore, these cells innervated the entire region of the striatum in the mouse brain. These mice also showed a significant level of recovery in terms of motor and non-motor function (Kriks et al. 2011).

Induced Pluripotent Stem (iPS) Cells

Induced pluripotent stem cells were first generated by inserting the genes for four transcription factors, known as Yamanaka factors, into fibroblast cells. These modified cells had an ES cell-like pluripotent nature and generated tremendous interest in the field of clinical transplantation. Under neuronal differentiation culture conditions, iPS cells can gave rise to specific neurons depending on the specific types of cytokines used for differentiation (Swistowski et al. 2012). The iPS-derived neurons showed inadequate engraftment and had poor functional recovery when they were transplanted into a PD mouse model. In iPS cells, there is the possibility that the viral genome and oncogenes could have integrated into the genome of the differentiated neurons, which may result in the transformation of the cells. A non-viral protein based human iPS cell-derived DA neuron showed higher engraftability in a PD rat model, which also showed a significant recovery from motor defects. It has been reported that human iPS cells are easily cultured and amenable for differentiation and transplantation in the clinical settings (Rhee et al. 2011).

Neural Stem Cells (NSCs)

NSCs were first introduced in a study by Altman and Das (1965). NSCs are multipotent in nature and can be obtained from both the fetal and adult brain. They can give rise to various cell types of the nervous system, such as astrocytes, oligodendrocytes, and dopaminergic neurons, if they are exposed to specific cues. For cell therapy, NSCs must first differentiate into A9 midbrain mesencephalic neurons before they can repopulate the striatum of a PD mouse model. Different reports suggest that mice can successfully recover from PD using fetal or human NSCs. NSCs-derived neurons can survive and migrate into target sites. These results suggest that NSCs can be used as an alternate source of cells in therapies treating PD and other neurological disorders (Schwarz et al. 2006).

Fetal Ventral Mesencephalic Tissue (FVMT)

The use of fetal tissue in PD cases was discouraged after adverse results were reported in a clinical trial involving PD patients. More recent studies, however, have provided encouraging results on graft survivals lasting for more than 4–12 years (Ma et al. 2010). Patients were found to be able to survive without L-DOPA treatments for such long periods. These cells restored various motor and non-motor deficits in the patients. A major problem with using fetal tissue is the contamination of DA neurons with serotonergic neurons, which may result in further deterioration and additional complications with the neurological problems. These additional problems would be the result of hyper-proliferation and activation caused by contaminating serotonergic neurons, but they can be mitigated by using a serotonergic blocker that can effectively restore normal behavior in the patients.

Induced Neurons (iN)

The most recent development in the field of cell therapy is the use of induced neurons (iN), which are human or mouse fibroblasts that have been transfected with a combination of genes, such as Brn2, Ascl1 and Myt1, that are referred to as the master transcription factors (Hong et al. 2009).

Once transfected, these cells differentiate into NSCs, which are characterized by the expression of NeuN, GFAP, and Tuj1. Functionally, these cells are characterized by their ability to form synapses that express synapsin and synaptotagmin and generate action potentials (Pang et al. 2011). DA neurons can also be generated by expressing the genes Lmx1a, Ascl1, Pitx3, Nurr1, Foxa2 and EN1. The induced DA neurons are positive for all of the necessary DA markers. These forcibly differentiated DA neurons could be successfully engrafted in the striatum and could alleviate PD symptoms when they were transplanted in 6-OHDA induced PD mice (Kim et al. 2011).

Mesenchymal Stem Cells (MSCs)

MSCs are multipotent and can differentiate into various cell lineages, including dopaminergic neurons (Trzaska et al. 2007). There are many sources from which MSCs can be isolated, such as bone marrow, adipose tissue, Wharton jelly, cord blood, fetal liver and dental pulp. These cells are easily propagated in culture, and their maintenance is also relatively less complicated when compared with neuronal cells. It has been shown that bone marrow-derived MSCs can be successfully engrafted in tissue and help to partially alleviate the motor and non-motor defects when they were transplanted into PD rodent models (Hellmann et al. 2006). It has also been shown that MSCs assist in the regeneration of brain cells, which shows that it also plays a paracrine role. Because MSCs are hypoimmunogenetic, allogenic sources of cells can be used for this type of therapy.

Limitations of Cell Therapy

Before planning a clinical trial in PD patients, the following information should be available: (a) a thorough patient evaluation, in terms of genetic background, nature of the mutation, severity of the disease and age, should be conducted, (b) there should be a well-defined protocol for the rescue procedure in case any complications arise as a result of the therapy, (c) any cell type used in a transplant procedure should be thoroughly characterized, and differentiated into DA neurons using well established cues, and finally (d) cells should not express any residual or exogenous antigens, and they should be safe for the patient (Lindvall et al. 2012). Some of the disadvantages for the different forms of cell therapies are described next.

ES Cells

The major limitations of ES cell-based therapy are teratoma formation, a less efficient differentiation rate and low engraftment potential. It is recommended that ES cells should be differentiated into DA neurons prior to transplantation. For safety reasons, the nonspecific-differentiated cells and undifferentiated ES cells should be removed from the DA neuron population prior to transplantation.

iPS Cells

The main limitation of iPS cells is that they are formed by using a retroviral vector system. Genes, such as the proto-oncogene *c-Myc*, can cause cancerous growth in the brain tissue if they are integrated into the genome of the patient. Furthermore, the retroviral genome can randomly integrate into the host genome, which may cause cells to transform into cancerous cells, and the viral capsid protein can elicit an immune response in patients.

FVMT

The limitation of using fetal tissue as a graft is that it contains a mixture of A8, A9 and A10 neurons, which can engraft in the SNpc, ventral tegmental and rostrorubral regions of the brain (Bayer et al. 1995). Only the A9 neurons are crucial for alleviating Parkinsonian symptoms. Contamination with A10 neurons can induce

dyskinesia in patients. Therefore, in this case, only purified A9 cells should be transplanted into the brain.

iN

Induced neurons are terminally differentiated cells; they cannot proliferate to generate the large number of cells required for a clinical transplantation. Therefore, these neurons must be generated in large quantities prior to transplantation.

MSCs

The propensity of MSCs to differentiate into neurons is low when compared with ES cells. A robust and efficient protocol for differentiating MSCs into DA neurons must be established. A pro-neural differentiated form of these cells is expected to improve the functional and behavioral responses in PD models (Jin et al. 2008).

Taken together, the currently available cell therapies do not fulfill their expected long-term therapeutic potential. Furthermore, in case of FVMT, postmortem brain analysis show that α-synuclein protein aggregates and lewy bodies had formed in the grafted tissue (Kordower et al. 2008). This finding suggests that the donor-derived DA neurons can also be affected by host cells. The mechanisms underlying this effect, however, are unknown.

In conclusions, the use of cell therapy for treating PD is in a very early stage of development. Long-term transplantation experiments need to be conducted in animal models to investigate the robustness of the behavioral recovery observed in the hosts. It is also necessary to determine if the transplanted cells form tumors in the host. Candidate cells for this type of therapy should be selected based on whether they meet the following requirements: they have a high propensity to differentiate into DA neurons, they have high engraftability, they provide long-term functional recovery, and they are non-tumorigenic.

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Presence of an Early Lineage Stem Cell Phenotype in Meningioma-Initiating Cells

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Prakash Rath, James M. Wilson, and Huidong Shi

Contents

Abstract

Meningiomas are a common form of adult brain tumors. Although most meningiomas are benign tumors that are associated with favorable outcomes, a small group of patients develop more aggressive manifestations which currently are more difficult to treat. It has increasingly been recognized that tumor stem-like cells play critical roles in tumor recurrence, angiogenesis, and invasion in malignant brain tumors. Several recent studies identified the stem-like features of sphere-forming cells in human meningiomas. These meningioma sphere cells expressed various progenitor cell biomarkers and can undergo differentiation if appropriate stimuli are applied. Moreover, these meningioma stem-like cells are more resistant to irradiation treatment, and tumorigenic in *in vivo* xenograft models. These new findings could lead to a better understanding of the development and etiology of meningioma formation and suggest that meningioma stem-like cells may serve as a novel target in therapeutically resistant meningiomas.

Introduction

Meningiomas are the most common primary intracranial tumor accounting for 35% of all primary brain and CNS tumors, with an incidence rate of approximately 7 per 100,000

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person-years in the United States. Meningiomas and its variants are well-characterized solid tumors, however, just a few years ago little was known if a stem cell phenotype was associated with meningioma development. Recently a handful of studies have reported the presence of a meningioma stem-like phenotype in benign and malignant variants, which suggests that developing directed therapies against this phenotype may be a promising approach for treating tumors that are aggressive or radio- and chemotherapy resistant. Another important aspect of understanding the presence of a stem cell phenotype would be to gain a deeper understanding of the development and etiology of meningiomas. More aggressive meningiomas such as atypical meningiomas generally have some malignant features such as an increase in mitotic activity and represent about 6–8% of all meningiomas (Wrobel et al. 2005). As tumor grade increases variants such as anaplastic meningiomas acquire more aggressive phenotypes and are thought to have a higher association with a stem-like phenotype. This is because stem cells share characteristics with higher-grade tumors such as the maintenance of self-renewal and the ability to differentiate, which suggests that high grade tumors are good candidates for a stem cell-based targeted therapeutic approach. In the following chapter we examine the current literature identifying and characterizing meningioma-initiating cells and discuss opportunities and challenges associated with targeting the meningioma stem-like phenotype.

Genetics and Epigenetics Associated with Meningioma Formation

According to the most recent WHO classification of meningioma, there are 16 different variants or subtypes, falling into three grade designations, namely Grade I (benign), Grade II (atypical), or Grade III (anaplastic/malignant) (Louis et al. 2007). Although the majority of meningiomas (90%) are benign and slow growing Grade I tumors, atypical and anaplastic meningiomas display more aggressive clinical behaviors, with a median survival time of anaplastic meningiomas being less than 2 years. The histopathological subtypes of meningioma are highly complex; Grade I tumors consist of meningothelial, fibroblastic, transitional, psammomatous, angiomatous, microcystic, secretory, lymphoplasmacyte-rich, or metaplastic subtypes. Grade II meningiomas are typically more aggressive clear cell and chordoid tumors, and Grade III tumors are usually rhabdoid or papillary subtypes (Pham et al. 2011). It remains to be determined what the genetic and molecular basis for the wide variety of biological behaviors and histopathological subtypes observed in meningiomas are.

Compared with other common brain tumors such as gliomas, the genetics and epigenetics of meningiomas are poorly understood and relatively understudied. Neurofibromatosis 2 (NF2), the homologous protein DAL-1 (differentially expressed in adenocarcinoma of the lung), and several tissue inhibitors of matrix metalloproteinases (TIMPs) have been identified as the tumor suppressor genes most commonly involved in the development of meningiomas (Pham et al. 2011). NF2, located on chromosome 22, has been implicated in more than half of spontaneous meningioma cases. Loss of heterozygosity of DAL-1 was found in more than 60% of sporadic meningiomas (Gutmann et al. 2000). Interestingly, both NF2 and DAL-1 were found to be associated with the protein 4.1 family as part of the actin cytoskeleton, which is certainly involved in the regulation of cell growth and proliferation in human tumors (Pham et al. 2011). TIMP1 and TIMP3 have been implicated in the aggressive behavior and invasion of meningiomas (Pham et al. 2011). These proteins can regulate the MMP activities and therefore play an important role in the regulation of cell proliferation, apoptosis, and angiogenesis. Genome-wide copy number analyses have identified additional genetic components that contribute to the more aggressive meningioma variants such as the loss of chromosomal regions 1p, 6q, 10, 14q, 18q, 22q or the gain of 1q, 9q, 12q, 15q, 17q and 20q (Weber et al. 1997; Watson et al. 2002; Wrobel et al. 2005; Keller

et al. 2009). Deletions in chromosome 9p21 which harbors tumor suppressor genes *CDKN2A* ($p16^{INK4A}$), $p14^{ARF}$, and *CDKN2B* ($p15^{INK4B}$) are the most common genetic alterations in high-grade meningiomas and are known to cause cell-cycle dysregulation at the G1/S phase checkpoint (Bostrom et al. 2001; Simon et al. 2007). With the development of next-generation sequencing technologies, whole genome, exome, and transcriptome sequencing in meningioma patient samples may provide new insight into the genetic alterations and pathway dysregulation of meningiomas, which will ultimately lead to a better understanding of the molecular signature for meningioma variants.

Epigenetic mechanisms such as DNA methylation also play an important role in tumorigenesis and progression. Tumor suppressor genes frequently inactivated by aberrant promoter hypermethylation in meningiomas include NDRG2, WNK2, DLC-1, MEG3, TIMP3 and many others (Lusis et al. 2005; Hankins et al. 2008; Jun et al. 2009; Barski et al. 2010; Zhang et al. 2010). A specific aberrant hypermethylation profile has also been associated with atypical and anaplastic meningiomas, suggesting that epigenetic change may be involved in malignant progression of meningiomas. In a recent study using genome-wide DNA methylation analysis, it was found that a subgroup of meningiomas is characterized by aberrant hypermethylation of a subset of genes in the early stages of tumorigenesis, thus highlighting the possibility of using DNA methylation as a means of predicting the potential malignancy of low-grade meningiomas (Kishida et al. 2012).

One of the consequences of aberrant genetic and epigenetic changes in meningiomas is activation of signaling pathways that promote cell proliferation and survival. Gene expression profiling has identified alterations in the Hedgehog and Notch signaling pathways in meningiomas (Cuevas et al. 2005; Baia et al. 2008; Laurendeau et al. 2010). Particularly, the Hedgehog and Notch pathways have been linked to the progression of meningiomas (Cuevas et al. 2005; Laurendeau et al. 2010). These signaling pathways are tightly regulated during the tissue development process and have been implicated in maintaining the pluripotency of normal and tumor stem cells. A further understanding of the signaling pathways involved in meningioma tumorigenesis may lead to the development and application of novel molecular treatments such as small molecule inhibitors or RNAi–based therapeutics.

Evidence for the Presence of Meningioma Initiating Cells

The cancer stem cell hypothesis suggests that subsets of rogue cells that reside within solid tumors are resistant to conventional therapies and are responsible for the recurrence of tumor growth. The presence of these cancer cells with stem cell properties has been well documented in many cancers including brain tumors, primarily in glioblastoma and glioma variants (Singh et al. 2004; Bao et al. 2006; Stiles and Rowitch 2008). Here we denote that cancer cells possessing the stem cell phenotype broadly as TICs or CSCs, referring to the tumor-initiating/cancer stem cell populations. Although the discussion is beyond the scope of this review, note that there are intricate differences between cells that possess tumor-initiation capabilities and cells that possess stem cell properties, as these are not mutually exclusive properties.

Literature examining the presence of TICs in meningiomas is relatively recent with the majority of studies appearing within the last few years (Hueng et al. 2011; Kalamarides et al. 2011; Rath et al. 2011; Hu et al. 2012). The impediment in identifying the meningioma stem cell phenotype compared to gliomas can perhaps be attributed to the fact that the overwhelming majority of meningiomas are benign, and the stem cell phenotype is more identifiable with the malignant and aggressive tumor characteristics.

One of the first papers to describe tumor stem-like cells in meningiomas was Hueng et al. (2011), who tested nine human benign and atypical meningioma samples for the presence of stem cell features such as self-renewal, differentiation, stem cell marker expression profiles, and

resistance to chemotherapy (Hueng et al. 2011). Cells that were able to form meningioma spheres in specially defined medium were characterized based on the expression of CD133, the putative stem cell marker in these studies, EMA, and Vimentin. Together with drug resistant gene expression profiling, chemotherapy and radiation therapy studies, they concluded that the meningioma spheres that arose from the primary tumor conferred more resistance than their serum grown counterparts. They followed up with tumorigenicity studies *in vivo*, which indicated that meningioma sphere cells were able to recapitulate the primary tumor from which they were derived from. This set of experiments suggests that meningiomas possess characteristics associated with stem cells, and in particular glioma stem cells, given the association with CD133.

Rath et al. (2011) took a similar approach to isolate meningioma-initiating cells by screening fresh meningioma samples and characterizing the cells that survived and multiplied in a mitogen containing stem cell medium. They employed a molecular genetics approach using microarray and pathway analysis technologies to gain a deeper understanding of the study population of cells that were isolated from an atypical meningioma specimen. The isolated cells were passaged as spheres in mitogen containing medium and possessed stem cell-like properties of self-renewal and differentiation. Tumorigenic and microarray studies showed that the cells were able to recapitulate some of the histological and molecular features of the parental tumor (Rath et al. 2011). The meningioma-initiating cells, or MICs as they were referred to, contained hallmark features of both meningiomas and cells undergoing an epithelial to mesenchymal transition (EMT) program (Weber et al. 1997; Simon et al. 2007). Studies have shown that EMT can generate cells with self-renewal properties, and possibly other features of stemness (Mani et al. 2008). A gene expression array analysis revealed that both CD44 and CD166 were up regulated in these cells, which were also identified in the primary and xenograft tumors, and confirmed by flow cytometry and immunohistochemistry. Pathway analysis identified a gene network that included many differentially expressed genes including the deregulated Wnt/β-catenin signaling pathway as a molecular growth mechanism of the MICs. To determine if CD166 and CD44 can be used as biomarkers for identifying a stem-cell like population in atypical meningiomas additional studies are necessary.

Hu et al. (2012) utilized a similar approach for characterizing and investigating the presence of tumor initiating cells in a rhabdoid meningioma, but began by culturing cells adherently for multiple passages before plating as sphere cultures (Hu et al. 2012). Flow cytometry and immunohistochemistry identified that CD105-positive cells could be maintained in culture much longer than their negative counterparts, and were able to recapitulate the parental tumor when injected into nude mice. CD105-positive cells were able to differentiate and maintain characteristics attributable to the parental tumor when tested via FISH analysis. Although CD105 may be a biomarker for a subset of cells defining certain histological meningioma subtypes, additional screening studies are necessary to determine if this may be used as a biomarker for TIC identification.

Kalamarides et al. (2011) have been investigating mouse models of meningiomas to identify the cell of origin with respect to major meningioma histological subtypes (Kalamarides et al. 2011). Through clever Cre-recombinase assays, they were able to show that a common event in meningioma development, the loss of the *NF2* gene, is time sensitive in driving prostaglandin D2 synthase (PDGS) positive primordial meningeal cells to a fibroblastic histological subtype, or towards a meningiothelial subtype. They concluded that the spatiotemporal locations of the meningiomas, such as the inner part of the dura mater or outer part of the arachnoid, are derived from this common PDGS positive progenitor cell. Studies such as this provide clues as to the cell of origin and the developmental window for meningioma formation, and will be insightful for developing a repertoire of

biomarkers for early detection and therapeutic paradigms in human analyses.

The identification of a stem or initiating cell presence can be linked to the markers and techniques that identify them. Although a large scale study has not been conducted to screen for the presence of stem cells, the growing literature of meningioma stem cell studies helps us build a character reference for identifying this subpopulation. Additionally, developing reliable identification tools will be invaluable for designing targeted therapies against this population of cells.

Targeting Meningioma-Initiating Cells

The goal of studies aimed at identifying CSC populations in meningiomas or other solid tumors is to be able to predict the outcome but more importantly to be able to design targeted therapeutics that will be effective in killing this population of cells directly or indirectly. Since the CSCs are resistant to conventional cytotoxic drugs, novel therapeutic approaches specifically targeting the CSC population should result in a better control of the disease. Therefore one might expect that combinatorial treatments involving both cytotoxic and targeted therapies, including those against CSCs, are probably required in order to ablate all cancer cells. Targeting CSCs will likely require both direct and indirect approaches. A directed approach includes the targeting of CSCs via monoclonal antibodies against cell surface molecules (CSC markers), targeting CSC developmental stem cell pathways to overcome their resistance to radiation and chemotherapy, or blocking their function by inducing differentiation (Binello and Germano 2011; Diaz and Leon 2011). Indirect targeting approaches will focus on the microenvironment and CSC niches (Binello and Germano 2011; Diaz and Leon 2011). Overall, it is expected that targeting the CSC population in meningioma will provides an opportunity to treat aggressive forms of disease that have poor patient outcome.

There are no reports yet on targeted therapies for stem-like cells in meningiomas. However, such studies in gliomas and other brain tumors may be used to guide the development of targeted therapeutics for meningioma initiating cells. For instance, EGFR is a well-known molecular target for multiple tumor types. Previous studies have demonstrated the ability of the anti-EGFR antibody to target the CD133+ CSC population, which acts as a radiosensitizer in brain tumor xenograft models (Diaz Miqueli et al. 2009). EGFR is overexpressed in more than 60% of meningiomas (Norden et al. 2007) and EGF is a mitogen that seems to be necessary for maintaining the proliferation of meningioma sphere cells in *in vitro* cultures. Small molecule inhibitors of EGFR and anti-EGFR antibodies can block the binding of EGF to EGFR. Two EGFR inhibitors, gefitinib (Iressa, Astra-Zeneca) and erlotinib (Tarceva, Genentech) have been used in clinical trials for recurrent meningioma, however, neither gefitinib nor erlotinib appear to have significant activity against recurrent meningioma (Norden et al. 2010). Although the direct clinical benefits were not observed in the two clinical trials, evaluation of EGFR inhibitors in combination with other molecular therapies may be warranted.

As discussed earlier, several development-related pathways such as the Hedgehog and Notch pathways are altered in meningiomas. Our microarray study of differential gene expression between a meningioma stem-like cells and normal neural stem cells also revealed significant changes in WNT pathway genes (Rath et al. 2011). These pathways play important roles in stem cell self-renewal and direct the stem cell differentiation, which represents an attractive approach for targeting CSCs. Small molecule inhibitors of the Hedgehog pathway such as GDC-0449 are currently in clinical trials for treatment of various cancer types and several trials completed so far show encouraging results (Gupta et al. 2010). The most clinically advanced Notch pathway inhibitors are γ secretase inhibitors. The activation of Notch pathway depends on the proteolytic activities of γ secretase, which cleaves Notch receptors releasing their intracellular domain. Currently, there are more than 30 clinical trials for Notch signaling inhibitor, RO4929097, listed on ClinicalTrials.gov. Several

other signaling pathways such as the PI3K/Akt and mTOR pathways can also play important roles in the regulation of self-renewal of CSCs. Both Akt and $p70^{S6K}$ are constitutively expressed and activated (phosphorylated) in benign meningiomas and play a role in signal transduction from PDGFR pathway. On the other hand, NF2-deficient meningioma tumors demonstrate elevated mTOR signaling (James et al. 2009). Collectively, these suggest that inhibitors of Akt and mTOR may also be used in targeting meningioma stem cells.

Meningioma stem cells reside in and are supported by the stem cell niche, which is not only an anatomic structural unit surrounding stem cells, but also a functional unit providing complex and dynamic interactions with stem cells. Therefore, there is an increasing interest in the possibility of targeting the CSC niche to disrupt the cancer stem cell functions. In glioma models it has been demonstrated that freshly isolated CD133+ glioma stem cells, but not the CD133-population formed highly vascular tumors in the brains of immunocompromised mice (Bao et al. 2006; Calabrese et al. 2007). We have also found that the meningioma stem-like cells form highly vascular tumors in xenograft models, suggesting that meningioma stem-like cells are capable of promoting angiogenesis for its growth. The CD133+ glioma stem-like cells can promote angiogenesis by secreting high levels of VEGF (Calabrese et al. 2007). The anti-VEGF antibody Bevacizumab (Avastin, Genentech) has been shown to inhibit the angiogenesis and suppress the growth of xenografts derived from CD133+ glioma cells (Calabrese et al. 2007). VEGF and VEGFR are expressed in meningiomas, and the level of expression increases with tumor grade. Expression of VEGF is increased two-fold in atypical meningiomas and ten-fold in malignant meningiomas when compared with benign meningiomas (Lamszus et al. 2000). This suggests that inhibitors of VEGF and VEGFR inhibitors may be promising agents for meningioma treatment, with the potential to inhibit angiogenesis and target the CSC niche. At least two on-going clinical trials of Bevacizumab in recurrent or progressive meningiomas are ongoing at this time, and a phase II clinical trial of small molecule inhibitors targeting VEGFR, sunitinib (Sutent, Pfizer) is currently recruiting patients with recurrent or inoperable meningioma.

In conclusion, the discovery of stem-like meningioma cells provides a window of opportunity to better understand this highly complex disease and identify new therapeutic targets for recurrent and aggressive forms of meningioma subtypes. However, further studies are needed to replicate and confirm the handful of studies which used a limited number of samples in order to generalize the concept of the existence of an early lineage stem cell phenotype in meningiomas. Although cell surface marker CD133 has been identified as the most common identifier among several studies, it is still questionable if CD133 is the most specific marker of CSCs in meningiomas. Other markers such as CD166, CD44, CD105, or PDGS warrant further investigations. Targeting the CSC population and their niches offers true potential for developing therapeutic strategies capable of extending patient survival, and will come to fruition as more studies in this area of brain tumor biology emerge.

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Isolation and Characterization of Cancer Stem Cells from Dog Glioblastoma

20

George Stoica and Gina Lungu

Contents

Abstract

There is increasing evidence in some malignancies that the tumor clone is heterogeneous (phenotypically and functionally) in regard to proliferation and differentiation. The cancer stem cell hypothesis implies that not all the cells in the tumor have the same capacity to proliferate and maintain the growth of the tumor. Only a relative small fraction of cells in the tumor, termed cancer stem cells (CSCs), possess the ability to proliferate and self-renew extensively. In the past decade, several groups have reported the existence of a CSC population in different human brain tumors from both children and adults. Our study demonstrated the presence of a CSC population from a dog with glioblastoma multiforme (GBM) that possesses a great capacity for proliferation, self-renewal, and differentiation. This cell line is aneuploid, forms neurospheres in culture, possesses CSC markers, and reproduces the original dog GBM when inoculated into the nude mouse brain. A comparative approach to the study of canine glioblastoma CSC provides an excellent animal model for human glioblastoma pathogenesis and development of novel therapies in both species.

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Introduction

Brain tumor stem cells are resistant to conventional radiation and pharmacological treatments (Bao et al. 2006; Liu et al. 2006). Development of more effective therapies for glial malignancies would be facilitated by the existence of *in vitro* and *in vivo* models that faithfully recapitulate the stem cell component of these lesions. The cancer stem cell hypothesis implies that tumors contain a minority of cells with stem-like properties, which have the ability to self-renew and sustain the growth of the tumor (Clarke and Fuller 2006). Tumor stem cells were first identified in hematopoietic malignancies, and more recently were also demonstrated in various solid tumors, including cancers of the brain such as glioblastomas, medulloblastomas, and ependymomas (Singh et al. 2004; Galli et al. 2004; Lee et al. 2006). Brain tumor stem cells in gliomas are defined by (i) the capacity to self-renew, (ii) the ability to initiate brain tumors upon orthotopic implantation and (iii) multipotency, that is, the capacity to differentiate into cells with a neuronal, astrocytic, or oligodendroglial phenotype (Pilkington 2005; Vescovi et al. 2006). However, multilineage differentiation is not a requirement of the CSC. It is more important that CSCs regenerate the cell types observed in the original tumor (Ward and Dirks 2007). In addition, brain tumor stem cells are characterized by the expression of neural stem cell antigens and the ability to grow as nonadherent spheres termed 'neurospheres' when cultured in the presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF) under serum-free conditions. Thus, stem-like cells in brain tumors share many characteristics with normal neural stem cells, supporting the hypothesis that brain tumors can arise from neural stem or progenitor cells (Vescovi et al. 2006). Recent studies further show that glioblastoma cells cultured under neural stem cell conditions can display heterogeneous growth characteristics and molecular profiles, suggesting that they may either arise from different cell types or from similar cells that have acquired different genetic alterations (Beier et al. 2007). Whether CSCs arise from normal stem cells, progenitor cells, or differentiated cells is not known at the present time. Nevertheless, CSCs in gliomas express CD133 and nestin that mark neural and progenitor cells (Galli et al. 2004). The factors governing glioma cell differentiation and migratory potential, and therefore tumor phenotype, are not completely understood. On the basis of gene expression profiling and by neurobiological criteria, two subtypes were identified recently among human glioblastoma cultures established under neural stem cell conditions. One cluster of CSCs expressed neurodevelopmental genes and displayed a full stem-like phenotype. The second cluster displayed an expression signature for extracellular matrix-(ECM)-related genes and only a restricted stem-like phenotype, fulfilling only in part the criteria considered typical of glioblastoma stem cells (Günther et al. 2008). Whether these differences reflect variations in stem-like cells present between individual glioblastomas and/or distinct emerging lineages remains to be established.

There is recent evidence generated from preliminary studies in solid tumors suggesting that CSCs reside in a niche. This hypothesis derives from reports on normal stem cells from various tissues, showing that stem cells exist within protective niches that are composed of a number of differentiated cell types (Fuchs et al. 2004; Pierret et al. 2007). This cellular microenvironment provides direct cell contacts and secreted factors that maintain stem cells in a quiescent state. Histological observations of mouse tissues suggest that neural stem cells lie within a vascular niche in which endothelial cells regulate stem cell self-renewal. In gliomas it is hypothesized that vascular endothelial cells provide such a niche for the glioma CSCs similar to the situation with normal neuronal stem cells (Calabrese et al. 2007).

In veterinary medicine there is a considerable literature that describes the occurrence of spontaneous tumors of the central nervous system in animals (Heidner et al. 1991; Stoica et al. 2004; Summers et al. 1995). Astrocytoma, of which glioblastoma multiforme (GBM) is the most malignant form, is one of the most common

neoplasms of the central nervous system in animals. Of the domesticated animal species, most examples are seen in dogs, with much lower occurrence in other species. Intracranial neoplasia occurs more frequently in dogs than in humans (14.5 per year per 100,000 canine population at risk compared with 4–5 per 100,000 humans) (Gavin et al. 1995).

Canine intracranial glioblastoma occurs most commonly in brachycephalic breeds, in particular the Boxer and Boston terrier. No sex predilection has been reported and they are recognized with greater incidence in animals over 6 years of age. Clinical signs caused by astrocytomas may vary with tumor location, and neurological deficits reflect direct nervous tissue involvement as well as secondary effects of peritumor edema, necrosis, hemorrhage, compression, herniation, or obstructive hydrocephalus. Cerebrospinal fluid findings in animals with astrocytoma are usually non-specific and include increased protein concentration and mixed-cell pleocytosis that reflect disturbance of the blood-brain-barrier. Computed tomography (CT) and magnetic resonance imaging are routinely available to veterinary practitioners for diagnosis and localization of intracranial lesions. Computed tomographic characteristics of some canine brain tumors have been reported to be similar to those in humans (Dickinson et al. 2010; Turrel et al. 1986; Thomas et al. 1996).

GBM, one of the most malignant and devastating forms of glioma, appears to have the highest incidence in dog (Summers et al. 1995). The highest incidence (30%) of this tumor occurs in the Boxer (Stoica et al. 2004). Despite progress in research on the molecular aspects of GBMs, the prognosis of these brain tumors continues to be dismal in humans and animal species. One reason for the lack of clinical advances is ignorance of the cellular origin of this disease and lack of understanding of the mechanisms of glioma cell migration and dispersal, which delays the application of molecular analyses to treatment and impairs anticipation of tumor biological behavior.

We have demonstrated that GBM isolated from a Boxer dog (D-GBM) has cells with phenotypical characteristics of CSCs. As a proof of concept, we characterized this cell line's morphophenotype and its specific stem cell markers such as nestin and CD133. CSCs demonstrated glial and neuronal differentiation when exposed to differentiation growth factors *in vitro*. An intracranial orthotopic model using nude mice was utilized for *in vivo* evaluation of D-GBM tumorigenicity. Immunohistochemistry results of nude mouse xenografts also demonstrated glial and neuronal differentiation. We reported the *in vitro* culturing of dog glioblastoma and the first demonstration of the presence of CSCs in dog GBM. Our data also demonstrate the similarity between human and dog GBM and emphasize the importance of studying dog spontaneous brain tumors in order to elucidate the mechanism(s) of tumor dispersal, post surgical reoccurrence, and resistance to therapy.

Dog Glioblastoma Morphology (D-GBM)

Glioblastoma in Boxer: Morphological Presentation and Similarity with Human Glioblastoma

An intracranial neoplasm was diagnosed by CT scan of an 8 year old, male Boxer dog. The necropsy revealed an intracranial tumor mass located within the left lateral ventricle (Fig. 20.1). A GBM was diagnosed on histopathological examination performed on frozen sections and formalin-fixed, paraffin-embedded tissue. The tumor consisted of a heterogeneous glial cell population, multifocal areas of necrosis with perinecrotic cellular pallisading, and marked neovascularization with glomeruloid pattern. The dog glioblastoma cellular heterogeneity consisted of areas showing oligodendroglial, gemistocytic, or fibroblastic astroglial differentiation, but an estimated one third of the tumor cells were undifferentiated. A disrupted and fragmented ependymal lining of the lateral ventricle was located at the periphery of the tumor mass confirming glioblastoma development

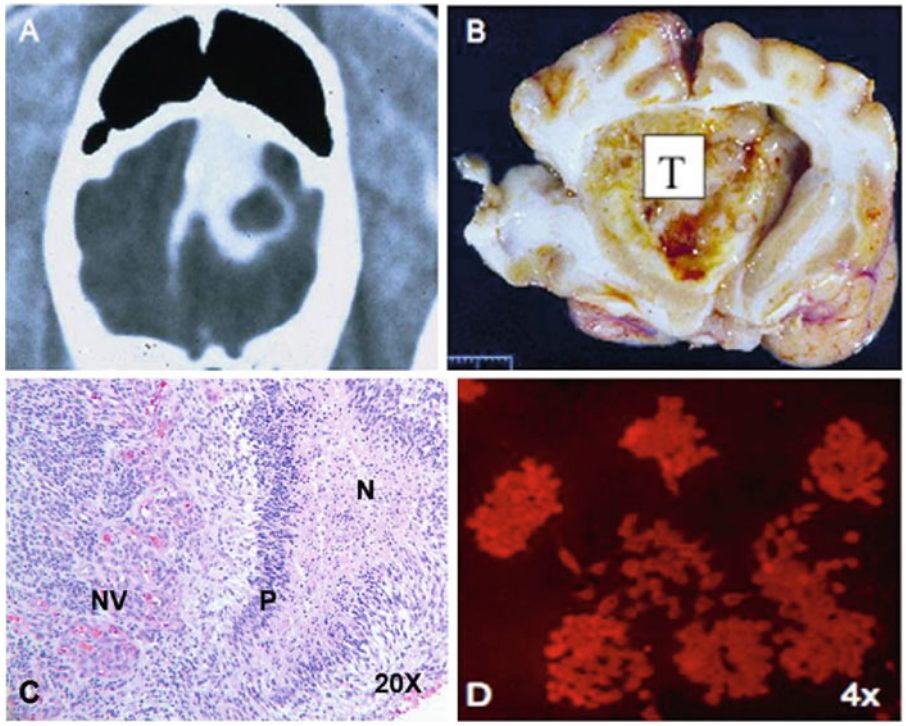

Fig. 20.1 (**a**) CT image of GBM from a Boxer dog. (**b**) Gross appearance of an intraventricular GBM. (**c**) Histological appearance of Boxer dog GBM. Necrosis (*N*), palisading (*P*), and neovascularization (*NV*) are evident. These are also the pathognomonic features of human GBM. (**d**) Nestin immunofluorescence of cultured D-GBM cells derived from a Boxer dog. Notice the spheroid pattern of growth that characterizes this glioblastoma

within the ventricle. These morphological features are characteristic and similar to human glioblastoma grade IV classification. In addition, this GBM showed intratumoral microglial infiltration and peritumoral astroglial activation.

In Vitro Study of D-GBM

In Vitro Culture of Dog GBM and Its Characterization

Necropsy was performed 1 h post euthanasia and a small segment of tumor was taken and processed for tissue culture. Our quest was to determine if dog gliomas possess CSC-like properties as previously reported in human gliomas. We were able to obtain glioma cultures (D-GBM) from a grade IV GBM isolated from a Boxer dog. Cultured D-GBM cells demonstrate neurosphere formation in both neurobasal media supplemented with FGF and EGF and also in DMEM supplemented with 10% FBS. All primary D-GBM cultures were composed of single cells, which gave rise to clonal spheres within a week. The non-attached neurospheres that grew in neurobasal medium supplemented with EGF and FGF were passed by simple mechanical dissociation. The tumor cells that grew in DMEM supplemented with 10% FBS formed neurospheres, which were partially adherent to the culture flask. They were lightly attached and we passed them by mechanical manipulation or light trypsinization. A cell line was established from this dog GBM, which has been passed 20 times to the present time. All the experiments using this D-GBM were performed with cells of early passage [up to five]. There were no differences in marker expression due to passage number and cells maintained a similar phenotype up to passage 20.

Dog Glioblastoma Cell Line Has a Capacity for Differentiation

Glioma stem cells are characterized by the expression of stem cell markers and the capacity for multilineage differentiation. CD133 and nestin are frequently used as markers for an undifferentiated state. When we analyzed CD133 expression by flow cytometry, we detected 8.52% $CD133^+$ cells derived from non-attached neurospheres maintained in neurobasal medium and 3.14% $CD133^+$ from cells maintained in DMEM medium supplemented with 10% FBS and partly adherent. D-GBM cells maintained in DMEM supplemented with 10% FBS showed neuronal cell differentiation (βIII tubulin labeling) and also astrocytic differentiation (GFAP labeling). In addition, the immunohistochemistry of xenografts showed strong nestin [a class VI intermediate filament protein and neural stem cell marker] expression as well as CD133 and CD34 [cell surface markers], beta tubulin class III [a neuronal filament marker], and GFAP [an astrocytic cell marker]. Their immunoreactivity in tumor xenografts demonstrated the existence of cancer stem cells with multipotent lineage.

Self-renewal of D-GBM Cells

Self-renewal is a critical feature of neural stem cells and CSCs. We performed limiting dilution assays to analyze whether single D-GBM cells have the capacity to form new spheres or colonies. Subsphere formation was observed for the non-adherent and for the partly adherent D-GBM cells. In this serial dilution assay, 53 clones, each consisting of 500–800 cells, grew out of the 53 original single cells. The clone formation rate was 100%. The primary clones were then dissociated and plated into new 96-well microplates to generate subclones. The subclone formation rate was 100%, with 122 subclones from 122 single cells.

Hypoxia Is a Factor in D-GBM Cells Migration

Matrigel migration assay was performed to evaluate the migration potential of D-GBM cells. Neurosphere cells were used for this procedure. Our data demonstrate a significant increase in tumor cell migration when kept under hypoxic conditions compared with cells kept under normoxic conditions.

Reverse-Transcriptase Polymerase Chain Reaction (PCR) Showing mRNA Expression of Pluripotency Markers

RT-PCR analysis of Boston terrier dog pilocytic astrocytoma grade I, as well as Boxer and Bull Mastiff glioblastoma grade IV, revealed the expression of CD 133, nestin, GFAP and βIII tubulin which was higher in Boxer D-GBM grade IV compared with Bull mastiff and Boston terrier tumors. CD133 and nestin were absent in grade I pilocytic astrocytoma cells. Normal dog brain tissue was used as a control (Fig. 20.2). These results confirmed the expression of CD133, nestin, βIII-tubulin, and GFAP in Boxer D-GBM grade IV by flow cytometry and immunohistochemistry demonstrating the differentiation potential of D-GBM CSCs.

Expression of Integrins in Dog Glioblastoma

The analysis of integrins was performed in D-GBM (grade IV) cells isolated from Boxer and Bull mastiff frozen tumor tissues, and pilocytic astrocytoma (PCA, grade I, WHO classification) isolated from a Boston terrier by RT-PCR. Our results demonstrate that grade IV GBMs express higher levels of α-5 integrin compared with grade I PCA tumor. Alpha-1 integrin was not detected in grade IV gliomas. Beta-3 integrin was highly expressed in Bull mastiff grade IV glioma compared with Boxer GBM grade 4 and Grade I PCA (Fig. 20.2).

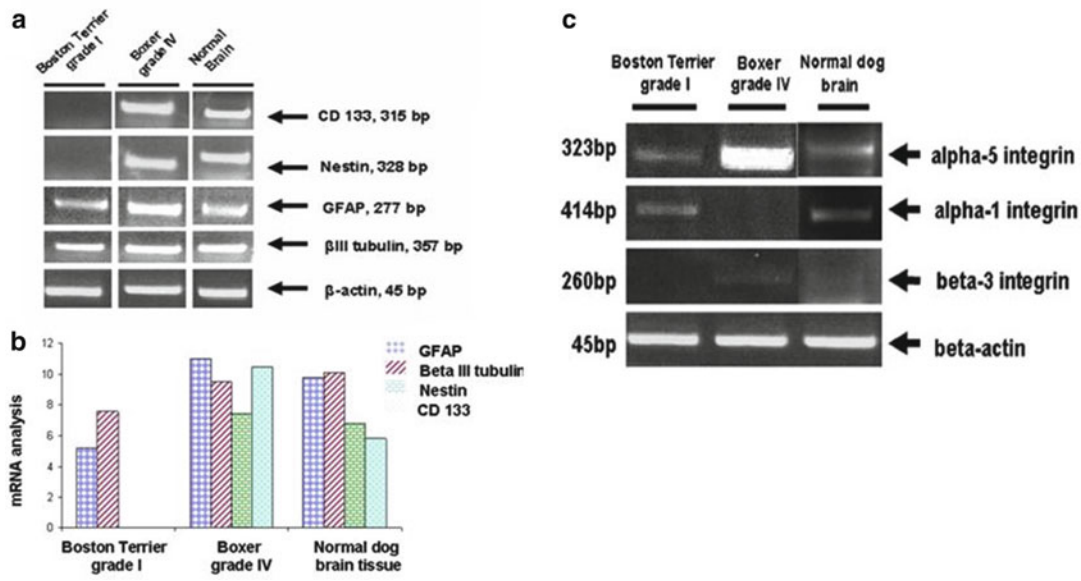

Fig. 20.2 Gene expression profile of dog brain tumor grade I and IV as analyzed by RT-PCR. Normal dog brain was used as a control. Total RNA was extracted from primary cell culture of a Boston terrier brain tumor grade I and Boxer grade IV, as well as from Bull Mastiff grade IV brain tumor tissue and normal dog brain tissue from SVZ. (**a**) Higher levels of CD133, nestin, βIII tubulin, and GFAP were found in Boxer grade IV tumor compared with Bull Mastiff grade IV brain tumor and Boston terrier grade I brain tumor. CD133 and nestin mRNA was not detected in Boston terrier grade I brain tumor. β-actin served as a loading control. (**b**) Quantitative analysis was performed by densitometry. RT-PCR was performed three times on each sample. (**c**) Differential integrin expression in dog brain tumor grade I and IV as analyzed by RT-PCR. Boxer and Bull Mastiff brain tumor grade IV express higher levels of alpha-5 integrin compared with Boston terrier brain tumor grade I and normal brain. Alpha-1 integrin was not detected in grade IV tumors. Beta-3 integrin level was highly expressed in Bull Mastiff grade IV tumor, compared with Boxer grade IV tumor and was not detected in grade I tumor and normal brain. β-actin served as a loading control

Nude Mice D-GBM Xenografts

In Vivo Growth of Glioblastoma Neurospheres

To address whether D-GBM cells are tumorigenic *in vivo*, cells were xenografted into the striatum of nude mice. Initially, three nude mice were inoculated intracranially with 1 x 10^6 D-GBM cells from dissociated neurospheres to assess the tumorigencity potential. We sacrificed these mice at 10 days post inoculation (dpi) due to severe neurological deficit. Large invasive tumors were detected on histopathological examination invading the cortex, corpus collossum, striatum and lateral ventricles. Next, for each cell line maintained in neurobasal and DMEM medium [adherent and partly adherent], three animals were injected with 200,000 cells and at least three with 1,000 cells. All mice that received 200,000 cells developed neurological clinical signs and were sacrificed at 25 dpi with tumors. The mice inoculated intracranially with 1,000 cells also developed tumors after a longer time (60 dpi). The tumor xenografts demonstrated the cardinal signature of GBM described in dogs and humans: cellular heterogeneity, marked neovascularization, and necrosis (Fig. 20.3). The morphology of tumors clearly reproduced the phenotype of the original dog tumor from which these cells were isolated. There was no morphological difference between the xenografts derived from neurosphere cells maintained in neurobasal or DMEM medium. The nude mouse xenograft was harvested and re-cultured in FGF- and EGF-supplemented neurobasal medium and also in DMEM

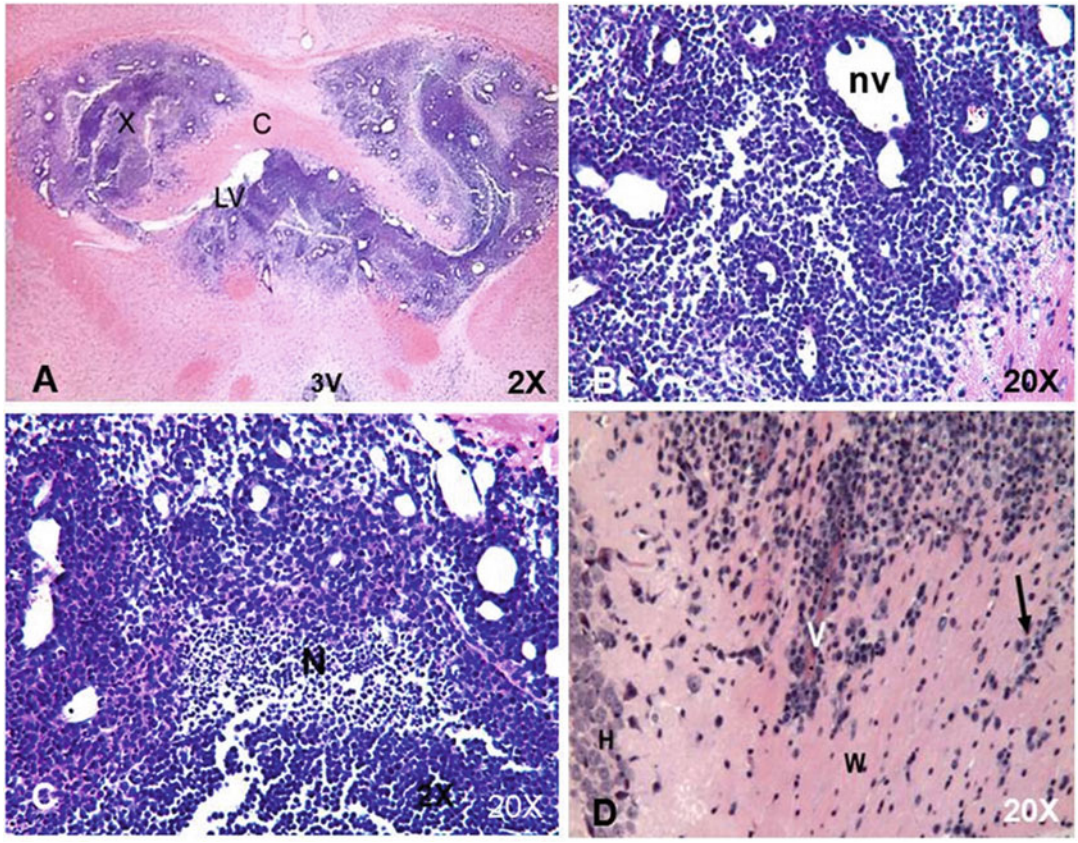

Fig. 20.3 Coronal section: H&E staining of the intracranial orthotopic inoculation of D-GBM cells in a nude mouse (1 x 10^6) sacrificed at 10 dpi. (**a**) Tumor xenograft (*X*) invading the putamen, corpus callosum (*C*) and lateral ventricle (*LV*), and third ventricle (*3v*). (**b**) Notice intense neovascularization (*nv*) within the fast growing tumor. Tumor vessels appear tortuous, hyperdilated, and irregularly shaped. (**c**) A necrotic center (*NC*) is evident within the tumor core, a characteristic feature of glioblastoma grade IV. (**d**) Notice the perivascular tumor cells invasion: (*v*) vessel, and migrating tumor cells within the white matter (*arrow*), and hippocampus formation edge (*H*)

containing 10% FBS. The cultured xenografts formed non-attached neurospheres in growth factor-supplemented neurobasal medium. Nude mouse xenografts cultured in DMEM with serum formed non-attached neurospheres, but there were also many attached, piled up colonies, which were loosely adherent and showed multilineage differentiation.

Discussion

Glioblastoma multiforme (GBM) is the most common and lethal primary malignant brain tumor in human and dog. GBM is a nonmetastatic tumor, but highly locally invasive, diffusely disseminating into the brain parenchyma and possessing cancerous cells outside the margin of therapeutic intervention (Stoica et al. 2004, 2009, 2011; Louis 2006).

The GBM infiltrative path into the normal brain is not random; it often follows white matter tracts and extends along perivascular spaces, the glial limitans externa, and the subependyma. Their diffuse infiltration suggests the activation of genetic and cellular programs that distinguish them from cells in the tumor core. Additionally, migrating tumor cells may activate the host brain microenvironment to facilitate tumor dispersal.

The D-GBM nude mouse xenografts clearly demonstrated the pattern of brain invasion by the CSCs. The inoculated tumor cell migration pathways followed the white matter of the corpus

collossum, glial limitans of lateral ventricles and the pial surface, and also perivascular venue as described in human gliomas (Louis 2006). The CD133- and nestin-expressing tumor cells were at the migration front in the nude mouse brain white matter inoculated with D-GBM cells. Glioma invasion is a combination of the ability of CSCs to migrate and their ability to modulate the ECM. The ECM of the brain is distinct from the ECM of most organs, being ill defined and scant. The matrix consists primarily of hyaluronic acid, except for the areas around the vessels and the pial surface (glial limitans) where there is well-defined basal lamina that include collagen. Glioma cell invasion preferentially involves the perivascular and subpial spaces, where basal lamina is well-defined, but also involves perineuronal and white matter locations in which the ECM is ill-defined. The nude mouse xenografts of D-GBM also demonstrated the differentiation capacity of the CSCs. Besides $CD133^{+}$ and nestin, tumor cell xenografts showed various degrees of immunoreactivity for CD34, βIII tubulin (neuronal marker), and GFAP (an astrocyte lineage marker), which imply a multilineage differentiation capacity of D-GBM *in vivo*. The differentiation capacity of D-GBM was also supported by RT-PCR results.

Our data demonstrate that D-GBM xenografts from nude mice showed an excessive vascularization pattern. These neovessels were incompletely formed, hyperdilated, and have irregular size and shape. Tumor cells appear to cluster and remain in close contact with the endothelial cells of the new vessels.

GBM is one of the most lethal primary brain tumors in human and animal species. This tumor is comprised of a phenotypically heterogeneous cell population (Stoica et al. 2009, 2011; Kleihues and Cavenee 2000). The mechanisms responsible for the GBM heterogeneity and the ability of tumor cells to migrate into the brain parenchyma are incompletely understood. Localized hypoxia appears to upregulate migration-associated genes, leading to migration of tumor cells away from a central hypoxic center. Necrosis is a central feature of the highest-grade malignant gliomas. No histological feature is more powerful in predicting poor prognosis (Kleihues et al. 2000). There is a vicious cycle involving necrosis-induced hypoxia and various angiogenic and growth factors that combine to foster the highly malignant state of glioblastoma. In the setting of rapidly dividing cells with high metabolic demands, small regions of necrosis may develop in areas where metabolic demands exceed supply. Another recently suggested possibility is that in response to hypoxia, small clones of tumor cells acquire molecular characteristics that encourage more active migration, thus clearing a central region more susceptible to necrosis. This hypothesis is based on work showing that glioblastoma tumor cells surrounding necrotic centers (pseudopalisades) are less proliferative and more apoptotic than adjacent cells (Brat et al. 2004). These perinecrotic cells also express hypoxia-inducible genes, such as hypoxia-inducible factor 1 alpha (HIF-1a), and *in vitro* studies have demonstrated that hypoxia increases cellular migration and gelatinase activity (Vaupel 2004; Lungu et al. 2007). Hypoxic cell death may lead to the release of growth factors from the dying cells, and some growth factors may remain intact within the necrotic zones. More importantly, hypoxia may allow the emergence of resistant and thus highly malignant clones of tumor cells. When selected molecules that are preferentially expressed in palisading cells are used as prognostic markers in glioblastomas, they correlate with shorter patient survival (Dong et al. 2005). This supports the notion that these perinecrotic regions are zones in which more malignant clones are actively selected. In addition, the interactions between hypoxia, necrosis, upregulation of growth factors, and selection of malignant clones might provide an explanation for the marked histological heterogeneity noted in glioblastomas and a possible explanation for the marked resistance to conventional cytotoxic therapies seen in clinical patients with glioblastoma (Louis et al. 2006).

Tumor progression leading to metastasis or invasion appears to involve equipping cancer cells with the appropriate adhesive (integrin) phenotype for interaction with the ECM. The integrins constitute a family of transmembrane

receptor proteins composed of heterodimeric complexes of noncovalently linked alpha and beta chains. Integrins function in cell-to-cell and cell-to-ECM adhesive interactions and transduce signals from the ECM to the cell interior and vice versa. Previous studies reported the expression of various integrins in brain tumors (Mizejewski 1999; Paulus et al. 1996). There have been few investigations of the expression of αvβ5 and αvβ3 in gliomas. Few data are available regarding the relationship between the expression of these integrins and angiogenesis and invasion in this type of tumor. Two αv integrins, αvβ3 and αvβ5, are necessary for cytokine- or tumor-induced angiogenesis. They mediate two distinct pathways of angiogenesis – expression of αvβ3 is required for fibroblast growth factor 2 (FGF-2) and tumor necrosis factor α-induced angiogenesis. The integrin αvβ5 is required for VEGF- and transforming growth factor β-induced angiogenesis (Friedlander et al. 1995). Hypoxia has been reported to stimulate expression of integrins αvβ3 and αvβ5 in retinal microvascular endothelial cells and to inhibit expression of integrin β1. The hypoxic induction of the αv integrin is partially mediated through VEGF induction in an autocrine-paracrine manner (Suzuma et al. 1998). The angiogenic integrins have been reported to not only mediate vascular cell migration but also to regulate metalloproteinase activity and cell proliferation. These specific increases in the tumor cellular integrin expression may contribute to neovascularization and invasion observed in dog glioblastomas.

Germinal regions, such as SVZ, have long been proposed as sources of gliomas. Many gliomas are either periventricular or contiguous with the subventricular zone, and they frequently express the progenitor-cell markers nestin and CD133. Recent studies suggest that human brain tumors are organized as a hierarchy and are maintained by a small number of tumor cells that have stem cell properties (Dirks 2008). Based on the anatomical location of the D-GBM isolated from a Boxer dog, intuitively we believe that the cells of origin for this glioblastoma multiforme most likely reside with the neural stem cells from the subependymal lateral ventricle region (SVZ). Histological observation derived from this tumor and others that were diagnosed in our laboratory demonstrated a similar intraventricular or periventricular location and fragments of ependymal lining cells were described within the tumor. In addition, the migration pattern in nude mouse xenografts is highly suggestive that D-GBM has a homing property directing migration from the site of implantation to the ventricular region. However, this observation is just correlative, but if the cell of origin for this type of tumors can be demonstrated to be derived from SVZ neural stem cells it will have a great impact on the future classification of brain tumors and novel therapeutic modalities.

In conclusion, we have demonstrated the *in vitro* culture of dog GBM and the presence of CSCs in dog brain tumors. Understanding the basic mechanisms underlying glioma cell invasion into the brain parenchyma requires improved and diverse experimental models of glioma invasion. The spontaneous dog glioma appears as an excellent animal model for investigating cancer stem cell invasion. A dog glioma model has a higher incidence and less restrictive rules than humans. The invasive nature of glioma cells is the obvious obstacle to effective therapy, making this issue of great significance.

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Role of Stem Cell Niche in the Development of Bone Metastases (An Update)

21

Nadia Rucci and Anna Teti

Contents

Abstract

Metastases represent the "point of no return" for tumor bearing patients since their occurrence determines a drastic fall of the chance of survival. Given the inexorability of the fate of metastatic patients, many efforts are being made to improve their management and increase their life expectancy and quality. The prevalence of tumor relapse to bone appears to be increasing over the years, likely due to a longer overall survival of patients. A large body of evidence indicates that the preference of tumor cells to metastasize to bone is an addressed event, which relies on specific interactions among them, the bone cells and the bone marrow microenvironment. The bone/bone marrow compartment is unquestionably a "fertile soil" for tumor growth, characterized by a high blood supply and the presence of countless growth factors which are released and activated during bone resorption upon stimulation by tumor cells. In the attempt to identify the crucial mechanisms inducing tumor recurrence in distant organs, recent evidence has demonstrated the key role played by the so-called Cancer Stem Cells (CSCs), a very small cell population in the tumor displaying self-renewal competence, differentiation potential and ability to recapitulate the phenotype of the tumor from which it derives. Cancer cells harboring stem properties have been characterized in several tumors, including those metastasizing to bone, and a causative correlation between the

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presence of the CSC pool in the bone/marrow and the relapse in this site has been suggested. Emerging researches strongly support the notion that CSCs could compete with the normal stem cells to inhabit the physiologic niche in the bone marrow, being primed by this niche for their proliferation and invasiveness.

Introduction

The relapse in bone is a common feature of many metastatic cancers, including solid and hematological malignancies. Bone metastatic events are known since long time and were initially thought to represent specific characteristics only of selected cancers, such as breast and prostate carcinomas or multiple myeloma (Coleman 2011). Although these tumors are still the most frequently relapsing in bone, it is clear that many other cancers are able to colonize this tissue with an increasing prevalence due to the better treatments that nowadays prolong the survival of patients (Coleman 2011). Furthermore, apparently cured tumors may relapse many years after their surgical removal, according to the new concept of tumor dormancy (Felsher 2006). It is believed that dormancy is most likely to occur especially in the bone marrow, where nests of cancer cells may stand in a quiescent condition for decades before recurring and spreading also to other organs (Felsher 2006). Therefore, the notion of bone metastasis is rapidly changing and an up-date is necessary to better understand the underlying cellular and molecular mechanisms, especially with a translational and clinical perspective. In this context, the hypothesis of the existence of a cancer cell stemness and the requirement for Cancer Stem Cells (CSCs) to interact with niche cells resident in the host organs emerged as essential mechanisms, on one hand inducing predilection for a tumor to form metastases in selected organs, and on the other hand to be responsible, in certain circumstances, for tumor dormancy and later relapse. In this article we will overview the general mechanisms underlying the development of bone metastases and will describe the most recent observations on the role played in this context by the CSC-niche interaction in the bone microenvironment.

Bone as Preferential Site of Metastases

Breast and prostate cancers show a marked preference to metastasize to bone, with a prevalence of at least 70%. Although with a lesser prevalence (30–40%), also carcinomas of the thyroid, kidney and bronchus commonly metastasize the bone (Coleman 2011), while the prevalence for other cancers is lower, even though it is rapidly increasing possibly because of the longer survival of treated patients (Coleman 2011). Relapse in the bone has a dramatic impact on prognosis, since once bone metastases are established the chances of survival dramatically drop, and the quality of life deteriorates due to the onset of a severe morbidity characterized by pain, spinal cord compression, fractures and, not last, life-threatening hypercalcemia (Coleman 2011; Clezardin and Teti 2007; Capulli et al. 2012).

One of the most intriguing challenge for scientists working in the oncology field is to explain the mechanisms of preferential colonization of a host site by tumor cells. In this regard, a large body of evidence suggests that the preference of tumor cells to metastasize to bone is not a casual but an addressed event, which relies on specific interactions among tumor cells, bone marrow microenvironment and bone cells. This concept is recapitulated by the so called “seed and soil theory” proposed more than 100 years ago by Paget (1889), which resulted to be particularly true for the bone metastasis. This theory emphasizes the importance of the host milieu for the selectivity of tumor cells to engraft a target organ, this event relying on a network of interactions among the tumor cells (seed) and the host microenvironment (soil). Bone is characterized by an extensive circulation and the bone matrix is an efficient storage for calcium and growth factors, including TGFbeta (Transforming Growth Factor beta), IGF-I and II (Insulin-like Growth Factor I and II), FGFs (Fibroblast

Growth Factors), PDGF (Platelet-Derived Growth Factor), BMPs (Bone Morphogenetic Proteins), among many others. These factors are released and activated during bone resorption, providing the fertile ground in which tumor cells can grow (Roodman 2004).

From a clinical and histopathological point of view, bone metastases can be classified as osteolytic, osteosclerotic (or osteoblastic) and mixed. Usually, the former are typical of breast cancer and multiple myeloma, where osteolysis is mediated by the osteoclasts rather than by the cancer cells themselves (Roodman 2004). In contrast, the nature of bone metastases in prostate cancer is preferentially osteoblastic (Coleman 2011). However, it is now well established that osteoclast activation is required not only in osteolytic but also in osteosclerotic metastases, and that the bone resorption phase is a prerequisite for the subsequent deposition of bone, so that an exacerbated bone resorption process is permissive for both types of metastases (Coleman 2011).

Physiology of Bone: "Virtuous" *Versus* "Vicious" Cycle

At variance with many other tissues, bone is continuously renewed during the lifetime of each individual by a process named bone remodeling (Teti 2011). Physiologic bone remodeling relies on a perfect balance between the activity of two main cells: the osteoclasts, arising from the monocyte/macrophage lineage, unique in their function of destruction of the tissue in which they reside, and the osteoblasts, cells of mesenchymal origin which deposit new bone matrix.

Several factors, both systemic and local, regulate bone remodeling, this regulation being also achieved by a mutual cross-talk between osteoclasts and osteoblasts/osteocytes, that regulate each other. Under physiologic conditions osteoblasts and osteocytes (the cells arising from the osteoblasts that remain entrapped in the bone matrix at the end of the bone formation phase) produce the pro-osteoclastogenic cytokine Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL). RANKL interacts with its cell surface receptor, RANK, expressed by osteoclast precursors, triggering intracellular signals that induce osteoclast differentiation (Teti 2011). Osteoblasts also produce osteoprotegerin (OPG), a RANKL decoy receptor with the same structure of the extracellular portion of RANK (Fig. 21.1a). Therefore, a balanced osteoclast differentiation relies on a correct RANKL/OPG ratio.

Macrophage-Colony Stimulating Factor (M-CSF) is another cytokine produced by osteoblasts, that interacts with its cell surface receptor, c-Fms, expressed by preosteoclasts, stimulating the expansion of this population. Mature osteoclasts polarize, adhere to the bone surface and, by a process of acidification and subsequent release of proteolytic enzymes, degrade the bone matrix. In doing so, they also release several growth factors previously stored in the matrix (i.e. TGFbeta, IGFs, FGF, PDGF, BMPs, etc.) or synthesized by the cell (S1P, TRAcP, HGF), that attract osteoblasts to the resorbed area, stimulating the deposition of new bone in the same site of previous resorption (Teti 2011; Fig. 21.1a). We propose to term this physiologic series of events characterizing bone remodeling "virtuous cycle" (Fig. 21.1a).

The virtuous cycle is drastically perturbed and transformed into a "vicious cycle" (Roodman 2004) when tumor cells engraft the bone (Fig. 21.1b). A critical event in the bone-tumor vicious cycle is the exacerbated osteoclast activity, induced by tumor cells through the deregulated secretion of a number of cytokines, which in turn increases the release of matrix-stored pro-tumoral factors.

Among the pro-osteoclastogenic cytokines released by tumor cells there is the ParaThyroid Hormone related Protein (PTHrP), whose production is triggered by TGFbeta. In turn, PTHrP induces RANKL and represses OPG production by bone marrow stromal cells and osteoblasts. Moreover, tumor cells produce molecules with marked pro-osteoclastogenic activity, including M-CSF, prostaglandin E2 (PGE2), interleukin (IL)-1 beta, IL-6, IL-8, IL-11 and the tumor necrosis factor (TNF) alpha, that enhance

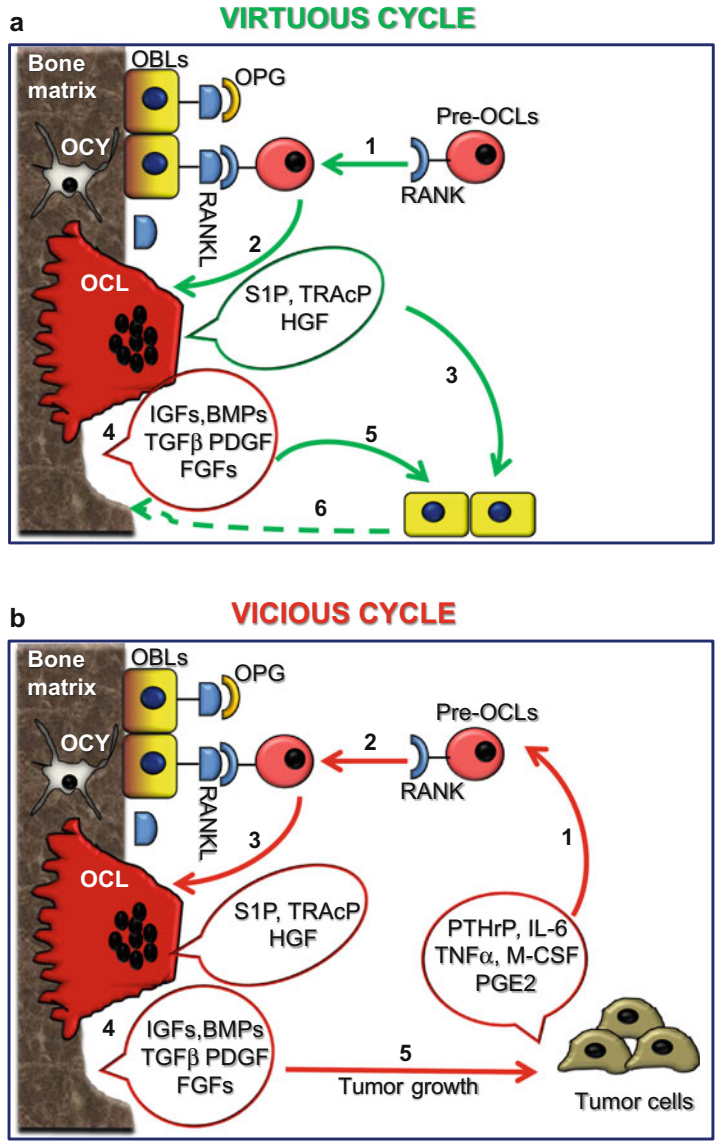

Fig. 21.1 The "virtuous" *versus* the "vicious" cycle. (**a**) The osteoblasts (*OBLs*) produce factors, such as RANKL and OPG, that regulate osteoclastogenesis. Osteocytes (*OCY*) also produce RANKL. Once differentiated, osteoclasts (*OCL*) resorb the bone, allowing the release of growth factors herein stored, such as IGF-1, BMPs, TGF-β, PDGF, FGFs, or the secretion of factors produced by the cell, such as Tartrate Resistant Acid Phosphatase (*TRAcP*), Sphingosine 1 Phosphate (*S1P*) and Hepatocyte Growth Factor (*HGF*). In turn, these molecules attract the osteoblasts in the resorbed area, thus eliciting the synthesis of new bone matrix (virtuous cycle). (**b**) Once reached the bone, tumor cells secrete factors (i.e. PTHrP, IL-6, TNF-α, M-CSF, PGE2) triggering osteoclastogenesis, with a consequent increase of bone resorption, eventually leading to the enhanced release of pro-tumoral factors which stimulate cancer cell proliferation (vicious cycle). Pre-OCLs = preosteoclasts

osteoclast formation (Fig. 21.1b). A recent work by Sethi et al. (2011) also described the relevance of the Notch/Jagged signaling in the context of the vicious cycle, demonstrating that Jagged1 produced by breast cancer cells promotes osteolytic metastases both directly stimulating osteoclast differentiation and activating the Notch signaling in osteoblasts, which in turn release the pro-osteoclastogenic cytokine IL-6.

Tumor Stem Cell Phenotype

The hypothesis of the CSCs conceived as a small subset of tumor cells developed from a restricted pool of tissue-specific stem cells is not a novelty, since it was already proposed more than 150 years ago (Cohnheim 1867). Consistently with recent findings, CSCs can be defined as a small fraction of cells, inside the tumor cell population, with unlimited renewal potential and with the ability of forming tumors in immunodeficient mice that recapitulate the heterogeneity of the original tumor (Al-Hajj et al. 2003; Polyak and Hahn 2006; Wicha et al. 2006).

A crucial concept in the CSC hypothesis is that only a very small population in the tumor displays the defining stemness properties of self-renewal and differentiation potential (Wicha et al. 2006). This hypothesis has been supported by pioneering *in vivo* experiments performed with leukemia cells, showing that the ability to transfer human leukemia into NOD/SCID immunodeficient mice was retained only by a small population of cancer cells (<1 in 10,000 leukemia cells), and that the leukemia produced by these cells recapitulated the histopathological phenotype found in the original tumor (Table 21.1; Bonnet and Dick 1997).

Normal and cancer stem cells share several properties, such as: (i) expression of common surface molecules, (ii) capacity to self-renew by asymmetric division, which allows to maintain the undifferentiated stem cell pool (without loss of the proliferative capacity) as well as the ability of daughter cells to differentiate, (iii) increased telomerase expression, and (iv) ability to migrate (Wicha et al. 2006). Moreover, similar to normal stem cells, CSCs can survive chemotherapy better than other cells in the tumor, as they replicate at a much slower rate and may constitutively express drug resistance transporters, with important clinical implications (Zhou et al. 2001). This property could also explain the inability of the chemotherapeutics to completely eradicate all tumor cells, since they can be effective only on those that are actively proliferating, leaving the quiescent counterpart undisturbed.

An interesting work by Al-Hajj et al. (2003), confirmed later by other researchers, described a possible phenotype which could identify breast CSCs. Starting from breast cancer biopsies, a subpopulation of cells with the highest tumorigenic potential was isolated and their characterization identified the following membrane receptor *status*: $CD44^{+}CD24^{-/low}/ESA^{+}$. CD44 and CD24 are two adhesion molecules, the former also associated with stem cells in normal breast tissue, while the latter is an epithelial marker. Other studies showed that the tumorigenic potential was increased in CSCs selected for their expression of aldehyde dehydrogenase (ALDH) (Table 21.1; Ginestier et al. 2007). The activity of ALDH is in fact associated with drug resistance, cell proliferation and response to oxidative stress. Moreover, it was estimated that as few as 20 tumor cells sorted from breast cancer samples according to the above described CSC phenotype were able to trigger orthotopic tumor growth when injected in immunocompromised mice, whereas a larger amount of cells depleted of this CSC pool was harmless (Ginestier et al. 2007).

Also in the largely heterogeneous prostate cancer cell population it has been identified a subset of cells with stem properties, characterized by the $CD44^{+}/alpha_2beta_1^{high}/CD133^{+}$ profile and present in approximately 0.1% of human prostate cancer cells. Moreover, this subpopulation was able to self-renew and proliferate, as well as to differentiate and recapitulate the phenotype of the

Table 21.1 Cancer stem cell (CSC) markers expressed in the indicated tumors

Tumor	CSC markers	References
Acute myeloid leukemia	CD34+/CD38+	Bonnet and Dick (1997)
Breast	$CD44^{+}/CD24^{-/low}/ESA^{+}/ALDH^{high}$	Al-Hajj et al. (2003), Wicha et al. (2006), Ginestier et al. (2007)
Prostate	$CD44^{+}/alpha_2beta_1^{high}/CD133^{+}/ALDH^{high}$	Collins et al. (2005), van den Hogen et al. (2010), Colombel et al. (2012)

tumor from which it derived (Collins et al. 2005). Similarly to breast CSCs, recent studies improved the selection of the prostate cancer stem pool by identifying ALDH activity once again as an important marker of stemness, since it strongly enhanced their ability to form distant metastases in preclinical orthotopic models (Table 21.1; van den Hogen et al. 2010).

Stem Cell Niches in the Bone Marrow

The hematopoietic stem cells (HSCs) home in dedicated microenvironments of the bone marrow, named niches, that regulate their survival, proliferation and differentiation. Two main niches support HSCs: the osteoblast niche and the vascular niche.

The osteoblast niche is represented by a subset of cells, named Spindle-shaped N-cadherin^{+}/CD45^{-} Osteoblasts (SNO), located next to the endosteal surface of bone, that have been discovered to be key players of HSC regulation (Yin and Li 2006). Their specific function is to retain the so-called Long-Term (LT)-HSCs in a quiescent *status*, as also demonstrated by the evidence that conditional ablation of osteoblasts leads to depletion of LT-HSCs (Visnjic et al. 2004).

Multiple signaling and adhesion molecules contribute to HSC-SNO interplay, such as the Notch, expressed by HSCs, whose interaction with its ligand Jagged1 expressed by osteoblasts inhibits HSC differentiation and enhances their self-renewal capacity (Varnum-Finney et al. 2000). Angiopoietin-1 (Ang-1), expressed by osteoblasts, promotes HSC quiescence while Tie2, a tyrosine kinase receptor expressed by HSCs, induces their adhesion to the osteoblasts (Arai et al. 2004). Osteoblasts also release the Stromal Derived Factor (SDF)-1, *alias* CXCL12, the receptor of which, CXCR4, is expressed by HSCs. It has been demonstrated that high levels of SDF-1 on the surface of osteoblasts favor HSCs to return to the osteoblast niche (Yin and Li 2006). Finally, SNOs express N-cadherin, which has a controversial role in the osteoblast niche. In fact, while N-cadherin knock-out mice do not show any defect of HSC behavior, HSCs expressing a dominant negative N-cadherin display a reduced ability to repopulate the bone marrow due to an impaired anchorage to osteoblasts (for review see Lilly et al. 2011).

HSCs interact not only with SNOs but also with sinusoidal endothelial cells, which constitute an alternative niche in the bone marrow, called the vascular niche (for review see Lilly et al. 2011). Indeed, it seems that the SNOs are primarily involved in maintaining a quiescent HSC microenvironment, while the likely role of the vascular niche is to regulate stem cell proliferation, differentiation and mobilization of the Short Term-HSCs (ST-HSCs) which leads to the amplification and subsequent differentiation of the various hematopoietic lineages (Yin and Li 2006). *In vivo* evidence of the vascular niche arose from the finding that deletion of Vascular Endothelial Growth Factor (VEGF) in adult mice inhibited regeneration of sinusoidal endothelial cells and prevented hematopoietic reconstitution of irradiated animals (Hooper et al. 2009).

Cancer Stem Cells and the Bone Marrow Niche

An interesting hypothesis, strengthened by increasingly experimental evidence, is that CSCs compete with the normal HSCs to inhabit their physiologic niche in the bone marrow. Therefore, CSCs could be primed for their proliferation and invasiveness by mechanisms similar to those employed by the bone marrow niches to regulate HSCs fate.

Pioneer studies, conducted with leukemia cells, demonstrated that both niches regulated CSCs survival and proliferation. Indeed, Ninomiya et al. (2007) showed that, after transplantation of leukemia cells in immunodeficient mice, CSCs were located close to the endosteal surface in the diaphyseal inner vascular region. In this context, it has also been demonstrated the crucial role of CXCR4, the expression of which is associated with poor outcome in patients with Acute Myeloid Leukemia (AML). Consistently, administration of an anti-CXCR4 blocking

antibody to immunodeficient mice previously engrafted with leukemia cells significantly reduced their presence in districts such as bone marrow, spleen and blood (Ninomiya et al. 2007). Another crucial factor of leukemia cells homing is the CD44, since treatment with anti-CD44 antibody reduced the engraftment of AML cells in immunodeficient mice (reviewed in Guerrouahen et al. 2011).

Recent evidence suggests that tumor cell dissemination to the bone marrow represents an early event for breast cancer progression associated with poor prognosis. Moreover, a study from Abraham estimated the percent of $CD44^{+}CD24^{-/low}$ tumor cells in breast primary tumors, and found that the higher prevalence of the CSC pool correlated with bone metastasis development (Abraham et al. 2005). Interesting findings arise also from Balic et al. (2006), who evaluated 50 bone marrow specimens from early breast cancer patients, showing in all of them a subset of tumor cells with the typical $CD44^{+}CD24^{-/low}$ stem phenotype, thus strengthening the hypothesis that these stem cells could be the initiating pool of bone metastases.

As far as prostate cancer is concerned, a recent study performed on human prostate cancers matched with bone metastases samples demonstrated that the frequency of prostate CSCs resulted to be higher in the bone metastases compared to the primary tumor, thus suggesting that these metastases are caused by the local expansion of the CSC pool (Eaton et al. 2010). In agreement with these observations, Colombel et al. (2012) have recently shown that the development of bone metastases correlated with the number of tumor stem-like cells present in the primary tumor.

A strong evidence of a physical interaction between tumor cells and the bone marrow niche comes from a recent work by Shiozawa et al. (2011), who demonstrated that prostate cancer cells compete with the HSCs for the occupancy of the endosteal niche after their transplantation in the bone marrow. Indeed, the ability of these cells to metastasize the bone in animal models is enhanced when the number of osteoblasts in the endosteal niche is increased by treatment with ParaThyroid Hormone (PTH). Moreover, the same mechanisms able to mobilize HSCs, such as the block of the CXCR4/CXCL12 signal or the treatment with Granulocyte-Colony Stimulating Factor (G-CSF), also mobilize prostate cancer cells, thus promoting bone metastasis development. This work clearly demonstrates that prostate cancer cells can usurp the HSC niches of the bone marrow and employ the same mechanisms of HSC regulation to enter and egress the niche. The same group recently published a paper (Joseph et al. 2012) showing that once reached the bone marrow, prostate cancer cells not only compete with the HSCs for the occupancy of the niche, but they also stimulate osteoblast or osteoclast differentiation (according to the type of prostate cancer cells injected) through the HSCs and the hematopoietic progenitor cells (HPCs), eventually leading to the development of mixed, osteoblastic or osteolytic lesions, respectively.

Beside the competition between tumor cells and HSCs in the lodge of the niche, other findings demonstrate the ability of HSCs to regulate prostate tumor growth in the bone marrow, by regulating the angiogenic switch (Okamoto et al. 2005). Moreover, an increase of bone marrow cellularity by treatment with PTH was demonstrated to stimulate prostate cancer cells local growth (Schneider et al. 2005).

Another molecule involved in the HSC-tumor cell cross-talk is Annexin II, which is expressed by endothelial and osteoblast cells and regulates HSC homing and engraftment. The study of Shiozawa et al. (2008) demonstrated that Annexin II signaling plays a crucial role in prostate cancer cell homing and metastatization to bone, regulating tumor cell adhesion to osteoblasts and endothelial cells. Moreover, blocking Annexin II or its receptor reduced the development of metastases in animal models.

Tumor Dormancy and Metastases

While it has been estimated that up to 90% of prostate cancer patients could develop bone metastases, long term follow-up studies indicate that about 30% of prostate cancer cases will

relapse in the bone within 10 years after radical surgery. Consistently, clinical evidence showed that although at the time of diagnosis 30% of breast cancer patients had micrometastases in their bone marrow, at least 50% of them did not develop clinical evident metastasis at 10 years follow-up. These are two clear examples describing a very crucial phenomenon related to tumor diseases, called tumor dormancy, that is the ability of several tumors apparently cured, to relapse even decades after their first diagnosis in the primary site. Tumor cell dormancy is described as "the prolonged latent phase that occurs in some patients between treatment and further evidence of disease progression" (Wicha et al. 2006).

The principal problem related to tumor dormancy is the presence/persistence of the disease at an undetectable level, so that it is very hard to identify patients harboring dormant cancer cells, with a consequent clinical underestimation. This is due to the fact that they are quite small in number and that there is not yet a specific panel of markers able to unequivocally identify the dormant phenotype of tumor cells.

Whether the identification of the signaling regulating tumor cell dormancy is a hot issue committing several researchers, from a clinical point of view it raises equal interest, if not greater, the identification of those mechanisms that trigger their "awakening", allowing tumor relapse.

Although the specific mechanisms driving tumor dormancy are still elusive, there are more and more evidence calling into question the CSCs described above. Therefore, it is thought that if cancer cells abandoning the primary tumor express a CSC phenotype matching with the requirements for their retention in a quiescent status by the hematopoietic niche, they can populate the bone marrow only upon microenvironmental changes permissive for CSC activation and amplification.

Bone is the preferred metastatic site for multiple myeloma and for solid tumors such as prostate and breast cancer *in primis* and to a lesser extent (approximately 30–60% of prevalence) for thyroid, renal and lung carcinomas. As the "seed and soil" theory teaches, the bone/bone marrow microenvironment is a quite suitable site to favor growth of metastases, due to (i) the high vascular supply, (ii) the presence of bone-stored growth factors eventually released after osteoclast activation and (iii) the presence of stromal and osteoblastic cells that stimulate tumor growth. A crucial aspect in skeletal metastases is the evidence that a small subset of cancer cells with stem features could be the principal players of tumor relapse. This hypothesis has received consensus especially for the bone colonization and metastasis formation by breast and prostate cancers. Cancer cells in bone marrow could also keep their latency, so that distant relapse may occur many years after successful primary tumor removal. This phenomenon has a dual meaning: it could make quite hard to completely eradicate the tumor, but it could also offer a large window for pharmaceutical prevention of the metastatic burst.

In conclusion, it seems well accepted that CSCs could play a role in the maintenance of minimal residual disease and consecutive metastasis of bone, by competing with the normal HSCs to inhabit the physiologic bone marrow niche. Therefore, the identification of the mechanisms regulating CSC behavior in the bone/bone marrow could represent an important target to improve our knowledge and advance the therapy to eradicate bone metastases.

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Treatment of Hemophilia A Using B Cell-Directed Protein Delivery

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Ali Ramezani and Robert G. Hawley

Contents

Abstract

Hemophilia A is an X-linked recessive genetic bleeding disorder caused by a deficiency or functional defect in coagulation factor VIII (FVIII). There is currently no cure for hemophilia A and the mainstay of treatment is FVIII replacement therapy by infusion of plasma-derived or recombinant FVIII at the time of bleeding. Although we and others have demonstrated successful correction of hemophilia A in a mouse model by hematopoietic stem cell (HSC) gene therapy, it remains to be determined whether the potential risks and toxicities associated with the approach will allow its translation into the clinic. Facing this obstacle, and in light of our recent data demonstrating the ability of B-lymphoid progeny of transduced HSCs to induce tolerance and direct sustained therapeutic levels of FVIII in hemophilia A mice, we have shifted our focus to mature antigen-specific memory B cells (MBCs) as an alternative target cell population for gene therapy of hemophilia A. MBCs are long-lived, resting cells that differentiate into long-lived, nondividing plasma cells while maintaining a reservoir of MBCs. Thus, these oligopotent progenitors share with HSCs the important property of self maintenance. We have devised a novel therapeutic approach that uses gene-modified MBCs that differentiate into FVIII-secreting plasma cells *in vivo*. This strategy uses antigenic immunization to generate antigen-specific MBCs which in principle could be isolated from the

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peripheral blood of patients, transduced with a FVIII-expressing lentiviral vector, and intravenously infused back into the recipient where they would recirculate through peripheral blood to places of antigen drainage (e.g., spleen), some of which would develop into long-lived FVIII-expressing plasma cells residing in the bone marrow. Periodic upregulation of circulating FVIII levels could be achieved if necessary using booster vaccinations (e.g., to tetanus toxin) to stimulate FVIII expression from long-lived antigen-specific MBCs and terminally-differentiated plasma cells.

Introduction

Hemophilia A is the most common severe hereditary bleeding disorder caused by a deficiency or functional defect in coagulation FVIII. It is inherited in an X-linked recessive pattern with an incidence of 1 in 5,000 live male births in all ethnic groups worldwide (Mannucci and Tuddenham 2001). In 30% of cases there is no family history of hemophilia and the disorder is caused by a spontaneous mutation in the FVIII gene. The disease is characterized by easy bruising and repeated bleedings into joints and muscles leading to chronic disabling arthropathy, although potentially fatal intracranial hemorrhage or retroperitoneal hematoma following trauma or surgery may also occur.

There is currently no cure for hemophilia A and the mainstay of treatment is FVIII replacement therapy by infusion of plasma-derived or recombinant FVIII at the time of bleeding. Alternatively, prophylactic regimens may be employed in which three to five infusions of FVIII are given per week in order to maintain adequate plasma levels of the protein (Blanchette et al. 2004). Although these therapies have increased the life expectancy of hemophiliacs significantly, they are inconvenient, expensive and—especially in the case of plasma-derived products, which are still widely used in economically developed countries and increasingly being used in Asia—have potential complications such as transmission of emerging blood-borne pathogens. More importantly, a serious complication of current protein replacement therapies is the development of inhibitory antibodies to FVIII (FVIII inhibitors) in ~25% of severe hemophiliacs, rendering the patients refractory to further FVIII infusions (Waters and Lillicrap 2009).

Critical Barriers to Correction of Hemophilia A by Gene Therapy

Hemophilia A remains an attractive candidate for somatic cell gene therapy for the following reasons: (1) It is a monogenic disorder; (2) Successful therapy only requires that the FVIII protein is secreted into the circulation; (3) A wide therapeutic window eliminates the need for precise regulation of FVIII production, with significant phenotypic improvement of patients obtained by 2% of normal plasma levels of the protein; and, (4) Genetically engineered FVIII-knockout mice and naturally FVIII-deficient dogs are convenient animal models for preclinical gene therapy experimentation.

Three phase I hemophilia A gene therapy trials have been initiated so far, all of which showed only minimal efficacy in some patients, indicating that much has to be improved in order to make this therapeutic modality a reality. The first trial was a non-viral *ex vivo* gene therapy targeting dermal fibroblasts that were implanted in the omental fat tissue of patients (Roth et al. 2001). The other two involved *in vivo* delivery of a murine leukemia virus (MLV) gammaretroviral vector (Powell et al. 2003) or a "gut-less" adenoviral vector (Chuah et al. 2004) into patients. The adenoviral trial was put on hold after the only patient enrolled showed hematological and liver toxicity (Chuah et al. 2004). Some patients from the other two trials had decreased FVIII usage for at least some duration (Roth et al. 2001). However, the lack of a clear-cut dose-response in these clinical trials and absence of placebo control groups makes it difficult to assess their efficacy.

The recent advances in gene transfer technology have accelerated progress toward a safe and efficient gene therapy for hemophilia A. A variety

of cell types (e.g., liver, muscle, hematopoietic) utilizing viral vectors derived from gammaretroviruses, lentiviruses, adenoviruses and adeno-associated viruses have been explored for hemophilia gene therapy (Petrus et al. 2010). Several of these strategies have resulted in amelioration of the bleeding disorder in hemophilia A mouse and canine models. However, as described above, these encouraging results have not yet been translated into correction of the bleeding disorder in hemophilia A patients. Furthermore, the use of integrating vectors required for long-term FVIII expression evokes concerns regarding the potential risks of insertional oncogenesis such that safer gene delivery vectors needed to be designed and utilized. Finally, preexistence or induction of an immune response to FVIII and/or the gene delivery vectors continues to be an important obstacle for in vivo gene therapy approaches in adults (Hu et al. 2011); an ideal solution would be to induce immune tolerance to FVIII without the procedure itself being immunogenic.

Enhancer/Promoter-Blocking Element FII/BEAD-A

Because insertional mutagenesis by conventional gammaretroviral vectors remains a concern in human gene therapy applications, much of our effort was focused on this issue (Ramezani et al. 2008a, b; Ramezani and Hawley 2010). Our hypothesis was that self-inactivating (SIN) gammaretroviral and lentiviral vectors devoid of transcriptional regulatory elements within their long terminal repeats (LTRs) and flanked by enhancer/promoter-blocking elements should be less likely to activate cellular proto-oncogenes than the conventional LTR vectors that are currently being employed in human gene therapy applications.

We developed a 77-base pair element, FII/BEAD-A (FB), which contains the minimal enhancer-blocking components of the chicken β-globin 5′HS4 insulator and a homologous region from the human T-cell receptor α/δ BEAD-1 insulator (Ramezani et al. 2008a). With a new flow cytometry-based assay, we showed that the FB element is as effective in enhancer-blocking activity as the prototypical 1.2-kilobase 5′HS4 insulator fragment. Importantly, we showed that the FB element inserted into the residual U3 region of the 3′ LTR of a SIN vector, was stably transferred to the 5′ LTR during reverse transcription, flanking the integrated transgene expression cassette. We then demonstrated using a recently established *in vitro* insertional mutagenesis assay involving primary murine hematopoietic cells that the already attenuated transforming potential of SIN gammaretroviral and lentiviral vectors could be completely eliminated under the experimental conditions employed, for both wild-type and hemophilia A genetic backgrounds. On the basis of these results, we proposed that the FB element-mediated enhancer-blocking modification represented a promising modification to dramatically improve the safety of any type of integrating vector for therapeutic gene transfer (Ramezani et al. 2008a).

Optimization of FVIII Transgene

In a subsequent study (Ramezani and Hawley 2009), we used our safety-enhanced RMSinOFB gammaretroviral vector to evaluate the efficacy of a newly bioengineered FVIII variant (eFVIII)—containing a combination of A1 domain point mutations (L303E/F309S) (Swaroop et al. 1997) and an extended partial B domain for improved secretion, plus A2 domain mutations (R484A/R489A/P492A) for reduced immunogenicity—toward successful treatment of murine hemophilia A. In cell lines, eFVIII was secreted at up to six-fold higher levels than our previous secretion-enhanced FVIII variant (sFVIIIΔB) (Moayeri et al. 2004). Most important, when compared with a conventional gammaretroviral vector expressing sFVIIIΔB, lower doses of RMSin-eFVIII-OFB-transduced HSCs were needed to generate comparable curative FVIII levels in hemophilia A mice after reduced intensity total body irradiation or nonmyeloablative chemotherapy conditioning regimens. Specifically, we

obtained comparable results by employing a more clinically-relevant HSC gene therapy protocol that: (1) Used a less genotoxic transgene delivery system; (2) Minimized vector copy numbers required per cell due to high-level eFVIII protein production; and, (3) Incorporated a nonmyeloablative transplant conditioning regimen.

Factor VIII Delivery by HSC-Derived B Cells

We recently extended our studies by directing FVIII synthesis to HSC-derived B-lineage cells using a safety-augmented lentiviral vector based on a nonpathogenic strain of simian immunodeficiency virus ($SIV_{mac1A11}$) and B lineage-specific immunoglobulin (Ig) transcriptional regulatory elements (EμPμ) (Ramezani et al. 2011). The rationale for this approach was the following: (1) Utilization of enhancer-promoter sequences that are inactive in HSCs should reduce the potential for insertional oncogenic transformation of these susceptible target cells; (2) FVIII secretion may be facilitated in differentiated B-lineage cells by the highly developed Ig secretory pathway in which the FVIII chaperone protein BiP (immunoglobulin-binding protein)/GRP78 is abundantly expressed to accommodate the increased rate of Ig production; and (3) FVIII expression in B cells could lead to induction of FVIII-specific peripheral immune tolerance (Scott 2010). Furthermore, since accumulating data indicate that lentiviral vectors are less genotoxic and thus pose less of an oncogenic risk than gammaretroviral vectors (Ramezani et al. 2008a), we have returned to a lentiviral vector platform (Ramezani and Hawley 2010) as a safer alternative to gammaretroviral vectors for our continued FVIII gene therapy studies.

More recently we reported the results of the above study (Ramezani et al. 2011), where we first examined the lineage specificity of the Ig transcriptional regulatory elements using a GFP reporter vector (FB.SIV-EμPμ-GFP). The vast majority of GFP expression was associated with $B220^+$ cells in peripheral blood, spleen and bone marrow, confirming B lineage-restricted activity of the EμPμ enhancer-promoter *in vivo*. Most important, we showed that transplantation of HSCs transduced with an analogous version of the lentiviral vector (FB.SIV-EμPμ-eFVIII) in which FVIII expression was placed under the control of the Ig transcriptional regulatory elements resulted in therapeutic levels of FVIII in the circulation of all transplanted mice for the duration of the study (6 months). Immunostaining of spleen cells showed that FVIII was selectively synthesized by $B220^+$ B cells and, as per design, by $CD138^+$ plasma cells. Subsequent challenge with recombinant FVIII elicited at most a minor anti-FVIII antibody response, consistent with significant suppression of host immune responsiveness to FVIII neoantigen (and lack of an overt cytotoxic immune response to FVIII-expressing B and plasma cells). All transplant recipients exhibited clot formation and survived tail clipping, indicating correction of their hemophilic phenotype. Therapeutic levels of FVIII could be transferred to secondary recipients by bone marrow transplantation, confirming gene transfer into long-term repopulating HSCs. Moreover, in pilot experiments, we showed that therapeutic FVIII levels could also be achieved in secondary recipients by adoptive transfer of HSC-derived splenic B cells for at least 4 weeks post transfer (the longest time point examined). These findings promoted us to further pursue B cell-directed protein delivery as a potential cell/gene therapy to treat hemophilia A.

Antigen-Specific Memory B Cell Gene Therapy for Hemophilia A

Although we and others have demonstrated successful correction of hemophilia A in a mouse model by HSC gene therapy (Moayeri et al. 2004; Ramezani and Hawley 2009; Doering et al. 2007), it remains to be determined whether the potential risks and toxicities associated with the approach will allow its translation into the clinic. Facing this obstacle, and in light of our recent data demonstrating the ability of B-lymphoid progeny of transduced HSCs to induce tolerance and direct

sustained therapeutic levels of FVIII in hemophilia A mice (Ramezani et al. 2011), we have shifted our focus to mature MBCs as an alternative target cell population for gene therapy of hemophilia A. MBCs are long-lived, resting cells that differentiate into long-lived, nondividing plasma cells while maintaining a reservoir of MBCs. Thus, these oligopotent progenitors share with HSCs the important property of self maintenance. We hypothesized that utilization of the plasma cell high efficiency protein synthesis machinery may allow production of sufficient amounts of FVIII to engender a therapeutic effect following delivery of a very small number of genetically-modified MBCs (Ramezani et al. 2011). Furthermore, studies by us and others have shown that B-cell-mediated FVIII antigen expression can lead to immune tolerance in hemophilia A mice (Ramezani et al. 2011; Scott 2010).

One of the most innovative translational aspects of our MBC gene therapy approach is the ability to isolate antigen-specific MBCs from the peripheral blood of patients, which continue to circulate for many years after infection or vaccination (Franz et al. 2011). [A procedure has already been reported that allows efficient transduction of human B cells with measles virus glycoprotein-pseudotyped lentiviral vectors (Frecha et al. 2009)]. Moreover, based on a transgenic mouse model of systemic erythropoietin delivery (Takacs et al. 2004) and our preliminary data, we predict that levels of circulating FVIII can be periodically increased *in vivo* if necessary by a secondary immunization step, equivalent to a tetanus booster shot. This approach is therefore a novel and yet logical extension of our own recent work for correction of hemophilia A. The following describes our key research findings and the related methodology on our MBC-directed gene therapy approach for correction of hemophilia A.

Ag-Specific MBC Targets

Using flow cytometry, we have characterized antigen-specific MBCs for use as the target cell population in our new hemophilia gene therapy approach. As a model antigen we are using 4-hydroxy-3-nitrophenylacetyl (NP) coupled to the T cell-dependent carrier chicken gamma globulin (CG) since the MBC response to NP has been very well characterized (Nishimura et al. 2011). In a pilot experiment, 6–10-week old hemophilia A mice were immunized with 200 μg NP-CGG (Bioresearch Technologies) intraperitoneally (i.p.) in alum adjuvant. Four weeks after the initial injection, the animals were rechallenged with 100 μg NP-CGG i.p. Spleens were harvested after another week and single cell suspensions were labeled for flow cytometry and cell sorting with predetermined optimal concentrations of NP-FITC and anti-B220-APC. Approximately 2% of the spleen cells stained positive with NP-FITC and anti-B220-APC (Fig. 22.1a), which were sorted and used for lentiviral vector transductions.

B Cell Culture and Lentiviral Vector Transduction Efficiency

Next, the conditions required for stable transduction of the MBCs were optimized using our SIV-derived lentiviral vectors (Fig. 22.1b). *In vitro* transduction of murine B cells can be performed with retroviral or lentiviral vectors preceded by activation of B cells with LPS or CD40L/IL-4 (Melo et al. 2002). LPS-activated B cell blasts have been transduced with retroviral vectors expressing antigens fused to the Ig heavy chain gene as a carrier molecule (Zambidis and Scott 1996). LPS-activated B cells have been shown to downregulate pathogenic T helper 1 cell immunity by triggering apoptosis of the T cells and/or inhibition of antigen presenting cell activity by the secretion of TGF-β (Tian et al. 2001). However, activated B cells can also exert pro-inflammatory activity because they often upregulate costimulatory molecules such as CD86. In addition, activated B cells have a short lifespan as they express high levels of Fas on their surface and are sensitive to apoptosis induction (Scott et al. 1996). On the other hand, tolerogenicity has also been achieved with resting B cells (Zambidis et al. 1997). Indeed, very recently, efficient tolerance induction with lentiviral-transduced B cells in a mouse autoimmune disease model was demonstrated

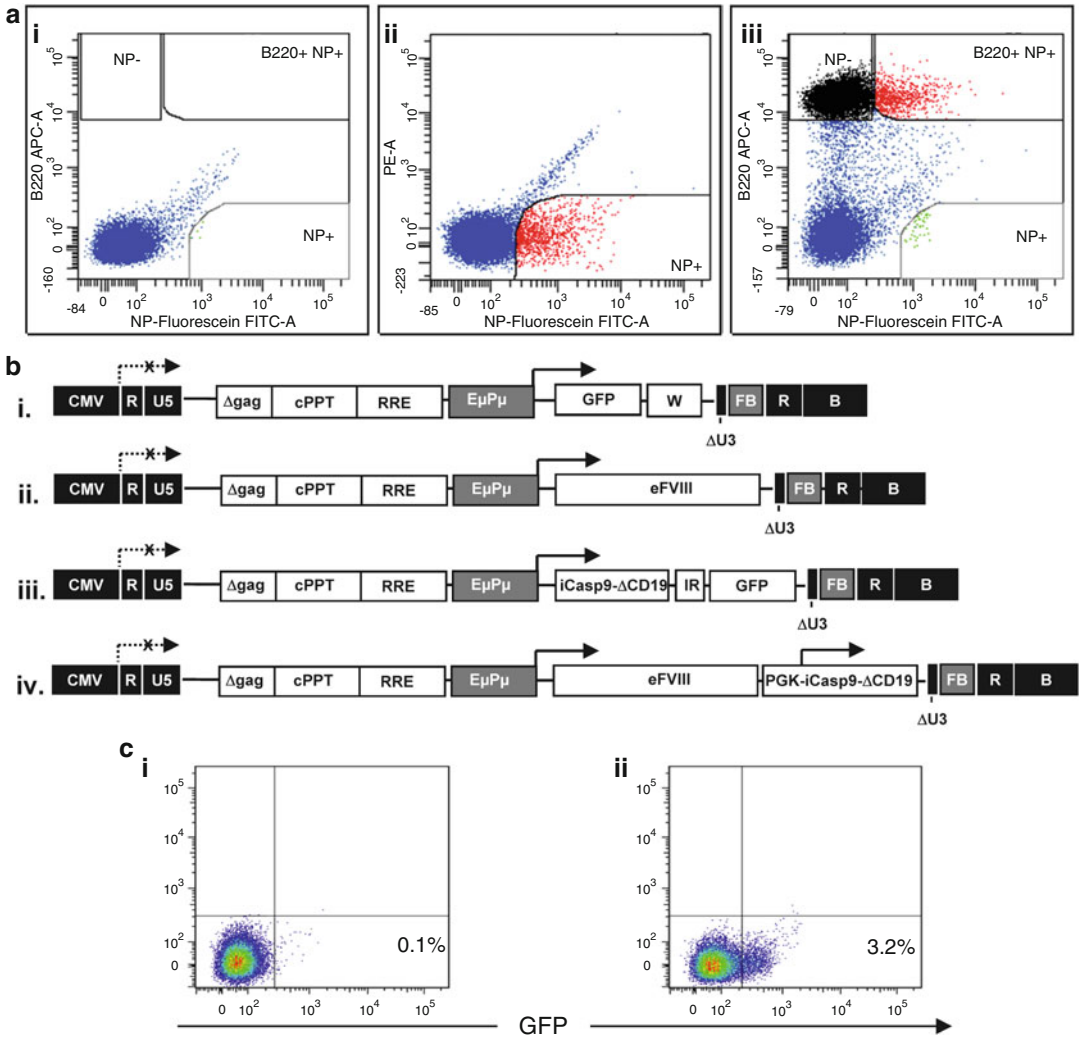

Fig. 22.1 (**a**) Frequencies and gating strategies for purification of antigen-specific MBCs. Total NP^+ (ii) or $B220^+NP^+$ (iii) cells were harvested from spleens 1 week after a second immunization with 100 μg of NP-CGG. Control unstained cells are shown in (i). (**b**) Schematic representation of the FB.SIV-EμPμ-GFP, FB.SIV-EμPμ-eFVIII, FB.SIV-EμPμ-iCasp9-IR-GFP, and FB.SIV-EμPμ-eFVIII-PGK-iCasp9 $SIV_{mac1A11}$-based SIN lentiviral vectors. Plasmid forms of the vectors are illustrated. (**c**) Detection of NP^+ B cells in recipient mice 3 weeks post transfer. Peripheral blood cells from control mice (i) and experimental mice (ii) were depleted of erythroid cells and analyzed by flow cytometry for GFP expression

(Calderon-Gomez et al. 2011). Therefore, because it is difficult to predict whether activated B cells might be pathogenic or not, our efforts were mainly focused on determining optimal culture conditions that allow efficient lentiviral transduction, cell engraftment, and tolerance induction in the absence of MBC activation. In a pilot experiment, 1×10^6 sorted NP^+B220^+ B cells were transduced with concentrated lentiviral vector particles: (1) for 4 h without any prestimulation; (2) overnight without any prestimulation; or (as a control), (3) overnight in the presence of 15 μg/ml LPS. Notably, our

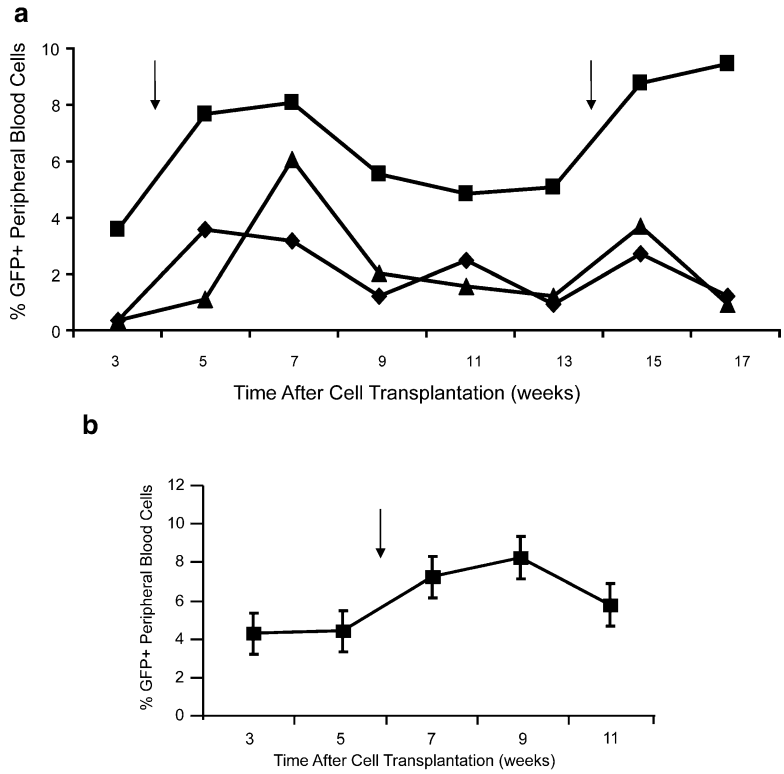

Fig. 22.2 (**a**) Engraftment of vector transduced antigen-specific MBCs. Cells were trasnsduced for 4 h (■), overnight (◆), or overnight in presence of LPS (▲), and transferred immediately afterwards into recipient mice (n = 3) preconditioned with a nonmyeloablative preparative regimen. Mice were boosted with NP antigen at the indicated times (*arrows*) and GFP^+ cells monitored for 17 weeks. (**b**) Antigen-specific MBCs were transduced for 4 h and transferred immediately afterwards into recipient mice (n = 5). GFP^+ cells monitored for 11 weeks. Mice were boosted with NP antigen at the indicated time (*arrow*)

results suggest that the short transduction protocol mediated the most efficient overall engraftment frequencies, with as high as 3% GFP^+ cells detected in the peripheral blood of recipient mice 3 weeks post transfer (Fig. 22.1c).

NP-Specific B Cells in the Recipient Spleen and Bone Marrow Following Antigen Recall

Expression of GFP in peripheral blood cells was monitored for up to 17 weeks, with the mice receiving i.p. booster immunizations of NP-CGG (100 μg) on weeks 4 and 14 (Fig. 22.2a). Preliminary data indicate that extended *in vitro* transductions lead to higher transduction rates; interestingly, this was associated with reduced numbers of circulating antigen-specific B cells in peripheral blood. In all cases, however, the transferred MBCs maintained their ability to recall and expand in response to subsequent antigenic challenges.

Long-Term Cell Engraftment Efficiency

Different culture and transduction conditions were compared as we monitored the transferred GFP^+ B cells in the peripheral blood of the recipient mice (for up to 17 weeks) (Fig. 22.2a). Based on the above preliminary data, the experiment was repeated exactly as before using the 4-h-transduction protocol followed by transfer of

1×10^6 cells. At 3 and 5 weeks post-transfer, GFP expression in peripheral blood of treated mice was analyzed by flow cytometry. At week 6, mice received an i.p. booster immunization of NP-CGG (100 μg) and were analyzed for GFP expression in peripheral blood by flow cytometry biweekly afterwards (Fig. 22.2b). This experiment confirmed the reproducibility of the results presented in Fig. 22.2a, since similar conditions for transduction, engraftment and booster immunizations were carried out in a larger ($n = 5$) cohort of mice (which is ongoing). We have observed ~2-fold increases in percentage of GFP^+ peripheral blood cells following each round of booster immunization.

FVIII Transgene Expression and Therapeutic Activity

To evaluate whether the NP-specific MBCs can be used to direct expression of human coagulation FVIII protein to their progeny plasma cells, we isolated NP-specific B cells as described above and transduced them with the eFVIII-expressing SIV-derived lentiviral vector, FB.SIV-EμPμ-eFVIII (Fig. 22.1b). 1×10^6 transduced cells (~8% transduction efficiency) were transferred into hemophilia A mice ($n = 12$). Preliminary results are highly encouraging as we could detect therapeutic levels of human FVIII in the plasma of all mice: with an average of 64 ± 22 ng/ml as early as 2 weeks following transfer, stabilizing around 48 ± 18 ng/ml (25% of normal) at 6 weeks post-transfer, the longest time point thus far (Fig. 22.3a). Our data clearly and reproducibly demonstrate: (1) successful isolation and transduction of antigen-specific B cells; (2) long-term engraftment of the transferred antigen-specific B cells and most importantly; (3) production of stable therapeutic levels of FVIII protein in hemophilia A mice that can be regulated by booster immunizations.

Based on our recent published data (Ramezani et al. 2011) and the data shown above, we anticipate induction of immune hyporesponsiveness using this approach. We are very encouraged to see sustained expression of FVIII in the plasma of all immunocompetent B cell recipients at the 6 week time point (and persistence of GFP^+ peripheral blood cells), which clearly indicates lack of a potent inhibitory immune response to FVIII as well as the absence of an overt cytotoxic immune response to FVIII-expressing B and plasma cells. This immune hyporesponsiveness has been obtained using NP^+B220^+ cells as well as a pool of NP^+B220^+ and NP^+B220^- cells (Driver et al. 2001).

In Vitro Safety Studies

In our studies, we employ a tissue-specific SIV-derived lentiviral vector with enhanced safety features that restrict transgene expression and decrease the likelihood of activating neighboring oncogenes. To provide an additional degree of safety, we have incorporated the conditional suicide system based on the inducible caspase-9 (iCasp9) safety switch into our MBC gene therapy approach (Straathof et al. 2005). iCasp9 can be activated using a specific chemical inducer of dimerization (CID) and is currently being evaluated in a Phase I clinical trial of adoptively transferred T cells (ClinicalTrials.gov Identifier: NCT00710892) (Straathof et al. 2005; Ramos et al. 2010). The FB.SIV-EP-iCasp9 lentiviral vector has been constructed (Fig. 22.1b) and the utility of the iCasp9 cassette tested in the mouse Sp2/0 B cell hybridoma. To induce apoptosis, the AP20187 CID, an analog of tacrolimus that binds the FK506-binding domain present in the iCasp9 product (B/B Homodimerizer, Clontech), was added at 50 nM to iCasp9-transduced Sp2/0 cells in culture. Apoptosis was evaluated 24 h later by FACS analysis, after cell harvest and staining with annexin V-PE (BD Biosciences) as we have done previously. In the absence of AP20187, the nontransduced, SIV-EP-GFP-, and SIV-EP-iCasp9-transduced Sp2/0 cells showed a spontaneous apoptotic rate of ~10%. Addition of AP20187 resulted in the apoptotic death of 60% of the iCasp9-positive cells by 24 h whereas the iCasp9-negative cells retained an apoptotic rate similar to that of controls (Fig. 22.3b).

In conclusion, all forms of medical treatment are accompanied by some risk of unwanted side effects. Nonetheless, irrespective of therapeutic

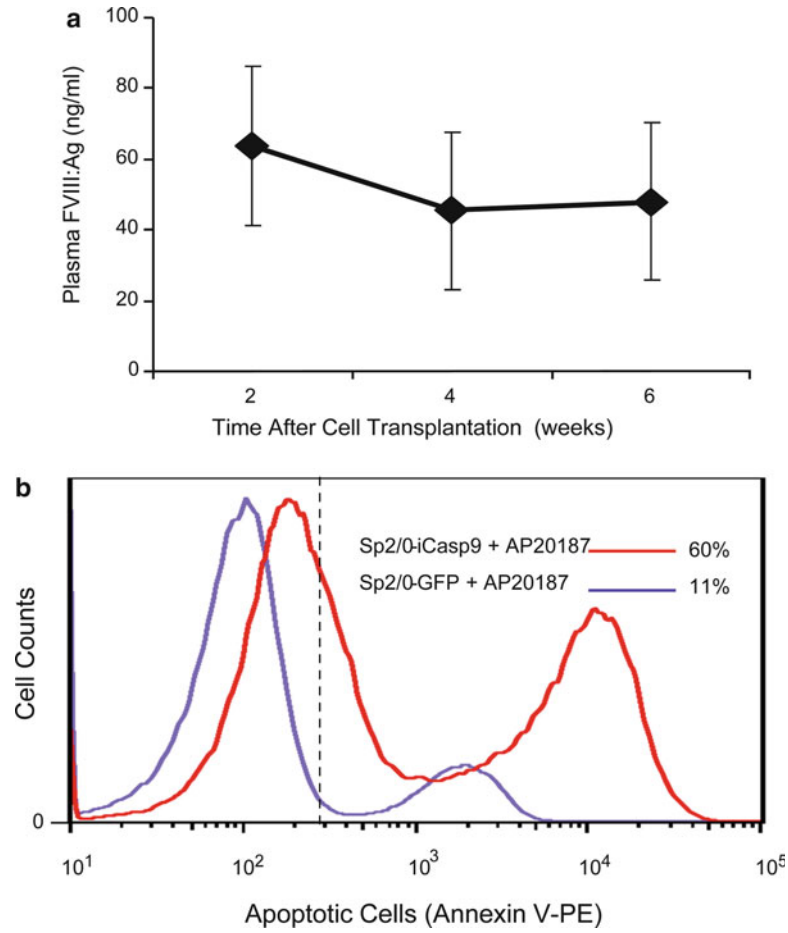

Fig. 22.3 (**a**) Human FVIII levels in the plasma of hemophilia A mice that received NP^+ MBCs trasnsduced with FB.SIV-EμPμ-eFVIII vector presented as mean values ± SDs at various time points after transfer (n = 12 mice). (**b**) *In vitro* apoptosis assay of iCAsp9-expressing Sp2/0 cells. Apoptosis was evaluated 24 h after exposure to AP20187 CID by FACS analysis, after staining with annexin V-PE

efficacy, a gene therapy approach will only become clinically acceptable if there is a low risk-to-benefit ratio. Hemophilia A remains an attractive candidate for gene therapy; but its clinical success requires overcoming a number of obstacles, necessitating development of a strategy that will achieve stable therapeutic FVIII expression and induce immune tolerance to the FVIII neoantigen while minimizing the risks of vector insertional mutagenesis or—in the case of somatic cell-based approaches—transplant-related complications. We believe that a "lower risk" strategy such as the MBC-based approach proposed herein will be more suitable for treatment of hemophilia A than HSC gene therapy, the latter of which remains a viable option for immunodeficiency patients without a matched donor. We anticipate that in sharp contrast to HSCs (and pre-B cells) which are known to be susceptible to insertional oncogenesis, use of MBCs as the therapeutic gene target cell population—especially in the context of our enhancer-blocking lentiviral vector backbone—will be associated with insignificant genotoxic risks. Irrespective of our findings in mice, because it is important to err on the side of caution when contemplating translation of gene therapy approaches to humans, to further augment the safety of our approach, we have also incorporated the iCasp9 "safety switch" in our lentiviral vector system that will allow us to eliminate gene-modified MBCs if necessary (Ramos et al. 2010); this safety feature is currently being evaluated in a clinical trial with adoptively transferred T cells (ClinicalTrials.gov Identifier: NCT00710892).

It should be underscored that there are also potential limitations associated with alternative hemophilia A gene therapy approaches, such as those involving i.v. injection of adeno-associated virus (AAV)-based vectors (Sabatino et al. 2011); e.g., certain AAV vectors are anticipated to require transient immunosuppression to some degree (Manno et al. 2006). Specifically, ~30% of the population already have neutralizing antibodies to AAV8 (Calcedo et al. 2009); in fact, neutralizing antibodies were detected in two of four patients who failed to show a therapeutic response in a recent AAV8 hemophilia B gene therapy trial (Ponder 2011); although permanent liver toxicity would seem not to be a significant concern with an AAV-based approach (Li et al. 2011), the risk of insertional mutagenesis still needs to be considered, especially if neonates are the target patient population.

In summary, the focus of this research not only addresses an important medical issue—i.e., the needs of hemophilia A patients for a safe and effective treatment strategy—from an academic perspective, we believe there is real potential for this highly innovative gene therapy approach to be translated to the clinic: (1) In humans, it would involve minimally invasive MBC collection from peripheral blood (Franz et al. 2011) and their transduction (Frecha et al. 2009); (2) It exploits the natural tolerogenic properties of B cells (Calderon-Gomez et al. 2011) involving B cell-mediated transgene delivery for tolerance induction in various systems, including the prevention/reversal of inhibitor formation in hemophilia A; and (3) It is also applicable to other disorders correctable by systemic distribution of recombinant proteins. Furthermore, the potential of engineered MBCs to induce immune tolerance to the newly expressed antigen could also be further investigated as a treatment strategy to correct autoimmune disorders.

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Part V

Stem Cell Transplantation

Reduction in the Risk Invasive Fungal Infection Relapse in Patients Undergoing Allogenic Stem Cell Transplantation Using Caspofunginas Secondary Prophylaxis

23

Paolo de Fabritiis

Contents

Abstract

Advancements in diagnosis and the development of new effective agents have improved the response of invasive fungal infections to appropriate therapies, allowing an increasing number of patients with hematologic malignancies to complete chemotherapy or to be submitted to stem cell transplantation. Prophylaxis with a number of antifungal agents has resulted efficacy against the majority of species, although the lack of conclusive results makes antifungal prophylaxis different from Center to Center. Caspofungin has excellent activity against Candida and Aspergillus species and a safety profile with reduced toxicities compared to other antifungal agents, resulting in one of the most interesting drug for both prophylaxis and therapy. Relapse of invasive fungal infection, however, remains a marker of poor prognosis, especially for patients candidates to stem cell transplant, because of the related risk factors. In recent years, the concept of secondary prophylaxis has emerged for patients more at risk, confirming that a history of invasive fungal infection is not a contraindication for allogeneic transplantation and that secondary prophylaxis could effectively offer a low-risk transplant related mortality. Although limited, studies using Caspofungin as secondary prophylaxis have demonstrated that this approach is feasible and effective in preventing relapse of fungal infections, allowing patients to complete the appropriate therapeutic strategy.

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Introduction

The need for antifungal prophylaxis to prevent invasive fungal infections (IFI) in hematological patients is still debatable, considering its potential role in inducing resistant strains and impairing the diagnostic accuracy of Aspergillus galactomannan (GM) antigen detection and its cost/efficacy ratio. On the other hand, a recent meta-analysis showed a reduction of IFI and mortality among patients with hematologic malignancies receiving antifungal prophylaxis (Glasmacher et al. 2003). Patients with acute myeloid leukemia (AML) are specifically considered for targeted antifungal prophylaxis because of the higher incidence of IFI and particularly invasive aspergillosis (IA) during induction chemotherapy.

The risk of developing invasive fungal infections (IFI) is particularly high in patients with hematological neoplasms, not only because of the disease-related immunodeficiency, but also as a complication of the newer therapeutic strategies and the increasing application of allogenic stem cell transplantation. The new molecules, intensively generated in the last few years, allowed both a more marked anti-neoplastic activity and the transformation of several incurable diseases in chronic types of evolution with a longer immunodeficiency and a consequent greater possibility of fungal infections.

Because of the limited therapeutic possibilities, they have been historically associated with high morbidity and mortality; in fact, the low number of antifungal agents (Amphotericyn-B represented the only available drug before 1990 years) and the poor diagnostic procedures have hampered the possibility of success. IFI also excluded many patients with leukemia from stem cell transplant approach, because exposed to a probable infection during the course of previous treatments and too much at risk of developing a fungal disease in the transplant context.

However, IFI remain a frequent cause of death in high risk patients, stimulating different prevention strategies with the aim to decrease the incidence, morbidity and mortality of developing such high life-threatening clinical situation. The impact of antifungal prophylaxis, both primary and secondary, is crucial and makes mandatory several considerations, since any prophylaxis involves a part of population that will never develop fungal infections and only a minority will benefit from it.

It is necessary, therefore, to identify the situations in which prophylaxis represents an effective advantage for the disease, making a balance between the clinical efficacy and the cost, particularly high for this category of agents.

Antifungal Prophylaxis in Patients with Haematological Disorders and in Stem Cell Transplant Recipients

Although the recent availability of some sensitive diagnostic procedures, such as galattomannan and beta-D-glucan determination, or fungal DNA identification through PCR techniques, early diagnosis of fungal infection remains difficult with the serious risk, as a consequence, of delay or even a cancellation of potentially curative chemotherapeutic strategies. In hematological patients, several risk factors for fungal infections have been identified, allowing the clinician to assign a risk level for each patient, contributing to the decision of starting an antifungal prophylaxis (De Pauw and Donnelly 2007; Segal et al. 2007).

The lack of conclusive results in the majority of studies makes antifungal prophylaxis different from Center to Center. This is certainly due to the many factors that can vary during treatments; however, some meta-analysis studies could amplify statistical evidence, showing that prophylaxis is associated to a reduction of IFI, but not to the whole mortality (Maertens 2007).

Fluconazole remains the largest utilized agent, since the study of Goodman et al. (1992). They demonstrated that in 350 stem cell transplant recipients, Fluconazole at 400 mg/day given intravenously up to neutrophil reconstitution, correlated with a significant IFI reduction and a lower mortality due to the fungal infection. Subsequent studies and meta-analysis could not

confirm these results, although indicated that a reduction of IFI and risk of death could be obtained at least in the categories of patients more at risk, such as stem cell transplant recipients (Kanda et al. 2000; Bow et al. 2002).

Itraconazole has also been used in prophylaxis since its wide spectrum of activity, including both Candida and Aspergillus species, and better plasma bio-availability. However, it is poorly tolerated and often interacts with other drugs, limiting its wide use.

Several studies confirmed a similar efficacy and tolerability between itraconazole and fluconazole (Glasmacher et al. 2006; Vardakas et al. 2005; Prentice et al. 2006) and that itraconazole may be used to prevent IFI, particularly if sustained by Candida non-albicans species.

A new generation oral azole, posaconazole, has recently shown a wide activity against resistant species such as Zygomicetes and Fusarium, associated to a low number of side adverse events (Nagappan and Deresinski 2007; Cornely et al. 2007). In high-risk patients, where the first generation azoles have demonstrated some limitation in efficacy and tolerability, posaconazole confirmed favorable characteristics to be utilized in antifungal prophylaxis.

Deoxycolate amphoterycin-B, considered the first developed major anti-fungal agent, has been proposed as prophylaxis in transplant recipients, although its liposomal form only was widely utilized, due to the low toxicity and good tolerability, as compared to the deoxycolate form. A recent study demonstrated that liposomal amphoterycin-B, administered by aerosols to prevent invasive pulmonary aspergillosis was efficacy both in the "intention to treat" and "on treatment" analysis (Rijnders et al. 2008).

Echinocandins have shown activity against Candida and Aspergillus spp together with a very low toxicity, representing a potential competitor to azoles for antifungal prophylaxis. However, a randomized study, comparing caspofungin and itraconazole in 200 leukemic or myelodisplastic patients, showed no differences in morbidity and mortality (Mattiuzzi et al. 2006). Furthermore, intravenous administration and high cost may represent a limit for a wider use in prophylaxis.

A different situation, however, might be represented by stem cell transplantation, where IFI remain a major cause of mortality (Wingard 1999; Hamza et al. 2004). If autologous stem cell transplant recipients are at risk early after transplantation because of neutropenia, allogeneic stem cell transplant recipients remain at higher risk for these infections after engraftment because of both developments of graft-versus-host disease (GVHD) and the long-term use of immunosuppressive agents for the prevention and treatment of GVHD. The combination of activity and minimal toxicity make caspofungin an ideal agent for prophylaxis in this setting and stimulated considerable interest in whether echinocandins may be also effective in preventing IFI in the period after engraftment.

Development of Caspofungin and Application as Primary Prophylaxis in Stem Cell Transplant Recipients

Caspofungin was the first echinocandin antifungal agent to gain FDA-approval for use in the USA. Echinocandins are lipopeptide molecules that specifically inhibit beta(1-3)-D-glucano synthesis, not present in mammalian cells. Beta (1-3)-D-glucano is essential to the cell-wall integrity of many fungi including Aspergillus and Candida spp. but lacks significant activity against Cryptococcus neoformans. Caspofungin may have some activity against dimorphic fungi such Histoplasma capsulatum and Coccidioides immitis, although no clinical data are available. Caspofungin is eliminated through the liver by hydrolysis and N-acetilation with a spontaneous chemical degradation to an open ring compound and there is no need to change dosage in renal insufficiency. While it is necessary to reduce the dosage of caspofungin in moderate hepatic insufficiency, there are no data in severe hepatic insufficiency; overall, it is well tolerated even at very high dosages. Caspofungin has poor bioavailability (not available in oral formulation), its plasma half life is between 9 and 11 h in human, thus supporting once daily i.v., dosing. To reach therapeutic plasma level it is necessary to administer a loading dose the first day of therapy (70 mg),

Table 23.1 Efficacy results: patients with candidemia

Overall response at end of IV therapy			
Analysis	Caspofungin 70/50 mg n/m (%)	Amphoterycin-B 0.6–1.0 mg/kg n/m (%)	Estimated difference adjusted for strata (95.0% CI)
MITT (n = 186)	66/92 (71.7)	59/94 (62.8)	10.0%* (−4.5, 24.5)
Evaluable Patients (n = 150)	57/71 (80.3)	51/79 (64.6)	15.2%** (−0.6, 31.01)

Mora-Duarte et al. (2002)
MITT Modified Intention to Treat
*p-value = 0.218; **p-value = 0.056

Table 23.2 Overall efficacy results

Overall response at end of IV therapy			
Analysis	Caspofungin 70/50 mg n/m (%)	Amphoterycin–B 0.6-1.0 mg/kg n/m (%)	Estimated difference adjusted for strata (95.0% CI)
MITT (n = 224)	80/109 (73.4)	71/115 (61.7)	12.7%* (−0.7, 26.0)
Evaluable Patients (n = 185)	71/88 (80.7)	63/97 (64.9)	15.4%** (1.1, 29.7)

Mora-Duarte et al. (2002)
MITT Modified Intention to Treat
*p-value = 0.0861; **p-value = 0.0346

followed by 50 mg daily. If the patient is over 80 kilos the dosage must be 70 mg every day. Several studies demonstrated that single doses of 150 and 210 mg produces a 2.4 and 3.2 increase in exposure without toxicity, and multiple doses of 100 mg/day produces 2.5 greater exposure than 50 mg. No pharmacokinetics alterations have been observed, related to age, gender, race or genetic polymorphism. In patients <18 years, pharmacokinetics is generally lower than in adults, with a direct correlation between age and blood levels.

Few clinically significant drug interactions require dose adjustment or monitoring:

Caspofungin and cyclosporine should only be used concomitantly in patients for whom the potential benefit outweighs the potential risk. Patients who develop abnormal liver function tests during concomitant therapy should be monitored and the risk/benefit of continuing therapy should be evaluated. Standard monitoring of tacrolimus blood concentrations and appropriate dosage adjustments are recommended for patients receiving both therapies. When caspofungin is co-administered with inducers of drug clearance, such as efavirenz, nevirapine, phenytoin, dexamethasone, or carbamazepine, use of 70 mg daily dose of caspofungin should be considered. Clinical studies in healthy volunteers show that the pharmacokinetic of caspofungin is not altered by itraconazole, amphotericin B, mycophenolate, nelfinavir, or tacrolimus. Caspofungin has no effect on the pharmacokinetics of itraconazole, amphotericin B, or the active metabolite of mycophenolate.

The use of Caspofungin has been approved (FDA, EMEA, EPARS) for the treatments of aspergillosis as salvage therapy (Maertens et al. 2004), candidaemia, oesophageal candidosys, disseminated candidosys, empirical therapy in febrile neutropenia, both in adults and children. The studies of comparison between caspofungin versus amphoterycin-B (Tables 23.1 and 23.2) and caspofungin versus liposomal amphoterycin-B for empirical anti-fungal therapy were used for registration (Mora-Duarte et al. 2002; Walsh et al. 2004).

The interest of focusing on caspofungin as a prophylactic agent was motivated by its spectrum of action against Candida and Aspergillus spp., its

safety record and the lack of toxicity. Because IFI are considered a major problem among patients undergoing induction treatment for acute leukemia, a randomized multicenter study comparing caspofungin versus antifungal prophylaxis according to investigator policy was completed and results recently published on Journal of Antimicrobial Chemotherapy. All patients receiving IFI prophylaxis according to local policy were prospectively included in the study while, to allow the comparison, caspofungin treatment was assigned through a centralized randomized procedure. Over a 2 years period, 175 patients were included, with an overall incidence of 32/175 (18.3%) IFI. No differences between the two groups were found in term of possible/probable/proven infections. Only one IFI-related death was recorded. The Authors concluded that, in this cohort of patients representative of a multicenter policy care, the incidence and mortality of IFI were lower than expected and the efficacy and safety of caspofungin were similar to other prophylactic regimens (Cattaneo et al. 2011).

The frequent interactions of antifungal agents with immuno-suppressive drugs (such as the calcineurin inhibitor tacrolimus) and the inability of patients to receive potentially toxic agents because of hepatic and/or renal dysfunctions, stimulated several investigators to evaluate caspofungin as primary prophylaxis in stem cell transplant recipients.

A recent study developed at MD Anderson Cancer Center evaluated retrospectively stem cell transplant recipients who were given caspofungin-based prophylaxis, with the objectives to evaluate effectiveness and tolerability as primary prophylaxis against IFI, and to determine whether any patient characteristics are independently associated with an increased risk of breakthrough IFI during caspofungin prophylaxis (Chou et al. 2007). The median duration of caspofungin administration was 73 days, and only one of the nine cases of breakthrough IFI occurred during the neutropenic period. Median time to development of IFI was 65 days after transplantation, and most of the infections developed 61–90 days after transplantation. These observations are consistent with previous studies of IFI in allogeneic stem cell transplant recipients, confirming the favorable safety profile and the lack of documented adverse effects.

A recent study assessed the effectiveness and tolerability of caspofungin in stem cell transplant recipients who are poor candidates for triazole or lipid amphotericin B prophylaxis due to renal or hepatic dysfunction, determining whether any patient characteristics are independently associated with an increased risk of breakthrough invasive fungal infection during caspofungin prophylaxis. One hundred twenty-three adult stem cell transplant recipients (117 allogeneic) received caspofungin 35–50 mg/day for up to 100 days after transplantation, as primary antifungal prophylaxis between 2002 and 2005, collecting data on host and transplant characteristics, such as transplant type, neutropenia, graft-versus-host disease (GVHD) and corticosteroid use, as well as evidence of breakthrough invasive fungal infections. Median time to engraftment was 12 days (range 6–26 days), 50 patients (40.7%) developed GVHD of grade 2 or greater and received corticosteroids for more than 21 days. Median duration of caspofungin prophylaxis was 73 days (range 10–100 days) and nine patients (7.3%) developed breakthrough invasive fungal infections, with a median time to development of 65 days (range 12–88 days). Only one case occurred during the neutropenic period before engraftment. Multivariate analysis showed that Pseudomonas co-infection ($p = 0.04$) and infliximab therapy ($p = 0.02$) were associated with breakthrough invasive fungal infections. By day 100, two of the five deaths (4.1%) were directly attributable to IFI, although no caspofungin-related adverse events were reported. (Chou et al. 2007).

The excellent tolerability is certainly explained by some pharmacological considerations: caspofungin is a poor substrate for cytochrome P450 or P-glycoprotein and it is not a cytochrome P-450 inhibitor or inducer, therefore lacking many of the drug interactions described during azole therapies. Studies in healthy volunteers demonstrated that caspofungin does not affect concentrations of agents such as Itraconazole, amphotericin-B or the active metabolite of mycophenolate. However,

concentrations of tacrolimus may be decreased by 20%, while cyclosporin may increase the AUC of caspofungin by 35% and significant elevations in liver enzyme levels in a proportion of normal volunteers. Recommendations, therefore, of a careful monitor of patients receiving P450 inhibitors with liver function tests, eventually considering dose reduction of caspofungin, is mandatory.

The Relapse of Invasive Fungal Infection

Use of new effective, less toxic antifungals and implementation of high resolution chest CT leading to earlier antifungal therapies have improved responses and survival allowing longer duration of cytotoxic chemotherapy including stem cell transplantation. These developments, however, have increased the risks of IFI relapse, especially for those agents, such as Aspergillus, that were not fully eradicated by previous antifungal therapy and may be reactivated through the mechanism of a latent sub-clinical infection.

Clinical factors that may affect relapse of invasive aspergillosis are probably similar between patients undergoing chemotherapy for acute leukemia and those receiving allogeneic stem cell transplantation. Regarding patients with leukemia, an analysis of the European registry showed that among 72 patients with a proven invasive fungal infection, 51 (71%) had invasive aspergillosis. Statistical analysis showed that predictors of relapse during subsequent immunosuppression were steroid use, failure to induce complete remission of the hematological disease, use of cytosine arabinoside, administration of more than 3 antibiotics, antibiotic use for more than 30 days and neutropenia >28 days (Sipsas and Kontoyiannis 2006).

Different is the knowledge of risk factors for Aspergillosis relapse during transplantation. Whether the above factors, with the adjunct of graft-versus-host disease, viral infections and anti-viral therapies may apply for relapsing Aspergillosis in stem cell transplantation, is unknown. The majority of studies include too few patients to detect risk factors of relapsing fungal infection, while in those with a larger recruitment, none of the risk factors examined affected relapse or the mortality rate (Fukuda et al. 2004).

Because transplant procedures and conditioning regimens are evolving rapidly, re-evaluation of relapse risk in subsequent series of patients is crucial: protocols of reduced intensity conditioning regimens, or the use of peripheral blood stem cells should be carefully evaluated, since the reduced neutropenia may play an important role in preventing fungal relapse (Fukuda et al. 2003).

The site of initial Aspergillus infection tend also to influence the relapse risk; Viollier et al. (1986) demonstrated that 11 of 18 leukemic patients with prior Aspergillus sinusitis, had relapse and infection dissemination during subsequent chemotherapy and Offner et al. (1998) reported relapse in the original location in the great majority of patients with relapsed Aspergillosis after transplantation. Relapse of IFI has a higher mortality rate than primary infection. Similarly, mortality is higher among patients with prior documented IFI, compared with patients with prior possible infection, probably reflecting the higher fungal burden in patients with proven IFI, particularly if Aspergillus driven.

Secondary Antifungal Prophylaxis in Patients Submitted to Stem Cell Transplantation

Recent studies have indicated hat IFI is not an absolute contraindication for subsequent allo-transplantation, although its management during immunosuppression remain challenging (El-Cheikh et al. 2010; Allinson et al. 2008). Controversies, however, on both the type of prophylaxis and the drug selection have limited a wider use, in the absence of a firm position from the major international guidelines. In a large retrospective study, Fukuda et al. (2004) demonstrated that the duration of antifungal therapy before transplantation, the resolution of radiographic abnormalities and conditioning regimens are important variables to consider for

minimizing the risk of IFI recurrence and mortality after allo-transplantation. Another retrospective study from the Infectious Disease Working Party of the European Group of Blood and Marrow Transplantation reinforced the concept that a previous IFI is not a contraindication for allo-transplantation, if the patient has a low-risk profile, characterized by short duration of neutropenia after transplant, early stage of the disease and more than 6 weeks from the start of antifungal therapy to the infusion of allogeneic stem cells (Martino et al. 2006).

The lack of a general consensus on the use of antifungal prophylaxis is particularly related to patients with acute leukemia or submitted to stem cell transplantation, where the incidence of IFI is less than 5%. On the contrary, in high risk patients, or in those with a previous history of IFI, secondary antifungal prophylaxis should be performed, despite the absence of results from controlled studies.

In a prospective, Italian, multicentre phase-II study of secondary prophylaxis, Caspofungin was given at standard dose to 18 consecutive patients undergoing allogeneic stem cell transplantation (de Fabritiis et al. 2007). All had a previous probable or proven fungal infection and were treated and followed with the same clinical protocol. Eighteen patients were submitted to the transplant with a median age of 40 years (19–58); the initial diagnosis was acute leukemia in the great majority of cases. Thirteen patients underwent stem cells collection from a related and five from unrelated donor. Peripheral blood (ten cases) and bone marrow (six cases) accounted for the majority of stem cell source. Standard and reduced intensity conditioning regimens were used in 14 and 4 transplants, respectively. All patients had a prior diagnosis of fungal infection, with residual or absent lesions at lung CT scan or abdominal ultrasound and the absence of clinical signs of fungal infection at the time of enrolment.

Eleven patients with probable and five with proven infection had a pulmonary localization; Aspergillus and Candida were the cause of infection in five and one patient of the proven group, respectively. Treatment of the previous IFI included liposomal amphoterycin-B in 66%, caspofungin in 12%, voriconazole in 16% and any combination of the above drugs in 6% of the treated cases. Median time between fungal infection and stem cell transplant was significantly longer in the group with proven infection, than the group with probable infection. Caspofungin was very well tolerated and no major side effects were recorded.

The effect of transplant procedure on previous fungal infection was evaluated at day 30, 180 and 360 from stem cell reinfusion, comparing to the pre-transplant status. Of the 18 patients evaluable at day 30, 4 were considered stable, 12 improved and 2 progressed. In particular, of the six patients with proven fungal infection, five were considered improved and one with a stable IFI. Fifteen patients were evaluable at day 180 because three deaths occurred before day 30. Two patients were considered stable and 11 still improved at day 180, while 2 patients had their previous IFI progressed. Eleven patients were evaluable at 1 year of follow up, because of three additional deaths observed within 180 days and one patient with a follow up ahead of time. No patient showed signs of previous IFI progression; in particular, two patients were stable and nine improved. Of the six evaluable patients with proven IFI, one was stable and five improved at day 180, while two were stable and four improved at day 360 (Table 23.3).

At 31 months of follow up, the probability of survival of the 18 patients submitted to allogeneic SCT with a previous IFI is 45%. Three patients died for leukemia relapse or progression; five patients died for transplant related complications with evidence of fungal infection in two. Transplant related mortality of the 18 patients was 28.6%.

Several other factors influence the outcome and survival of transplanted patients at risk of IFI. The use of reduced intensity conditioning (RIC) regimens has been shown to reduce short-term TRM and in retrospective studies with a relatively large number of patients, RIC showed a reduced risk of invasive aspergillosis progression after transplant (Martino et al. 2006); however, fungal infections in the long-term follow-up may be less influenced by the use of non-myeloablative regimens, mainly because of

Table 23.3 Evaluation of previous fungal infection during follow-up

	Probable	Proven
Day + 30 (n = 18)		
Stable	3	1
Improved	7	5
Progressed	2	–
Day + 180 (n = 15)		
Stable	1	1
Improved	6	5
Progressed	2	–
Day + 365 (n = 12)		
Stable	0	2
Improved	5	4
Progressed	–	–
Not reached	1	–

De Fabritiis et al. (2007)

the prolonged immune-suppression (Fukuda et al. 2003; Junghanss et al. 2002). Immune dysfunction after transplantation, although variable in duration and severity, remains one of the major causes of fungal infection susceptibility. The restoration of immune function is critical and efforts to enhance the immune system by several modalities are currently being explored as a treatment for IFI.

Caspofungin is not the only agent used for secondary prophylaxis in stem cell transplantation. Cordonnier et al. (2010) evaluated voriconazole for secondary prophylaxis of previous proven or probable invasive fungal infections, in a 12-month, open-label, non-comparative study conducted in 45 patients undergoing allogeneic transplantation.

The crude survival rate at 12 months was 75.6% and the 1-year cumulative incidence of invasive fungal infections with death as a competing risk in the modified intent-to-treat population was 6.7 ± 3.6% Three patients developed an invasive fungal infection during the course of the study, all occurring within 6 months after transplantation. One of the infections was a proven recurrence of a previous proven Candida Albicans candidemia, one was a proven recurrence of a previous probable Scedosporium prolificans infection, which ultimately led to death, and the third case was a new probable Mucor spp. Zygomycosis in a patient with previous probable aspergillosis. These infections occurred at days 3, 16, and 66 post-transplant, respectively. Four hundred forty-five adverse events were reported. They were considered severe in 24 (53%) patients and serious in 23 (51%). Two patients discontinued the study as a result of adverse events, in both cases hepatotoxicity. The majority of the adverse events were considered to be unrelated to voriconazole. Although the main limitation is the open-label design, the positive results justify future comparative studies of voriconazole in this setting, recruiting adequate numbers of patients into randomized controlled trials.

The only comparison between antifungal agents employed in secondary prophylaxis was reported by Vehreschild et al. (2009) in a retrospective study including 448 patients with haematological malignancies collected and analysed in the Multinational Case Registry of Secondary Antifungal Prophylaxis, a project of the ID Working Party of the German Society for Hematology and Oncology. Of the 75 evaluable patients, 28 received caspofungin and 47 itraconazole. Characteristics of patients included a greater number of underlying disease progression, incomplete response of initial IFI at baseline and allogeneic stem cell transplantation in the group of patients receiving caspofungin.

There was no difference in the occurrence of breakthrough IFI as well as the overall mortality between both groups. Both itraconazole and caspofungin were equally effective in preventing second episodes of IFI, although risk of breakthrough infection was high in both groups. From the great majority of the studies it can be concluded that secondary prophylaxis in high-risk patients with a history or persistence of IFI, undergoing allogeneic transplantation is safe and effective. The use of caspofungin results in a very low toxicity and shows a low incidence of breakthrough infections. This allows stem cell transplant to proceed as scheduled, even if signs of florid fungal disease are still evident.

In conclusion, since amphotericin B deoxycholate had been considered the gold standard for the treatment of fungal infections in early 1960, several other agents have been produced and their activity confirmed in controlled studies.

The interest in minimizing detrimental effect of IFI has conducted scientists and industries worldwide in a common effort to produce and validate an extraordinary number of antifungal agents both in prophylaxis and treatment of the most aggressive form of fungal infections. The agents and type of therapy that are used today could be completely abandoned, therefore, in a near future.

Therapeutic approaches of hematological disorders, on the other hands, are evolving continuously, both in strategies and chemotherapies. Better supportive therapies have allowed more powerful anti-leukemic agents and a more profound immunosuppression. In addition, development of new drugs for diseases such as multiple myeloma, myelodisplastic or myeloproliferative syndromes has prolonged duration of treatment and survival, increasing the risk of fungal infections. The identification of specific marker or gene, responsible for the clinical manifestation of some disorders, such as acute promyelocytic leukemia, or chronic myeloid leukemia has allowed, on the contrary, a targeted therapy and a marked reduction of opportunistic infections with an extraordinary improvement of survival and quality of life. The hope of all patients and hematologists is that this approach could be feasible in the totality of disorders.

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24 Hematopoietic Stem Cell Transplantation in Elderly Patients with Myelodisplastic Syndrome and Acute Myelogenous Leukemia: Use of Busulfan/Fludarabine for Conditioning

Nelson Hamerschlak, Marcos de Lima, and Fábio Kerbauy

Contents

Abstract

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are, typically, diseases of the elderly, with a 5–15% long-term survival rate and a prognosis that worsens beyond 60 years of age. The survival rate is low, regardless of the chemotherapy regimen used. Bone marrow transplantation was traditionally not used in this age group, because of the high mortality rate due to the conditioning regimen. However, survival and leukemia-free survival with lower recurrence rates can be improved with immune therapy provided by reduced-intensity and reduced-toxicity preparative allogeneic bone marrow transplantation regimens, with encouraging results in studies using busulfan and fludarabine. The effective antileukemic effect provided by cytoreduction, together with the immunological graft versus leukemia effect, with long-term disease control, leads us to believe that a myeloablative regimen without significant toxicity could be successfully used in selected elderly patients.

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Introduction: Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) in the Elderly

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are typically diseases of the elderly, with a median age at diagnosis of

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65 years (Champlin 2011). AML usually becomes more aggressive as age advances, and long-term survival ranges from 5 to 15% (Estey and Döhner 2006). The results obtained in the treatment of AML in patients over 55 years are still very discouraging, and the prognosis worsens at ages beyond 60 years (Döhner et al. 2010). Little has been added to chemotherapy regimens in the past 40 years. The number of patients with long-term survival is very low, regardless of the regimen used for remission induction or consolidation (Cancer and Leukemia Group et al. 2006; Burnett et al. 2009).

Bone marrow transplant, traditionally, was not an alternative for patients in this age group, due to various factors including:

- Presence of co-morbidities, which are common among these patients, and affect the patient's tolerance to the conditioning regimen.
- Changes in the metabolism of different pharmaceutical agents in elderly patients, which interferes with the exposure and toxicity of systemic chemotherapeutic agents and also has an impact on the incidence and severity of chronic graft versus host disease (GVHD) caused by the release of cytokines by the damaged organs.
- Increased incidence and severity of GVHD due to an altered immune response, mainly related to the stimulatory activity of antigen-presenting cells.
- Reduced ability of the T cells of elderly individuals to mediate the graft-versus-leukemia phenomenon.

Historically, myeloablative transplantation in elderly patients has been little used, since it was associated with a high mortality rate due to the conditioning regimen (Döhner et al. 2010). However, several studies show that improved survival and leukemia-free survival with lower recurrence rates can be obtained with the immune therapy provided by reduced-intensity allogeneic bone marrow transplantation after a first complete remission had been achieved (Champlin 2011). Studies show encouraging results in elderly patients with AML or high-risk MDS (Döhner et al. 2010), regardless of age (Farag et al. 2011). However, the reduced-intensity conditioning (RIC) is associated with higher relapse rates than myeloablative regimens (Farag et al. 2011).

A recent study by Farag et al. (2011) was conducted with a population aged between 60 and 70 years, based on the Center for International Blood and Marrow Research data. The study compared 94 AML patients receiving non-myeloablative bone marrow transplantation in first remission, with a group of 96 patients treated with induction chemotherapy and consolidation in CALGB (Cancer and Leukemia Group B) protocols. The authors found a lower risk of relapse and longer leukemia-free survival in the transplanted group compared to the patients receiving conventional chemotherapy treatment. However, they were unable to demonstrate an increase in overall survival (Farag et al. 2011).

The retrospective study of Farag et al. (2011) is important because prospective studies are very difficult to carry out in this field, due to difficulties related to patient selection, variability among transplant institutions in terms of health care conditions for the older population, and the need for commitment to more intensive treatment regimens, on the part of the patient, their families and caregivers (Champlin 2011). The effective antileukemic effect provided by cytoreduction, together with immunological reconstitution, with long-term disease control, leads us to believe that a myeloablative regimen without significant toxicity could be successfully used in elderly patients, and could lead to improved leukemia control.

A comparative study of the busulfan and intravenous fludarabine (BuFlu) and busulfan and cyclophosphamide (BuCy) regimens demonstrated better overall survival, leukemia-free survival, and lower toxicity in the group of AML patients receiving BuFlu. Based on this study, the group from the MD Anderson Cancer Center proposed an analysis of 79 patients with AML and MDS in the sixth through eighth decades of life (Alatrash et al. 2011). The data confirmed that for selected patients, the use of such treatment is beneficial.

Historical Use of Busulfan and Fludarabine for Conditioning in Stem Cells Transplantation

Busulfan is a bifunctional alkylating agent, first described by Haddow and Timmis (1953). Since the demonstration of its potent antitumor effects, busulfan has been used extensively in the treatment of malignant diseases, especially neoplastic diseases of the blood and myeloproliferative diseases. It was used for a long time as a palliative low-dose oral therapy, and frequent monitoring of blood count was recommended (Canellos 1985; Hughes and Goldman 1991).

Santos and Tutschka (1974) investigated the use of busulfan in a murine model of aplastic anemia. The experience with this model was used to introduce high-dose chemotherapy based on oral busulfan for pre-transplant conditioning in non-human primates, and then in patients undergoing autologous and allogeneic bone marrow transplantation (Peters et al. 1987; Geller et al. 1989; Grochow et al. 1989; Dix et al. 1996; Vaughan et al. 1991). Since then, high doses of busulfan, most commonly in combination with cyclophosphamide (BuCy), have proven to be effective as an anti-leukemia treatment, when used together autologous or allogeneic hematopoietic stem cells. A comparison of BuCy and cyclophosphamide (CY) combined with total body irradiation (TBI) for the preparation of patients with neoplastic disease of the blood undergoing allogeneic bone marrow transplantation has shown that the BuCy regimen was better-tolerated, with similar odds of survival and recurrence, when compared with the regimen based on CY-TBI (Clift et al. 1994).

Therapy with high-dose busulfan has several advantages for use in myeloablative treatment before transplantation, although some controversy remains. Firstly, when using chemotherapy alone for the conditioning of patients undergoing bone marrow transplantation, dependence on a radiation unit – which generally has limited capacity to provide the necessary treatment on a fixed schedule – is avoided. Secondly, high doses of radiation are very toxic, especially to the lungs, and may require special protection measures (such as the use of a protector screen). In general, this excessive toxicity is not observed in chemotherapy. Thirdly, the treatment regimen based on radiation may be administered only to patients who have not previously received radiation. Many patients with lymphoma, Hodgkin's disease and leukemia have had extensive prior exposure to radiation for the control of locally aggressive disease, in sites such as the mediastinum, neck and central nervous system (CNS). Additional radiation as part of the pre-transplant conditioning therapy may cause irreversible and often fatal toxicity in such cases. However, most patients who have previously received irradiation can safely receive a regimen based on busulfan, provided the acute toxicity of the radiotherapy (usually within 2–4 months of radiotherapy) has decreased.

Fourth, in selected patients with leukemia recurrence following allogeneic bone marrow transplantation, a second transplant can still offer a chance to control the disease in the long term, or even complete cure (Champlin et al. 1985; Sanders et al. 1988). Due to the subclinical toxicity (which is irreversible), TBI may be used only once in the patient's lifetime, whereas combination chemotherapy can be administered after the treatment regimen based on previous TBI.

Toxicity

Unfortunately, the administration of oral busulfan has several drawbacks. When used in combination regimens in high doses, severe side effects are often observed in the liver, which prevents its use at doses higher than 8–10 mg daily: 2–3% of patients develop busulfan-induced pulmonary fibrosis (Collis 1980; Koch and Lesch 1976; Oakhill et al. 1981), occasionally severe, and in some cases, irreversible myelosuppression was also observed after prolonged administration (Canellos 1985; Hughes and Goldman 1991). Several investigators have reported veno-occlusive disease (VOD) of the liver, leading to the most severe side effect, fatal liver failure (Geller et al. 1989; Grochow et al. 1989;

Dix et al. 1996). Neurological disorders, such as tonic-clonic seizures and the occurrence of nausea and vomiting, are also common (Marcus and Goldman 1984; Martell et al. 1987; Sureda et al. 1989; Vassal et al. 1990). It is impossible to predict whether patients will develop liver failure, and it is not yet known whether liver failure occurs due to the toxicity caused by systemic administration of busulfan, or whether it is mainly due to the first pass effect. Based on the limited data available on the pharmacokinetics of busulfan, it appears that patients who absorb a large fraction of the ingested dose, with a prolonged high plasma concentration of busulfan, are at higher risk of developing serious side effects (Grochow et al. 1989; Dix et al. 1996).

Another disadvantage of oral administration of busulfan is that patients develop severe nausea and vomiting shortly after taking the medication – within half an hour to 2 h – and it is virtually impossible to accurately determine the amount of dose that is lost through vomiting. Furthermore, intestinal absorption of any given dose may be influenced by the patient's nutritional status, by the concomitant administration of other drugs that affect the intestinal microenvironment, by consumption of food close to the time of drug taking, and finally by inherent biological variability in intestinal absorption between different patients (Benet and Sheiner 1985).

Oral busulfan in high doses carries an inherent safety problem, due to the possible danger of inadvertent overdose, and the risk of sub-optimal dosing, causing relapse or persistent malignancy after the bone marrow transplant. The acute toxicity of oral administration of high doses of busulfan in combination with cyclophosphamide is described in detail elsewhere (Tutschka et al. 1987; Peters et al. 1987; Geller et al. 1989; Grochow et al. 1989; Dix et al. 1996; Clift et al. 1994; Vaughan et al. 1991; Marcus and Goldman 1984; Martell et al. 1987; Sureda et al. 1989; Vassal et al. 1990). VOD (veno-occlusive disease) is dose-related and only rarely observed in patients with the area under the curve (AUC), i.e., the area under the plasma concentration, of less than 1,500 mol/min (Dix et al. 1996).

Intravenous busulfan can be used dissolved in DMA/PEG400/dextrose (dimethylacetamide and polyethylene glycol 400) in water, in a daily dose, and is able to produce an area under the plasma concentration curve (AUC) equivalent to the oral formulation, in combination with fludarabine. It is expected that the dose of 3.2 mg/kg body weight will produce a median AUC of about 5,000 mM/min, based on a phase II study comparing the AUC obtained after the increase in intravenous doses of busulfan with that obtained after oral dose of 1 mg/kg body weight (Russell et al. 2002).

The combination of fludarabine and busulfan therapy, as a non-myeloablative conditioning regimen prior to transplantation, has produced encouraging results in patients who would not otherwise be eligible for myeloablative conditioning therapy (Shapira et al. 2004). Fludarabine is an effective drug against neoplastic blood diseases, and has been shown to be less toxic than cyclophosphamide in several studies. It inhibits the repair of DNA cross-links induced by alkylating agents and has a synergistic action with busulfan (Russell et al. 2002). These authors used a combination of intravenous busulfan (total dose of 12.8 mg/kg body weight for 4 days) and fludarabine (total dose of 250 mg/m^2 for 5 days), both agents given once daily, for 4 days, to approximately 75 patients with hematological disorders undergoing allogeneic hematopoietic stem cell transplantation (Russell et al. 2002). The safety data indicated that this regimen is well-tolerated and has no unexpected side effects, allowing a consistent engraftment and good antitumor effect. In addition, Russell et al. (2002) obtained limited pharmacokinetic data that support the previous idea that busulfan, in this dosing interval, presents a linear pharmacokinetic.

Current Treatment Protocol with Busulfan and Fludarabine

The intravenous (IV) dose of busulfan that has been used is derived from pharmacokinetic data from a phase II study with high doses of busulfan and cyclophosphamide therapy, together with data obtained from the above-mentioned study by J. Russell (Calgary) (Russell et al. 2002).

The dose of 3.2-mg/kg body weight was extrapolated from previous experience, administering 0.8 mg/kg every 6 h, with a total of 16 doses. The dose of 0.8 mg/kg had previously been shown to be pharmacokinetically similar to that achieved with 1.0 mg/kg orally. The dose of 3.2-mg/kg body weight is equivalent to a dose of 130 mg/m^2. This dose can be predicted as being able to produce the target median AUC of between 5 and 6,500 μM/min, and should be considered safe and associated with a low (10%) risk of VOD. Thorough analysis of the pharmacokinetics of IV administration of busulfan in relation to the weight of the dose (real weight versus ideal adjusted weight versus ideal weight) provided the information that the dosage based on body surface area derived from the actual weight could provide a more consistent dose of IV busulfan in adults (Vaughan et al. 1991). Therefore, the current protocol incorporated a change in dosing strategy in relation to the previous pattern, from using the ideal body weight for the administration of doses of busulfan to using body surface area derived from the actual body weight, even in patients who are more than 20% above the ideal body weight. The combination of IV administration of busulfan and fludarabine for myeloablative doses had not been used prior to the studies of Calgary and Houston, including the phase III publication comparing busulfan and cyclophosphamide and busulfan and fludarabine, showing the superiority of the latter.

The main cytotoxic actions of busulfan and fludarabine occur through alkylation of DNA, and by inhibiting the repair of DNA damage, respectively. We are learning that modulating the dose of busulfan by calculating the AUC definitely interferes in the aggressiveness of the disease/status performance, and therefore the patients' evolution, providing better therapy that is tailored to each situation or group of patients, to be analyzed based on the individual. The combination BuFlu is gradually becoming the preferred conditioning regimen for patients with AML and MS. It is also the regimen of choice at the MD Anderson Cancer Center, and at our transplant center at the Albert Einstein Hospital, São Paulo, Brazil, for elderly patients with myeloid diseases and good performance status (Alatrash et al. 2011).

Prognosis and Follow-Up of Patients

The key study analyzing the regimen of reduced toxicity with IV BuFlu in hematopoietic stem cell transplantation in patients with AML and SM in the elderly was published by Alatrash et al. (2011), representing researchers of the MD Anderson Cancer Center, in Houston (USA), and the Albert Einstein Hospital, in São Paulo (Brazil). The study included 79 patients aged 55 years or over, selected retrospectively from three studies carried out at these institutions using IV BuFlu (Alatrash et al. 2011).

Patients with adequate performance status and no major organ dysfunction were included and classified according to prognosis, based on cytogenetics. The conditioning regimen was fludarabine (40 mg/m^2) and busulfan 130 mg/m^2 administered in 3 h between days −6 and −3. Tacrolimus and minidoses of methotrexate were used to prevent GVHD. Antithymocyte globulin was used in patients with mismatched related donors or unrelated donors. The main characteristics of patients treated in the study by Alatrash et al. (2011) are presented in summary form in Table 24.1, in comparison with the results by Farag et al. (2011) and the results of both in Table 24.2.

The findings are promising, including overall survival, leukemia-free survival and incidence of GVHD. They allow us to continue using this treatment regimen in elderly patients, and to recommend its use. The remaining challenge is the high rate of recurrence: this should be further studied with various strategies, to better prepare pre-transplant patients and eliminate minimal residual disease, using new drugs in the conditioning (such as clofarabine), and using immunomodulating agents, or even vaccines in the post-transplant period. Recurrence was main cause of death (in 58%) (Alatrash et al. 2011). These results warrant continued investigation of this regimen.

Table 24.1 Some of the pretransplant patient characteristics selected from the studies by Alatrash et al. (2011) and Farag et al. (2011)

		Myeloablative reduced toxicity (Alatrash et al.)	Non-myeloablative (Farag et al.)
		n = 79	n = 94
Age		58 (55–76)	63 (66–70)
Gender	Male	49 (62%)	55 (59%)
	Female	30 (38%)	39 (41%)
Cytogenetics	Favorable	4 (5%)	2 (2%)
	Intermediate	50 (63%)	59 (75%)
	Adverse	20 (25%)	17 (23%)
	Unknown	5 (6%)	–
Graft received (donor)	Related	41 (52%)	
	Unrelated	38 (48%)	
Graft type	Bone marrow	38 (48%)	7 (7%)
	Peripheral blood stem cells	41 (52%)	87 (93%)

Table 24.2 Some of posttransplant outcomes selected from the studies by Alatrash et al. (2011) and Farag et al. (2011)

		Myeloablative reduced toxicity (Alatrash et al.)	Non-myeloablative (Farag et al.)
		n = 79	n = 94
Overall survival	Complete remission 1	71%	37%
	Complete remission 2	44%	
	Active disease	32%	
Leukemia-free survival	Complete remission 1	68%	32%
	Complete remission 2	42%	
	Active disease	30%	
	Acute graft-versus-host disease	37%	39%
	Chronic graft-versus-host disease	34%	39%

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Co-transplantation of Islets with Mesenychymal Stem Cells Improves Islet Revascularization and Reversal of Hyperglycemia

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Aileen King and Chloe Rackham

Contents

Abstract

Allogeneic islet transplantation offers the possibility to treat selected patients with Type 1 Diabetes Mellitus (T1DM). Limited availability of human pancreatic islets is a major obstacle to the more widespread use of islet transplantation as a therapy for the majority of patients with T1DM. This is exacerbated by extensive islet cell death during the early post transplantation period, which increases the number of islets required to achieve insulin independence. Additionally, suboptimal vascular engraftment contributes to the long term decline in graft function and survival. Mesenchymal Stem Cells (MSCs) play a major role in tissue repair through localized immunosuppressive effects and the release of soluble trophic factors to affect neighboring cells, making them excellent candidates for improving the engraftment and survival of transplanted islets. MSC-based modulations to the islet transplantation procedure therefore have the potential to reduce the number of donor islets required for each transplant recipient, which is likely to help in overcoming the problems associated with human islet donor shortage.

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Introduction

Type 1 Diabetes Mellitus (T1DM) is an autoimmune disease, resulting from the destruction of the pancreatic beta cells, which are the only cell type in the body which both synthesize and secrete the blood glucose lowering hormone insulin. Symptoms of hyperglycemia (elevated blood glucose concentration) present when approximately 70–80% of the total beta cell mass is destroyed by the patient's own immune system, at which point the majority of people are treated with daily subcutaneous insulin injections.

Although, it is possible to maintain reasonable glycemic control with exogenous insulin therapy, fluctuations in blood glucose concentration mean that chronic secondary microvascular complications can occur. Furthermore, a small subset of patients with T1DM suffer from hypoglycemia (low blood glucose concentration) unawareness, which is extremely dangerous and potentially life-threatening. For these patients, an alternative treatment is allogeneic islet transplantation. Islets are small clusters of highly vascularized endocrine cells, which include the pancreatic beta cell; therefore islet transplantation represents a clinical therapy for restoring beta cell mass in patients with T1DM. For the small subset of patients with hypoglycemia unawareness, allogeneic islet transplantation is a safer alternative to insulin therapy as it can restore hypoglycemia awareness and stabilize blood glucose control, thus substantially increasing the patient's quality of life. Allogeneic islet transplantation is also associated with improved glycemic control and reduced secondary complications compared to exogenous insulin therapy, thus it would be beneficial for the majority of patients with T1DM to be given a transplant.

Islet transplantation requires that the pancreatic islets are isolated from the surrounding exocrine component of the pancreas by collagenase digestion. This means that the islets can be transplanted to the patient without the surrounding exocrine pancreas, which makes up ~98% of the pancreas and is not needed for the purpose of beta cell replacement therapies. The isolation procedure disrupts the islet vasculature and therefore the transplanted islets need to revascularize during the post transplantation period to restore their blood supply. Unfortunately, inadequate engraftment (the adaption of the transplanted cells to their implantation organ with regards to revascularization, reinnervation, and reorganization of other stromal components), immunological rejection, suboptimal function of the transplanted islets and extensive cell death during the early post transplantation period taken together with the scarcity of donor human islet material prevents the widespread application of clinical islet transplantation. For patients who are given an islet transplant, these obstacles also limit the longevity of the islet graft and therefore the time period over which the transplant recipient is rendered insulin independent. Although some transplant centers have had successful outcomes with single-donor transplantations, the large majority of islet transplant recipients require islets from multiple donors, further exacerbating the problem of donor shortage. Thus, efforts to increases the success rates of single-donor transplantation are clearly warranted to make better use of the donor islets that are available. In addition, islet transplant recipients currently require chronic immunosuppression, which is associated with unwanted side-effects including painful mouth ulcers, peripheral edema and poor wound healing, as well as deleterious effects on the transplanted islet cells. Alternative immunosuppressive regimens or beta cell replacement strategies that could be carried out in the absence of immunosuppression, would therefore improve the quality of life for transplant recipients.

Mesenchymal stem cells (MSCs) are adult progenitor cells which give rise to differentiated mesenchymal cell types and can be expanded to clinically efficacious numbers *in vitro*. MSCs offer a host of immunomodulatory properties, which may be utilized in an allogeneic islet transplant setting, to reduce non-specific immune mediated islet cell death during the early post transplantation period. In the longer term, the immunosuppressive properties of MSCs mean

that they have the potential to prevent recurrent autoimmunity and allograft rejection, as demonstrated in a number of preclinical studies. In addition to their immunosuppressive properties, MSCs play a major role in tissue repair through the release of soluble trophic factors to affect neighboring cells (Xu et al. 2008). A number of experimental studies have demonstrated the potential for MSCs to stimulate the revascularization of transplanted islet cells (Figliuzzi et al. 2009; Ito et al. 2010; Rackham et al. 2011; Sordi et al. 2010), thereby promoting their function and survival in the longer term. These functional properties make MSCs excellent candidates for improving the longevity of transplanted islets, thereby making better use of the donor islets that are available for human islet transplantation.

Islets of Langerhans

The pancreas is divided into two major components. The majority of the pancreas (approximately 98%) is exocrine tissue, which serve as the major source of digestive enzymes in the body and ensures the breakdown of lipids, proteins and polysaccharides. The endocrine component of the pancreas consists of small clusters of cells called islets of Langerhans; first described by Paul Langerhans in 1869. These highly vascularized endocrine mini-organs are of limited size and are dispersed throughout the exocrine pancreas, as opposed to being merged into a single solid organ. The human pancreas contains one to two million islets of Langerhans, which contribute 1–2% of the gland. Approximately 55% of the endocrine cells in human islets are insulin secreting beta cells, 38% glucagon secreting alpha cells and 10% somatostatin secreting delta cells. The endocrine composition within the islets is known to vary between species, with rodent islets having a significantly higher proportion of beta cells than human islets (Cabrera et al. 2006). There is a complex interplay between endocrine cells within the islets, which ensures the fine-tuning of blood glucose homeostasis, maintaining blood glucose concentrations between 55 and 125 mg/dl in healthy individuals.

The specific organization of cells within the islet suggests a coupling between morphology and function, with two major mechanisms by which both endocrine and non-endocrine cells within the islets communicate with each other. The first is via paracrine interactions, in which a secretory product from one cell moves a short distance through the interstitial fluid to reach a target cell. The second mechanism is via the islet vascular system, with the co-ordinated function of all islet cell types ensuring optimal blood glucose homeostasis.

The islet microvasculature has been described as glomerular-like since its initial description by Paul Langerhans in 1869. The islets of Langerhans are one of the most vascularized organs in the body, with the developmental process ensuring that every beta cell is no more than one cell away from a capillary Endothelial Cell (EC) (Lammert et al. 2001). The close association between beta cells and islet ECs ensures that beta cells are able to respond rapidly to fluctuations in blood glucose concentration; secreting insulin into the circulation and maintaining tight blood glucose homeostasis (Lammert et al. 2001). The vascular density of the endocrine pancreas is higher than that of the surrounding exocrine pancreas, with wider more tortuous vessels present (Brissova et al. 2006). Islet ECs have 10× as many fenestrae as ECs in the exocrine pancreas, which allows for the rapid trans-endothelial transport of insulin into the circulation (Lammert et al. 2001).

Islets become disconnected from their vascular supply when they are isolated from the pancreas by collagenase digestion, for subsequent transplantation. This means that they are avascular during the immediate post transplantation period (Menger et al. 1989), until they have revascularized. Experimental studies have shown that the revascularization process takes 10–14 days to be completed and that it does not restore the dense vasculature of endogenous pancreatic islets. The rate at which islets revascularize is important to prevent excessive hypoxia-related cell death (Davalli et al. 1996).

Moreover, the insufficient blood supply to transplanted islets contributes to their inadequate function and impairs the longer term survival of the transplanted cells. Improvements in the rate or overall extent to which transplanted islets revascularize would therefore help to increase the longevity of transplanted islets, making the islet transplantation procedure more efficient.

MSCs have a number of angiogenic properties and have been shown to enhance the revascularization of transplanted islets in a number of preclinical studies (Figliuzzi et al. 2009; Ito et al. 2010; Rackham et al. 2011; Sordi et al. 2010), which correlated with improved islet function and capacity of the graft to reverse hyperglycemia. We will discuss the functional properties of MSCs and recent experimental studies which highlight the significant potential MSC-based therapies have for improving allogeneic islet transplantation outcome.

Mesenchymal Stem Cells

MSCs are pluripotent stromal cells, with the potential to give rise to cells of diverse lineages (Pittenger et al. 1999). The pioneering work of Friedenstein et al. (1968), lead to the identification (Prockop 1997) of plastic adherent cells termed colony forming unit fibroblasts, residing within the bone marrow, with the ability to differentiate into bone (Friedenstein et al. 1968). The finding that these cells were in fact able to differentiate into other mesodermal cell lineages, including tendocytes, chondrocytes and myoblasts lead to the popularization of the term 'Mesenchymal Stem Cells' by (Caplan 2007) and the ability of cells to differentiate into adipocytes, chondroblasts and osteoblasts *in vitro* now forms part of their classification as MSCs (Dominici et al. 2006). MSCs typically have a spindle-shaped fibroblast-like morphology and their classification also requires that they are plastic-adherent when maintained in standard culture conditions and that they express typical cell surface markers. MSCs should also lack the expression of typical hematopoietic and EC markers (Dominici et al. 2006). However, it is noteworthy, that the classification of human and murine MSCs does vary to some extent (Abdi et al. 2008) and the levels of expression of specific markers can vary between MSC populations, particularly after extended subculture (Meirelles et al. 2006).

The bone marrow contains two sources of stem cells; hematopoietic stem cells (HSCs), which renew components of the blood and MSCs which replace mesenchymal tissues. Although bone marrow MSCs represent a rare population of cells making up only 0.001–0.01% of all nucleated cells they can be extensively expanded in an undifferentiated state *in vitro* (Pittenger et al. 1999) and grown up to cell numbers that are clinically efficacious. MSCs were initially isolated from the stromal fraction of the bone marrow (Prockop 1997), however it has now become clear that they reside in the stroma of virtually all vascularized post natal tissues (Meirelles et al. 2006). MSCs are classified as stromal cells, which are cells of non-lymphoid origin, forming the framework of each organ; they also have the capacity to secrete stromal components. This subset of adult stem cells plays an important role in tissue repair and regeneration. By expressing various molecules, MSCs can support the adhesion, proliferation and survival of distinct cell subsets. Most adult tissues additionally contain reservoirs of tissue specific stem cells that can contribute to tissue repair and maintenance following some form of trauma, ageing or disease process.

MSCs can be derived from virtually all vascularized post natal tissues including the brain, spleen, liver kidney, lung, bone marrow, muscle, thymus and pancreas (Meirelles et al. 2006). It is thought that the distribution of MSCs is related to their existence in a perivascular nice, with evidence suggesting that they are resident in vascular walls (Meirelles et al. 2006), thus supporting growing evidence for their relationship with pericytes. Under normal physiological conditions MSCs appear to play a role as vascular supportive cells. In general, MSCs derived from different tissues have a very similar morphology and immunophenotype to bone marrow derived MSCs. However, there is

some evidence of variable differentiation capacities, suggesting tissue specific functions for different MSC populations (Meirelles et al. 2006). It is also important to consider that the functional properties and differentiation potential of stem cells is influenced by extracellular cues and the local microenvironment, as well as the intrinsic genetic programs within the cells. MSCs derived from different tissue sources have been shown to secrete varying levels of trophic factors and have different functional properties (Park et al. 2009), thus at present we cannot predict the efficacy of MSCs derived from different sources to improve islet transplantation outcome with any certainty. Although, MSCs derived from the kidney, pancreas and bone marrow have all been shown to have a positive influence on transplantation outcome in diabetic rodents or non-human primates, indicating a certain degree of flexibility in the choice of tissue source.

Mesenchymal Stem Cells and Beta Cell Replacement Strategies

The capacity for MSCs to differentiate into cells of diverse lineages means that they have emerged as a useful tool for a number of clinical applications involving tissue engineering or cell replacement. MSCs have been investigated in a variety of disease models including T1DM for a number of reasons, which include: (1) their capacity for tissue regeneration (Sordi and Piemonti 2010); (2) their anti-inflammatory and immunomodulatory properties (Abdi et al. 2008) and; (3) the positive paracrine influences they are capable of exerting upon adjacent cells including islet cells through the secretion of bioactive molecules (Xu et al. 2008). Emerging evidence suggests that MSCs derived from different tissue sources can help to improve the outcome of islet grafts in diabetic recipients, with most therapeutic mechanisms involving beneficial effects on the immune system and engraftment.

Recent experimental studies have emphasized the positive influence that MSCs can exert on islet function and survival in syngeneic islet transplantations, thus making better use of donor islets that are available. Additionally, studies have focused on the positive effects MSCs exert in an allogeneic transplantation setting, with evidence to suggest that MSC infusions or co-transplantation with islets can delay or prevent graft rejection. Thus, research to date highlights the capacity for MSCs to help in addressing the major obstacles, which limit the widespread application of islet transplantation: (1) the scarcity of islets available for transplantation; (2) the high rates of islet cell death; and (3) the need for life-long immunosuppression.

Regenerative Properties of Mesenchymal Stem Cells

The regenerative properties of MSCs, as well their capacity to promote tissue repair, mean that they have the potential to aid the engraftment of transplanted islet cells, which are subjected to a number of stresses during the isolation, pre-transplant and post-transplant period. Adequate revascularization is essential for the survival of any vascularized tissue, thus the angiogenic properties of MSCs alone are likely to have a positive influence on the engraftment of transplanted islets. The release of anti-inflammatory cytokines, anti-apoptotic, angiogenic and mitogenic factors all function together to create a niche, which is permissive for the repair of damaged tissue.

Additionally, the capacity of MSCs to migrate specifically to injured tissues, including islets (Lee et al. 2006), means that they have the potential to increase the regeneration of any remaining endogenous beta cells in the pancreas of the transplant recipient. Studies have shown that MSCs express chemokine receptors that enable them to migrate towards islet-secreted chemokines, thus partially explaining reports that MSCs have the ability to migrate to injured tissues. Interestingly, it has been suggested that hypoxia can also increase the migratory capacity of MSCs (Hung et al. 2007), which may be important during the immediate post transplantation period when the islets are most vulnerable to

hypoxic stresses (Davalli et al. 1996). The migratory capacity of MSCs is important as it may allow some flexibility in the route of MSC administration to islet transplant recipients, allowing for repeated infusions if this proves to be of benefit to transplantation outcome. Indeed, Berman and colleagues have shown that repeated intravenous infusions of MSCs results in the reversal of islet allograft rejection episodes (Berman et al. 2010).

At present, there is conflicting evidence regarding the capacity of MSCs to cause endogenous pancreatic beta cell regeneration, with some studies supporting this as a therapeutic mechanism of MSCs (Lee et al. 2006) and others that do not (Berman et al. 2010; Urbán et al. 2008). Interestingly, Urbán and co-authors suggest that MSCs need to be administered together with bone marrow cells to effectively achieve beta cell regeneration. Thus, the mechanism through which MSCs cause endogenous beta cell regeneration is likely to be complex, involving factors related to the presence of additional cell types, as well as MSC dose and timing/route of administration. A different, but potentially very useful application of MSC-based therapies for the treatment of T1DM, is to use the MSCs as 'feeders for pancreatic islet transplants' (Sordi and Piemonti 2010). With this approach, co-transplanted MSCs essentially act as 'islet helper cells', providing the transplanted islets with trophic support by creating a milieu of cyto-protective, anti-inflammatory, angiogenic and immunomodulatory factors that are likely to enhance the function and survival of the transplanted islet cells.

Co-transplantation of Mesenchymal Stem Cells for Enhanced Islet Graft Revascularization and Function

The revascularization process is highly complex, involving the digestion of the vascular wall by proteases and the migration, proliferation and differentiation of ECs. It is thought that ECs from the implantation organ of the transplant recipient (host ECs), as well as donor intraislet ECs from the transplanted islets both contribute to the formation of functional blood vessels within the transplanted islet tissue (Brissova et al. 2004; Linn et al. 2003; Nyqvist et al. 2005). Additionally, bone marrow derived ECs are another minor source of ECs, which are thought to contribute to the revascularization of transplanted islets. The revascularization process is a highly regulated process that occurs in response to tissue demand, thus during the early post transplantation period, the islets themselves express angiogenic factors that initiate the process.

There is substantial evidence showing that islet graft revascularization is suboptimal irrespective of the transplantation site used for both rodent and human islets (Lau and Carlsson 2009; Mattsson et al. 2002). Islets implanted beneath the renal capsule, spleen or islets transplanted intraportally all show reduced EC density, which correlates with impaired beta cell function and curative capacity of the graft. A number of experimental studies have shown that MSCs enhance the revascularization of islets transplanted intraportally (Ito et al. 2010) or beneath the kidney capsule (Figliuzzi et al. 2009; Rackham et al. 2011; Sordi et al. 2010), correlating with enhanced islet function. Importantly, MSCs derived from different tissue sources, including bone marrow (Figliuzzi et al. 2009; Ito et al. 2010), pancreas (Sordi et al. 2010) or kidney (Rackham et al. 2011) have all been shown to exert similar effects; enhancing the EC density of the transplanted islets, which is consistent with a more effective revascularization. The re-establishment of a blood supply to the transplanted islets is important for ensuring the adequate delivery of oxygen and nutrients, but also for allowing the rapid distribution of insulin and other hormones from the endocrine cells to peripheral target tissues. Increasing evidence emphasizes the importance of the islet vasculature, not only as a transport system, but also because of paracrine interactions between islet ECs and beta cells, which ensure the optimal function of both of these cell types.

MSCs are likely to influence the revascularization process through several mechanisms. They secrete a range of angiogenic factors, including vascular endothelial growth factor, IL-6, IL-8,

hemopoietic growth factor and platelet-derived growth factor (Park et al. 2009; Sordi et al. 2010), which are known to enhance islet revascularization (Brissova et al. 2006). Hypoxia is thought to stimulate the angiogenic potential of MSCs (Hung et al. 2007), indicating that the microenvironment surrounding co-transplanted islets and MSCs during the initial post transplantation period, is likely to provide the appropriate stimuli for MSC-modulated improvements in the rate or overall extent to which islets revascularize. Experimental studies in rodents have shown that ECs from the host implantation site rarely migrate into the transplanted islet tissue because of difficulties associated with islet ECM degradation, but also because the transplanted islets may express high concentrations of angiostatic factors preventing capillary in growth. MSC co-transplantation may help to enhance the revascularization process through the secretion of matrix metalloproteases (MMPs) (Ding et al. 2009), which facilitate migration of host-derived ECs into the islets (Johansson et al. 2008) by degrading the extracellular matrix (ECM).

In addition to their angiogenic effects *in vivo*, MSCs have been shown to maintain normal islet organization and morphology, in contrast to the abnormal islet structures observed in mice transplanted with islets alone beneath the kidney capsule (Rackham et al. 2011). The maintenance of normal islet size was also associated with enhanced EC numbers, suggesting that MSC co-transplantation may favorably alter the microenvironment to which islets are implanted, allowing for a more effective revascularization.

MSCs show tremendous phenotypic plasticity, with the ability to acquire tissue specific characteristics when subjected to the appropriate stimuli (Choi et al. 2005). Thus, an additional mechanism through which MSCs have been shown to improve islet graft revascularization is by directly differentiating into ECs themselves at the graft site (Ito et al. 2010). In the same study, Ito and colleagues also quantified the number of ECs present within the graft and showed that there were significantly higher numbers of ECs in MSC co-transplanted mice compared to control islet-alone recipients. Thus, MSCs have the potential to offer an additional source of ECs that may contribute to the revascularization process.

In a co transplant setting, the close proximity of MSCs to islet cells means that the array of angiogenic factors, as well as anti-apoptotic, anti-inflammatory, immunomodulatory and mitogenic factors all function together to create a niche which is permissive for the regeneration of islet cells, which are likely to have suffered substantial damage during the pre- and post-transplantation period. MSCs are known to have a host of paracrine properties that are potentially beneficial for islet graft revascularization and islet graft function, as discussed in more detail below.

Paracrine Properties of Mesenchymal Stem Cells

Paracrine interactions between cells occur when a secretory product moves a short distance through the interstitial fluid to reach a target cell. MSCs have been shown to secrete a number of soluble bioactive factors, which mediate trophic effects on the target cell (Xu et al. 2008). Notably, the paracrine influences of MSCs are likely to play an important role in their ability to promote tissue regeneration and repair, but are distinct from the capacity for MSCs to differentiate into other cell types including ECs and injured tissues. The secreted soluble factors may have a direct effect, influencing the target cell themselves (i.e. the beta cell), or indirect by modulating the activity of surrounding cells (such as ECs).

A number of studies have demonstrated the importance of soluble mediators, by utilizing a transwell co-culture system in which direct cell-cell contact between islets and MSCs can be prevented, but the diffusion of paracrine factors is allowed due to the separation of these cell types using a semi-permeable membrane (Jung et al. 2011; Park et al. 2009). Additionally, MSC-conditioned media experiments can also help in determining the importance of MSC-secreted factors (Jung et al. 2011; Park et al. 2009).

In addition to the array of angiogenic factors that MSCs have been demonstrated to secrete, thereby enhancing islet graft revascularization; experimental data also supports the hypothesis that MSCs may enhance islet graft function and survival by secreting immunomodulatory cytokines, which modulate the immune system to prevent graft rejection (Berman et al. 2010; Ding et al. 2009; Longoni et al. 2010; Solari et al. 2009) and inflammation mediated-islet cell dysfunction and death. Amongst the many soluble mediators indicated, Transforming Growth Factor-β, Hepatocyte Growth Factor, indoleamine 2,3-dioxygenase (IDO), MMP-2 and MMP-9 seem to be important and have been shown to suppress T and B cell activation and/or proliferation (Ding et al. 2009).

The paracrine activities of MSCs are known to be influenced by the microenvironment in which they reside. Recent studies suggest that pro-inflammatory cytokines, such as IFN-γ, IL1-β and TNF-α, which are present during the immediate post transplantation period, can upregulate the immunosuppressive properties of MSCs. Thus, the paracrine activities of MSCs are likely to be highly dependent upon the specific conditions in which the MSCs are cultured or those where the MSCs localize to *in vivo*.

The islet isolation procedure disrupts the complex cytoarchitecture of islets and causes the loss of trophic support from the surrounding ductal cells, ECM proteins and that provided by ECs lining the capillaries of the islet vasculature. Therefore, the additional trophic factors provided by MSCs are particularly important during the immediate post transplantation period before the revascularization process is completed, as well as during the pretransplant period where the islets are subjected to hypoxic stresses during culture. Thus, the paracrine mechanisms, through which MSCs have been shown to improve islet transplantation outcome in a co-transplant setting, should also be considered as a potential way to improve islet quality, function and survival during the pretransplant culture period.

Indeed, recent studies have indicated that MSC-derived trophic factors can improve islet quality after culture, as shown through reduced ADP/ATP ratios and DNA fragmentation, enhanced glucose-stimulate insulin secretion and increased expression of the anti-apoptotic molecules Bcl-xL and Bcl-2 (Park et al. 2009) *in vitro*. Additionally, streptozotocin-induced diabetic mice transplanted with islets precultured in MSC-conditioned media had better glycemia than control mice, which should be expected as the quality of islets prior to transplantation is thought to have an important impact on the success of islet transplantations.

It is clear that there are a number of paracrine mechanisms by which MSCs have been shown to exert their therapeutic efficacy in animal models of diabetes, however, it has additionally been suggested that direct cell-cell contact is important for maximizing the beneficial effects of MSCs on islet function (Jung et al. 2011). Efforts to ensure the close proximity of islets with MSCs, so that direct cell-cell contact is allowed during the pre- and post-transplantation period are therefore likely to maximize the potential for MSCs to improve islet graft revascularization and function.

Co-localization of Islets with Mesenchymal Stem Cells

It seems likely that the most efficient use of MSCs in terms of islet function and engraftment will ensure that they are localized to the same site as the islets. From this perspective, it is important to consider that there are site-specific differences in terms of the way in which transplanted islets adapt to their microenvironment. Clinical islet transplantations have almost exclusively been done through intraportal islet infusion using the percutaneous approach, with subsequent embolisation of islets in the portal vein, since the early pioneering work of the late Dr. Paul Lacy. Although, allogeneic human islet transplantation at the intraportal site can be considered a success, experimental and clinical research has more recently investigated the potential of alternative transplantation sites, due to a number of problems associated with the intraportal

transplantation site, as detailed in a recent review by (Espes et al. 2011). Briefly, these include the loss of islets due to the instant blood-mediated inflammatory response (IBMIR), which is particularly problematic because of the islet-blood contact that occurs following intraportal infusions. The portal vein also has high concentrations of immunosuppressive drugs, which can be detrimental to islet function. Furthermore, the liver parenchyma has a far lower oxygen tension than that of the endogenous pancreas, which further contributes to the suboptimal islet function seen after transplantation.

Certainly for intraportal transplantation, co-localization of islets with MSCs necessitates the use of composite MSC-islets, in which islets would be coated with a layer, or indeed multiple layers of MSCs, prior to their infusion. Alternative sites may offer some flexibility in terms of the way in which the MSCs should be administered, due to the fact that the islets are not dispersed throughout the vasculature at extra-hepatic sites; potentially allowing for co-transplantation strategies in which islets are transplanted as a pellet/cluster with the MSCs transplanted immediately adjacent to this. It is however important to consider that it may be beneficial to avoid transplantation strategies which implant islets as clusters, as this may lead to central necrosis of central parts of the graft. Thus, it may well be that the use of composite MSC-islets is the best option at all transplantation sites, because this method allows for islets to be spread out at the implantation site.

MSC-dependent immunomodulation depends upon both cell-cell contact and MSC-secreted soluble factors. Thus, it seems likely that transplanting composite MSC-islets would also maximize the potential for MSCs to exert their immunosuppressive functions, as well as their capacity to improve islet function and engraftment. Furthermore, the use of composite MSC-islets may improve the survival of the co-transplanted MSCs *in vivo*, as the islets provide a surface for MSCs to attach to; which may prolong the time period over which MSCs exert their beneficial effects during the post transplantation period.

Immunomodulatory Properties of Mesenchymal Stem Cells

Treatment of T1DM should ideally address both the insulin deficit and also the autoimmune response to the cells expressing insulin. Therefore, the immunomodulatory properties of MSCs are of great interest in the context of islet transplantation. MSCs have been shown to create a beneficial microenvironment through the secretion of anti-inflammatory and immunosuppressive molecules, which can modulate the host's immune system in a positive way, helping to prevent allogeneic graft rejection. Additionally, the immunosuppressive properties of MSCs are exerted through direct cell-cell interactions with T- and B-cells, as well as natural killer and dendritic cells. MSCs are also able to increase the numbers of CD4+CD25+ and CD4+CD25+FoxP3+ regulatory T cells and reduce the proliferation of both CD4+ and CD8+ T cells, which could potentially reduce or prevent recurrent autoimmunity.

MSCs have been described as hypoimmunogenic cells, due to their lack of expression of MHC class II molecules and most of the classical co-stimulatory molecules. This is beneficial in terms of immune rejection as it enables them to escape recognition by the host's immune system. In theory, MSCs have the potential to reduce or prevent the need for life-long immunosuppression, which has deleterious effects on transplanted islet cells, whilst producing serious unwanted side effects for the patient. In support of this hypothesis, Longoni et al have demonstrated the synergistic effect of MSCs with immunosuppressive drugs in mice (Longoni et al. 2010). Furthermore, there is evidence to suggest that intravenous MSC infusions may help to prevent allogeneic graft rejection in non-human primates (Berman et al. 2010). Thus, MSC co-transplantation strategies have

great potential to increase the longevity of islet grafts, which means that the transplant recipient remains insulin independent for a greater time period following transplantation. This, taken together with the potential for a reduced requirement for immunosuppressive drugs, may significantly improve the quality of life for islet transplant recipients, as well as increasing the efficiency of allogeneic human islet transplantation and therefore enabling the more widespread application of islet transplantation as a therapy for T1DM.

Despite encouraging preclinical data regarding the application of MSC-based modulations to the islet transplantation procedure; as with all stem cells, it is important to consider the potential side-effects and safety outcomes with the clinical use of MSC infusions/co-transplantation strategies. To date, reports of adverse events are certainly low, but some of the potential problems with MSC therapies are discussed below.

Clinical Trials and Safety Issues Concerning the Use of Mesenchymal Stem Cells

Numerous preclinical and clinical studies have demonstrated the safety and toxicity free-effects of MSC transplantation or intravenous infusion in different diseases, including cardiovascular disease, renal disease, Crohn's diseases, cirrhosis, osteogenesis imperfect, graft-versus-host disease and T1DM, as detailed at http://www.ClinicalTrials.gov.

It is important to consider the potential safety issues that may arise from the clinical use of MSCs, which by their very own definition are known to show phenotypic plasticity and hence could have the potential to form tumors in patients. A number of studies with murine MSCs have shown that they can undergo malignant transformation *in vivo*, but the risks associated with culturing human MSCs seem to be lower. Berman and colleagues showed that there were no chromosomal abnormalities in MSCs derived from the iliac crest aspirate of nonhuman primates through to passage 11 (Berman et al. 2010), supporting observations for human MSCs. To date there have been no reports of any malignant transformation of human MSCs in clinical trials aiming to treat autoimmune diseases (Abdi et al. 2008). However, the extent to which the capacity for tumor formation varies between species, tissue source and passage number is currently unknown, emphasizing the need for increased quality control testing and standardized characterization of MSC phenotype and functional properties. Furthermore, there is currently little information regarding the longer term effects of MSCs, with regards to tumourogenicity, but also the potential concern that MSCs may transdifferentiate into unwanted tissues, such as bone. For example, a recent study by Duprez et al. (2011) showed that syngeneic transplantation of their mouse MSC-islets resulted in bone formation underneath the kidney capsule (Duprez et al. 2011). However, it was noted that human MSCs are less likely to differentiate into bone *in vivo* unless the appropriate stimuli are present.

Despite some of the safety concerns regarding the use of MSCs clinically, it is clear that MSCs have a number of functional properties that make them excellent candidates for improving islet transplantation outcome: (1) They can be derived from virtually all vascularized post natal tissue, which may increase the supply of these cells for clinical use; (2) They can be rapidly expanded *in vitro* to numbers that are clinically relevant; (3) They are 'hypoimmunogenic' avoiding recognition by the host immune system; and (4) In addition to their positive cytoprotective and angiogenic effects, they also have the potential to help prevent graft rejection and recurrent autoimmunity. Although other cell types have been shown to have positive trophic or immunological effects on islet graft function, the capacity for these cell types to be expanded *in vitro* to clinically efficacious numbers is more limited. Furthermore, most other cell types do not inherently possess all of these major functional benefits, which together have great potential to address a large number of the problems associated with clinical allogeneic islet transplantation. Thus, MSCs have good potential to maximize the use

of the donor islets that are available, which taken together with their immunomodulatory functions, could potentially enable the more widespread application of allogeneic islet transplantation as a treatment for T1DM.

In conclusion, MSC-based therapies represent an attractive strategy to enhance islet graft function and revascularization, thus improving the efficiency of islet transplantation and potentially reducing the number of donor islets required for each transplant recipient. These should help to overcome the significant problem of human islet donor shortage within the clinical islet transplantation field, enabling the more widespread application of allogeneic islet transplantation as a therapy for T1DM.

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Significance of Interleukin-7 Receptor Alpha Polymorphisms in Allogeneic Stem Cell Transplantation

26

Klaus Müller, Zaiba Shamim, and Lars P. Ryder

Contents

Abstract

Recipients of haematopoietic allogeneic stem cell transplantation (HSCT) are at risk of severe complications due to prolonged post-transplant immune deficiency as well as graft versus host disease (GvHD), that are a major obstacles in the course of HSCT. Interleukin-7 (IL-7) is a cytokine with combined haematopoietic and immunoregulatory functions, essential for normal T cell development in the thymus as well as T cell homeostasis in the periphery. Accordingly, IL-7 is considered a strong candidate molecule in immuno-inflammatory disorders.

IL-7 signals through the IL-7 receptor (IL-7R), which consists of an α-chain (CD127) and the γc-chain (CD132). The IL-7Rα chain is also utilized by the receptor for the cytokine, thymic stromal lymphopoietin (TSLP). TSLP is involved in the thymic differentiation of regulatory T cells and modulation of the Th1/Th2 balance through effects on dendritic cells.

We have identified a number of single nucleotide polymorphisms (SNPs) in the exons of IL-7Rα including rs1494558 (+510C/T in exon 2), rs1494555 (+1237A/G in exon 4), and rs6897932 (+2087T/C in exon 6). These all give rise to amino acid substitutions. Both rs1494558 TT and rs1494555 GG genotypes of the donor are associated with adverse outcome in HSCT, including acute graft versus host disease (aGvHD) and increased treatment related mortality. Moreover, an association between

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the T allele of rs6897932 and increased frequency of relapse was shown, whereas the C allele was associated with increased risk of grade III–IV aGvHD. Interestingly, the C allele of this SNP has been associated with multiple sclerosis in a number of studies, and it is associated with increased plasma levels of soluble IL-7Rα, suggesting a biological mechanism. These findings may be of clinical significance in relation to the potential use of IL-7 treatment in HSCT and other conditions with lymphopenia. Furthermore, knowledge of the IL-7Rα genotypes of the donor may help to guide dosage adjustments of GvHD prophylactic medication, including cyclosporin A.

Introduction

Recipients of haematopoietic allogeneic stem cell transplantation (HSCT) suffer from a prolonged post-transplant immune deficiency that results in significant morbidity and mortality, caused by complete bone marrow aplasia and injured epithelial barriers, primarily affecting the gastrointestinal tract. Following this, the patients may suffer from a prolonged deficiency in the adaptive immune system with depression of both T and B cell functions. Infectious complications with virus, bacteria and fungi are major obstacles during the course of HSCT.

Another major challenge is the development of acute and chronic graft versus host disease (GvHD), which is initiated by alloreactive donor T cells. Although GvHD may be sought controlled by immunosuppressive treatment this may severely aggravate immunodeficiency and the risk of infections, and reduce the graft versus leukaemia effect (GvL), leading to increased risk of relapse in patients transplanted for haematological malignancies. Accordingly, further understanding of the pathways controlling immune recovery after HSCT is considered a key point in the improvement of transplant regimens.

Interleukin-7

Interleukin-7 (IL-7) is a cytokine with combined haematopoietic and immunoregulatory functions. IL-7 is essential for normal T cell development in the thymus as well as T cell homeostasis in the periphery. IL-7 signals through the IL-7 receptor (IL-7R), which is a heterodimer consisting of an α-chain (CD127) and the γ-common (γc)-chain (CD132). Binding of IL-7 to the IL-7Rα chain on cell surfaces leads to heterodimerization with the γc-chain, and triggering of JAK3, followed by phosphorylation of Janus kinase-1 (JAK1) and ultimately activation of STAT3 and STAT5. IL-7/IL-7R signalling also activates anti-apoptosis pathways by up-regulating the anti-apoptotic proteins Bcl-2 and Bcl-xL as well as lung Kruppel-like factor (Ponchel et al. 2011).

The expression of IL-7R is tightly controlled during thymopoiesis, being expressed on double–negative thymocytes, absent on double-positive thymocytes and then re-expressed on single-positive thymocytes (Mazzucchelli and Durum 2007). IL-7 is central for T cell development in the thymus, controlling the availability of the chromatin for V (D)J recombination. The fundamental significance of the IL-7 axis on T cell development is demonstrated by the fact that deleterious mutations of IL-7Rα leads to T-B+NK +phenotype in patients with severe combined immunodeficiency (SCID) (Puel et al. 1998). Animal studies have shown that IL-7 deficiency is related to impaired thymic function and reduced numbers of T cells, and IL-7 treatment in mice after HSCT partially corrects thymic defects including thymic hypocellularity (Chung et al. 2001).

IL-7Rα is also expressed on most mature T-cells and is essential for the peripheral homeostasis of T cells. In particular high IL-7R expression is seen in recent thymic emigrants and other naive T- cells, while the receptor is expressed at relatively low levels on FOXP3 + T regulatory cells (Tregs) (Mackall et al. 2011).

The CD127 chain is shared with another cytokine receptor, binding the cytokine Thymic

Stromal LymphoPoietin (TSLP). The TSLP receptor is a heterodimer consisting of CD127 and CRLF2 (Cytokine Receptor Like Factor 2) which is closely homologous to CD132. TSLP is produced by epithelial cells and has a key role in the induction dendritic cells with low IL-12p40 release, that drive the generation of induced Foxp3+ Tregs and TH2 cytokine responses (Ziegler and Artis 2010) and it has been much incriminated as a factor involved in the allergic inflammation (Brown 2011).

IL-7 is available as a result of continuous production by epithelial and stromal cells in the thymus, bone marrow and intestine (reviewed by Ponchel et al. 2011). A markedly increased IL-7 concentration is present during T cell deficiency caused by immune system disorders or induced by clinical interventions such as chemotherapy and HSCT. Moreover, elevated IL-7 concentrations have been shown in HIV (Napolitano et al. 2001), and in severe combined immunodeficiency (SCID), normalizing as the number of T cells recovers (Holm et al. 2005). Increased IL-7 levels during lymphopenia appears to be due to decreased receptor mediated clearing rather than increased production of IL-7 (Guimond et al. 2009).

IL-7 Receptor α Single Nucleotide Polymorphisms

Based on the known functions of IL-7 and TSLP, we hypothesized that genetic variations in the exons of IL-7Rα in the donor might influence the process of immune reconstitution after HSCT with potential impact on the risk of infections, acute and chronic graft versus host disease (GvHD), and treatment related mortality. Others and we identified a number of single nucleotide polymorphisms (SNPs) in the exons of the IL-7α gene (Shamim et al. 2003; Teutsch et al. 2003). Among these SNPs, rs1494558 (+510C/T in exon 2) and rs1494555 (+1237A/G in exon 4) give rise to amino acid substitutions in the extracellular region, while rs6897932 (+2087T/C in exon 6) results in aminoacid substitutions in the transmembrane region of the folded protein. These locations of the SNPs suggest a functional impact on the binding of IL-7 and TSLP, and the interaction with γc-chain/CRFL2, and/or the signalling through the receptor (Fig. 26.1). The genes encoding TSLP and CRLF have also been shown to be polymorphic but a database search does not

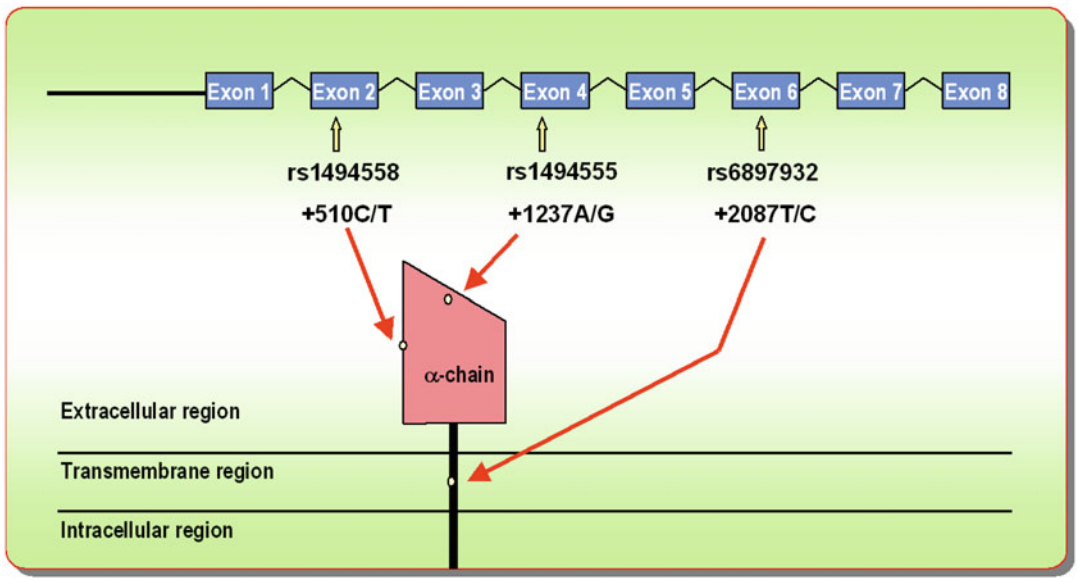

Fig. 26.1 IL-/Rα SNP positions and locations of immunoacid substitutions in the folded IL-7Rα chain

reveal non-synonymous SNPs. Genetic association has been found between SNPs located in the promoter region of CRLF2.

Studies in Haematopoietic Stem Cell Transplantation

Our initial study of a Danish HSCT cohort indicated higher treatment related mortality in MUD transplants with donors carrying the rs1494555 G (Shamim et al. 2006). The same pattern was seen for SNP rs1494558 T, which is in close linkage disequilibrium with rs1494555, and both alleles were associated with early onset chronic GvHD, but unrelated to the risk of relapse of leukaemia. In transplants with sibling donors the same pattern was seen, although the differences were not significant. In a subsequent study of a British-French cohort of mixed MUD and sibling donor transplants the same survival pattern was seen, and in this study the risk alleles were significantly associated with grade 3–4 acute GvHD as well (Shamim et al. 2011a).

Similar findings were seen in a multi-centre study encompassing 590 MUD transplants (Shamim et al. 2009). This larger study also allowed an appropriate analysis of the rarer HAP2 IL-7 haplotype, that has been associated with a protective effect in inflammatory diseases such sarcoidosis (Heron et al. 2009), inhalation allergy (Shamim et al. 2007), asthma (Kurz et al. 2006), multiple sclerosis (Lundmark et al. 2007; Gregory et al. 2007), and type 1 diabetes (Todd et al. 2007) as well as T-cell recovery in HIV-patients during combination anti-retroviral therapy (Rajasuriar et al. 2010). In this HSCT multicenter study rs6897932T (tagging HAP2) was significantly associated with increased risk of relapse of leukaemia, although this did not translate into reduced overall survival, probably due to a reduced risk of aGvHD in these patients (Shamim et al. 2009).

Functional Aspects of IL-7Rα Polymorphisms

Although the data from these three studies suggest an impact on IL-7R genotypes on alloreactivity in HSCT, the biological mechanisms that may explain these findings are not yet fully understood. A main clue in the search for the underlying mechanism is derived from the fact that associations between IL-7R SNPs and the clinical outcome were restricted to *donor genotypes,* while no effect of *recipient* genotypes has been observed. This points to a key role of haematopoietically derived, CD127 expressing cells, bringing T cells and dendritic cells of donor origin into focus.

Studies in mice and humans have shown that T cell reconstitution after HSCT is dependent upon extrathymic expansion of mature T cell populations as well as thymopoiesis (Dulude et al. 1997; Mackall et al. 1997). Moreover, reduced thymic function has been associated with increased occurrence of cGvHD, and there is evidence that IL-7 may preferentially stimulate the homeostatic proliferation of T cells in transplanted mice. To what degree IL-7 may induce GvHD is still controversial since an increased as well as an unaffected rate of GvHD has been observed in IL-7 treated mice (Alpdogan et al. 2003; Sinha et al. 2002; Chung et al. 2008).

In a group of HSCT patients, thymic function was determined by peripheral blood quantification of T cell receptor excision circles (TRECs), which are by-products of T cell receptor gene rearrangement that occurs during thymocyte ontogeny. In this study a reduced expression of both IL-7Rα and protein Bcl-2 even years after transplantation was noticed, and the authors concluded that the inability to reconstitute the naive T cell population was caused by defects in the IL-7/IL-7Rα pathway rather than impaired thymic function (Poulin et al. 2003).

Accordingly, IL-7Rα SNPs may modulate IL-7R signalling/activity and thereby influence

the ability of IL-7 to sustain de novo production of T cells in the thymus, with impacts on the rate of T cell recovery, diversity and tolerance and the release of FOXp3 positive T cells, of major importance for alloreactivity and the risk of infections in the post-transplant period. Secondly, altered IL-7 sensitivity may increase the risk of GvHD by expanding the population of alloreactive donor T cells in the periphery.

We noted a trend towards faster immune reconstitution for rs1494555 AA suggesting increased thymic production of T cells. Indeed, recent data by Broux et al. have indicated an association between numbers of circulating recent thymic emigrants in MS patients and an IL-7Rα promoter SNP (*rs*11567685) (Broux et al. 2010). However, analysis of TREC levels in CD4 and CD8 T cells in samples derived from the patients before and 1 year after transplantation did not indicate any effect of exon IL-7R SNPs on thymic T cell release (Shamim et al. 2010), and genotyping for rs11567685 did not reveal any significant associations with TREC levels (unpublished data).

Another possible mechanism to be considered, relates to the role of dendritic cells in the pathogenesis of GvHD and GvL. Induction of GvHD and GvL involves complex interactions in which dendritic cells and effectors from the immune system play a major role prior to the establishment of the allogeneic immune response. Although studies in experimental animals suggest that the presence dendritic cells of host origin is sufficient to mount an allogeneic immuneresponse after HSCT, alloantigens can also be presented by dendritic cells of donor origin and these may further augment CD8 T cell mediated GvHD (Mohty 2007; Schlomchik et al. 1999). Since maturation of dendritic cells is modified by TSLP through binding to the IL-7/CRLF2 receptor expressed on these cells, it is very likely that IL-7Rα SNP donor genotypes may modify interactions between donor derived dendritic cells and T cells and thereby influence alloreactivity and outcome in HSCT.

The locations of IL-7Rα SNPs suggest a functional impact at different levels: (1) interference with the binding of the ligands (IL-7 or TSLP), (2) impact on the interaction between γc-chain/TSLPR and IL-7Rα, (3) altered signalling through the receptor and (4) impact on the shedding of IL-7R from the cells. Different forms of IL-7Rα have been identified, including the membrane bound form (737 base pair), and a soluble form (IL-7Rα) (643 base pair) (Goodwin et al. 1990; Crawley et al. 2010), and both forms effectively bind the ligand. sIL-7R has been found to inhibit IL-7 activity *in vitro*, suggesting a role as a decoy receptor. Others and we have found an association between reduced serum levels of sIL-7R and HAP2 (tagged by rs6897932T) in healthy individuals (Hoe et al. 2010), and sIL-7R levels are also reduced post-transplant in transplants with donors having this genotype (Shamim et al. 2011a, b). Accordingly, the observed association between rs6897932 genotypes and outcome in HSCT may be related to variable levels of IL-7 decoy receptors.

Translational Aspects of IL-7Rα SNPs

Based on the known function of IL-7, this cytokine pathway appears a promising candidate for pharmacological optimization of immune reconstitution after HSCT. However, the clinical use of IL-7 may be complicated by the potential of IL-7 to aggravate GvHD. Clinical studies of patients with metastatic cancer have shown that administration of IL-7 may increase the number of CD8 and CD4 T cells, and decrease the percentage of regulatory T cells (Rosenberg et al. 1997) and IL-7 treatment of HIV patients has also shown a sustained increase in naive and central memory CD4 and CD8 T cells (Sereti et al. 2009; Levy et al. 2009). Thus the clinical use of IL-7 in HSCT may be complicated due to potential effects on immunoregulation and alloreactivity, and it is conceivable that this may vary depending on IL-7Rα genotypes of the donor. IL-7 is currently being tested in HSCT in patients receiving a T cell depleted graft (ClinicalTrials.gov identifier: NCT00684008). The results of this trial will be important to understand the therapeutic potential of IL-7 and the risks of this treatment. Future studies should address whether IL-7Rα

Table 26.1 Associations between IL-7Rα genotypes donors and outcomes in three studies of HSCT

	I	II	III
Cohort	Danish	British-French	NMDP[c]
N	195	116	590
Donor (N)	SIB (120) MUD (75)	SIB (93) MUD (23)	MUD (590)
Diagnosis	Benignant + Malignant	Benignant + Malignant	Malignant
Year of transplant	1988–2003	1987–2005	1988–2004
Donor genotype vs. with outcomes:			
Overall survival			
rs1494555 G	↓ (MUD)	–	–
rs1494558 T	(↓) (MUD)	–	–
rs6897932 C	–	–	–
Relapse			
rs1494555 G	–	–	–
rs1494558 T	–	–	–
rs6897932 C	–	–	↓
Treatment related mortality			
rs1494555 G	↑ (MUD)	(↑)	–
rs1494558 T	(↑) (MUD)	↑	–
rs6897932 C	–	–	–
Acute GvHD			
rs1494555 G	–	↑	↑[a]
rs1494558 T	–	↑	↑[a]
rs6897932 C	–	–	↑[a]
Chronic GvHD			
rs1494555 G	↑ (MUD)[b]	–	↑[a]
rs1494558 T	(↑) (MUD)[b]	–	↑[a]
rs6897932 C	–	–	–

I (Shamim et al. 2006), II (Shamim et al. 2011a) and III (Shamim et al. 2009). Recipient genotypes were not associated with outcome in any of these studies

SIB Sibling donor, *MUD* Matched Unrelated Donor

[a]Significant in univariate analysis, borderline significant in multivariate analysis

[b]Early onset chronic GvHD by day 100

[c]National Marrow Donor Programme

genotyping and monitoring IL-7 and sIL-7R levels may help to guide dosage adjustments in patients treated with IL-7. Moreover, risk-profiling based on non-HLA related polymorphisms, including IL-7Rα SNPs, may prove important for optimizing donor selection, and guiding the dosage of GvHD prophylaxis, such as cyclosporine A, based on donor IL-7Rα genotypes may be of potential importance. Prospective studies that include a range of candidate protein- and genetic markers in addition to IL-7R SNPs SNPs, e.g. TSLP and CRLF2, should address these questions.

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