Stem Cells and Cancer Stem Cells 9
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 9 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 and 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 82 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 six subheadings: Umbilical Cord Blood Stem Cells, Induced Pluripotent Stem Cells, Embryonic Stem Cells, Hematopoietic Stem Cells, Stem Cells in Tumors and Cancer, and Stem Cells for Regenerative Medicine for the convenience of the readers.

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

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

M.A. Hayat

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Part I

Umbilical Cord Blood Stem Cells

Cryopreservation of Hematopoietic Stem Cells from Umbilical Cord Blood for Transplantation

1

Vicente Mirabet and Pilar Solves

Contents

Abstract

Umbilical cord blood (UCB) has become an alternative to bone marrow and peripheral blood as a source of hematopoietic progenitors (HSC) for transplantation. Since the first cord blood transplantation was performed in Paris in 1988, knowledge of biologic characteristics of UCB has greatly improved. UCB banks have greatly facilitated transplantation activity worldwide. The main objective of UCB banks is to select, cryopreserve and store high quality UCB units. Because UCB transplantation may be lifesaving and a particular UCB unit may be the only potential source of HSC for a patient hematopoietic reconstitution, quality of UCB stored in a bank must be strictly assured. Volume reduction and cryopreservation are the standard method for the processing and storage of HSC from UCB intended for transplantation. The main risk in cell cryopreservation is the loss of cell viability. This aspect is especially important for UCB that contains a significant lower HSC content as compared to bone marrow or peripheral blood. The formation and growth of ice crystals and the consequences of osmotic stress on cell membranes can lead to irreversible cell damage. Thus, efforts to avoid it must be implemented in the different phases of cell processing. Dimethyl sulfoxide is the most used cryoprotector for maintaining progenitor cell viability for a long period. In this chapter cryopreservation methodology for UCB banking is exhaustively explained.

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Introduction

The long-term haematopoietic reconstitution of a Fanconi anaemia patient with cord blood in 1988 showed the effectiveness of umbilical cord blood (UCB) as a source of haematopoietic progenitors (HSC) for transplantation (Gluckman et al. 1989). Other authors had previously shown the potential of cord blood for a stable and durable hematopoietic graft (Broxmeyer et al. 1989). Currently, UCB has become a common source of haematopoietic progenitors for transplantation in the treatment of haematological malignancies, deposit diseases, marrow failure, immunodeficiencies, hemoglobinopathies and inherited metabolic diseases. Some advantages that have contributed to the increasing transplant UCB activity are: faster availability than for bone marrow/peripheral blood, reduced risk of infectious disease transmission, better biological characterization and immaturity of immunological subset that leads to less frequent and less severe graft versus host disease with preserved graft versus malignancy effect. These advantages have led many transplant centers have clearly opted for cord blood as regular source of haematopoietic progenitors for transplantation. From the first UCB transplantation until now, clinical results have improved with experience and the higher quality of cord blood units transplanted, supporting the efficacy and safety. Some disadvantages are the lower cell dose and delayed immunologic reconstitution and engraftment (Brunstein 2011).

UCB banks have greatly facilitated transplantation activity worldwide. The main objective of UCB banks is to select, cryopreserve and store UCB units while maintaining a high quality in all the successive processes. Today more than 400,000 UCB units are available in more than 50 UCB banks. The probability of finding an HLA matched donor depends on the number of UCB units stored. Although the optimal number of UCB units to cover the needs of allogeneic transplantation is not really known, it has been estimated in 50,000 for a population of 60 million inhabitants (Querol et al. 2009). Storage space in liquid nitrogen required for the high number of UCB units that will be stored for many years can be a limiting factor for UCB banking development. In order to overcome this and other problems as the cryoprotector toxicity, UCB collections are usually reduced to a standard volume before freezing. Cryopreservation is the standard method for the storage of HSC from UCB intended for transplantation. The main risk in cell cryopreservation is the loss of cell viability. The formation and growth of ice crystals and the consequences of osmotic stress on cell membranes can lead to irreversible cell damage (Mazur 1970; Meryman 2007). Thus, efforts to avoid it must be implemented in the different phases of cell processing. This chapter reviews the critical points, which should be taken into account in designing the UCB cryopreservation protocols under the clinical setting.

Volume Reduction of Umbilical Cord Blood

Dr Rubinstein designed in 1995 the volume reduction as an important step in the UCB processing strategy (Rubinstein et al. 1995). Currently, most banks perform UCB volume reduction before cryopreservation, following different techniques. Volume reduction methodology must guarantee high haematopoietic cell recovery (quantity and quality) and red blood cell (RBC) depletion by reducing the UCB units to a standard volume. The procedure must ensure the sterility of the samples by maintaining the system closed. Another important advantages are the reduction of cryoprotector quantity in the final product and the minimization of ABO incompatibility by reducing the red blood cell content.

Hydroxyethyl starch (HES) sedimentation was the first method developed for cord blood volume reduction by the New York Cord Blood Bank (Rubinstein et al. 1995). The HES method is a manual technique that requires two centrifugation steps. HES addition has the effect of increasing the red blood cells sedimentation rate. HES is added to the anticoagulated UCB to a final concentration of 1.2%, in a 1:5 volume ratio. After mixture centrifugation (50×g for 5 min at

10 °C), leukocyte rich supernatant is transferred to a 150 ml plasma bag and centrifuged to 400×g for 10 min. Plasma supernatant is then transferred to another bag and the pellet containing haematopoietic progenitors is resuspended in plasma to a total volume of 20 ml. Top and bottom (TAB) was a method originally developed for the peripheral blood component separation in blood banks and transfusion centres. In order to partially automate the procedure, some authors adapted TAB methodology for the UCB volume reduction purpose. The cord blood units were collected in triple bags and hard centrifuged to 2,000–4,000g ensuring that the bags were well supported to prevent disruption of the buffy coat layer. UCB collections are stratified into plasma, Buffy coat (BC) containing haematopoietic progenitors and red blood cells concentrate. The supernatant plasma and sedimented red cells are expressed by TAB devices as Optipress (Baxter Health Care) and Compomat (Fresenius Hemocare) leaving the BC in the primary bag (Solves et al. 2010).

Process automation could be critical to facilitate good tissue practices (cGTP) compliance for UCB banks. In order to better standardize the process and save time, two automatic devices have been developed specifically for cord blood volume reduction: SEPAX and AXP. Sepax was the first one and is currently implemented in many UCB banks, while AXP has been more recently developed. Basically, this methodology consists of a specific processing kit and a device. They work in an automatic way and reduce systematically to a standard volume without influence of technician (Solves et al. 2010).

Umbilical Cord Blood Cryopreservation

Cryoprotective Solution

Haematological reconstitution after cord blood transplantation requires efficient cryopreservation method to reduce the loss and the functional damage of pluripotent haematopoietic progenitor cells. The particular characteristics of UCB collections as the limited and often scarce number of progenitors contained makes mandatory to optimize cryopreservation and storage techniques.

Selection of Cryoprotectant

The word cryoprotectant is used to refer to substances, which protect cells from freezing injury. One of their most important properties is to lower the freezing point. Two kinds of cryoprotectant can be distinguished depending on their ability to enter inside the cell or not:

- Penetrating: dimethyl sulfoxide (DMSO or Me_2SO), glycerol, ethylene glycol (EG), 1,2-propanediol (PROH), propylene glycol, etc.
- Non-penetrating (impermeable to cell membrane): sugars (sucrose, glucose, trehalose), polymers (dextran, hydroxyethyl starch), etc. Generally, they are used in association with penetrating cryoprotectants, to prevent intracellular freezing (Allen et al. 1976).

The most widely used cryoprotectant in HSC and specifically in cord blood banks is DMSO. This is a hygroscopic polar compound, having a higher affinity for water than to protein, originally used as a solvent. Then, competition between cellular biomolecules and cryoprotectants for hydration may govern the partitioning of water to cellular constituents. Its cryoprotective effect was described by Lovelock and Bishop (1959). As a waterlike solute, it has the ability to reduce de amount of ice formed; and, because of its large molar volume, occupies a proportionally larger volume than intracellular salts, thus protecting against excessive cell shrinkage (Meryman 2007).

Dimethyl sulfoxide is used at a final concentration of 10% and can be diluted in normal saline or culture medium (e.g. M199 or DMEM) with human serum albumin. Standard cryopreservation solution for UCB collections consists of DMSO 10% in dextran 40 (Rubinstein et al. 1995). This solution is commercially available in 7 ml vials. Considering that DMSO has been related to adverse effects after infusion (Cox et al. 2012), some authors have proposed a reduction of its presence in the cryoprotective solution, by simply decreasing its percentage (Galmes et al. 2007) or compensating this reduction with the addition of other, less toxic, cryoprotectants

(Son et al. 2010; McCullough et al. 2010; Rodrigues et al. 2008). This problem is well solved in UCB banking, because previous volume reduction to 20–25 ml reduces the DMSO quantity to be added usually to 5 ml.

Addition of Cryoprotectant

In general, DMSO is well-tolerated for HSC. Nevertheless, it has a potential cell toxicity which is time, temperature, and concentration dependent. Some authors have shown that the toxicity of this cryoprotectant, at the final concentration used in cord blood banks, is unlikely to be of practical importance even at ambient temperatures (Rowley and Anderson 1993; Hunt 2011).

Considering the importance of maintaining a relatively small volume in stored cord blood units (≈25 ml), in order to optimize storage space, two strategies can be used for the addition of the cryoprotective solution to cell suspension:

- A small starting specimen volume.
- A high concentrated cryoprotective solution.

In the first case, because of the limitations of nucleated cell number in UCB units, the implementation of measures to drastically reduce their volume can lead to a decrease in cell recovery which may jeopardize their clinical utility. Then, cryoprotective solution is usually added at a high concentration of cryoprotectant (from 20 to 50%). To minimize the risk of cell damage during this step, the Rubinstein's method is widely used (Rubinstein et al. 1995). In this protocol, the loading of DMSO 50% solution (usually 5 ml) is performed with cooled cell suspensions, over the course of 15 min. However, cold reduces transmembrane diffusion rate to this cryoprotectant, as it is temperature dependent. Some authors have observed a fivefold decrease in permeability when cells were incubated at 1.5ºC, compared to that at 20ºC (Hunt et al. 2003a; Woods et al. 2000). Then, the addition of DMSO at room temperature over 5 min has been proposed. The goal during this step is to avoid exceeding osmotic tolerance of cells. Before starting freezing, the cell suspension has to be in equilibrium, which is reached when the concentration of cryoprotectant in the specimen is sufficient to prevent adverse effects of ice crystals and osmotic stress. Then, introducing a short holding period is recommended (Hunt et al. 2003b). These last authors have determined the ideal osmometric response of $CD34^+$ cells from UBC over the range 160–1,800 mOsmol/kg and have observed, as others, that these cells are more tolerant to shrinkage (with hypertonic solution) than to swelling (with hypotonic solution) (Meryman 2007).

Freezing Process

During cooling, the cell suspension remains unfrozen at a temperature lower than its normal freezing point, because of the presence of cryoprotectant. This state is defined as supercooling. The cell injury during freezing process has been explained by the two-factor hypothesis (Mazur 1970). According to that, using too rapid cooling rates, the intracellular water nucleates and forms lethal intracellular ice. Otherwise, with slow cooling rates, cells are dehydrated in response to the osmotic gradient produced by the increased concentration of the unfrozen fraction in the extracellular solution. This occurs because a growing ice crystal excludes any solutes present in the solution at the interface between ice and liquid. Excessive cell dehydration may lead to injury due to high concentration of electrolytes, which is called 'solution effect'. The presence of a non-penetrating cryoprotectant in the cryoprotective solution may reduce the effect of this event on cell. In addition, the cooling rate also influences the growth kinetic of ice crystals, slower cooling rates yield larger ice crystals (Meryman 2007).

Cooling at 1–2ºC/min is suitable for a wide range of human cells, including HSC from UCB. It can be achieved by using:

- Controlled-rate freezing: with programmable automatic devices. The process is stepwise designed using cooling ramps at adequate rates in each step. It allows to develop a shock cooling when crystallization starts (between −5°C and approximately −15°C) and then, the released latent heat of fusion is fully dissipated. The temperature is continuously registered by

placing the equipment's probe in the same type of container and conditions (composition of cryoprotective solution and volume) than the specimen. The main disadvantage of these computer-assisted devices is their high cost.

– Uncontrolled-rate freezing or passive cooling: placing the bag with the UCB in a −80 °C mechanical freezing (Galmés et al. 1999; Shlebak et al. 1999). Once reached −80 °C, the frozen bag may be passed to lower temperature (vapour or liquid nitrogen) for its storage. It is simpler and less costly but requires a previous validation of the procedure in order to determine the parameters which assure its reproducibility. This methodology is effective for large scale UCB cryopreservation achieving good cell viability (Itoh et al. 2003).

Vitrification has been proposed as an alternative method to conventional freezing of cell suspensions. This process requires the use of a high concentration of cryoprotectant and ultra rapid cooling rate. With these conditions, the viscosity of the cell suspension is increased and becomes a glassy solid, preventing ice nucleation and, therefore, avoiding the risk associated to ice crystals. Nevertheless, it is difficult to achieve a cooling rate fast enough considering the volume of UCB units (≈25 ml). Kurata et al. (1994) have shown a high cell viability after vitrifying cells from UCB but processing a small specimen ($1.5–2 \times 10^6$ nucleated cells suspended in 60 μl) which is not useful in routine practice in UCB banks but it allows us to attain high cooling rates. When high cooling rates are used, the thawing process is the main concern. The higher the cooling rate, the higher is the warming rate required to achieve high cell survival (Mazur and Seki 2011). In our experience, we have achieved cooling rates near to 450 °C/min by direct immersion of bags in liquid nitrogen, concentrating UCB in a final volume of 10 ml (unpublished data). The aluminium cases used to protect UCB units do not interfere significantly with cooling rate. We used a mixture of ethylene glycol (25%) and DMSO (15%) as cryoprotectant solution. Thawing was performed by immersion for a few seconds in a water bath at 65 °C (warming rate of ≈2,000 °C/min) and reported acceptable cell recovery and viability, but this last parameter decreased significantly during the washing step for the elution of cryoprotectants. Further research on this concern is needed to design protocols which maintain cell membrane integrity and its ability to respond to osmotic excursions.

Storage

Umbilical cord blood units are intended to be stored for long time. Some authors have observed that the viability of HSC kept in mechanical freezers at −80 °C decreases progressively with the length of storage (Galmés et al. 1999; McCullough et al. 2010). Then, ultra low temperatures must be undoubtedly used. Liquid nitrogen has been widely used with this aim, because it yields an environment at a constant temperature of −196 °C. Nevertheless, this liquid medium may represent a vehicle for the diffusion of contaminant agents. During storage handling, the condensation and freezing of atmosphere above the nitrogen containers can generate crystals which sediment inward and these crystals may trap environmental microorganisms (Mirabet et al. 2012). In addition, Tedder et al. (1995) have demonstrated the transmission of viral agents released into the liquid nitrogen. Thus, the bags used to contain UCB units during storage must be protected (e.g. double bagging) to prevent the risk of contamination due to a faulty seal, leak or breakage of these bags, especially when a liquid medium is present. Unlike the exhaustive microbiological monitoring for environmental conditions during UCB processing, storage is not usually considered as a critical point of potential contamination risk in professional standards for cell banking. Tanks containing only nitrogen in vapour phase have been designed, as an alternative to avoid the presence of a liquid medium during storage. However, while the temperature is constant inside the liquid nitrogen, in the case of vapour there is a risk of temperature gradient from the bottom to the top of the tank. So, special measures must be observed during handling the specimens in this kind of containers to minimize the risk of temperature excursions.

As we said before, UCB units are included in aluminium cases, which provide some advantages: maintaining a homogenous thickness in the cell suspension during freezing, protection against breakage during handling, improving heat exchange during freezing and thawing and facilitating organization in storage containers.

According to current knowledge, expiration date of UCB collections stored in liquid nitrogen at temperatures below −150 ºC is not known. High efficient recovery for multipotential progenitors from up to 23.5-year cryopreserved cord blood has been shown (Broxmeyer et al. 2011).

Thawing and Washing

Submersion in a shaking water bath at 37 ºC is the most common method to attain a high enough warming rate which assures cell viability, avoiding the growth of the innocuous small ice crystals generated during freezing, which might be detrimental for cells (Meryman 2007). This phenomenon is known as recrystallization. The water must contain a germicide product with wide range of antimicrobial action to minimize contamination risk during this stage. Once the UCB is completely thawed, the bag is removed and the outer surface carefully cleaned with an antimicrobial product, with special attention to the ports to be used later for connections.

As we said before, in case of assaying vitrification protocols, a higher warming rate must be used (e.g. increasing the water bath up to 65 ºC, with an exhaustive control of the process to avoid cell damage due to overwarming).

Elution of Cryoprotectant

The implementation of a washout protocol (assuring optimal cell recovery) after thawing the cell suspension may reduce infusion-related toxicity associated to compounds from cryoprotectant solution (Rodríguez et al. 2004; Windrum et al. 2005). This step involves the higher risk for cell viability because cells experience further osmotic excursions. Even more, it is considered more of a challenge than the addition process because of the more limited ability of cells to swelling than to shrinkage, as we commented before. Sometimes, osmotic stress is induced during the addition phase and its consequences are only shown when the cells are newly subjected to osmotic stress, during elution (returning to isotonicity from a hypertonic cryoprotective solution). In addition, this process is specially time-consuming and requires experienced personnel. Hunt et al. (2003a) have suggested the incorporation of a post-centrifugation dilution phase in two steps to restricting cell swelling within a safe level.

A slow continuous dilution of cryoprotectant with rapid mixing of thawed cell suspension with washing solution is a commonly used protocol. The use of closed automated cell processors for the washing of thawed UCB units facilitates the unloading of DMSO with non-significant reduction in cell recovery (Rodríguez et al. 2004). These devices allow nearly instantaneous mixing as the washing solution is added and also volume control. As in the case of cryoprotectant addition, time to reach equilibrium must be respected at each stage. A solution of normal saline containing 10% dextran−40 and human albumin at 5% is commonly used as washing solution for UCB cells and, finally, to suspend cells for infusion (Rubinstein et al. 1995). Dextran acts as a buffer preventing damage related to osmotic excursions. In addition to reduction in cryoprotectant concentration, other products as cell lysis residues, red cells, and non-mononuclear cells may be eliminated, then increasing the quality of the infused product. Washing the cells inevitably leads to a certain degree of cell loss ranging from 10 to 20%. In fact, hematopoietic cells recovery (total nucleated cells and/or $CD34^+$ cells) after thawing and washing in different studies is around 80% (Solves et al. 2008; Regan et al. 2010).

Granulocytes are less tolerant to cell volume excursions than HSCs (Law et al. 1983) but some adverse reactions have been related to these cells (Cox et al. 2012).

Cell Viability Assessment

Satisfactory hematopoietic recovery after HSC transplantation depends on the quantity and quality of the cell suspensions delivered from the umbilical cord blood bank. So, considering the limitation in HSC quantity in UCB and the several centrifugation steps at which cell suspension is underwent, efforts for efficient cell recovery are of high concern. Following, we described two parameters which can be tested in order to determine the quality of cell suspensions. In both of them the results are observer-dependent, then an accurate interpretation requires high degree of consistency and expertise.

Membrane Integrity

Cell membrane is the structure by which the cell establishes a relationship with its environment and is of great importance for cell function. In addition to osmotic responses, cell membrane plays an important role in engraftment. As HSC are subjected to volume excursions in response to osmotic events during processing, testing the cell membrane integrity is a significant parameter to evaluate cell quality. The following dyes are widely used with this aim:

- Trypan blue: it is based on the ability of viable cells to exclude this stain. Then dead cells are stained blue when viewed by light microscopy while cells with intact membrane remain unstained (Mascotti et al. 2000).
- Acridine orange/propidium iodide: the first is a metachromatic permeating dye that binds to nucleic acids, generating green (double stranded DNA) or red (single-stranded DNA or RNA) fluorescence. The second is a non-permeating dye but, if the cell membrane is damaged, readily penetrates and binds to nucleic acids causing orange to red fluorescence. Then, using both of them simultaneously, viable cells appear stained green and non-viable cells bright red to orange, when viewed under fluorescent microscopy (Mascotti et al. 2000). Ethidium bromide may be used instead of propidium iodide, maintaining the same criterion to evaluate cell viability.
- 7-Amino-actinomycin D: it is a nucleic acid dye which is excluded by viable cells but can penetrate if cell membrane is damaged. It is used for flow cytometry, thus requiring sophisticated devices. One of its advantages is that can be used in conjunction with other fluorochromes without interference, which is very common in flow cytometry Therefore, it is possible to determine simultaneously the proportion of CD34-positive cells in the sample (using specific monoclonal antibody) and the percentage of these cells which can be considered alive.

Colony Forming Assay

The hematopoietic recovery potential of HSC depends on their ability to engraft, grow and differentiate. Thus, a method to assay in vitro the potential of cell proliferation and differentiation of colony forming units (CFU) has been designed. The cells are seeded in plates with methylcellulose, a semi-solid matrix, and culture medium containing a combination of growth factors (e.g. GM-CSF, IL3, SCF and EPO) supporting the growth of erythroid progenitors (burst-forming unit-erythroid, BFU-E), granulocyte/macrophage progenitors (colony-forming unit-granulocyte, macrophage, CFU-GM) and multi-potential colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). These plates are then incubated during 2 weeks at 37 ºC, 5% CO_2, in humidified atmosphere. After this time, colonies (defined as aggregates of more than 40 cells) may be scored under an inverted microscope (Solves et al. 2008).

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Expansion of Mesenchymal Stem Cells Derived from Umbilical Cord in Media Containing Human Serum (Method)

2

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Contents

Abstract

The regeneration of damaged or diseased tissues or organs is one of the most ambitious and challenging fields in modern medicine. In this context, stem cells and especially mesenchymal stem or stromal cells (MSC) have proven to offer great promise as these cells are capable for extensive self-renewal and display a multilineage differentiation potential. Over the last decade the human umbilical cord and other birth- associated tissues have been found to be a rich and valuable source of MSC. The production of therapeutically significant cell numbers still remains to be one of the major challenges in clinical applications. Therefore biotechnological protocols need to be established to guarantee a reproducible and safe isolation and expansion of the cells. In this chapter an overview of techniques for the isolation of MSC from the tissue of the human umbilical cord is given and different expansion strategies are presented. In this context the composition of the culture media with regard to xeno-free culture conditions and the usage of growth factors as a proliferation trigger are discussed. Furthermore, different strategies for MSC expansion are briefly described, whereby the expansion in bioreactor systems using microcarriers is highlighted.

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Introduction

Today human mesenchymal stem cells (MSC) isolated from different sources are widely used in the area of tissue engineering as they obtain a high potential for cell-based therapies and tissue regeneration. Nevertheless, human MSC derived from bone marrow (bmMSC) are still considered as "golden standard" (Moretti et al. 2010), but bone marrow as a source for MSC presents several disadvantages: an invasive and painful collecting procedure, with a high risk of infection, a low frequency of MSC (approximately 0.001–0.01% (Moretti et al. 2010; Rodrigues et al. 2010)) and a varying quality depending on the donor's age. Thus, for example adipose and postnatal tissue gained more and more attention for the application in Tissue Engineering. MSC from e.g. the human umbilical cord tissue present an alternative with promising results, since the tissue is easily accessible; it obtains a high frequency of MSC, with a high proliferative capacity.

Today, MSC are involved in many clinical trails regarding the treatment of many different diseases such as diabetes, liver cirrhosis, non-union bone fractures, Parkinson's disease, autoimmune diseases, immune system diseases and many more. At the U.S. National Institutes of Health more than 200 clinical trails are listed involving MSC (November 2011). Most studies are currently in phase II. Depending on the study and the disease, MSC can be injected intravenously or intramuscular. If MSC are injected intravenously, generally an amount of about one to two million cells per kilogram body weight is used (Wagner et al. 2010a). By multiplying this number with the average body weight of 80 kg, an amount of 80–160 million cells is necessary for one single treatment of one patient. However, a few studies implement several treatments per patient. This example indicates the need for huge amounts of MSC from the same donor. Moreover, proliferation of MSC seems to be highly inconsistent. Therefore, the use of these cells in a low passage is favourable. Besides, the high risk of contamination by carrying out multiple passages, proliferation under non-physiological culture conditions may result in mutations and chromosomal aberrations, even though these effects have rarely been examined in scientific studies using human MSC (Rosland et al. 2009). Until today, no tumour formation has been observed in clinical trails with MSC (Wagner et al. 2010a). Additionally, bmMSC exhibit a very low in vitro proliferative capacity, thus they can only be expanded for a limited time (7–10 passages (Wagner et al. 2008), 8 passages (Wagner et al. 2010b), ~15–20 population doublings), before reaching senescent state. In contrary to bmMSC, we could expand our isolated ucMSC at least up to passage 20, without loss of proliferation and differentiation capacity (Moretti et al. 2010; Wagner et al. 2010a). Besides, no tumour factors, such as TERT, could be verified up to the clinical relevant passage 10 (unpublished data).

Another hurdle to be crossed using MSC in clinical trails is the very low vital incorporation of only 5% (e.g., injection in a porcine heart after a 14 day period) (Rodrigues et al. 2010). Therefore, intensive studies have been performed over the last decade to examine the role of growth factors concerning the proliferation and survival of MSC. Yet, an ideal growth factor for the use with MSC has not been found. Most factors are pleiotrophic, causing multiple changes, e.g., in motility, proliferation, morphogenesis and survival (Rodrigues et al. 2010). Well studied growth factors are for example the transforming growth factor beta (TGFβ), the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF).

Since static amplification of MSC is a labour-intensive procedure with a high risk of contamination, bioreactors can be applied in order to maximize ex vivo expansion of MSC. The use of large-scale bioreactors assures controlled and stable cultivation conditions, which is essential to guarantee a safe and constant expansion of MSC and minimize the risk of infection. Different bioreactor systems have been developed as an alternative to standard culture flasks, including spinner flasks, rotating-, perfusion- or wave systems, combined with microcarrier cultures. The use of microcarrier cultures seems to be the most effective method to expand adherend cells under stirred or flow conditions.

In clinical human trails a fast and reliable ex vivo amplification of MSC is needed to fulfil the high demand of autologous cells. To achieve this goal within a few passages, expansion triggers such as various growth factors can be applied. Additionally, the use of bioreactors in combination with microcarriers shows multiple advantages.

Isolation of Umbilical Cord Mesenchymal Stem or Stromal Cells

As stated before ucMSC present many advantages compared to bmMSC. In particular high amount of cells can be obtained from one single human umbilical cord (UC) and the obtained MSC exhibit a higher proliferation activity. Therefore, the preparation of large amounts of MSC for clinical trails in a shorter period of time becomes possible. UC are mainly obtained from term-delivery (38–40 weeks) by Cesarean section patients, since this procedure carries the lowest risk of contamination. For the transportation from the operating room to the laboratory the UC is usually stored in a transportation buffer containing saline, antibiotics and fungicides. Two methods to obtain ucMSC from the UC are well described in literature: The enzymatic digestion and the explant culture. Variations in the protocols concerning for example the removal of arteries and veins are existent.

Enzymatic Digestion

The isolation of MSC from the UC tissue is mainly been carried out by an enzymatic digestion using different protocols. Briefly, the human UC was disinfected in Betadine solution followed by 100% ethanol wash (Koliakos et al. 2011) or directly rinsed several times with sterile PBS (Weiss et al. 2006). Generally, all UC were obtained from full-term Caesarian section births and the blood from arteries and the vein removed. Most protocols describe a full removal of the arteries and the vein (Weiss et al. 2006; Koliakos et al. 2011). Thereafter, the UC was cut into small fragments (for example 2–4 cm lengths (Weiss et al. 2006) or 2–5 mm^3 pieces (Koliakos et al. 2011)) using a forceps and a scalpel or scissors. The tissue pieces were immersed in an enzymatic cocktail containing hyaluronidase, collagenase type I and trypsin for 45 min (Weiss et al. 2006) up to 1 h 30 min (Koliakos et al. 2011) at 37°C with gentle agitation. Then, the UC tissue pieces can be removed (Weiss et al. 2006) or passed through a 70 μm nylon mesh (Koliakos et al. 2011). The cell suspension was centrifuged and the obtained pellet resuspended in fresh media.

Another approach has lately been described by Tsagias et al. (2011). They isolated MSC using the total length of umbilical cord without removing the vessels and/or mincing the cord. The isolation has been carried out in a sterile bag under gentle agitation. One major disadvantage of the enzymatic digestion method is that bovine collagenase can not be used, if the isolated cells are intended for transplantation. The use of bovine products involves the risk of prion infection or other yet unidentified zoonoses. This limits the clinical applicability. Only GMP grade collagenase, for example collagenase NB 6 produced in *Clostridium histolyticum* can be used if the cells are isolated for transplantation purposes. However, this biotechnologically produced collagenase makes the enzymatic isolation of cells a very expensive procedure.

Explant Culture

As previously described (Moretti et al. 2010; Majore et al. 2011) we have used a protocol to isolate MSC from the whole human UC tissue in an explant culture approach (Fig. 2.1). We obtained umbilical cords from term-delivery (38–40 weeks) by Cesarean section. In a first step, blood from arteries and the vein was removed by flushing the UC with phosphate buffered saline (PBS) enriched with 5 g/l glucose, 50 μg/ml gentamicine, 2.5 μg/ml amphotericin B, 100 U/ml penicillin and 100 μg/ml streptomycin, in order to minimize the risk of contaminations. The UC was manually cut in pieces of about 0.5 cm^3. After this process, the small tissue pieces were transferred into a

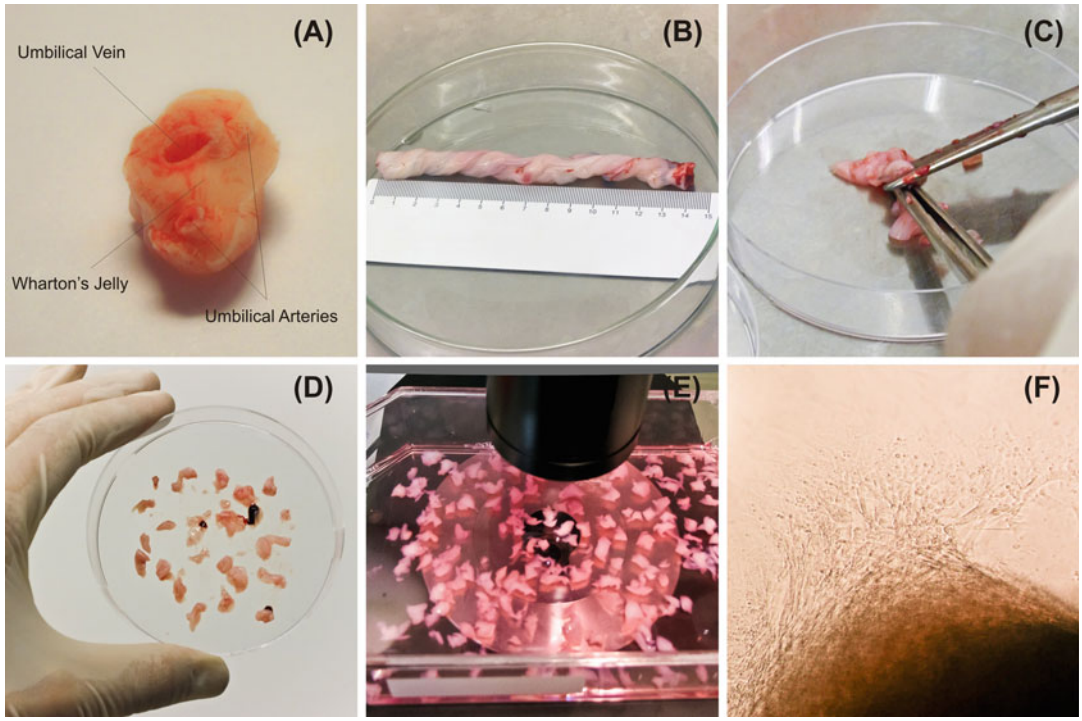

Fig. 2.1 Isolation of MSC from whole UC tissue. Human umbilical cord (cross section (**a**) and one third segment (**b**)) is first cut into 0.5–1 cm^3 large pieces (**c**+**d**) which are incubated as explant cultures in standard growth medium (**e**). After approx. 10 days of culture, cells start to grow out of the segments (**f**). After approx. 14 days of culture, the tissue pieces are removed and adherent growing cells can be sub-cultivated

175 cm^2 culture flask and incubated under standard conditions (37 °C in a humidified atmosphere with 5% CO_2) in αMEM supplemented with 10–15% of human serum and 50 μg/ml gentamicine. The medium was changed every second day. A beginning outgrowth of adherent cells from several tissue pieces could be observed after approx. 10 days. After 2 weeks UC tissue pieces were removed and the adherent cells were harvested by using accutase enzymatic treatment. The cell suspension was centrifuged at 200 g for 5 min, the cell pellet was resuspended in growth media (αMEM supplemented with 10% of human serum and 50 μg/ml gentamicine) and subcultured until 80% of confluence. Within this approach unstable factors such as the batch-to-batch variability of collagenase and its narrow activity parameters can be eliminated. Furthermore, a GMP conform automation of this isolation process is conceivable.

Cell Aging

Within human clinical trails large-scale expansion is an indispensable step previous to the treatment. However, MSC under non-physiologic in vitro culture conditions can only be expanded for a limited time. The simplest parameter for the documentation of a long-term study is to give the number of cell passages. After multiple passages MSC reach senescent state, also called "Hayflick limit". This phenomenon is defined as "an essentially irreversible arrest of cell division" (Wagner et al. 2010a). The cells are irreversibly kept viable in the G1-phase of the cell cycle involving the suppression of genes for cell cycle progression. Generally, senescence is accompanied by a change in morphology; cells increase in size and display flattened morphology, also called "fried egg phenomenon" (Wagner et al. 2010a).

Furthermore, many studies indicate that human senescent MSC are characterized by their loss of differentiation capacity (Wagner et al. 2010b). One marker to confirm senescence is to measure the β-galactosidase (β-GAL) activity, which is known to be associated with senescence in vitro. Research experiments have confirmed that β-GAL activity increases after multiple passages of a MSC culture, but no difference could be found between MSC from young and old donors. This evidence shows that β-GAL can not be used as a marker for cell aging in vivo, but on the contrary as a reliable marker for senescence correlated to cell aging in vitro (Sethe et al. 2006). For the verification of β-GAL the substance 5-brom-4-chlorine-3-indolyl-β-D-galactosid (X-Gal) is transformed to the blue indigo dye.

It is also documented that senescence goes along with a telomere shortening. The loss of telomere repeats might be associated with the regulation of cell life and tumour suppression (Sethe et al. 2006). However, it is still controversially discussed if the loss of telomere repeats initiates senescence or if it is an effect of this process (Wagner et al. 2010b). Nevertheless, the identification of telomere length could be used as a safety marker to assess the state of cellular aging. Various methods, such as Southern hybridization, flow cytometry or quantitative PCR can be applied to determine telomere length (Wagner et al. 2010a).

It is non-controversial that long-term cultivations induce changes in gene expression, whereas this phenomenon depends on many factors; for example the technique for cell isolation, the culture media and the cell culture methods have a strong influence on the gene expression profile (Wagner et al. 2010a). Within many long-term studies of especially murine or rat MSC malignant transformation phenotypes could be observed. But is effect seems to be rather exceptional for human MSC. This issue is still under debate, since few studies report a spontaneous malignant transformation during a long-term cultivation (Lepperdinger et al. 2008; Wagner et al. 2010b). However, Wagner et al. (2008) compared the gene expression profiles of human bmMSC isolated in two different laboratories, long-term cultured in different media and finally analysed with different microarray platforms. The results showed a high similarity of senescence-associated gene expression. In conclusion, this specific changes might be suitable to detect cellular aging and therefore be implemented a method for quality control (Wagner et al. 2010a). Besides a change in the genetic profile of long-term cultures often have functional consequences for MSC, which might be relevant for the application in clinical trails. It could for example be shown that long-term cultures of MSC result in a reduction of hematopoiesis supportive activity. Moreover, the influence of a long-term cultivation on the secretion of growth factors and cytokines for fibroblasts could be demonstrated (Wagner et al. 2010b). Summing up all these phenomena, the need for ex vivo expansion of human MSC with only few passages becomes obvious. Therefore, it is necessary to establish an in vitro expansion routine and to set up analytical methods to determine the cellular state in order to guarantee a safe and constant cell quality.

Medium Composition

The Use of Fetal Calf Serum, Human Serum and Other Xenofree Media

Nowadays, most experiments with human MSC are still carried out in media containing 10–20% fetal calf serum (FCS) (Lepperdinger et al. 2008). However, the use of animal, or in particular bovine sera raises various safety concerns. They exhibit a high risk of infections with e.g. viruses or prions. Moreover, the transmission of yet unidentified zoonoses might deactivate the host immune system with biomolecules, which are foreign to human (Lepperdinger et al. 2008; Hatlapatka et al. 2011). Additionally, FBS contains further undefined elements, which might vary in quantity. These factors cause a high variability of FBS, affecting a stable proliferation (Rodrigues et al. 2010). Alternatives to FCS have been introduced several years ago; for example, freshly frozen plasma platelets or platelet lysate have been used. Comparisons with FCS showed

that MSC cultivated in media containing human platelet lysate obtain enhanced proliferation rates. Besides, MSC did not show any change in morphology, immunophenotype, differentiation capacity or genetic profile (Lepperdinger et al. 2008). In our laboratory we use another alternative to FCS: Allogenic human serum (HS) (Lavrentieva et al. 2010; Moretti et al. 2010; Hatlapatka et al. 2011; Majore et al. 2011). We investigated an expansion method for ucMSC under xeno-free conditions using low glucose media (α-MEM) and 10% HS. Our experiments proved that ucMCS cultivated in α-MEM containing 10% HS display an enhanced proliferative potential and do not change their morphology over at least eight passages. On the contrary, the same cells cultivated in α-MEM containing 10% of FCS exhibit distinct "fried egg" morphology accompanied by a diminished proliferation and a loss of MSC surface marker expression (Hatlapatka et al. 2011).

Another attempt of the commercial industry is the replacement of serum and other animal supplements using synthetic ingredients, thus reducing the variability of the medium. Different companies propose a well growth of MSC in their synthetic medium. Experiments in our laboratory with synthetic medium from Mesencult also showed promising results. Compared to our standard medium (α-MEM containing 10% HS), we could obtain higher cell numbers and a more uniform fibroblast-like morphology (unpublished data). However, their proprietary compositions limit the application in clinical trails. The most promising alternative in the design of a serum-free medium is the parallel use of various growth factors (Rodrigues et al. 2010).

Proliferation Trigger

Multiple growth factors are widely known. Some of them only enhance proliferation others do affect differentiation as well. However, the main goal is the discovery of a growth factor, which improves the *ex vivo* expansion of MSC and increases the survival after transplantation. The growth factor TGFβ for example is known to increase proliferation, but also induces chondrogenesis (Bonewald and Dallas 1994; Longobardi et al. 2006). A similar function has the bone morphogenetic protein (BMP), a factor belonging to the TGFβ superfamily. MSC overexpressing BMP 2 show enhanced proliferation, but also a strong differentiation into the osteogenic lineage. BMP 3 e.g., another factor of this family, could increase the proliferation by a threefold magnitude. Within the bone morphogenetic proteins 2, 4, 6 and 7, the BMP 2 is known to have the greatest impact on differentiation (Luu et al. 2007; Stewart et al. 2010).

Another well studied group of growth factors is the fibroblast growth factor family (FGF), which is involved in wound healing and angiogenesis. This family consist of various factors, whereas FGF 2, also known as b-FGF (basic fibroblast growth factor) is most studied. The applicability of this factor as a trigger for MSC proliferation has been studied by several groups. It was shown that MSC displayed a markedly increased proliferative potential in the presence of FGF 2 with a prolonged life span with up to 70 doublings. Furthermore an enhanced multilineage differentiation potential (e.g., chondrogenic and osteogenic) of MSC under the influence of FGF 2 could be detected (Tsutsumi et al. 2001; Bianchi et al. 2003; Solchaga et al. 2005; Sotiropoulou et al. 2006). However, some results are conflicting and only few of the studies included the examination of pheno- or genotypic alterations or other critical parameter, like cell senescence or apoptosis. In these studies it could be shown that the use of FGF 2 led to a critical alteration of MSC morphology and gene expression profiles. Additionally, an increased expression of HLA class I molecules and a down-regulation of the CD44 expression was observed. Since CD44 is involved in cell-cell and cell-matrix interactions, the authors hypothesized that the expansion of MSC under the influence of FGF 2 may lead to a less effective engraftment of the cells after transplantation (Sotiropoulou et al. 2006). Furthermore it could be shown that the use of FGF 2 led to a drastically elevated level of apoptotic and senescent cells within only four passages and the number of apoptotic cells further increased upon switching from FGF-2

supplemented to non-supplemented media in P5 (Hatlapatka et al. 2011).

The vascular endothelial growth factor (VEGF), originally found to be a potent mediator of angiogenesis and vasculogenesis, was also shown to trigger the proliferation of MSC in vitro as well as to enhance the survival of transplanted cells. In a rodent model it could be shown that the co-injection of MSC with VEGF to hearts after myocardial infarction increased cell engraftment and resulted in better improvement of cardiac functions (Pons et al. 2008). However, it is still under debate, whether VEGF enhances incorporation and survival of MSC or if it triggers paracrine effects on surrounding endothelial cells, which improves angiogenesis.

All growth factors described above are known to enhance proliferation, but also support differentiation into a particular lineage. This effect is advantageous for generating differentiated cells prior to transplantation. But it can be counterproductive if the transplantation requires rather undifferentiated cells. The addition of growth factors initiates differentiation, which competes with expansion, resulting in lower or insufficient cell numbers. Furthermore it should be carefully tested, if the use of a growth factor as a proliferation trigger may lead to unintended and undesired side effects (e.g., high numbers of only low quality cells), which led to the search of growth factors, which only promote proliferation and has no influence on differentiation or other critical parameters. Signals released by the epidermal growth factor receptor (EGFR) enhance proliferation, motility and survival of MSC, but do not trigger differentiation (Tamama et al. 2006). Ligands binding to this receptor are the epidermal growth factor (EGF) and the heparin-binding EGF. However, studies using EGF could not verify and enhanced survival of MSC.

Since growth factors affect proliferation and differentiation at different rates, often synergistic pairs of growth factors are used to obtain the optimal, desired result. The combination of for example FGF-2, $TGF\beta_1$ and the platelet-derived growth factor (PDGF-BB) show the best outcome in MSC proliferation, while maintaining the phenotype and differentiation potential (Ng et al. 2008). Other growth factor combinations resulted in an enhanced MSC survival after incorporation. Nevertheless, using growth factors to increase proliferation, one fear remains; MSC and surrounding cells might escape the cell cycle and result in tumour growth. Therefore, the development of a pinpoint delivery and a time controlled release of the growth factors are urgent (Rodrigues et al. 2010).

In summary the application of growth factors shall fulfil the following criteria: (1) Increase proliferation, while retaining the phenotype and differentiation potential, (2) enhance MSC survival after transplantation and (3) replace serum and animal-components in the media, in order to reduce the variability.

Expansion Methods

Extended expansion in culture leads to loss of multi-potentiality and senescence (Javazon et al. 2004). A criterium for maintenance of immature phenotype is the availability of appropriate extracellular substrates to which the cells adhere. Intracellular signals triggered by the interaction of adhesion molecules with extracellular matrix components are critical for cell survival both *in vivo* and *in vitro*. Using conventional expansion methods cells grow in monolayers T-flask cultures. Expansion and transplantation usually requires repeated trypsination. However, cyclical disruption of cell-extracellular matrix contact by trypsination either during cell expansion or immediately prior to cell transplantation may impair cell viability and facilitate a specialized form of apoptosis called anoikis and, therefore, leads to a limitation of cell expansion (Reddig and Juliano 2005). Furthermore, trypsination not only contains an associated safety risk but causes high labor costs, is a time coming and space requiring procedure and does not scale well.

Cell Factory

As an approach to reduce trypsination steps required for expansion and therefore diminish costs, contamination risks and change of cell phenotype Cell Factory systems (e. g. Nunc) were developed. Cell factories are especially

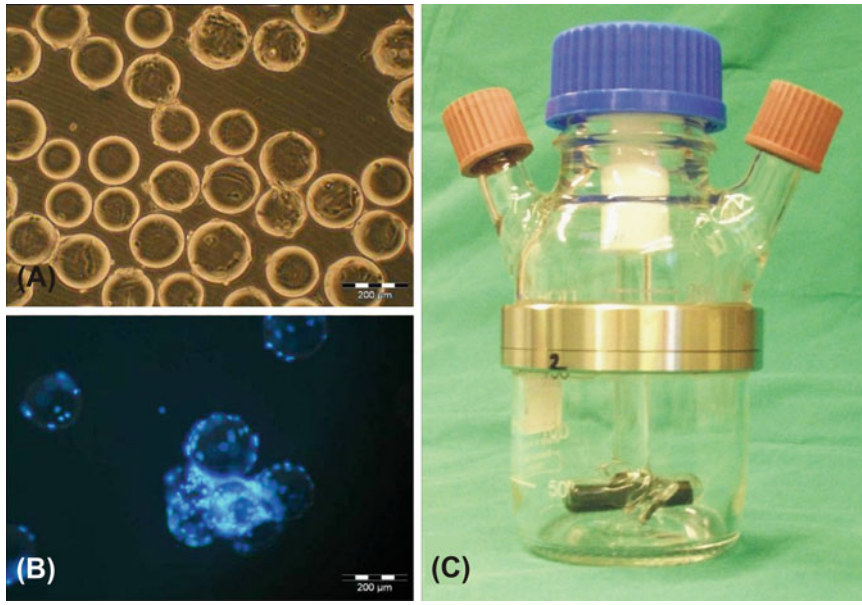

Fig. 2.2 Expansion of MSC on microcarriers. Cells are seeded on microcarriers ((**a**) and DAPI stained cell nuclei (**b**), both 100× magnified) and cultivated in spinner flasks (**c**)

designed for large scale cell culture. They provide a large growth surface in limited space areas. Some benefits are the low risk of contamination and the easy to handle multi-layer stacks. Cell Factory systems are available with 1, 2, 10 and 40 trays. Due to the height of the 10 and 40-chamber Cell Factory systems, cells cannot be viewed with common microscopes. Nevertheless, expansion remains difficult since trypsination is still required. Additionally, the system it is not suitable for cultivation under dynamic conditions in a bioreactor. Such a bioreactor technology should provide several criteria: (I) large surface area for cell expansion (II) monitoring and controlling of cultivation parameters online (III) closed system to avoid contamination (IV) scaleability (V) economical running costs. The use of bioreactors in for cell expansion enables a more precise monitoring and regulation of culture conditions by controlling various physiological parameter such as oxygen supply, pH values and mass transfer.

Microcarrier

As an alternative approach to expand MSC is the cultivation on microcarrier beads in a spinner flask. Spinner flasks originally developed for mass cultures of mammalian cells have been established for more than 25 years (Levine et al. 1977). Several protocols have been shown to be highly suitable for MSC expansion using microcarriers in spinner flask bioreactors (Fig. 2.2) (Frauenschuh et al. 2007; Rubin et al. 2007; Yang et al. 2007; Sart et al. 2009). Cultivation in spinner flaks comprises of following steps: (1) cell seeding on microcarrier beads (2) addition of cells on microcarriers in cell culture medium to a spinner flask (3) cultivation under dynamic conditions and constant stirring and controlled culture conditions (4) harvest of cells/microcarriers from the culture, and if necessary (5) detachment of cells from the carrier.

Porous microcarriers were originally developed for mammalian cells recombinant protein production in stirred vessels. Microcarrier beads offer a beneficial surface-volume ratio and high cell densities. The beads in general range between 100 and 400 µm in diameter and are less likely to get eluted during filtration to separate cells from media. Surface area, bead size, cell adhesion and cell spreading are all critical factors that may modulate cell

attachment and ultimately their expansion potential. Thus, cell seeding procedures were achieved, concluding, that there is no dynamic seeding strategy up to date to satisfactory load beads with MSC since only about 25% of cells in suspension adhere. Additionally, serum free condition leads to a diminished cell attachment due to lack of important cell adhesion promoting proteins. Consequently, MSC seeding under static conditions (over night) combined with a precoating of the microcarriers before inoculation is a widely used method for providing high seeding densities, which clearly correlates with the expansion rate.

Gelatine based microcarriers are widely used for cell expansion. In order to expand MSC for clinical purposes under xeno-free conditions microcarriers made from other materials such as cellulose, polystyrene, collagen or glass were intensively investigated as a feasible alternative.

Microcarrier performance differs depending on format, coverage with cells, expansion behaviour and recovery of the cells. Hence, choice of the carrier type used needs to be made according to the intended application (Santos et al. 2011).

After cultivation MSC can be detached from the microcarriers by enzymatic digestion. Contrariwise, MSC on microcarriers can also be directly implanted, depending on the material properties of the beads (such as biocompatibility), without the requirement of detaching the cells. This might even be a favourable method for some medical applications, as the beads are relatively easy to handle and the additional enzymatic treatment is avoided. Furthermore, MSC loaded microcarriers are better located after injection in the target tissue. Cultivation of MSC on microcarriers also support the in vitro differentiation along an osteogenic and chondrogenic lineage. Yang et al. (2007) described a subcutaneous transplantation of BM-MSC on microcarrier beads. After expansion the cells showed a significantly less apoptosis the trypsinized cells. In the long term, BM-MSC expanded on microcarrier beads induced de novo trabecular bone formation in vivo (Yang et al. 2007).

Spinner Flask and Wave-Type Bioreactor for Mesenchymal Stem or Stromal Cells Expansion

MSC on microcarriers are suitable for suspension cultivation in bioreactors. In the following two systems will be described, the spinner flask and wave-type bioreactor. When cultivated on microcarriers in these bioreactors, cells are exposed to the simulated microgravity environment (Gao et al. 1997). Microgravity-induced bone lose was described as a respond to the gravity and, consequently mechanical loading absence during space flights. MSC, cultivated in microgravity were demonstrated to decrease their osteogenic differentiation while adipogenesis was increased (Klein-Nulend et al. 2003). Gene expression analysis supported these findings, showing significant decreases in osteogenic and chondrogenic gene expression and increase in adipogenic expression profile (Dai et al. 2007). Thus, bioreactor systems where MSC are cultivated on microcarriers should consider the fact that in certain conditions cells can be exposed to lower gravity which, in turn, may affect their differentiational capacity, which is favourable for MSC expansion since they maintain their primitive characteristics. Spinner flaks are glass or disposable plastic vessels with an included central magnetic stirrer shaft. The addition or removement of new medium, cell suspension or gas supply is enabled by two side arms at the vessel. MSC can be cultivated in a spinner flask when seeding on microcarriers or other scaffolds, which are injected by threading onto long needles embedded in the side are stoppers.

The wave type bioreactor was first described by Singh (1999). It is composed of a disposable, flexible and sterile plastic bag that sits on a rocking thermo-platform. The bag is constantly and gently rocked. Different rocking mechanism ensure a complete precipitation-free suspension, good nutrient distribution and, due to the constantly renewed surface that increases oxygen transfer without causing shear damage to the cells.

For MSC cultivation in wave-type bioreactors microcarriers are commonly used as described above. In a wave type bioreactor the MSC loaded

microcarriers were also mixed, but the procedure is more gentle compared to spinner flask cultivation as there is no stirrer and, consequently, less mechanical forces involved. Furthermore, the periodically incline and decline motion of the bag provides a higher oxygen transfer within the culture medium (Rodrigues et al. 2011). Up to date two wave-type bioreactor systems are commercially available: the Cultibag system (Sartorius Stedim) and the Cellbag system (GE, Heathcare).

For cultivation cells are suspended in a minimal volume and introduced in a plastic bag, the bag gently rocked for a several times for equal distribution. Incubation without rocking overnight promotes cell attachment on the beads. Afterwards, the medium volume gets increased and rocking commenced. Santos et al. recently described a xeno-free method for MSC expansion using a wave type bioreactor (Santos et al. 2011). They were able to achieve expansions of 16- to 18-fold over 14 days, which is a significant improve compared to spinner flask cultivation expansions. Timmins et al. (2012) even improved the yield by application of appropriate microcarrier types (Timmins et al. 2012).

It can be concluded, that for future investigations it is essential to understand key culture parameters for MSC expansion in order to guarantee maintenance of multilineage differentiation potential and non tumoric properties after long term cultivation. For clinical application it is necessary to develop new cultivation approaches. Standard cultivation on 2D plastic surfaces under static conditions is not suitable for the production of therapeutical amounts of MSC regarding economical and qualitative concerns. Bioreactor systems seem to be a promising tool to successfully address this question.

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Transformation of Human Umbilical Cord Blood Cells to Support Neuro-Regeneration in the Diseased Brain

3

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Contents

Abstract

Loss of function may ensue when internal bodily mechanisms fall short of healing a damaged tissue. Regenerative medicine has recently been established as an innovative discipline to restore such failing systems by either replacing impaired parts and/or by stimulating the body's own repair processes. Gross transplantation of entire organs, where possible, is now being changed with more sophisticated methods that involve cells or even genes only. Stem cells have the ability to self-renew and to differentiate into various tissues and therefore can be used to substitute lost cells. When genetically modified to express growth and trophic factors, they are also able to fulfill the task of promoting self-healing. Neuro-science is one of the fastest emerging fields of biology and medicine, however neuro-degenerative disorders still pose an insurmountable clinical challenge despite massive pioneering studies. This chapter presents the latest developments in gene-cell therapy and dissects advances in the use of human umbilical cord blood for the treatment of various diseases of the brain.

Introduction

Regeneration refers to the fundamental properties of the body. Promoting restoration of lost or damaged body structure is inherently linked with the problem of improving the quality of life in degenerative diseases, and after ischemic

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or traumatic injuries. Some tissues have very good self-healing potential, however other organs such as the central nervous system (CNS) are technically unable to regenerate *per se*.

Levels of Transplantation

Replacing a bodily part can take place at several levels: transplantation of entire organs (*e.g*., heart, kidney, liver) has long been successfully used, however with the advancement of technologies there has been attention not to use entire structures. Administering hematopoietic cells has been widely performed to treat a number of congenital blood disorders, including aplastic anemia, β-thalassemia, sickle cell anemia, and they are also employed during anti-cancer treatments to maintain a pool of poly-potent hematopoietic cells of bone marrow following chemo-therapy, *etc*. Current interest in more minimally invasive technologies (*i.e*., cells and genes instead of whole organs) has increased: development of innovative technologies, generation of new scientific knowledge to stimulate restitution, intensive introduction of interdisciplinary knowledge (especially physics and chemistry), and (pre-)clinical trials offer great prospects for the development of regenerative medicine.

Problem with the Nerves

The brain is a special structure: it cannot be replaced in whole or parts. Unlike other organs in which cells are generally uniform and have similar if not the same functions (*e.g*., all cardio-myocytes contract in every part of the heart, *etc.*), neurons in different areas of the CNS have diverse characteristics, and play special roles such as motor functions, sensations or cognition, and the human brain is highly individualized (personal memories, feelings, *etc.*). Restoring lost or dysfunctional nerve cells apparently pose a challenging task. Current therapeutic strategies to treat various neuro-degenerative disorders involve (1) transplantation of cells (cellular therapy), (2) delivering "therapeutic genes" whose products are intended to prevent the development of pathological symptoms, to correct (inherited) genetic defects, and/or to stimulate restoration of lost brain cells, and (3) a combination thereof (gene-cell therapy).

Gene-Cell Therapy

Gene-cell therapy is entering clinical practice for the correction of mutations in, or failed expression of genes. The method involves genetic modification of cells prior to transplantation, *i.e*., providing functional copy of a gene to correct the performance of the ineffective gene.

Vectors

Delivering the needed genetic material into the transplanted cells requires the use of genetic vehicles (vectors), whose integral part is an expression cassette, usually consisting of a promoter, the recombinant gene and the signal for termination of transcription. The most common expression vectors are based on viruses or plasmids. Viral vectors are genetically-engineered viruses (adeno-, adeno-associated, herpes, retro-, lenti-viruses, *etc.*) carrying modified viral genomes that have been rendered non-infectious: they also contain the trans-gene of interest, and enter target cells utilizing evolutionary perfected mechanisms based on receptor-mediated recognition followed by penetration through the cellular lipid-bilayer. In contrast, plasmids are delivered into cells by various physico-chemical methods across the plasma membrane: once entered, they can either integrate into the host genetic material, or may stay separate from it and can replicate independently of the chromosomal DNA. With the help of genetic engineering techniques it is possible to create genetic vectors expressing virtually any recombinant genes.

General Clinical Implications

Gene therapy of diseases caused by inherited or acquired gene mutations are based on the functional correction of the disease. For instance, the

treatment of X-linked adreno-leyko-dystrophy, a fatal neuro-degenerative disease, involves transplantation of autologous hematopoietic stem cells in which the mutated gene is corrected by lenti-viral vectors (Cartier et al. 2009). X-linked adreno-leuko-dystrophy is caused by mutations of the *abcd1* gene encoding an adenosine-triphosphate-binding transporter localized in the membrane of peroxisomes in oligo-dendrocytes and microglia. This protein is responsible for the peroxisomal degradation of very-long-chain fatty acids and its deficiency results in a severe brain demyelinating disease. The clinical investigation revealed that infusion of the autologous CD34+ cells that have previously been genetically modified by lenti-viral vector encoding wild-type *abcd1* may stop progressive cerebral demyelination in patients.

Another important aspect of gene and cell therapy in regenerative medicine is to supplement or increase the level of bio-molecules. Administering animal-derived (or more recently human recombinant) proteins is proven to be very effective in the treatment of maladies in which the lack of a certain molecule (*e.g.*, insulin, erythropoietin, calcitonin, parathyroid hormone, growth hormones, glucagon, *etc.*) is the underlying pathology. A significant drawback of such readily available proteins as replacement therapy is their extremely high price, as well as the need for a lifetime drug use in that externally supplementing bio-molecules does not eliminate the cause of the disease. An alternative to intra-venous or sub-cutaneous administration of proteins is delivering recombinant genes via cellular carriers/vectors that can function as producers of these molecules in the diseased body. Transplantation of genetically modified cells expressing the cloned genes is justified not only by the possibility of a prolonged secretion of therapeutic molecules, but also the stimulating effect of transplanted (stem/progenitor) cells on the regeneration of the affected organ.

Gene-Cell Therapy in Neuro-Degeneration

The possibilities of gene-cell therapy for neuro-degenerative diseases have been intensively studied. However, many developments are still at the pre-clinical level, or (at best) entered clinical trials. Utilization of these approaches in a daily medical routine is also marred by several other factors: physicians may face ethical and/or legal issues when using embryonic or fetal cells, risk of malignant transformation of transplanted stem cells, infection of the recipient by unknown microorganisms from the serum and animal cells, immunological response to allogeneic cells, uncontrolled production of recombinant proteins, *etc*.

Current goal is to slow down the degenerative processes and to promote neuro-regeneration. Prepared for transplantation, cells must have predictable and reproducible properties: high viability, migration potential, ability to integrate into host tissues and differentiate into desired cell types, restore tissue matrix in the target organ and form a guide path for the growth of axons, participate in the process of myelination, possess trophic and neuro-protective properties, *etc*. Cells for genetic modification can be obtained from the patient (for autologous transplantation) or healthy human donors (allo-transplantation).

Umbilical Cord Blood Cells (UCBCs)

Administering stem, progenitor or differentiated cells are actively investigated as a way to deliver growth and trophic factors that inhibit the death of parenchymal cells and stimulate the regeneration of the body. The most promising in this sense are UCBCs. The basis for the use of these cells is their suitability for allogeneic and autologous transplantation for humans, low immunogenicity, accessibility, ease of production and storage. An important factor is the lack of legal, ethical and religious taboos associated with transplantation of embryonic stem cells. In addition, umbilical cord blood is rich in hematopoietic stem and progenitor cells (Gluckman 2000; Gluckman and Locatelli 2000; Ballen et al. 2001; Laughlin 2001) which can are great sources of numerous growth and trophic factors, and can also give rise to specialized cells of various tissues and may stimulate angiogenesis as well. Ample experimental evidences confirm the potential use of

UCBCs in ischemic and degenerative diseases (Chen et al. 2001). In addition the safety of cord blood allo-transplantation was also convincingly demonstrated for the treatment of various forms of lymphoma (Yang et al. 2010).

Trophic/Growth Factors

To improve their therapeutic efficacy, UCBCs have been genetically modified as a delivery system of trophic/growth factors and adhesion molecules to support survival of parenchymal cells, stimulate cell proliferation, restoration of intercellular contacts and formation of lost tissue structures. There is great interest in the use of genetically modified UCBCs to stimulate regeneration of cardiomyocytes and blood vessels in coronary artery disease and heart attack. Transplantation of UCBCs transfected with human vascular endothelial growth factor (VEGF) gene has been shown to activate angiogenesis in chronic limb ischemia in rats (Ikeda et al. 2004) and myocardial infarction in mice (Chen et al. 2005).

It is known that the effect of a single pro-angiogenic molecule is not sufficient for the development of functional blood vessels (Kano et al. 2005). The effectiveness of gene-cell therapy, therefore, can be enhanced by the transfection of cells with a combination of therapeutic genes. VEGF and fibroblast growth factor (FGF) are considered to be the most promising for the induction of therapeutic angiogenesis (Al Sabti 2007) due to pronounced synergistic effect between VEGF and FGF_2 on endothelial cells. Currently there is a number of clinical trials underway that test the safety and efficiency of direct gene therapy strategies using plasmid vectors expressing VEGF and/or FGF_2 (http://clinicaltrials.gov). Importantly, VEGF and FGF_2 are also considered as potential therapeutic molecules for the treatment of certain neuro-degenerative diseases.

VEGF

It is known that increased VEGF gene expression plays an important role not only in the growth of blood vessels, but also in the navigation of axons (Miao et al. 1999). Axotomy, in turn, increases expression of VEGF and its nuclear receptor VEGF-R_1 (or *Flt*-1) in lumbar spinal cord motor neurons (Islamov et al. 2004). In addition, VEGF stimulates the proliferation of neuroblasts (Jin et al. 2002), and controls neuronal survival both *in vitro* and *in vivo* (Matsuzaki et al. 2001). VEGF expression directly activates estrogen receptors (Klinge 2001) and hypoxia-inducible-factor (HIF)-1 (Lambrechts et al. 2003). Numerous studies have also demonstrated links between amyotrophic lateral sclerosis (ALS) and VEGF (Ilan et al. 2003). Reduced expression of VEGF results in ALS-like progressive degeneration of moto-neurons (Lambrechts et al. 2003). The absence of HIF_1-responsive element (HRE) in the promoter of VEGF gene ($vegf^{\delta/\square}$) is characterized by progressive degeneration of motor neurons in mice resembling ALS (Oosthuyse et al. 2001). The $^{G}93^{A}$ substitutional transgenic mouse expressing human mutant copper/zinc super-oxide dismutase-1 (SOD_1) gene (also found in a hereditary form of ALS) shows attenuated hypoxia-induced expression of VEGF (Murakami et al. 2003). When crossing $vegf^{-/\delta}$ and $^{G}93^{A}$ transgenic mice their resulting progeny dies at an early age due to severe degeneration of motor neurons (Lambrechts et al. 2003). VEGF promotes the survival of moto-neurons expressing the mutant *sod*1 gene (Li et al. 2003), however there is no current comprehensive under standing of the mechanism of VEGF-dependent neuro-protection.

FGF2

FGF_2 is a growth factor with significant neuroprotective effects: it plays a wide role in the molecular and cellular mechanisms of neuroregeneration. FGF_2 belongs to the family of morphogens that control the induction and specification of tissues during embryo-genesis and is regarded as an essential component in culture media for the directed differentiation of neural cells *in vitro* (Weiss et al. 1996; Johansson et al. 1999; Kojima and Tator 2002; Martens et al. 2002). FGF_2 is strongly implicated in neuro- and glio-genesis from stem cells. Upregulation of neurogenic gene expression with FGF_2 promotes the migration and maturation of neurons derived

from transplanted neural stem cells (NSCs) (Vergano-Vera et al. 2009). FGF_2 and its receptors contribute to the creation of an important micro-environmental niche that promotes neurogenesis in the adult and aged brain (Mudo et al. 2009). Exposure to FGF_2 selectively induces the astrocyte precursors to proliferate, forming clusters of vimentin (+) cells, which differentiate into astrocytes (Lin and Goldman 2009). FGF_2, interacting with receptors on the surface of astrocytes, is a signal to modulate the phenotype, which results in their proliferation. FGF_2 promotes conversion of *olig*$_2$-expressing progenitor cells into pre-oligo-dendrocyte progenitor cells (Hu et al. 2009) and inducts oligo-dendrocyte progenitors in dorsal forebrain (Naruse et al. 2006). NSCs cultured with FGF_2 and epidermal growth factor (EGF) generate neuro-spheres: these freely floating, non-adherent spherical clusters of neurogenic structures include cells expressing glial markers (Dromard et al. 2007). Schwann cells are able to produce FGF_2, the expression of which increases during damage of peripheral neurons (Santos-Silva et al. 2007). FGF_2 has an effect on reorganizing axonal cytoskeleton by inducing phosphorylation of type-B ephrin (Bruckner et al. 1997; Chong et al. 2000), a tyrosine-kinase that controls the directional growth of axons in the developing and regenerating spinal cord, with subsequent activation of Rho-GTPases that regulate intra-cellular actin-dynamics. The rationale for FGF_2 in gene modification of UCBCs is, therefore, based on its neuro-protective effects and involvement in differentiation of various cell types via specialized signaling system.

Neuro-Degeneration, Neuro-Protection and Neuro-Genesis

Neuro-degenerative diseases are accompanied by loss of neurons, degeneration of axons, disruption of communications in neural networks, and impaired control functions. These include irreversible changes to cholinergic neurons in Alzheimer's dementia (AD), moto-neurons in the spinal cord and brain stem in ALS and spinal muscular atrophy, dopaminergic neurons in the *substantia nigra* in Parkinson's disease (PD), GABA-ergic neurons of the basal ganglia in Huntington's chorea, *etc*. Maintaining the viability of neurons that entered into a pathological process, and restoration of inter-cellular connections in neural networks with neuro-trophic factors, growth factors and adhesion molecules can greatly increase the quality and length of life of patients after neural injuries, ischemic stroke and neuro-degenerative diseases affecting different structures and types of brain cells in the CNS.

Problems with the Blood–Brain Barrier (BBB)

A promising approach for treating patients suffering from neuro-degenerative diseases could be gene therapy to correct aberrant gene expression that is responsible for disease development, stem cell transplantation to replace lost neurons, and a combination of gene and cell therapy: transplantation of genetically modified cells (including stem cells) that express neuro-trophic and neuro-protective factors, such as brain-derived neuro-trophic factor (BDNF), glial cell line-derived neuro-trophic factor (GDNF), insulin-like growth factor (IGF), and VEGF (Hester et al. 2009; Lunn et al. 2009). The neuro-protective effects of these molecules were individually demonstrated both *in vitro* and in vivo. It was also determined that a combination of several neuro-trophic factors may have a more pronounced effect on the survival of nerve cells. However, such experimental studies and clinical trials failed to significantly stimulate neuro-regeneration. One of the key problems is drug delivery to brain tissue. Currently, scientists are exploring different ways for delivery of biologically active molecules into the CNS through the BBB.

Trans-migration of BBB by mono-nuclear cells during inflammatory reactions in response to various cytokines and activated adhesion molecules may be exploited in gene-cell therapy of neuro-degenerative maladies. Having the potential to enter the brain, umbilical cord blood mononuclear cells (UCB-MCs) are now extensively studied as cell-based vectors, designed to carry

therapeutic molecules for stimulation of neuro-regeneration.

Promising Progress in ALS

ALS was first described in 1874 by French doctor Jean-Martin Charcot, which became famous in 1939 when baseball legend Henry Louis "Lou" Gehrig succumbed to the disorder. This malady is also named after the late sportsman (Lou Gehrig's disease).

Clinico-Pathological Characteristics

The term amyotrophic refers to the muscle atrophy, weakness, and fasciculation that signify disease of the lower motor neurons, whereas lateral sclerosis indicates the hardness to palpation of the lateral columns of the spinal cord in autopsy specimens, where gliosis follows degeneration of the cortico-spinal tracts. Apoptosis of spinal motoneurons in ALS may be induced by any neural noxa, such as glutamate excito-toxicity, mitochondrial dysfunction, oxidative stress, axonal transport defects, accumulation of toxic intracellular aggregates, aberrant glial reactivity, *etc*. As a result of the spinal moto-neuronal death a progressive skeletal muscle weakness and atrophy throughout the body leads to the muscle paralysis and eventually affects diaphragm muscle. Due to lack of effective therapies, the patients die from respiratory failure within 3–5 years after the onset of paralytic symptoms. Since its description in the late nineteenth century, there has only been a very limited progress in effective treatment options.

The cause of neuro-degeneration in the most common (sporadic) form of ALS is not known, but some cases of familial (inherited) forms of the disease are linked to dominant mutations in the *sod*1 gene (21q22.1-q22.2). Its product, SOD_1, is the main enzyme of antioxidant defense, localized in every compartment of the cell, including the nucleus, cytosol and mitochondria. It is a homo-dimer composed of two subunits, each containing Cu-binding domain, Zn-binding domain and a disulfide bridge. There are more than 100 known mutations of SOD_1: most of them are point-mutations resulting in the replacement of one of the 153 amino acid residues of the protein. The glycine-to-alanine substitution at position 93 ($^{G}93^{A}$) is a common form: mice with this variant express human mutant SOD_1, and characterized by progressive degeneration of motor neurons, similar to human ALS. Homozygous $^{G}93^{A}$ rodents demonstrate progressive paralysis of skeletal muscles, and die at the age of 4–5 months (Gurney et al. 1994). These mammals, therefore, are used as a common animal model in ALS.

Gene-Cell Approach

Multi-cistronic (multi-gene) vectors have recently been delivered to UCB-MCs for dual expression of any pair/combination of neuronal L_1 cell adhesion molecule (L_1CAM), VEGF, GDNF, FGF_2, erythro-poietin, anti-apoptotic protein *Bcl*2, as well as pluri-potency associated (master transcription regulator) genes *oct*4 and *sox*2. When transplanted into transgenic $^{G}93^{A}$ mice, these genetically modified cells migrated in the spinal cord and, depending on the combination of any of these protein-pairs expressed, differentiated into macrophages, endothelial cells or astrocytes. In addition, over-production of L_1CAM in UCB-MCs also increases cell viability, while genetic modification with VEGF enhances differentiation into endothelial cells and provides neuro-protective effects for motor neurons (Rizvanov et al. 2008).

The beneficial effect of paired-expression of these molecules has gained other grounds as well. Injecting VEGF-FGF_2 dual expressing plasmid into peripheral nerve-stumps stimulated regeneration of axons and restored loco-motor activity of the limb in sciatic nerve injury model in rats (Masgutov et al. 2011). Human embryonic kidney (HEK)-293 cells *in vitro,* transfected with plasmids expressing VEGF-FGF_2, secrete recombinant proteins into the culture medium which enhance the proliferation of human umbilical vein endothelial cells (Salafutdinov et al. 2010).

Xeno-transplantation of VEGF and FGF_2-producing human UCB-MCs into $^{G}93^{A}$ transgenic mice resulted in their differentiating into endothelial and astrocyte-like cells (Rizvanov et al. 2011). It is known that astrocytes have neuro-trophic

effects on moto-neurons, but their function is impaired in ALS (Lepore et al. 2008), and therefore supplementing normal astrocytes in the spinal cord would have a therapeutic implication. In addition, the local increase of VEGF and FGF_2 concentration in the area of neuro-degeneration might prevent apoptosis of motor neurons.

Viability of Genetically Modified Stem Cells

Simultaneous over-expression of neuro-trophic factors and neural adhesion molecules in genetically modified cells increase their targeted migration, survival and duration of neuro-trophic factor secretion in target cells. In such gene-cell therapy approach it is reasonable to expect more pronounced neuro-protective/neuro-genic effect of transplanted cells due to the additive and synergetic power of the two or more therapeutic molecules.

Cell-Survival

Genetically modified stem cells maintained long survival after transplantation. Regardless of the type of the expression vector, they continued to be present after 2 weeks in the circulatory system of transgenic $^{G}93^{A}$ mice and were found not only in the spinal cord, but also in other parenchymal organs (*e.g.*, lungs, liver, spleen). UCB-MCs, transfected with L_1CAM, were detected in the spinal cord of transgenic mice up to 3 months after transplantation.

Safety of Transfection and Gene-Expression

Transfection efficiency and duration of transgene expression by plasmid vectors have technical limitations. Currently, utilization of recombinant adeno-associated viruses (AAV), which are not linked with known human diseases and are considered to be biologically safe, is one of the most promising approach. Unlike other viral vectors, they also lack problems with immunogenicity and pro-oncogenic effects. Because viral transduction can be also controlled, gene-cell therapy based on AAVs might be a safer method than direct gene therapy.

Induced Pluri-Potent Stem (iPS) Cells

Along with the use of genetically modified stem cells expressing therapeutic genes, attention has been focused on induced pluripotent stem (iPS) cells. These are obtained via reprogramming fully differentiated cells by transfection of certain stem cell (pluri-potency)-associated genes, such as master transcriptional regulators *oct*4 and *sox*2, to revert adult somatic cells to pluri- or toti-potency. These de-differentiated iPS cells do not only have properties that of other (*e.g.*, embryonic) stem cells, but their use also avoids any immunogenic responses as they are developed from a patient's own somatic cells.

Recently, iPS cells were generated from an 82-year-old woman diagnosed with ALS (Dimos et al. 2008), and were successfully induced to transform into motor-neurons. Moreover, de-differentiation of genetically modified UCB-MCs with pluri-potency associated genes has been hypothesized to enhance their therapeutic power by increasing the proportion of progenitor and pluri-potent cells. Therefore, transient genetic alteration of UCB-MCs with master transcription regulators might be a promising strategy for regenerative medicine, in particular to stimulate neuro-regeneration during ALS.

In conclusion, genetic modification of UCB-MCs may be useful in (1) improving the viability of transplanted cells in the recipient, (2) delivering specific growth and trophic factors that will stimulate regeneration and enhance the viability of cells undergoing pathological degeneration, and (3) directed differentiation of transplanted cells into various parenchymal cells (*e.g.*, macrophages, endothelial cells, astrocytes, *etc*). In addition, UCB-MCs are able to penetrate through tissue barriers, including BBB, therefore they can be considered as prime candidates as vehicles to deliver therapeutic molecules to the areas of interest. These growth/trophic factors may affect target cells by several modes of action. In an autocrine manner it is possible to control viability, migration and targeted homing, as well as directed differentiation of transplanted cells. Through paracrine and endocrine mechanisms, trophic and neuro-protective effects can be

exerted on target cells in areas of degeneration (Rizvanov et al. 2011).

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Clinical Application of Umbilical Cord Blood-Derived Stem Cells for Tissue Regeneration

4

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Abstract

Cord blood transplantation has been utilized as an alternate allogeneic stem cell source for more than two decades for the treatment of a variety of malignant and nonmalignant diseases, including hematologic malignancies (most prominently acute leukemias), marrow failure syndromes, hemoglobinopathies, and inherited lysosomal and peroxisomal storage diseases. Advantages of cord blood transplantation as compared to other allogeneic stem cell sources include greater HLA disparity without an increase in graft-versus-host disease (GVHD), faster availability and lower risk for somatic mutations and transmitting infections by latent viruses. Cord blood also contains multiple populations of stem cells and progenitors. In addition to hematopoietic stem and progenitor cells, non-hematopoietic stem cells with a variety of different regenerative potential have been identified in cord blood. The multitude composition and the plasticity of cord blood stem cells have elicited great interest in the therapeutic application of cord blood and its derived stem cells in tissue regeneration in non-hematopoietic organs. This chapter will discuss the development of cord blood transplantation as an autologous source for acquired diseases such as brain injury, and as a gene replacement therapy for inherited genetic diseases focusing on recessive dystrophic epidermolysis bullosa.

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Table 4.1 Number of unrelated CBT reported to Eurocord Registry according to diagnosis and recipient age

Diagnosis	Children (≤16 years, n = 1,609)	Adults (>16 years, n = 1,136)
Acute lymphoblastic leukemia	579 (36%)	269 (23.7%)
Acute myelogenous leukemia	257 (16%)	356 (31.3%)
Secondary acute leukemia	38 (2%)	63 (5.5%)
Myelodysplastic syndrome	20 (7.5%)	197 (8.5%)
Chronic myelogenous leukemia	40 (2.5%)	119 (10.5%)
Chronic lymphocytic leukemia	–	16 (1.4%)
Hodgkin/non-Hodgkin lymphomas	31/– (2%)	97/32 (11.4%)
Myeloma	–	20 (1.8%)
Solid tumors	9 (0.7%)	5 (0.4%)
Histiocytosis	60 (3.5%)	1 (0.1%)
Congenital and acquired bone marrow failure syndromes	157 (9.5%)	50 (4.4%)
Hemoglobinopathies	7 (0.5%)	–
Primary immunodeficiencies	170 (10%)	1 (0.1%)
Metabolic diseases	126 (8%)	5 (0.4%)
Other disease	11 (0.8%)	5 (0.4%)

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Introduction

For over 40 years, allogeneic stem cell transplantation (AlloSCT) has been successful as a potential curative treatment option for patients with malignant and non-malignant diseases (Liao et al. 2011). Unfortunately, only 25% of patients in need of AlloSCT have a human leukocyte antigen (HLA)-matched sibling donor. Furthermore, although the National Marrow Donor Program (NMDP) in the United States and other international registries have registered more than 13 million unrelated adult donors, 50–70%of patients continue to be unable to find a matched adult donor. Related and unrelated human umbilical cord blood is an increasingly utilized alternative source of hematopoietic stem cells (HSCs) (Cairo et al. 2008). Cord blood is collected by gravity and venipuncture from the placenta after birth. Once routinely discarded, cord blood can now be easily harvested through puncture of the umbilical vein either from the delivered placenta or during the third stage of labor (Cairo et al. 2005).

The idea to use cord blood arose in the early 1980s, when experiments using blood from near-term mice demonstrated hematopoietic reconstitution in lethally irradiated mice (Kurtzberg 2009). Further, cord blood-derived HSCs were found to have a higher proliferative capacity than those in bone marrow (BM) and sufficient doses of HSCs and hematopoietic progenitor cells (HPCs) were contained in a single collection of cord blood (Cairo and Wagner 1997). Importantly, cord blood can be cryopreserved, from which functional HSCs may be recovered after >20 years of cryo-storage (Kurtzberg et al. 2005).

In 1988, a 5-year old boy with Fanconi anemia underwent the first UCB transplantation (UCBT) from his HLA-identical newborn sister (Gluckman et al. 1989). In 1993, the first unrelated UCBT was performed in a 3-year old with refractory T-cell acute lymphoblastic leukemia (ALL). Currently, over 25,000 UCBTs have been performed worldwide with >500,000 UCB units available in >40 UCB banks. Recently the Center for International Blood and Marrow Transplant Research (CIBMTR) noted a growing trend in the utilization of UCBT which now account for approximately 40%of unrelated donor transplants in pediatric recipients (Liao et al. 2011). UCBT has been utilized in a variety of malignant and non-malignant conditions (Cairo et al. 2008). Table 4.1 lists the unrelated UCBT reported to the Eurocord Registry by diagnosis.

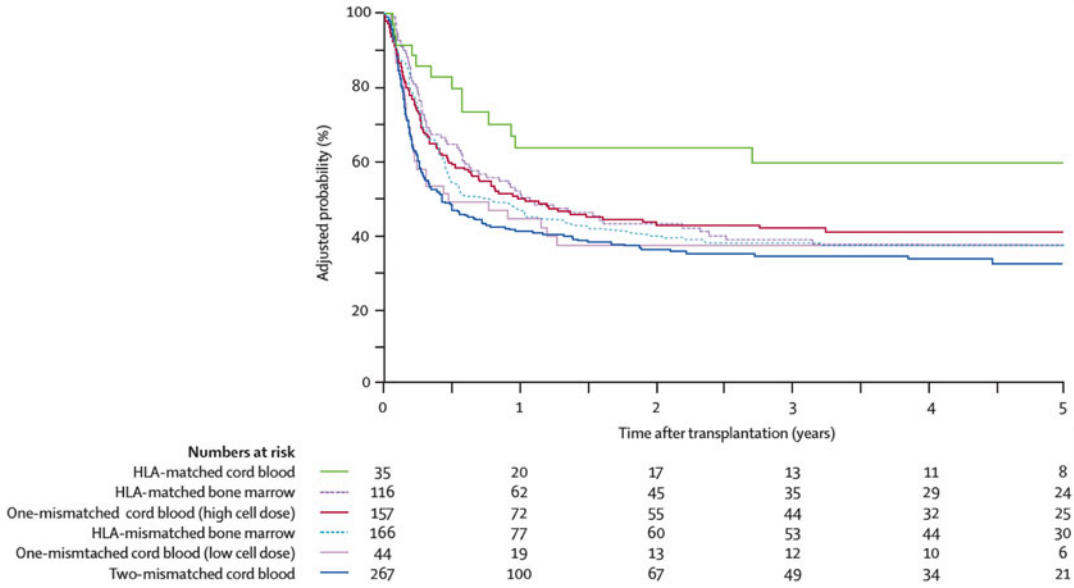

Fig. 4.1 Probability of leukemia-free survival after bone marrow and cord blood transplantation adjusted for disease status at transplantation (Reprinted from Eapen et al. 2007, Copyright (2007), with permission from Elsevier)

Cord Blood Transplantation for the Treatment of Malignant Disorders

UCBT has been used successfully in the treatment of numerous pediatric malignancies, including ALL, acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), neuroblastoma, Hodgkin lymphoma, and non-Hodgkin lymphoma (Liao et al. 2011). Two initial trials by Kurtzberg et al. (1996) and Wagner et al. (1996) demonstrated in 25 and 25 patients, respectively, that cord blood could be used successfully as an alternate allogeneic stem cell source in pediatric recipients with malignant and non-malignant diseases. The Cord Blood Transplantation (COBLT) study was the first multi-center trial of unrelated UCBT. The study utilized cord blood from public UCB banks and enrolled 191 patients with hematological malignancies. Two-year overall survival (OS) was 49.5% with a cumulative incidence of relapse at 2 years of 19.9% (Kurtzberg et al. 2008). The Eurocord/European Blood and Marrow Transplant (EBMT) group reported 2-year OS and leukemia-free survival (LFS) of 49 and 42%, respectively, in 95 children receiving unrelated UCBT for AML.

While there have been no randomized control studies comparing outcomes of unrelated cord blood versus peripheral blood stem cells (PBSCs), several retrospective studies suggest that outcomes are similar (Liao et al. 2011). A CIBMTR retrospective study of unrelated donor graft sources in pediatric acute leukemia patients found that 5-year LFS was similar among patients receiving fully matched unrelated marrow transplant and those receiving one or two antigen-mismatched UCBT (Fig. 4.1) (Eapen et al. 2007).

Cord Blood Transplantation for the Treatment of Non-malignant Disorders

UCBT has been successfully utilized in a variety of non-malignant diseases, including hemoglobinopathies, bone marrow failure syndromes, and inborn errors of metabolism. A retrospective Eurocord/EBMT review of patients with Fanconi anemia demonstrated a high incident of primary graft failure (40%) with 40% of patients alive at 3 years post-transplant (Gluckman et al. 2007). A small number of reports have examined UCBT

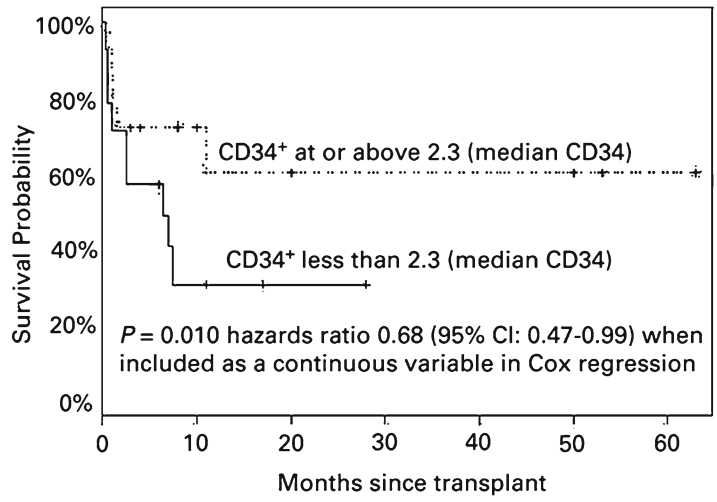

Fig. 4.2 Probability of overall survival (OS) by CD34+ cell dose. Probability of OS by CD34+ cell dose as determined by Kaplan-Meier and Cox's regression multivariate analysis in patients receiving UCB transplantation. CD34+ at or above 2.3×10^5 (––––); CD34+ less than 2.3×10^5 (____) (P = 0.010), (>2.3 vs <2.3 × 105/kg).in patients receiving cord blood transplantation (From Styczynski et al. 2004)

in both matched sibling and unrelated donors in patients with sickle cell disease. UCBT from a related donor was associated with a low incidence of graft failure and high levels of donor chimerism, while outcomes from unrelated UCBT in patients with the sickle cell disease remain dismal due to non-engraftment and transplant-related mortality (Bhatia et al. 2011 [abstract]; Ruggeri et al. 2011).

In patients with metabolic errors due to single gene defects, engrafted donor cord blood cells are able to correct the defect, leading to life-long enzyme replacement therapy (Kurtzberg 2009). Recent studies found that 1-year, 3-year, and 5-year survival probabilities in patients with lysosomal and peroxisomal storage disorders were 79, 62.7, and 58.2%, respectively (Eapen et al. 2010; Martin et al. 2006). Further, as donor cells can cross the blood–brain barrier, neurological and cognitive function is stabilized and even improved, particularly if transplant is performed by 2 years of age.

Advantages/Limitations of Cord Blood as a Donor Source

UCBT has multiple advantages over other unrelated donor graft sources, including greater HLA disparity without an increase in GVHD, faster availability (<2 weeks as compared to 51 days for BM or PBSC through NMDP), and easier recruitment of minority donors (Liao et al. 2011). Limitations include higher risk of graft failure with decreased ability for post-transplant donor-derived cellular therapies, limited numbers of cells, lower rates of neutrophil engraftment, and delayed median times to neutrophil and platelet recovery as well as immune reconstitution. Delayed engraftment and immune reconstitution may be due to a decreased number of immature CD34+ progenitor cells or leading to more severe post-transplant infections and longer hospital stays (Szabolcs and Cairo 2010).

Due to limited blood volumes collected from typical cord blood units, less than 12%contain sufficient cells for a 60 kg recipient. TNC/kg and CD34+ cell dose/kg are directly linked to transplant outcome, with thresholds of 25–30 million nucleated cells/kg typically required for engraftment and significantly poorer survival outcomes in patients receiving $<1.7–2.3 \times 10^5$ CD34 cells/kg (Fig. 4.2) (Styczynski et al. 2004; Wagner et al. 2002). In order to expand the use of UCBT to adults and adolescents, multiple strategies have been developed, including using 2 UCB units, eicosanoid-based strategies to enhance engraftment, and ex vivo expansion of progenitor cells (Liao et al. 2011).

Co-transplantation of two partially HLA-matched unrelated donor cord blood units (D-UCBT) has been employed, with increased engraftment, decreased relapse, and higher 3-year EFS when compared to single UCBT recipients

(Barker et al. 2005). Intriguingly, though engraftment rates improve with double-cord unit transplantations, only one of the units serves as the source for hematopoietic engraftment and factors that predict UCB dominance remain unknown. Unfortunately D-UCBT is associated with increased rates of acute and chronic GVHD and costs double that of a single UCBT.

Several methods have been employed to enhance engraftment. Eicosanoids, particularly PGE_2, have been demonstrated to enhance homing of stem cells to the marrow, through up-regulation of the CXCR4 receptor, a critical component of HSC homing (Brenner et al. 2004). Further eicosanoids are thought to increase self-renewal and increase survival of HSCs through regulation of survivin and Wnt/β-catenin (Goessling et al. 2009). Ex vivo expansion of peripheral blood progenitor cells to facilitate engraftment started with cytokine-mediated expansion methods, including stem cell factor (SCF), thrombopoietin (TPO), and granulocyte colony-stimulating factor (G-CSF) (Shpall et al. 2002). Unfortunately, this approach led to only slight increases in stem cell numbers and translated to minimal improvements in neutrophil recovery. Current approaches target stem cell self-renewal, particularly the Notch signaling system. This approach involves culturing UCB stem or progenitor cells in the presence of the extracellular domain of the Notch ligand Delta-1. An ongoing Phase I trial includes co-transplantation of a non-manipulated cord blood unit with progenitor cells from a second unit that have undergone Notch-mediated expansion. Time to neutrophil engraftment was significantly shorter in these patients as compared to patients receiving D-UCBT without expansion (Delaney et al. 2010). Numerous ongoing studies are underway to further elucidate the molecular pathways involved in D-UCBT, increasing engraftment, and ex vivo expansion of stem or progenitor cells.

Stem Cells and Tissue Regeneration

Stem cells are a unique subset of cells which possess the biological property of self-renewal. The cardinal feature of self-renewal is the ability of a cell with long-term differentiation capability to give rise to progeny with the same potential to differentiate into various cell types. In order to do this, a stem cell must be able to undergo asymmetric division and possess the ability to avoid replicative senescence. Asymmetric division refers to the process wherein a stem cell divides to form two different daughter cells, both a more differentiated cell and one that retains the ability to self-renew.

The hematopoietic stem cell was the first described and has consequently become the prototypical stem cell. Since then, however, stem cells have been defined in a variety of tissues including germline, skin, mammary tissue, gut, and neural tissues. These stem cells have been recognized to play critical roles in the long-term maintenance and regeneration of their associated tissues. The remarkable regenerative property of human stem cells is highlighted by the success of hematopoietic stem cell transplantation as a therapeutic modality for a variety of diseases (Orkin and Zon 2008).

Stem and progenitor cells are thought to exhibit a hierarchical organization with stem cells residing at the peak and possessing the greatest ability to self-renew (Orkin and Zon 2008). As progenitor cells develop more functional differentiation they become restricted in their ability to self-renew. While self-renewal is mediated by a number of cell-intrinsic factors, there is increasing evidence that the specialized niche microenvironment of the stem cell is highly influential in directing asymmetric division and regulating cell cycle entry (Orkin and Zon 2008).

Embryonic stem (ES) cells are a class of stem cells derived from the inner cell mass of the blastocyst stage of early human development. Compared to adult stem cells, they are more mitotically active and can give rise to structures of endodermal, mesodermal, and ectodermal derivation. By virtue of their indefinite capacity for self-renewal and pluripotency, they harbor great potential for clinical applications in regenerative medicine. However, their clinical application has been challenged with ethical consideration.

A major breakthrough in the field of stem cell biology came through the work of Takahashi and Yamanaka in 2006 when they showed that forced

expression of transcription factors Oct4, Sox2, Klf4, and c-Myc was sufficient to reprogram somatic cells to possess features of self-renewal. These so-called inducible-pluripotent stem cells (iPS) displayed characteristics similar to ES cells including surface markers and the ability to differentiate into cell types from all three germ layers.

The development of iPS cells, in combination with current technologies on gene correction and directed stem cell differentiation, has provided exciting possibilities of generating autologous cells for cell- replacement therapy for a variety of degenerative diseases. However, for an ultimate clinical translation of iPS cells, many issues have to be solved, including the safety of iPS cells, efficient differentiation of iPS cells into any desired cell lineage and development of novel stem cell delivery system so that the transplanted cells could efficiently integrate to function in the recipient tissue. Moreover, Zhao et al. (2011) has recently revealed that transplantation of autologous iPS cells derived from fetal fibroblasts into the matched mice unexpectedly resulted in immune-rejection, in contrast to matched ES cells. This finding has raised a cautionary note for the therapeutic use of iPS cells, and many questions remain to be further explored with regard to the underlying difference between iPS cells and ES cells.

A significant advantage of UCBT over other sources of stem cells including iPS cells in tissue regeneration, is the readily availability of UCB. This is particularly important for some diseases such as hypoxic ischemic injury, where the most effective rescue therapy is likely within few hours or days after the insult. For the inherited metabolic diseases such as Krabbe disease, better prognosis has been observed in those patients that were diagnosed and treated early in the course of the disease (Escolar et al. 2005). Such early treatment would not be realistic with the iPS therapy, as derivation of iPS cells, characterization and directed differentiation would respectively consume a long period of time. Cord blood could be more effectively bridged with the iPS development for a potentially improved cellular therapy. First of all, cord blood mononuclear cells (MNCs) or the cells enriched in CD34 or CD133 represent an efficient cell source for generation of iPS cells. It is also noteworthy that UCB, being neonatal cells, is expected to carry minimum somatic mutations and infections, presenting them as a more reliable source for iPS cells as opposed to other adult cell types. Secondly, as discussed below, a number of primitive stem cells have been isolated from UCB, some of which showed the ability to differentiate into cells representative of all three lineages thus could be used as an alternative stem cell source to ES or iPS cells in tissue regeneration. Currently, these cord blood-derived primitive stem cells have been actively investigated in various animal models and some populations have been produced under good manufacturing practice (GMP) grade for the upcoming clinical investigation. The experience learned from such cell therapy, including the optimal timing, dosing, administration as well as mechanism of function will be invaluable to the future of iPS based cell therapy. Last but not least, there is a potential combination of AlloSCT (cord blood) with iPS development in the treatment of genetic diseases.

Cord Blood Derived Stem and Progenitor Cells

Cord blood is a rich source for stem cells and progenitors (Liao et al. 2011). In addition to the HSCs that undoubtedly are significant in reconstituting blood lineages after myeloablative therapy, monocytes constitute about 5–10%of cord blood MNCs and bear some unique features in their immaturity in the immune and inflammation stimulatory functions, as compared to those originating from BM or peripheral blood. Monocytes have been shown to participate in angiogenesis via several mechanisms including degradation of the extracellular matrix, secretion of pro-angiogenic cytokines, promotion on the migration of endothelial cells and differentiation into endothelial cells. Such angiogenic and anti-inflammatory effects have likely played an important role in the cord blood transplantation therapy, especially in ischemic diseases.

Besides the HSCs, a number of non-hematopoietic stem cell populations have been isolated from cord blood (Liao et al. 2011). McGuckin et al. (2008) has previously described the isolation of an embryonic-like stem cell (CBE) population from cord blood, by immunomagnetic removal of CD45, CD33, CD7 and CD235a positive cells (Fig. 4.3a). The resultant lineage negative cells, which represent 0.1–1% of total MNCs of each UCB, express ES cell markers Oct4, Sox2, SSEA3/4, TRA-1-60 and TRA1-81. CBEs have been shown to differentiate into neural cells, and according to the authors, were able to give rise to cells representative of all three germ layers. Habich et al. (2006) applied a similar immunodepletion method to remove the CD34 positive cells from cord blood. Following stimulation by epithelial growth factor (EGF), the UCB-MNC$^{CD34\ neg}$ cells were able to undergo spontaneous aggregation and differentiation toward neural lineage, where neural stem cell lines could be established. Recently, using multi-parameter FACS analysis, Kucia et al. (2007) isolated very small embryonic-like (VSEL) stem cells (CXCR4^{pos} Lin^{neg}CD45^{neg}Oct4^{pos}SSEA4^{pos}) from UCB. Very small embryonic-like stem cells represent about 0.02% of total MNCs of cord blood and could also be isolated from BM and peripheral blood.

In addition to the isolation of stem cells based on the cell surface markers as described above, different populations of stem cells have also been derived from cord blood based on their plastic adherent properties. A well known stem cell population from cord blood is mesenchymal stem cells (MSCs). The therapeutic values of MSCs have been related to their immunomodulatory function and the ability to differentiate into cells mainly within mesoderm lineages. It has been shown that the derivation efficiency of MSCs from cord blood was lower than that from other sources such as BM and adipose. However, human placenta represents another novel source for MSCs. Recently, Celgene Cellular Therapeutics (CCT) announced the U.S. FDA approval for the clinical use of PDA-001, human placental derived MSCs. PDA-001 cells exhibit moderate levels of HLA Class I, are undetectable for HLA Class II, and do not express the co-stimulatory molecules CD80 and CD86. Preclinical studies on co-transplantation of PDA-001 with cord blood in immunecompromised mice indicated that PDA-001 cells facilitated cord blood cell engraftment and reconstitution (Robert Hariri, MD, PhD, personal communication).

Cord blood is also a promising source of endothelial cells for the cell therapy of vascular diseases. Endothelial progenitor cells (EPCs), also named endothelial colony forming cells (ECFC), could be identified based on the outgrowth of cobblestone-like adherent colonies between day 7 and 14 following plating of UCB MNCs on collagen I coated plastics. The ECFCs have shown the ability to form de-novo blood vessels when seeded in either a collagen fibronectin or matrigel matrix and implanted subcutaneously in immunocompromised mice, suggesting their potential therapeutic treatment of patients with impaired vascular function.

By plating cord blood MNCs on tissue culture plates in the presence of high concentration of fetal bovine serum and dexamethasone, Kogler et al. (2004) derived a novel stem cell population with intrinsic pluripotent differentiation potential, named unrestricted somatic stem cells (USSCs) (Fig. 4.3b). USSCs have been considered an earlier cell type of MSCs and can be distinguished from MSCs by their broader differentiation capability and differential expression of DLK1 and a set of HOX genes. USSCs possess the abilities to differentiate *in vitro* into bone, cartilage, adipocytes, hematopoietic cells and neural cells, and *in vivo* into myocardial cells, purkinje fibers and hepatic cells. Preclinical studies have suggested the therapeutic value of USSCs, as a single pure population in the promotion of bone healing, relief of neural injury and improvement of recovery from myocardial infarction. In addition, USSCs constitutively produce a series of hematopoiesis-supporting cytokines, and co-transplantation of USSCs in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice enhanced *in vivo* homing of both unselected and selectively amplified CD34 positive cord blood cells.

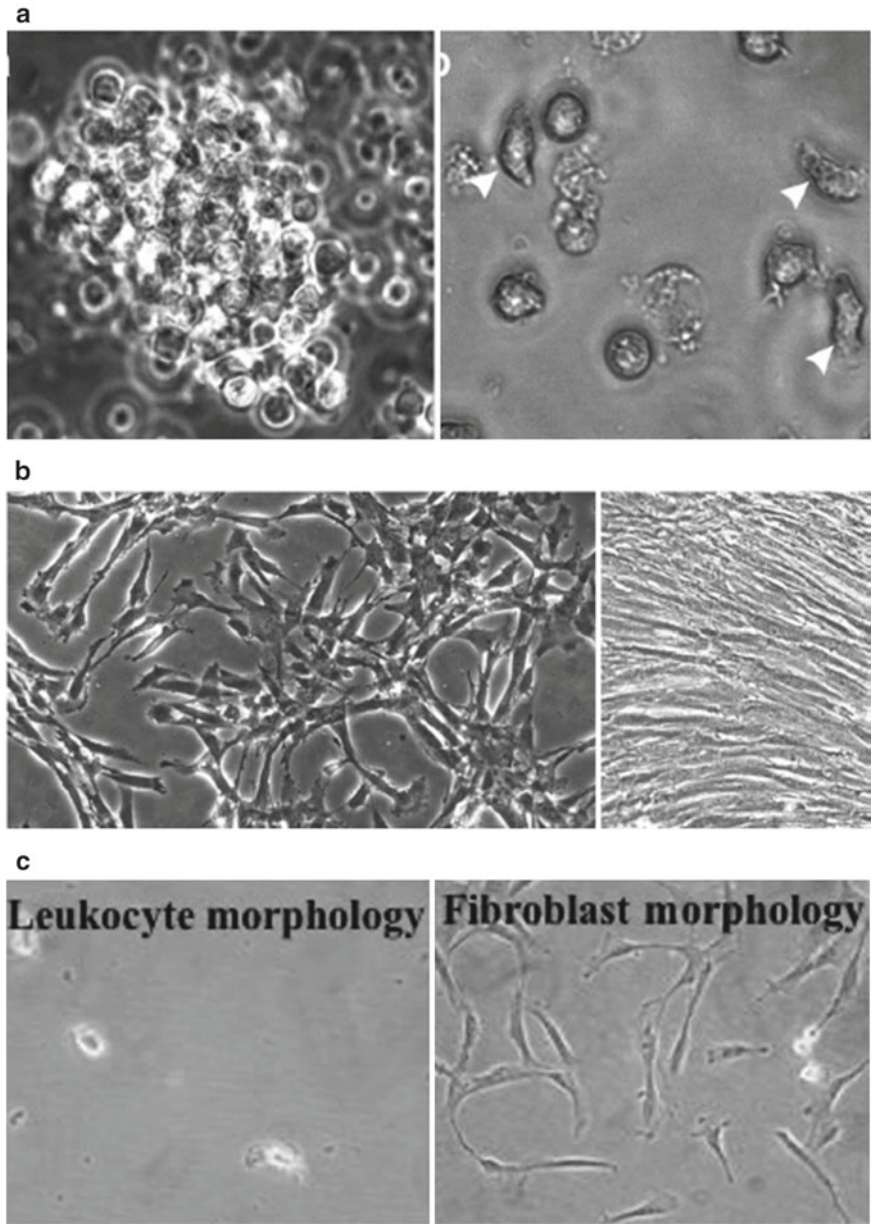

Fig. 4.3 The existence of multiple primitive stem cells from cord blood. (**a**). Harvesting and expansion of CBEs in serum-free media. *Left*, aggregates of embryonic-like stem cells following the expansion phase formed in SF-TPO medium 16–48 h after isolation from cord blood. *Right*, expected initial shape of the differentiated, collagen-adhered, neural progenitors after 1–7 days in SF-EFS medium (Reprinted by permission from Macmillan Publishers Ltd: *Nature Protocols*, McGuckin et al. 2008, copyright (2008)). (**b**) *Spindle-shaped* USSCs when plated at low density (*left*) and high density (*right*) (From Kogler et al. 2004). (**c**) Freshly isolated MLPCs and cells cultured less than 3 days exhibited a leukocyte-like morphology (*left*) and converted to a fibroblast-like morphology following extended culture (*right*) (Reprinted from van de Ven et al. 2007, Copyright (2007), with permission from Elsevier)

Van de ven et al. (2007), in our group, also characterized multi-lineage progenitor cells (MLPC) from cord blood by non-particular based negative cell selection followed by plastic adherence. MLPCs exhibit two distinct morphologies in cell surface phenotypes, a leukocyte-like morphology

on freshly isolated cells (positive for CD45, CD34, CD133, CD13, CD29, CD44, CD73, CD90, CD105, CD9 and SSEA-3/4) and a fibroblastic morphology ($CD45^-$, $CD34^-$, $CD133^-$, $CD13^+$, $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD9^+$) upon continued culturing (Fig. 4.3c). MLPCs can be differentiated into cell types representative of all three germinal layers. Comparative microarray analyses indicated that MLPCs were also more quiescent and primitive, and less committed to lineage than MSCs.

Cord Blood Regenerative Therapy in Brain Injury

Brain damage resulting from perinatal hypoxic-ischemic encephalopathy (HIE), often as a consequence of birth asphyxia, is a major cause of acute mortality, chronic neurologic disability and large health care expenditures in infants and children. Despite tremendous improvements in obstetric and neonatal care, the incidence of HIE remains at about 2.5 (1.2–7.7) of every 1,000 full-term births. About 20–50% of asphyxiated babies die within newborn period and up to 25% of the survivors exhibit permanent neuropsychological handicaps including mental retardation, cerebral palsy and epilepsy. To date the only clinically approved intervention is hypothermia for moderate to severely asphyxiated term babies.

Earlier experience on allogeneic unrelated cord blood transplantation for the patients with inborn errors of metabolism demonstrated that cord blood cells, both hematopoietic and non-hematopoietic in origin can engraft in the patients' central nervous system, providing the missing enzyme and facilitating neural cell repair (Escolar et al. 2005). Based on these observations, clinical studies have been undertaken to investigate the feasibility of cord blood infusion in children with acquired neurological diseases. Sun et al. (2010) reported the result of a pilot study on intravenous infusion of autologous cord blood in 184 pediatric patients who had their cord blood banked at birth and were subsequently diagnosed with acquired neurological disorders. This investigation has proven the safety and feasibility of autologous cord blood infusion in those pediatric patients. The efficacy of the approach however requires further evaluation through a randomized placebo controlled clinical trial. Duke University is also conducting an ongoing clinical trial on autologous cord blood transplantation in HIE infants, with or without whole body cooling (Michael Cotten, MD, personal communication). In this clinical trial, fresh-volume reduced cord blood was infused to infants immediately after diagnosis with HIE, and at 24, 48 and 72 postnatal hours respectively. Preliminary results on this experience have also demonstrated feasibility of UCBT in neonates with HIE (Michael Cotten, MD, personal communication).

While the human clinical studies start to address the safety and feasibility of UCBT, a number of animal studies involving the models of asphyxia and stroke have provided potential mechanisms of action for cord blood transplantation. With a few exceptions, most of the pre-clinical transplantation studies showed varied levels of histological and/or functional improvements in the damaged brain. Due to the poor survival of the transplanted cells in the brain, by-stander effects have been postulated for functional recovery after cord blood transplantation, including the release of trophic or neuroprotective factors, anti-inflammatory function and modulation on periphery immune response (Liao et al. 2011).

The outcomes of UCBT are likely to vary depending on the severity of the brain damage, cell dose, routes of administration and importantly timing of administration relative to the stage of the brain injury. A severe acute asphyxial episode has been shown to trigger a cascade of events leading to cellular energy failure, cellular injury and ultimately cell death. This cascade involves primary and secondary energy failure and a latent phase in between. Brain damage starts during initial cerebral ischemia, which results in deprivation of energy substrates i.e. oxygen and glucose to brain tissue. The damage then worsens during the recovery phase after resuscitation. During this latent phase, which may last up to 8 h, accumulation of excitatory neurotransmitter, uncontrolled opening of excitatory glutamate receptors, accumulation of intracellular calcium and subsequent activation of

intracellular mechanisms have been observed, leading to secondary delayed energy failure, and cellular necrosis and apoptosis. After the secondary phase of injury there is another chronic phase of injury where there is further loss of brain cells.

For such an acute injury, an ideal rescue therapy is the intervention before the delayed cellular death. However, this therapeutic window is narrow and often challenged by the timely diagnosis on the onset of primary energy failure. In a neonatal rat model of HIE, Pimentel-Coelho et al. (2010) demonstrated that intraperitoneal injection of cord blood cells 3 h after hypoxic-ischemia resulted in improvements in sensorimotor reflexes at certain days of examination. The improved outcomes were associated with reduced caspase-3 cleavage, activated microglia and macrophages at various brain regions. This study showed that cord blood cells have anti-apoptotic and anti-inflammatory actions during the acute phase of the injury. In addition, functional neurological improvements have been reported in HIE animal models following cord blood or its-derived stem cell transplantation at 24-, 72-h, 7 or 10 days post HI. In an adult rat model of stroke, behavioral recovery was observed following iv injection of BM stromal cells even 1 month after stroke. In this study reported by Shen et al., BM stromal cells engrafted at the site of injury and showed evidence of neural differentiation. The treatment also reduced scar thickness, and increased the number of proliferating cells and oligodendrocyte precursor cells along the subventricular zone in the ipsilateral hemisphere. It appears that the transplanted cells at the acute phase play a neuroprotective role by relieving inflammation, apoptosis and/or promoting endogenous neurogenesis. On the other hand, in a delayed stage with diminished inflammation, the engraftment of transplanted stem cells and potential differentiation into neural cells, in addition to the paracrine effect, are likely to be important for the regeneration of the damaged brain tissue. Meanwhile, Borlongan et al. (2004) concluded using a rat model of stroke that central nervous system entry of peripherally injected cord blood cells was not required for the role of neuroprotection, provided that neurotrophic factors released from cord blood could enter the brain. Gornicka-Pawlak et al. (2011) recently compared therapeutic effectiveness of freshly isolated cord blood MNCs and other neural committed progenitors or stem cells in experimental rats 3 days after focal brain injury. The highest effectiveness, demonstrated by enhanced functional recovery and reduced lesion volume was observed in the group treated by cord blood MNCs. As human cells were not detected in the rat brain, the authors contributed the benefits of stem cell transplantation to neuroprotection at the acute phase of brain injury. Due to the complex composition of UCB, the neuroprotective role of UCB is likely to be more multifactorial than other defined sources of stem cells.

The above animal studies suggest that infusion of autologous UCB in infants with HIE within few hours or days after diagnosis provides neuroprotection. However, as the stem cell treatment may function via different mechanisms as the injury progresses, a combined therapy should be considered in the future clinical setting. Multiple dosing of stem cells, combined administration of cord blood-derived stem cells as described above, or transplantation of iPS derived mature neural cells will likely have beneficial effects on the repair and regeneration of the damaged brain.

Cord Blood Regenerative Therapy in Recessive Dystrophic Epidermolysis Bullosa (RDEB)

Recessive dystrophic epidermolysis bullosa (RDEB) is a severe congenital skin disease caused by mutations in the gene encoding type VII collagen (COL7A1) at the dermal-epidermal junction (Fig. 4.4). Depending on the severity of the COL7A1 mutations, the anchoring fibrils in RDEB patients are reduced in number and demonstrate abnormal morphology, or in severe cases barely detectable. Patients with RDEB suffer from severe defects of anchoring fibrils resulting in impaired dermal-epidermal connections, which leads to an unrelenting clinical course characterized by chronic, progressive, and mutilating scarring. Further, patients are at high risk for systemic

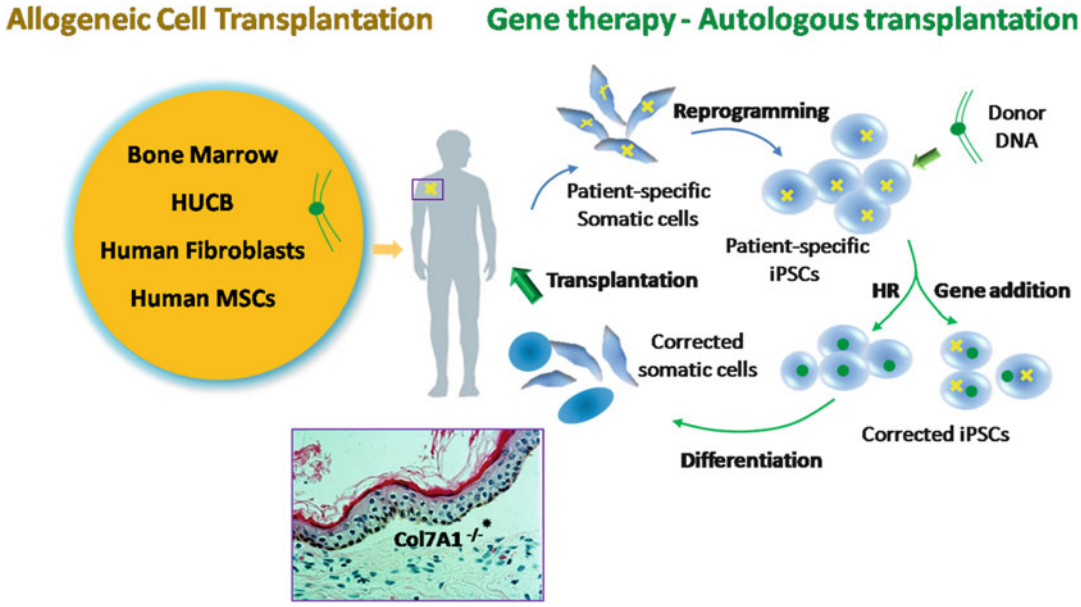

Fig. 4.4 Potential cell therapy for the patients with RDEB. The skin of the patients with RDEB showed a separation of epidermis from dermis, due to mutations in the *COL7A1* gene. Allogeneic transplantation of bone marrow, cord blood, fibroblasts or MSCs has now been respectively investigated in experiment models and human subjects with RDEB. Meanwhile, combination of technologies including iPS development, gene therapy (homologous recombination or gene addition) and directed differentiation has provided a potential to rescue patient's own cells for autologous transplantation

sequelae including scarring alopecia, pseudosyndactyly, esophageal stenosis, dental deformities, cardiomyopathy, and squamous cell carcinoma. Most importantly, patients with EB have a significantly shortened lifespan (20–30 years).

Currently, there is no cure for treatment for patients with RDEB. However, significant progress has been made to develop gene-, protein- and cell-based therapy for patients with RDEB, with a common goal to replenish the affected skin in RDEB patients with functional COL7A1 protein. Among these, autologous and allogeneic cell therapies have provided promises in patients with RDEB, by providing a sustained production of COL7A1 from the transplanted cells (Fig. 4.4). Korbling et al. (2002) previously demonstrated that donor allogeneic hematopoietic cells can differentiate into epithelial cells that engraft in the human recipient skin. To determine whether AlloSCT could serve as a gene-replacement therapy for this genetic skin disease, wildtype murine hematopoietic progenitor enriched cells ($SLAM^+$) were infused into unconditioned Col7A1 knockout mice (Tolar et al. 2009). Donor skin engraftment and a limited COL7A1 production were observed in 3 out of 13 recipients, suggesting that adoptive transfer of COL7A1 producing hematopoietic cells may rescue the underlying basement membrane defect (Tolar et al. 2009).

The concept of gene replacement was further tested in a pilot clinical study of allogeneic BM or UCBT following myeloablative chemotherapy in seven RDEB patients (Wagner et al. 2010). Five patients showed increased COL7A1 deposition at the dermal-epidermal junction and exhibited clinical improvement. Donor cell chimerism was observed in the recipients' skin, including not only the $CD45^+$ hematopoietic cells, but also non-hematopoietic, non-endothelial donor cells in the vicinity of the epidermal-dermal junction (Wagner et al. 2010). However, there was no distinct anchoring fibril formation in the recipient skin. There also seemed to be a linear relationship between the baseline level of recipients' mutant COL7A1 protein and the extent of skin

improvement following transplantation, implying that transplantation may contribute to the improvement in the skin of RDEB patients through stimulating the recipients' baseline mutant protein expression.

The above preclinical and clinical investigations have demonstrated a beneficial effect of AlloSCT for the treatment of RDEB. However, the level and/or durance of wildtype COL7A1 protein from the transplanted cells may be limiting for a complete rescue of the disease. A critical question is the identity of the transplanted cells that are able to engraft and secrete wildtype COL7A in the skin. As the hematopoietic cells do not express COL7A1, the non-hematopoietic, non-endothelial donor cells that were observed in the vicinity of the epidermal-dermal junction in the recipient skin might be the source for the wild type COL7A1. However, it is unknown whether these non-hematopoietic, non-endothelial donor cells were the products of trans-differentiation from the hematopoietic stem cells or were the rare non-hematopoietic stem cells existing in the BM or UCB. Nevertheless, supplementation of an expanded population of such cells with properties of engraftment and secretion of wildtype COL7A1, together with AlloSCT, may provide a further rescue to the defective anchoring fibril formation in the skin of the patients with RDEB. It has been demonstrated that MSCs express COL7A1 and could reverse the RDEB phenotype in a murine model of RDEB.

Our previous experience demonstrated that reduced-intensity conditioning (RIC) regimens (many fludarabine [FLU] based) utilized prior to AlloSCT are able to minimize the cytotoxic/morbid effects of past myeloablative conditioning (MAC) regimens (Satwani et al. 2005). The rationale behind using very RIC vs. MAC prior to AlloSCT is to induce donor chimerism to correct the underlying genetic deficiency, while keeping the acute or long-term toxicities and other long-term effects minimal. In particular, in patients with RDEB, reducing the risk of developing acute mucositis, infection and aGVHD is critical to reducing stem cell transplant-related morbidity and mortality in this high-risk population who have concomitant co-morbid features prior to allogeneic stem cell therapy. Theoretically, AlloSCT has opened possibilities in the recipients to receive any donor or donor iPS-derived somatic cells or organs for enhanced tissue regeneration. In the scenario with RDEB patients who have undergone AlloSCT and established donor chimerism, the recipients are then able to accept donor or donor iPS-derived skin grafts without immune-rejection.

In conclusion, 24 years have been passed since the first successful UCBT using sibling HLA-matched UCB to treat a boy with Fanconi anemia. Over the years, much has been learned from both clinical and preclinical experience in the biology of UCB, the measures to enhance immune reconstitution and the transplantation regimen to reduce the UCBT associated toxicity. Cord blood offers many practical advantages such as relative ease of procurement, minimal risk to the donors and the ability to bank the screened and HLA-typed samples for immediate private or public use. Moreover, cord blood also possesses a primitive ontogeny, has not been exposed to immunological challenges and is expected to carry minimal somatic mutations. It has also been demonstrated that UCB contains multiple populations of non-hematopoietic stem cells and progenitors including MSCs, endothelial progenitors and rare populations of primitive stem cells capable of multilineage differentiation. These UCB-derived stem cells are currently under investigation clinically and pre-clinically as either independent stem cell sources or supplementary populations to UCBT in regeneration therapy. As discussed in this chapter, autologous infusion of UCB for acquired diseases such as brain injury, or allogeneic UCBT for inherited genetic diseases has shown promise as an approach for tissue regeneration. However, many questions remain to be investigated, including the mechanism of action, the functional cell type, optimal therapeutic window and route of injection. Ultimately, cell replacement is a major challenge faced by the whole field of stem cell regenerative medicine.

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Part II

Induced Pluripotent Stem Cells

5 Differentiation of Induced Pluripotent Stem Cells into Oligodendrocytes: Increased Efficiency of Selecting Oligodendrocyte Precursor Cells Using A2B5 Monoclonal Antibody

Yasuhito M. Tokumoto

Contents

Abstract

Induced pluripotent stem cells (iPSCs) from somatic cells, lead to speculation that this technique might become a novel source of oligodendrocytes for transplants in regenerative medicine and tools of drug discoveries. However, the efficiency of *in vitro* differentiation into oligodendrocytes was lower from iPSCs than either from embryonic stem cells (ESCs) or from neural stem cells (NSCs). It was thought that using a mouse monoclonal antibody A2B5 to select cells of oligodendrocyte lineage from iPSC-derived cells could increase the *in vitro* differentiation efficiency of oligodendrocytes.

Introduction

Demyelination is associated with many central nervous system (CNS) disorders, including multiple sclerosis, stroke, spinal cord injury, brain trauma, abuse of drugs such as alcohol and cocaine, Alzheimer's disease, and aging (Bartzokis 2004). As demyelination is mainly caused by defects in oligodendrocytes, the replacement of lost or damaged oligodendrocytes by cell transplantation may represent an effective therapeutic approach for such conditions (Martino et al. 2010). In animal models, transplantation of neuronal precursor cells (NPCs) or oligodendrocyte precursor cells (OPCs) has been shown to effectively remyelinate the brain cells and the spinal cord. In those experiments, myelinating

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transplants are conventionally obtained from NSC-derived cells or ESC-derived cells. However, there are numerous religious and ethical concerns surrounding the use of these cells for human therapy. Moreover, as both NSC-derived cells and ESC-derived cells are essentially allografts, the possibility of immune rejection remains a significant complication. However, recent development of iPSC technologies provided possible solutions (Takahashi and Yamanaka 2006). *In vitro* analysis revealed that iPSCs exhibit almost the same lineage-specific differentiation efficiency as ESCs (Inoue and Yamanaka 2011). As iPSCs can be induced from the recipient's own tissue, it is expected that iPSCs-derived NPCs and OPCs will be able to be used as autologous sources for transplantation therapies (Inoue and Yamanaka 2011). Several groups including us have been trying *in vitro* differentiation of iPSCs into oligodendrocytes. This review will focus on the progresses of *in vitro* induction methods of oligodendrocytes from iPSCs.

Induction of Oligodendrocyte from Induced Pluripotent Stem Cell (iPSC) In Vitro

As there are so many effective neuronal lineage-directed *in vitro* differentiation protocols from ESCs or NSCs, trials of *in vitro* induction of oligodendrocyte from iPSCs were carried out very early. The appearance of oligodendrocytes in iPSC-derived cells was first reported in the Japanese patent application forms of iPSC technology in 2005 (Yamanaka et al.; P2005-359537, P2008-283972A). At that time, they differentiated mouse embryonic fibroblast (MEF)-derived iPSCs (induced by Oct4, Sox2, c-Myc and Klf4 overexpressions) into oligodendrocytes. The appraisal of oligodendrocyte was based on the cellular morphology and the recognition with mouse monoclonal antibody O4. O4 antibody recognizes sulfatide that is synthesized primarily in the oligodendrocytes in the CNS and is used as a conventional cell surface marker of oligodendrocytes. Although they showed a photograph of O4-positive ($O4^+$) and multi-process with membrane-like structure bearing oligodendrocytes, they did not describe the detailed protocols and the efficiency of differentiation into oligodendrocytes.

Wernig et al. (2008) reported the appearance of mouse iPSC-derived oligodendrocyte in culture. They induced mouse iPSCs from MEFs (E13.5) and adult tail-tip fibroblasts (TTFs) through ectopic expressions of Oct4, Sox2, c-Myc, and Klf4 *via* retrovirus vectors. They used the NPCs development protocol optimized to ESCs (Okabe et al. 1996). The iPSCs were transferred to nonadherent culture dishes where they readily formed floating embryoid bodies. Four days later, embryoid bodies were transferred to tissue culture surface and allowed to spread on this substrate. The next day, the medium was switched to serum-free media. After 1 week, the cells formed clusters of neuroepithelial-like cells that could be isolated and propagated in bFGF containing media. To obtain oligodendrocytes from these iPSC-derived NPCs, bFGF were omitted from the medium. Seven days later, although $O4^+$ oligodendrocytes with multi processes appeared in their culture, Wernig et al. (2008) simply mentioned that their appearances were "rare".

Miura et al. (2009) evaluated the neural-differentiation capacity of 36 mouse iPSC lines derived in 11 different ways. In 36 iPSC lines, 12 were induced from MEFs, 11 were induced from TTFs, 10 were induced from hepatocytes, and 3 were induced from gastric epithelial cells. For *in vitro* neural differentiation, they used the secondary neurospheres method (Okada et al. 2008). In this method, embryoid bodies were prepared in the presence of all-trans retinoic acid (RA). To culture primary neurospheres, day 6 embryoid bodies were dissociated and cultured in suspension media in hormon mix serum free medium supplemented with B27 and bFGF for 7–8 days. To culture secondary neurospheres, primary neurospheres were dissociated with TrypLESelect (Invitrogen) and cultured in the same culture medium. For neural differentiation, secondary neurospheres were plated onto poly-L-ornitine/fibronectin-coated coverslips and cultured without bFGF for 5–6 days. The appraisal of oligodendrocyte was based on the cellular morphology and the expression of 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase). CNPase is one of the most abundant myelin proteins and is

expressed in differentiated oligodendrocytes in CNS (Fulton et al. 2010).

Twenty-six from 36 iPSC lines differentiated into oligodendrocytes *in vitro*. As CNPase is a cytoplasmic protein, anti-CNPase antibody staining could not show membrane structure of oligodendrocyte. So, the *in vitro* morphology of oligodendrocyte that they showed in this paper was multi-proccessed web-like structure. Miura et al. (2009) did not mention the efficiency of *in vitro* oligodendrogenesis of each iPSC line. In this study, they also evaluated the teratoma-forming propensity of their iPSC lines. After labeling with lentivirus mediated Venus gene transduction, iPSC-derived secondary neurospheres were transplanted into the right striatum of 6-weeks-old NOD/SCID mice which were then examined for tumor formation up to 45 weeks. Although 22 iPSC lines formed tumor in mouse brains, 14 iPSC lines did not form tumor even 45 weeks after the transplantations. They called these non-tumor forming iPSC lines as safe-iPSC lines.

Subsequently, Tsuji et al. (2010) evaluated the therapeutic potential of these safe-iPSC lines for spinal cord injury. One of the safe iPSC lines, 38C2, which was established from MEF by the introduction of four factors (Oct4, Sox2, c-Myc, and Klf4), was used in the spinal cord injury model experiment. *In vitro* neural differentiation of the 38C2 iPSC-derived secondary neurosphere resulted 3.7% of CNPase$^+$ oligodendrocytes. The 38C2 iPSC-derived secondary neurospheres that had been prelabeled by lentivirus to express both luciferase and RFP were transplanted into mice contused spinal cords 9 days after injury. Thirty-five days after the transplantation 14.4% of grafted cells were differentiated into oligodendrocytes. Further histological analysis showed that at 42 days after injury (33 days after the transplantation), 38C2 iPSC-derived cells myelinated host neuronal fibers. Same experiment was carried out on myelin basic protein (MBP)-null *shiverer* mice, a severely dysmyelinating mutant mice that lacks the major dense line of CNS myelin (Fulton et al. 2010). Myelinating potential of the grafted 38C2 iPSC-derived secondary neurosphere cells was confirmed, exhibiting MBP deposits and major dense line, using electron microscopy.

Onorati et al. (2010) reported the *in vitro* direct differentiation of oligodendrocytes from mouse iPSC-derived NSCs. They induced iPSCs from newborn mouse skin fibroblasts with the introduction of four factors (Oct4, Sox2, c-Myc, and Klf4). For NSC differentiation, iPSCs were dissociated and plated onto gelatin-coated tissue culture dish in N2B27 serum- free medium. Cells were maintained for 12 days, and then plated on non-coated tissue culture flask in the serum-free medium supplemented with N2 supplement, EGF and bFGF. After 2–3 passages, monolayers of NSCs were generated. For *in vitro* oligodendrocyte differentiation from NSCs, they used the protocol that was established by Glaster et al. (2007). This method induced 20% of O4$^+$ oligodendrocyte from ESC-derived NSCs. iPSC-derived NSCs were dissociated and plated on a laminin-coated culture dish in serum-free medium with N2 supplement, forskolin, bFGF, and PDGF for 4 days to support the glial precursor stage. Then, oligodendrocyte differentiation was induced by a 4-days growth factor withdrawal in the presence of T3 and ascorbic acid. They reported that 25% of iPSC-derived cells expressed both oligodendrocyte differentiation markers, NG2 and O4$^+$. Unfortunately the morphology of their NG2$^+$/O4$^+$ double positive cell was branched but not with the web-like processes and no membrane like structure. It was not typical form of oligodendrocyte *in vitro*.

Tokumoto et al. (2010) compared the efficiency of terminal differentiation of oligodendrocytes from mouse iPSCs and from mouse ESCs. We used a MEF-derived germline-competent iPSC line that was established by Yamanaka et al. (2005) with the introduction of four factors (Oct4, Sox2, c-Myc, and Klf4; Okita et al. 2007). The *in vitro* oligodendrocyte differentiation protocol which we used had already been established by Brüstle et al. (1999). In the original report, this protocol differentiated 38.3% of ESC-derived cells into O4$^+$ oligodendrocyte *in vitro*. To form embryonic bodies, trypsin-dissociated iPSCs or ESCs were suspended in 15% fetal calf serum containing ES medium and seeded on non-coated bacterial culture dishes. Four days later, the floating embryonic bodies were transferred to tissue culture dishes, and the next day the medium

was changed to ITS/fibronectin serum-free medium. During 7 days culture, cell monolayers grew from attached embryonic bodies. Subsequently the attached cells were dissociated with trypsin and seeded on poly-L-ornithine/fibronectin coated tissue culture dish in the serum-free medium supplemented with N2 and bFGF for 4 days. Then, EGF was added and the culture carried out another 4 days. Finally, the medium was changed to the serum-free medium supplemented with N2, bFGF, and PDGF and cells were cultured for 4 days (glial precursor stage). For the induction of the oligodendrocyte terminal differentiation from iPSC-derived or ESC-derived glial precursor stage cells, bFGF and PDGF were withdrawn and thyroid hormone (3, 3′, 5-triiode-L-thyronine; T3) was added to the medium. After 7 days, the oligodendrocyte differentiation efficiencies were evaluated by O4 antibody staining. The results demonstrated that only 2.3% of iPSC-derived cells differentiated into $O4^+$ oligodendrocytes compared with 24.0% of ESC-derived cells.

We also tried *in vitro* oligodendrocyte differentiation from human iPSC line (Ogawa et al. 2011a). The human iPSC line which we used was established from adult human fibroblast by Yamanaka et al. (2005) with transduction of three genes (Oct4, Sox2, and Klf4; Takahashi et al. 2007). The *in vitro* human oligodendrocyte differentiation experiment was carried out by following the procedure that was established by Nistor et al. (2005). In the original report, 85% of human ESC-derived cells were differentiated into $O4^+$ oligodendrocytes *in vitro*. In this procedure EGF was used as the major mitogen of oligodendrocyte lineage cells, but PDGF was depleted. The reason may be that differentiated $O4^+$ oligodendrocyte induced by this procedure showed characteristic starfish-like morphology *in vitro*. To form embryoid bodies, collagenase-dissociated human iPSCs were suspended in 50% human ES medium/50% glial restriction medium with bFGF and cultured in non-coated bacterial dishes overnight. On day 2, EGF was added to the medium. On day 3, RA was added to the medium. On day 4, the medium was exchanged to glial restriction medium containing EGF and RA. Cultures continued for another 7 days. On day 11, the medium was exchanged to glial restriction medium containing EGF, and cultured for 17 days. On day 28, embryonic bodies were transferred into a growth-factor-reduced matrigel coated culture dishes and allowed to spread on this substrate for 2 weeks. For the terminal differentiation into oligodendrocyte, EGF was depleted from the medium. After 7 days, cells were subjected to O4 antibody staining. Less than 0.01% of human iPSC-derived cells showed $O4^+$ starfish-like morphology.

Czepiel et al. (2011) induced mouse iPSCs from MEFs (E14) through ectopic expressions of Oct4, Sox2, c-Myc, and Klf4 via retrovirus vectors and tried oligodendrocyte differentiation from iPSC both *in vitro* and *in vivo*. To induce the differentiation into oligodendrocyte lineage, iPSCs were trypsinized and cultured as embryonic bodies in 15% fetal calf serum containing medium for 8 days. Then, embryonic bodies were dissociated and cultured in serum-free medium supplemented with N2 supplement, bFGF and EGF for 7–8 days. These cells were referred to as iPSC-derived NSCs. For oligodendrocyte-lineage differentiation, iPSC-derived NSCs were plated on poly-D-lysine/laminin coated coverslips and cultured in serum-free medium supplemented with N2 supplement, PDGF, bFGF, and EGF for 2 days, then the medium exchanged to serum-free medium supplemented with N2 supplement and PDGF for another 2 days. The cells in this stage were referred to as iPSC-derived OPC. To enhance the terminal differentiation of oligodendrocytes, T3 and NT-3 were added for 6 days. Using this protocol, 18% of iPSC-derived cells differentiated into MBP^+ mature oligodendrocytes *in vitro*. Czepiel et al. (2011) examined whether their iPSC-derived OPCs were able to form proper myelin around nude axon *in vitro*. They cocultured the iPSC-derived OPCs with a pure, 2 weeks-old culture rat dorsal root ganglion (DRG) neurons for 2 weeks and found extensive myelin formation by the expression of MBP. For *in vivo* myelination assay, they used cuprizone treated demyelinated mouse model. Mice receiving intracerebral cell suspensions containing iPSC-derived OPCs (mini-Ruby labeled) did not form teratoma even after a period of longer than 24 weeks. Before the implantation,

iPSC-derived OPCs were sorted with anti-PDGF receptor antibody. Both unsorted cells and sorted cells were used in the experiments. Four weeks after implantation, the surviving unsorted implants were in the majority of undifferentiated nestin-positive NSCs, but all the surviving sorted grafts developed into mature MBP$^+$ oligodendrocytes *in vivo*.

Jang et al. (2011) induced oligodendrocytes from human iPSCs *in vitro*. They induced iPSCs from fibroblasts of X-linked adrenoleukodystrophy patients through ectopic expressions of Oct4, Sox2, c-Myc, and Klf4 via retrovirus vectors. Differentiation of oligodendrocytes was performed as described by Kang et al. (2007). iPSC colonies were detached by collagenase type VI and embryoid bodies were formed by incubating ES cell culture medium without bFGF supplemented with dorsomorphin (BMP2 inhibitor) and SB431542 (TGF-β inhibitor). After 4 days, the embryoid bodies were plated on matrigel-coated culture dishes in serum-free medium including N2 supplement and bFGF. After 5 days, the neural rosette structures were mechanically isolated and suspension-cultured in bFGF to make spherical neural masses. These spherical neural masses (containing NSCs) were replated onto matrigel-coated culture dishes and differentiated into OPCs in serum free medium supplemented with N2 supplement, bFGF and PDGF for 2 weeks. During this period, 73.1–79.2% of human iPSC-derived cells generated A2B5$^+$ cells (described in the next section) and 68.0–90.3% of cells became PDGF receptor expressing cells. Two weeks after plating, terminal differentiation of oligodendrocytes was induced by withdrawing the growth factors and adding T3 for 3–4 weeks. MBP$^+$ multi-processed oligodendrocytes with membrane-like structures were observed but the authors did not show the efficiencies of oligodendrocyte terminal differentiation *in vitro*.

Mouse Monoclonal Antibody A2B5

A2B5 mouse monoclonal antibody (IgM subtype) producing A2B5 hybridoma cell was established from mouse spleen cells that had been immunized by intrapertoneal injections of 8-day-chicken embryo retina cells (Eisenbarth et al. 1979). It recognizes the plasma membrane of the cell bodies of chicken retina neurons. The antigens of A2B5 antibody are c-series gangliosides, GT3, GQ1c, GP1c, and GH1c (Saito et al. 2001). A2B5 antibody also recognizes glial-restricted precursors in rat embryos (Rao and Mayer-Proschel 1997) and cancer stem cells in adult human glioma (Ogden et al. 2008). The recognition of OPC by A2B5 antibody in rat optic nerve was first reported by Abney et al. (1983). The principal A2B5-reactive c-series gangliosides in cultured OPCs were prepared from rat cerebral are GT3 and its O-acetylated derivative, O-acethyl GT3 (Farrer and Quarles 1999). As both of them are down-regulated during terminal differentiation from OPCs into oligodendrocytes, differentiated oligodendrocytes become A2B5$^-$ cells. A2B5 antibody can be used for cell purification procedures, including for the immunopanning purification of OPCs from rat optic nerve (Barres et al. 1992), the immunopanning purification of glial-restricted precursors from neural tube of mouse embryo, and mouse ESC-derived cells (Mujtaba et al. 1999), and magnetic beads cell-sorting of OPCs from rat NSC-derived cells (Cizkova et al. 2009). Although A2B5 antibody can recognize OPCs in rat, mouse, rabbit, and humans, it is not used for immunohistochemical applications. Because, the antigens of A2B5 antibody are destroyed by the tissue fixation procedures.

A2B5-Immunopanning Purification of Oligodendrocyte Precursor Cells (OPCs) from iPSC-Derived Cells

In our previous study (Tokumoto et al. 2010), although the *in vitro* differentiation efficiency of O4$^+$ oligodendrocytes from mouse iPSC-derived cells was lower than that from mouse ESC-derived cells, the differentiation efficiencies of A2B5$^+$ cells (OPC candidates) in the glial precursor-stage were almost the same (14.1% of iPSC-derived cells and 12.6% of ESC-derived cells). We speculated to the possibility that some paracrinical effects in the iPSC-derived cell culture

might have prevented the terminal differentiation from iPSC-derived A2B5$^+$ OPC into O4$^+$ oligodendrocytes. So, we tried to purify A2B5$^+$ cells from the glial precursor-stage of iPSC-derived cell culture by A2B5-immunopanning procedures (Barres et al. 1992). The iPSC-derived glial precursor-stage cells were dissociated with trypsin and purified on the A2B5 antibody-coated panning plate. The recovery rate of purified A2B5$^+$ cells was 1.9% and the purity was 99% (Ogawa et al. 2011b). Because OPCs can be cultured in clonal density (Barres et al. 1992), we plated iPSC-derived A2B5$^+$ cells onto poly-D-lysine coated culture flasks in clonal density with serum-free medium containing N2 supplement, bFGF and PDGF. We cultured these cells for another 7 days, during which time the number of cells increased more than tenfold. Subsequent staining with the A2B5 antibody revealed that 88.1% of cells were still A2B5$^+$. To induce the terminal differentiation into oligodendrocytes, the withdrawal of the growth factors and the addition of T3 were carried out for an additional 7 days. Staining with O4 antibody revealed that 43.5% of A2B5-immunopanned cells were O4$^+$ oligodendrocytes. Keeping in the terminal differentiation culture for another 2 weeks, 62.3% of O4$^+$ cells also expressed mature oligodendrocyte marker protein MBP, with multi-processed membrane-like structures (Ogawa et al. 2011b). We succeeded in improving the *in vitro* differentiation efficiency into oligodendrocytes ~20-fold by purifying A2B5$^+$ cells from iPSC-derived cells prior to inducing the oligodendrocyte terminal differentiation process.

Although the A2B5-immunopanning purification procedure works satisfactorily, there are several weak aspects to be improved. First, ~50% of OPCs in mouse CNS are A2B5$^-$ (Fanarraga et al. 1995), and not only glial-restricted precursor cells but also some portions of mouse mature astrocytes are also recognized by A2B5 antibody (Magoski et al. 1992). It means that we may loose 50% of OPCs (A2B5$^-$) during the purification and cannot avoid possibilities of the contamination of astrocytes from mouse iPSC-derived cells. Second, without OPCs in CNS, there are many organs and cells that are recognized by A2B5 antibody (Saito et al. 2001). The A2B5 antigens, c-series gangliosides have also been detected in tissues and cells of extra-neural origin, including swine kidney, human lung, rat liver, cat erythrocytes, and pancreatic islet cells (mouse, human, chicken). As iPSCs are pluripotent, even in the oligodendrocyte-directed differentiation procedures, there are possibilities of the appearances of non-neural A2B5$^+$ cells *in vitro*. To get more highly purified OPCs from mouse iPSC-derived cells may require immunoselection with other OPC-specific antibodies in combination with A2B5 antibody.

PDGF-receptor α (PDGFRα) is another cell differentiation marker molecule on the cell surface of OPCs in the CNS. Using anti-PDGFRα antibody, Watkins et al. (2008) had succeeded to purify OPCs from the cortex of newborn mouse by immunopanning procedures. The purity of the brain-derived mouse OPCs was higher than 95%. So, we decided to use anti-PDGFRα antibody in our immunopanning procedures in combination with A2B5 antibody. First, the mouse iPSC-derived glial precursor-stage cells were dissociated with trypsin and purified on the anti-PDGFRα antibody-coated panning plate, then purified again on the A2B5 antibody-coated panning plate. However the O4$^+$ oligodendrocyte differentiation efficiency of PDGFRα$^+$/A2B5$^+$ double-positive iPSC-derived cells was not different from than that of A2B5$^+$ single positive cells (Tokumoto, unpublished result). We also tried differentiation from the PDGFRα$^+$/A2B5$^-$ single positive cells into oligodendrocytes *in vitro*. These cells were cultured in serum-free medium supplemented with N2 supplement and T3. The results demonstrated that there was no O4$^+$ oligodendrocytes differentiation from PDGFRα$^+$/A2B5$^-$ single positive iPSC-derived cells (Tokumoto, unpublished result). In these PDGFRα$^+$/A2B5$^-$ single positive cells, some cells could proliferate and increase their number under the oligodendrocyte terminal differentiation culture condition (in the absence of growth factors). Because the reduction of the cell density of the inoculation repressed the proliferation of those cells and caused cell death, some autocrine factors might support their survivals and proliferations (Tokumoto, unpublished result).

Although the number of the known cell surface molecule which can be used for purification of living OPC is not extensive, there are possibilities of the appearance of OPC specific new cell surface markers. The known OPC markers are used for identification of dissected OPCs from the CNS tissues. In the dissection procedures, because the intensive proteinase-treatments cannot be avoided, most of the plausible protein antigens on the OPC cell surface are destroyed. It makes difficult to find a suitable cell surface specific antigen on the cell surface of OPC. However, for the preparation of OPCs from iPSC-derived cell cultures such hard proteinase treatments are not needed. OPCs in culture are not attached tightly onto the surface of the culture dish. To harvest OPCs from a culture dish, very mild trypsin treatments are always enough, and in some cases, EDTA treatment without proteinases or the simple mechanical shaking can be used for this purpose (Louis et al. 1992). For such gently prepared OPCs, intact protein cell surface markers can be used for purification of OPCs from iPSC-derived cells *in vitro*. More than 300 clusters of differentiation markers (CD markers) have been established. Systematic screening of CD markers on OPCs may give a new candidate of antibody which can be used for the purification of OPCs from iPSC-derived cells.

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Epigenetic Reprogramming Without Genetic Modification: Use of Sendai Virus Vectors for Generating Safe Induced Pluripotent Stem Cells

6

Noemi Fusaki

Contents

Abstract

Since the first report of induced pluripotent stem cells (iPSCs), considerable effort has focused on developing more efficient methods for generating iPSCs without foreign gene insertions. Here, we show that the Sendai virus (SeV) vector, an RNA virus vector that carries no risk of integrating into the host genome, is a practical solution for the efficient generation of safer iPSCs. We improved the Sendai virus system for generating iPSCs by introducing temperature-sensitive mutations so that the vectors could be easily removed at non-permissive temperatures. Using these vectors enabled us to efficiently produce viral/factor-free iPSCs from both human fibroblasts and blood cells. The production of iPSCs from blood cells is minimally invasive and has the added advantage of taking less time than conventional methods. The resulting iPSCs expressed human embryonic stem cell markers, exhibited pluripotency, and contained no exogenous genes. We successfully applied this method to the generation of patient-specific iPSCs and also to further an understanding of non-integrating gene therapy. It is envisaged that the efficient generation of transgene-free iPSCs using SeV vectors will accelerate understanding disease mechanisms, discovery of new drugs, and the realization of regenerative medicine in the near future.

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Introduction

Many methods have been developed to express foreign genes in order to reprogram or deprogram the phenotype of cells. A popular method is the use of retroviral and lentiviral based gene delivery vectors. A disadvantage of this approach is that they integrate into the host genome and thus have the associated risk of tumorigenesis (Okita et al. 2007).

Safer and more efficient reprogramming methods have been explored since the first report of the generation of human induced pluripotent stem cells (iPSCs) (Takahashi et al. 2007; Yu et al. 2007). Toward this end, several techniques have been used for obtaining integration and/or transgene-free iPSCs, including the use of plasmids (Okita et al. 2008; Yu et al. 2009), the Cre/loxP system (Soldner et al. 2009; Sommer et al. 2009), adenoviruses (Stadtfeld et al. 2008), piggyBac (Woltjen et al. 2009; Kaji et al. 2009), minicircle vector (Jia et al. 2010), and proteins (Zhou et al. 2009; Kim et al. 2009). However, these methods suffer from low efficiency, require repetitive induction, and/or produce insufficient excision of integrated vectors. Synthetic modified mRNA may solve the problem, but the reagents must be added every day (Warren et al. 2010). Thus, more efficient and simple methods are needed to generate human iPSCs without foreign gene insertions or remaining factors for both clinical applications and basic studies.

An alternative technology involves the use of Sendai virus (SeV) vectors. SeV, a member of the *Paramyxovirdae* family, is an enveloped virus with a single-stranded, negative-sense, non-segmented RNA genome of ~15 kb (Lamb and Kolakofsky 2001). Importantly, recombinant SeV vectors replicate only in the cytoplasm of infected cells and do not go through a DNA phase or integrate into the host genome (Li et al. 2000). SeV vectors have proven to be efficient for the introduction of foreign genes in a wide spectrum of host cell species and tissues, and studies have focused on their application to gene therapy for cystic fibrosis, critical limb ischemia, vaccines for AIDS, and other areas (Nagai et al. 2011).

This review will show that efficient generation of iPSCs using non-integrating SeV vectors, modification of SeV vectors that can be easily removed from the cells, and the potential application of transgene-free iPSCs for gene therapy and as models to study disease.

Non-integrating Sendai Virus Vectors

Sendai virus (SeV) is a respiratory virus of mouse and rat and classified as mouse parainfluenza virus type I belonging to the *Paramyxoviridae* family. SeV was first isolated in Tohoku University located at Sendai, Japan in 1953 and thus also called Hemagglutininating Virus of Japan (HVJ). It is an enveloped virus of 150–250 nm in diameter whose genome is a single chain of RNA (15,384 bases) in the minus sense. The nucleotide sequencing of the entire paramyxovirus genome was first achieved for SeV in the mid 1980s. Six genes coding for viral proteins are situated sequencially on the genome in the following order starting from the 3′ end; Nucleocapsid protein (NP), Phosphoprotein (P), Matrix protein (M), Fusion protein (F), Hemagglutinin-Neuraminidase (HN), and Large protein (L). NP forms the core nucleocapsid complex with the genome RNA; P protein is the small subunit of RNA polymerase; M protein supports the envelope structure from inside; F protein fuses the viral envelope with cell membrane when the virus invades the cell; HN protein recognizes the cell surface receptor, sialic acid; L protein is the large subunit of RNA polymerase. Since SeV infects cells by attaching itself to its receptor, sialic acid containing glycoproteins, on the cell surface, it can infect a wide range of cell types of many animal species. Activation of F protein by a protease is needed for the infection process. After infection, the virus goes through genome replication and protein synthesis, and then daughter virus particles are assembled and released (Lamb and Kolakofsky 2001).

SeV was first applied for the cell fusion that was especially contributed to the technology to produce monoclonal antibodies. The application of SeV fusion technology has been extended to

tools for gene delivery such as HVJ-liposome. Furthermore, SeV virus has been developed as a vector. This is the superior feature that Sendai virus (SeV) vectors replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells and do not go through a DNA phase nor integrate into the host genome (Nagai et al. 2011).

Since they can infect to wide spectrum of host cell species and continuously replicated in cytoplasm after infection, the expression level is extraordinary high in most mammalian and avian cells. To increase safety, non-transmissible and deficient type of SeV vector was developed (Li et al. 2000). The F-protein deficient vector does not reproduce the infectious viral particles after infection.

SeV itself is not pathogenic for primate including humans and has these characters described above, therefore SeV vectors have been intended to apply to clinical studies of gene therapy for cystic fibrosis, critical limb ischemia or vaccines for AIDS and so on (Nagai et al. 2011).

Sendai Virus Vectors for Generating Human Induced Pluripotent Stem Cells (iPSCs)

To apply the SeV vectors to generate iPSCs, non-transmissible F-deficient (ΔF) and temperature-sensitive (TS) SeV vectors were first used to generate iPSCs to decrease cytotoxicity. The efficiency of transduction measured by using green fluorescent protein (GFP)-encoding control SeV vector showed that almost all of the infected human dermal fibroblast cells expressed GFP at the low multiplicities of infection (MOI, number of viral particles per cell) of three (Fig. 6.1a).

Then, the cDNAs encoding the reprogramming factors, OCT3/4, SOX2, KLF4, and c-MYC (Takahashi et al. 2007), were cloned into ΔF/TS-SeV vectors at different sites, designated 18+, HMN, PM, MHN and L, to control the expression level (polar effect: Tokusumi et al. 2002) (Fig. 6.1b). An exogenous gene expressed most strongly when inserted upstream of the NP gene and most weakly when inserted downstream of the L gene (Fig. 6.1b). An anti-SeV blot confirmed an equivalent level of infection for each SeV vector (lower panels). Since AT-rich regions are known to decrease the expression of target genes in SeV vectors (Stuttering sequence: Ferrari et al. 2007), silent mutations of codons to reduce the AT content without altering the amino acid coding sequence were prepared for wild-type c-MYC (designated rev-c-MYC).

Using these vectors, optimum conditions were determined for generation of iPSCs. Induction of human iPSCs was carried out as described previously (Takahashi et al. 2007). Briefly, 1×10^6 neonatal human foreskin-derived fibroblasts (BJ), or adult human facial dermal fibroblasts (HDF) were plated onto 100 mm dishes and infected with OCT3/4, SOX2, KLF4 and/or c-MYC by a series of SeV vectors at an MOI of 3. One week after infection, cells were collected and re-plated on mitomycin C- treated MEF feeder cells. The next day, the growth medium was changed to primate ES medium supplemented with 10 ng/ml bFGF. The conditions used to generate SeV-iPSC are summarized in Fig. 6.1c. Lane numbers correlate with the conditions listed in the column under the Figure. Each dot represents one experiment. Reprogramming efficiency was calculated as the number of alkaline phosphatase (ALP)-positive, ES-like colonies formed per number of infected cells seeded (Fig. 6.1c). Typical colonies are shown in the lower panel. Reduction in numbers of seeded cells on MEF improved the induction efficiency (Fig. 6.1c, no. 2, 3). AT-revised c-MYC also enhanced the reprogramming efficiency depending on the expression level of c-MYC (rev-c-MYC: no. 4–6). All genes inserted at HNL showed the maximum reprogramming efficiency of 1–3% (Fig. 6.1c, no. 7). In contrast, the efficiency of reprogramming decreased approximately 100 fold when c-MYC was absent (Fig. 6.1c, no. 1). This result is consistent with the efficiency of reprogramming achieved by retroviral induction without c-MYC (Nakagawa et al. 2008). No iPSC colonies were observed when KLF4, but not OCT3/4 or SOX2, was moved to sites other than 18+ or HNL (Fig. 6.1c, no. 8, 9), suggesting that the expression level of KLF4 is critical for efficient induction of iPSC reprogramming.

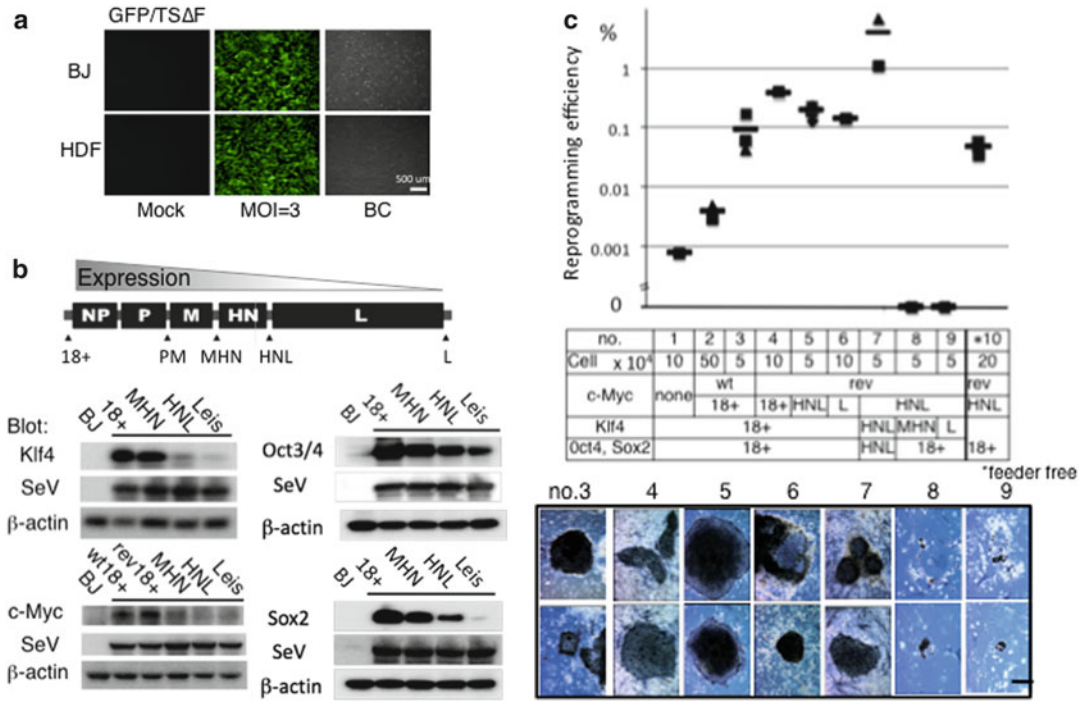

Fig. 6.1 (**a**) Efficient induction of GFP cDNA by TSDF/SeV in BJ and HDF at an MOI of 3. *BC*: bright contrast. (**b**) Schematic presentation of SeV vector genomes. Reprogramming genes were inserted at 18þ, PM, HN, HNL and Leis (*L*), respectively. The expression levels of inserted genes decreased depending on the inserted site (polar effect) as shown by Western blotting on day 3 after infection. Anti-SeV blot was performed to confirm equal infection efficiency of the vectors. (**c**) The reprogramming efficiency with SeV vectors. iPS colonies were determined by ALP positive and ES-like morphology. Lane numbers correlate with the conditions listed in the column under the figure. Each dot represents one experiment. The bars represent the average efficiency for each condition. Lower: Typical ALP-positive colonies (scale bar: 100 μm)

We also successfully induced iPSCs using SeV vectors without feeder cells (Fig. 6.1c, no. 10). These cells may keep their undifferentiated state depending on exogenous gene expression. Taken together, these results show that the efficiency of iPSC induction by SeV vectors with c-MYC significantly surpassed that achieved by retroviral transduction (Fusaki et al. 2009).

Modification of Sendai Virus Vectors for Easy Removal

SeV vectors used for cell reprogramming can be diluted during the robust division of cells that occurs when iPSCs are being established. Alternatively, SeV vector–positive cells can be removed using an anti–SeV-HN antibody (Fusaki et al. 2009). These transgene-free iPSCs showed the expected expression of hES markers and pluripotency. However, a more efficient shutdown of viral replication is needed to lighten the effort and save the cost of long periods of cell culture. The use of temperature-sensitive (TS) SeV vectors is considered the best approach to address these issues. We generated SeV vectors that were more temperature sensitive than conventional ones by introducing point mutations into polymerase-related genes (Fig. 6.2a). We then used these modified vectors to generate viral/factor-free human iPSCs by using two different strategies that included (1) replacing the c-MYC–carrying vector only or (2) replacing all reprogramming vectors with the TS vectors (Fig. 6.2d) (Ban et al. 2011). Details of these strategies are described later.

The SeV RNA polymerase comprises the phosphoprotein (P) and the large protein (L), and formation of the P-L complex is required for RNA synthesis (Lamb and Kolakofsky 2001). Mutations in P or L confer temperature sensitivity to the virus (Bowman et al. 1999; Feller et al. 2000). Although a conventional non-transmissible F protein-deficient (ΔF)/TS vector was demonstrated to have low cytotoxicity at temperatures above 37°C (Inoue et al. 2003), it still expressed the GFP gene at 39 °C, albeit at slightly lower levels (Fig. 6.2b: conv. TS). Thus, we generated a greater number of TS vectors using a combination of known point mutations in the P and/or L genes and screened for GFP gene expression in infected cells at various temperatures. The TS vectors obtained were (1) P2 vectors (D433A, R434A, and K437A), which contain a charge-to-alanine mutation in the L-binding domain of the P protein (Bowman et al. 1999); (2) TS7 vectors (Y942H, L1361C, and L1558I); (3) TS13 vectors (P2 and L1558I); and (4) TS15 vectors (P2, L1361C, and L1558I), as indicated in Fig. 6.2b. In this study, we chose to evaluate candidate vectors with combined mutations, because a single mutation appeared to be insufficient to confer temperature sensitivity (i.e., the Y942H, L1558I, or L1361C vector). Furthermore, by using TS vector candidates with combined mutations, the occurrence of WT revertants, which are occasionally observed in RNA viruses (Lamb and Kolakofsky 2001), was less likely. In contrast to the conventional TS vector, TS7 and TS13 SeV vectors expressed GFP at 32 and 35 °C, and weakly at 37 °C, but not at non-permissive temperatures of 38 or 39 °C. The TS15 vector exhibited the greatest temperature sensitivity as GFP expression was barely detected at 37 °C (Fig. 6.2b). We confirmed that there was no GFP expression after transfection of cells with the TS7 and TS13 vectors after temperature-shift treatment to 39 °C, even when the infected cells were then cultured at 37 °C for >1 month with several passages (Fig. 6.2c). None of the vectors was cytotoxic, and all infected cells were attached and live, with or without temperature-shift treatment. Thus, we used these TS vectors to generate human iPSCs.

We used two different strategies to generate human iPSCs using the TS vectors including (1) replacing the c-MYC–carrying vector only or (2) replacing all four gene-carrying SeV vector mixtures with the TS vectors (Fig. 6.2d). Our previous study showed that the exogenous c-MYC inserted between the HN and L positions in the SeV vector (HNL-MYC) persisted in the infected cells longer than any other vectors carrying OCT3/4, SOX2, or KLF4 at the 18+ position. When c-MYC was inserted at the 18+ position, selective retention of the c-MYC–carrying vector was not observed. This was apparently due to the prolonged replication of the HNL-MYC vector (Fusaki et al. 2009). Because GFP expression with the new TS vectors was relatively weak compared with that obtained using conventional vectors (Fig. 6.2b), we initially inserted HNL-MYC into the TS vectors (strategy 1) so that the initial levels of expression could be restored by the polymerases supplied in trans from the other vectors (OCT3/4, KLF4, and SOX2). In addition, it was hoped that by using this strategy, the viral vectors might easily disappear when the remaining vector was HNL-MYC in the TS vector alone (Fig. 6.2d). Using these vectors at a MOI of 3, we obtained colonies from human fibroblast cells that were alkaline phosphatase (ALP)-positive and exhibited human embryonic stem (ES) cell-like morphology at about 28 days after induction. We then monitored the amount of SeV genome present during reprogramming and cell expansion using quantitative RT-PCR (qRT-PCR). Surprisingly, replacement of the HNL-MYC vector into only one of the four reprogramming factor mixtures using the TS vectors resulted in a marked decrease in all viral genomes after the appearance of the iPSCs (Ban et al. 2011). As expected, expression of c-MYC on the TS vectors was correlated with the viral genome. Then individual colonies were isolated, and the remaining SeV genome in each colony was evaluated. During cell expansion, the vectors were diluted, and most colonies were only partially positive for SeV (Fig. 6.2e, middle panel). At passage 4, 80% of the colonies were negative for the viral genome for the TS13-HNL-MYC and TS15-HNL-MYC vectors, and by passage 10, all colonies were negative for the vector (Fig. 6.2e,

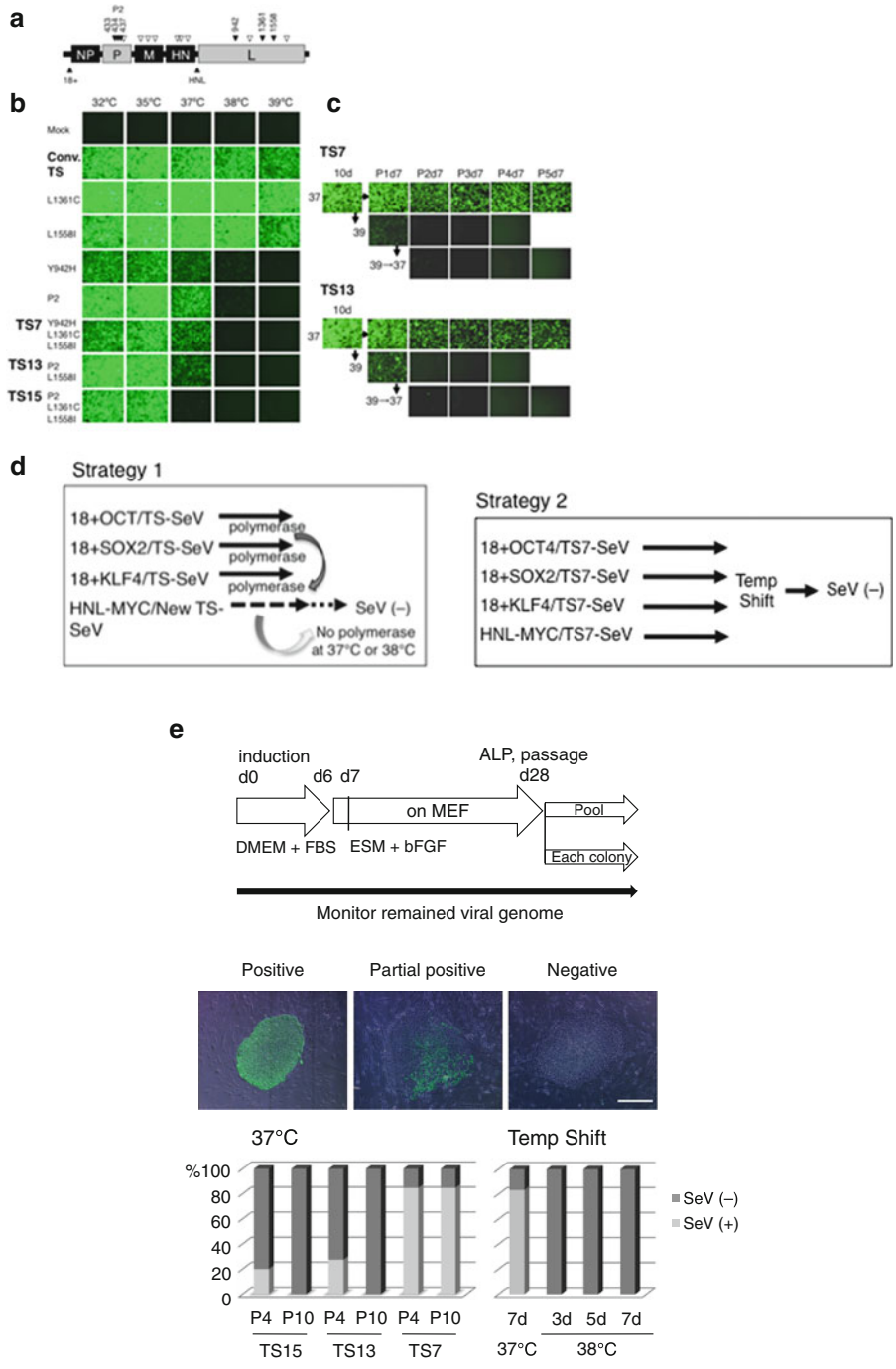

Fig. 6.2 (**a**) Point mutations were introduced into the polymerase-related genes P (P2: 433, 434, and 437) and/or L (942, 1361, and 1558), as indicated in the schematic structure of the ΔF/SeV vector. Open angles indicate conventional mutations in the previous TS vector; closed angles, newly introduced mutations. (**b**) Confluent LLC-MK2 cells were transduced with each SeV vector carrying GFP at an MOI of 5 and cultured at the indicated temperatures (32, 35, 37, 38, and 39°C). Green fluorescence was compared at 3 days after infection. (**c**) To confirm the irreversible inactivation of gene expression by temperature-shift treatment, infected cells were cultured at 37 °C for 10 days and then split into two groups, one group was kept cultured at 37 °C and the other group

bottom panel). Because the number of SeV-negative colonies had not increased by passage 10 using the TS7 vector, although the number of partial negative colonies had increased, we subjected the iPSCs to a temperature shift of 38 °C (Fig. 6.2e, bottom right panel). Incubation of cells at 38 °C for 3–5 days was sufficient to obtain SeV-negative iPSCs with no changes in the expression of human ES cell (hESC) marker genes (Ban et al. 2011). Western blot and qRT-PCR analyses of iPSCs obtained by this strategy revealed no detectable viral genome or protein expression in the established iPSCs at late passage numbers. More importantly, copy numbers of OCT3/4, SOX2, KLF4, and c-MYC genes in SeV-generated iPSCs were the same as those in parental cells. In contrast, copy numbers of these genes in retroviral-generated iPSCs were verified and reflected the level of vector integration (Ban et al. 2011).

In the second strategy, we obtained viral-free iPSCs from human fibroblasts using TS13 or TS7 vector mixtures consisting of four reprogramming factors (i.e., 4F/TS13 and 4F/TS7, respectively) at an MOI of 30 at 37°C with (TS7) and without (TS13) temperature-shift treatment (Fig. 6.2d, strategy 2). In this strategy, we used the higher MOI of 30 because we could not obtain iPSCs at a lower MOI, most likely because weak expression of TS vectors at 37°C means a lack of expression of supplemental polymerases from the mixed conventional vectors. We used this approach to successfully obtain iPSCs that expressed hES markers and pluripotency by using the 4F/TS13 and TS7 vectors. The TS7 vectors were less temperature-sensitive than the TS13 vectors thus the temperature-shift treatment described above was effective in removing the vectors (Ban et al. 2011).

iPSCs from Cord Blood CD34+ Cells

This method was also used to generate iPSCs from CD34+ cord blood (CB) cells because the SeV vector provides a highly efficient gene transfer into human CB-derived hematopoietic stem cells (Jin et al. 2003). CD34+ CB cells are the earliest somatic stem cells and are expected to lack postnatal genomic aberrations caused by irritants from the environment or UV irradiation. These cells correspond to hematopoietic stem cells and progenitors with less epigenetic modification related to hematopoietic differentiation. These unique features suggest that CD34+ CB cells might be an ideal cell source for generating gold standard iPSCs. However, the risk of foreign gene integration (Giorgetti et al. 2009; Takenaka et al. 2010) needs to be overcome for future clinical applications. We successfully obtained viral/factor-free CB-iPSCs by the TS SeV vectors, and examined the advantages of using the TS SeV vector (Ban et al. 2011).

For the TS7 vectors an MOI of only 2 was sufficient to generate iPSCs from human CB stem cells, possibly because of the high efficiency of gene transfer into this cell type (Jin et al. 2003). We infected freshly isolated mononuclear cells with the GFP construct at an MOI of 2 and found that GFP expression was limited to the CD34+ fraction (43% of the CD34+ cells were GFP + cells), whereas no GFP + cells were found in the CD34– fraction at 2 days after SeV infection. The rate of SeV infection determined by GFP expression in CD34+ cells was increased up to 100% if we used SeV vectors at an MOI of 20. This fraction corresponds to hematopoietic stem cells or progenitors. We used freshly isolated purified CD34+ CB cells

Fig. 6.2 (continued) was cultured at 39°C for 28 days, with cells passaged every 7 days. Similarly, cells infected with a TS vector treated at a non-permissive temperature of 39°C for 7 days were also cultured for a further 28 days at 37°C, with cells passaged every 7 days, to evaluate GFP expression. (**d**) Strategy used to obtain vector/factor-free iPSCs with an SeV vector mixture. (**e**) (*Upper*) Procedure for reprogramming using SeV vectors. (*Middle*) Typical staining of iPSC colonies with anti-SeV antibodies. (*Left*) At passage (P) 1, many colonies were positive for SeV. (*Middle*) After several passages (P4), many colonies were partially positive. (*Right*) Colonies were found to be negative for SeV at P10. (*Lower*) Ratio of SeV-positive colonies. (*Left*) Randomly chosen colonies were expanded independently, and the existence of SeV vectors was evaluated by qRT-PCR at P4 and P10. (*Right*) Temperature shift to a non-permissive temperature of 38°C effectively removed SeV vectors from the iPSC colonies generated using TS7 vectors. The culture dishes at P4 were split and transferred to culture at 37 or 38°C for the number of days indicated

(purity of CD34+, 96–99%) or the frozen CD34+ CB cells (RIKEN BioResource Centre; purity of CD34+, 97–99%), thawed 1 day before infection, for SeV infection. These cells were treated with a mixture of SeV vector consisting of SeV TS7-OCT3/4, -SOX2, -KLF4, and -c-MYC. Infected cells were incubated under hematopoietic cell culture conditions, followed by cocultivation on mitomycin C (MMC)-treated SNL cells on day 4 or day 10. hESC-like colonies expressing SSEA-4 appeared after 14 days of cocultivation with SNL (18 days after SeV infection), whereas no colonies were obtained after transfer onto SNL on day 10. To eliminate remaining SeV virus constructs, hESC-like colonies were subjected to heat treatment at 38 °C for 3 days and recloned. SeV-positive cells detected with anti-SeV HN (envelope) antibody constituted 60–80% of total cells within hESC-like colonies before heat treatment (at passage 2). SeV constructs were not detected by qRT-PCR at passage 4 after heat treatment. The virus-negative ESC-like clones exhibited expression of hESC markers, a global gene expression profile similar to that of hESCs, and a normal karyotype. The established cell clones tested positive for embryoid body–mediated in vitro differentiation potential as well as for in vivo teratoma formation in SCID mice. All clones tested were able to give rise to cells of all three germ layers as determined by immunocytochemistry and cell morphology studies. The efficiency of generating iPSCs is higher with SeV (>0.1% at an MOI of 2) than with episomal vectors (Chou et al. 2011; Hu et al. 2011). The major advantage of using non-integrated SeV vectors is the capacity for potent and robust protein-expressing that does not require optimization of the transfection medium. Our results show that only 1×10^4 CD34+ cells, corresponding to ~5 mL of CB, are needed to obtain 10 independent iPSC clones with the SeV vector system. The rest of the CB can be sorted and used for regular bone marrow transplantation therapy. Additional advantages of the SeV vector system are that there have been no reports of pathogenicity associated with SeV vectors in primates and that the safety of these vectors is further enhanced by the F-deficiency that makes the vector non-transmissible (Nagai et al. 2011).

In the study, we have confirmed that the SeV vectors were not reactivated or detected in iPSCs at late passages or after temperature-shift treatment. It has been suggested that TS mutations in P and L affect polymerase activity and promote degradation of the virus vector after treatment at non-permissive temperatures (Feller et al. 2000). Based on our findings (Ban et al. 2011), we believe that the capacity of the TS SeV vector to eliminate the SeV construct from reprogrammed cells by temperature-shift treatment gives this system the potential to accelerate future clinical applications of iPSCs generated from cells obtained by less invasive or even noninvasive methods.

iPSCs from Peripheral Blood Cells: Activated T Cells

The initial methods for generating human iPSCs used a skin biopsy (Takahashi et al. 2007), requiring local anesthesia and suturation. It is preferable to use less invasive or noninvasive methods to generate iPSCs, especially patient-specific iPSCs. Activated T cells in peripheral blood provide an ideal cell source for such an approach because these cells are efficiently infected by SeV vectors (Okano et al. 2003) and samples are readily available from routine blood examinations. We recently demonstrated that transgene-free iPSCs can be efficiently generated from a small amount of human peripheral blood within 1 month of blood sampling by activating T cells with a combination of anti-CD3 and IL-2 treatment and using TS mutants of SeV vectors encoding human OCT3/4, SOX2, KLF4 and c-MYC (Seki et al. 2010) (Fig. 6.3). These T cell–derived iPSCs were named TiPS cells (TiPSCs) by K. Fukuda, Keio University (Seki et al. 2010). Although murine T cells are not efficiently reprogrammed using only four factors (Eminli et al. 2009; Hong et al. 2009), human activated T cells efficiently express exogenous genes when transduced by SeV vectors. Thus, the combination of activated T cells and TS SeV mutants made it possible to easily and noninvasively generate patient-specific TiPSCs that did not harbor T cell receptor (TCR) or immunoglobulin gene rearrangements.

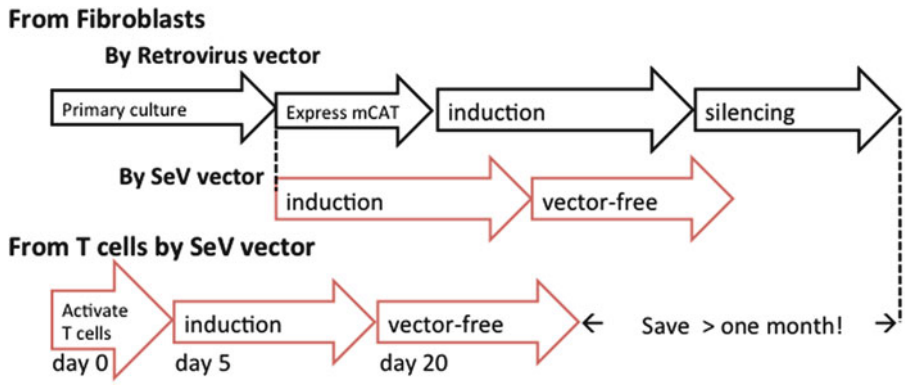

Fig. 6.3 Advantages of using SeV vectors to generate iPSCs from blood cells. In addition to generating iPSCs with an integration-free phenotype, this approach has the advantage of being non-invasive with respect to obtain source cells and in enabling a shorter period of time to establish iPSC lines

The efficiency of reprogramming by other approaches using retroviral (Brown et al. 2010) or lentiviral (Loh et al. 2010; Staerk et al. 2010) vectors to express the four factors, human OCT3/4, SOX2, KLF4 and c-MYC, was extremely low (approximately 0.0008–0.01%). Even if these methods used less peripheral blood and did not require the pharmacological pretreatment of patients, the problems associated with genomic insertion of transgenes and low reprogramming efficiency remain and preclude their wide use in clinical applications of iPSCs. Generating iPSCs with TS-mutated SeV vectors easily erases residual genomic viral RNA from the target cells (Ban et al. 2011), and is significantly more efficient (~0.1%) than protocols in which iPSCs are generated from T cells with retroviral or lentiviral vectors. Furthermore, compared with methods using skin fibroblasts with retrovirus vectors, this method also has the advantage of shortening the induction period to 1 month (Fig. 6.3).

Patient-Specific iPSCs and Gene Therapy

In collaboration with Kumamoto University, funding from the Japanese Ministry of Health, Labor and Welfare has enabled us to successfully establish hundreds of patient-specific iPSC strains from 84 fibroblast and blood samples from 53 patients with intractable diseases including neural, metabolic, muscular, skin, bone, and other inherited diseases (Era, et al., manuscript in preparation). The aim of this project is to generate a bank of transgene-free patient-specific iPSC lines for disease research and drug screening. It is expected that TS SeV vectors will contribute to the discovery of new drugs and therapy options for these intractable diseases by enabling an efficient supply of non-integrated transgene-free bona fide iPSCs.

Non-integrating patient-specific iPSCs were used to correct α1-antitrypsin deficiency (A1ATD) as a result of our collaboration with Wellcome Trust Sanger Institute and University of Cambridge (Yusa et al. 2011). A1ATD is an autosomal recessive disorder found in 1 out of 2,000 individuals of North European descent and represents the most common inherited metabolic disease of the liver. It results from a single point mutation in the A1AT gene (the Z allele; Glu342Lys) that causes the protein to form ordered polymers within the endoplasmic reticulum of hepatocytes. The resulting inclusions cause cirrhosis for which the only current therapy is liver transplantation. The increasing shortage of donors and harmful effects of immunosuppressive treatments impose major limitations on organ transplantation, making the potential of human iPSC-based therapy highly attractive. A1ATD patient-specific iPSCs were established using the TS SeV vectors described above and then zinc

finger nucleases (ZFNs) and piggyBac technology was used to correct the mutated sequences. The corrected patient-specific iPSCs successfully differentiated into functional hepatocytes in vitro and in mice. This approach is novel because the established iPSCs and corrected cells contain no exogenous genes. These results provide the first proof of principle for the potential of combining human iPSCs with genetic correction to generate clinically relevant transgene-free cells for autologous cell-based therapies. TS SeV vectors have also been used to generate transgene-free disease-specific iPSCs from elderly patients with type 1 and type 2 diabetes (Kudva et al. 2012). These iPSCs will be also useful for disease modelling and molecular diagnosis.

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Differentiation and Use of Cardiomyocytes Derived from Human Pluripotent Stem Cells

7

Gabor Foldes and Maxime Mioulane

Contents

Abstract

A long-standing need in the field of regenerative medicine for improved, humanized cell models for disease has led to a focus on human pluripotent stem cell derivatives. Functional cardiomyocytes, with therapeutic and drug screening potential, have recently been differentiated from human embryonic and induced stem cells. These cells show electrophysiological, contractile, and calcium handling profile similarities with their *in vivo* counterparts with relatively immature phenotype resembling foetal or diseased cardiomyocytes. Depicting cardiogenic pathways has helped to enhance cardio-differentiation protocols, including increased yield, scaling up, subtype preference and maturation in order to match the strict conditions of therapeutic application. However, severe limitations still hamper their use in clinic. On the other hand, human pluripotent stem cell-derived cardiomyocytes seem excellent candidate for drug testing in high content/throughput screening and for disease modelling. The proximity of these cells to the adult/diseased cardiac phenotype, their scalability and the possibility to convert somatic cells isolated from patients into cardiac cells, has permitted the investigation of

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heart congenital diseases and makes possible future personalized medicine.

Making New Human Cardiomyocytes

Key Cellular Processes Driving Cardiomyocyte Formation from Human Pluripotent Stem Cells

Since the derivation of the first human embryonic stem cells lines in 1998 and induced pluripotent stem cells in 2007, several laboratories have generated functional cardiomyocytes from pluripotent cells. First authentic human cardiomyocytes have been generated by differentiation of hESC in cell aggregates called embryoid bodies (EBs) in serum-free media, as seen by the expression of the cardiac marker α-actin and microscopy observation of spontaneous beating cells (Itskovitz-Eldor et al. 2000). The molecular signature and the contraction profile of clusters comprised of human embryonic stem cell-derived cardiomyocytes (hESC-CM) were characterized further by Kehat et al. (2001). The differentiation protocol using EBs in serum-containing media became the gold standard method for the production of hESC-CM. The serum contains TGF-β superfamily related molecules, which are known to induce cardiac mesoderm formation. However, the serum paradoxically retards cardiac differentiation (Passier et al. 2005) and, more importantly, yields few cardiomyocytes with great batch-to-batch variability. Kehat et al. reported 8.1% of differentiated aggregates with spontaneous beating activity (Kehat et al. 2001). Finally, only 1% of the cells are comprised of cardiac myocytes within serum-induced differentiated EBs (Laflamme et al. 2007). Commitment into the cardiac lineage results from the interaction of the pre-cardiac mesoderm with endodermal factors. Co-culture of hESC with the mouse endoderm-like cell line END2 yields culture with 25% of cardiomyocytes in serum-free media (Passier et al. 2005) with, interestingly, increased ventricular subtype compared to differentiated EBs. A different approach relies on the sequential addition and removal of Activin-A and BMP-4, both morphogens of the TGF-β superfamily produced by the developing endoderm. This differentiation protocol functions in hESC in dense monolayer culture and produces an average of 30% of cardiomyocytes (Laflamme et al. 2007). The induction of cardiogenesis using the bivalent role of Wnt signalling in the embryonic mesoderm was achieved by Yang et al. (2008), in a three stages differentiation protocol where primitive streak-like cells, induced by Activin-A and BMP4, are exposed to the Wnt antagonist DKK1. Up to 50% of cardiomyocytes were produced using this method. Improved differentiation protocols with enhanced cardio-differentiation include the control of the EB size (Burridge et al. 2007) and the use of defined factors in serum-free media (Burridge et al. 2011). In order to stimulate cardiogenesis, inhibition of PI3K and p38-MAPK pathways, exposure to ascorbic acid, and the NFAT and Cyclophilin D inhibitor Cyclosporine A have been shown successful. Finally, induced trans-differentiation into cardiomyocytes (α-MHC-GFP) has been achieved in post-natal cardiac or dermal mouse fibroblasts transduced with *GATA-4*, *MEF-2c* and *TBX-5* (Ieda et al. 2010). This raises the possibility to generate functional cardiomyocytes from somatic tissues without the need of stem or progenitor cells.

Gene and Protein Profile of Human Pluripotent Stem Cell-Derived Cardiomyocytes

Appearance of spontaneously beating activity is concomitant with the expression of structural cardiac genes and proteins including tropomyosin, troponin I, troponin T, α/β-actin and β-myosin heavy chain (β-MHC), atrial natriuretic factor, calcium handling proteins and genes for potassium channels, and actins and actin-regulatory genes (Cao et al. 2008). Automaticity of clusters of hESC-CM typically occurs 9–25 days following induction of differentiation. Interestingly, the first beating cardiac myocytes contract at day 17 post-conception in the human embryo (Snir et al. 2003). In total, serum-induced differentiation of human EB consists of a down-regulation of 2,389 genes and an up-regulation of 1,012 genes as shown by a transcriptional profiling study (Cao et al. 2008). Cardiac-specific transcripts levels in hESC-CM

and human tissue (from patient with heart disease, foetal and neonatal heart) are highly similar (Asp et al. 2010). However, the high level of β-MHC, the slow shortening velocity isoform of the contractile protein, compared to α-MHC in hESC-CM evokes a foetal-like phenotype. Interestingly, the failing and the hypertrophic cardiomyocyte undergo a regression to the foetal phenotype with MHC isoform switched. Nevertheless, if the isoform switch can contribute to the contractile dysfunction in rodent where the α form is largely predominant in the normal adult heart, this is unlikely to be extensive in the healthy human adult where the β isoform represents already 90% of the total MHC protein (Miyata et al. 2000).

Electrophysiological Characteristics

Developmental changes in action potential and ion channel gene expression profiles of hESC-CM were characterized over time in culture (He et al. 2003; Sartiani et al. 2007). According to these reports, early hESC-CMs (before day 40) acquire a sino-atrial node-like phenotype characterized by short action potential. At this stage, ions channels controlling diastolic potential and action potential repolarisation are expressed at low levels. Molecular and functional expression of ion channels undergoes developmental processing during the intermediate stage (from day 40 to day 90). From 90 days of differentiation, both the molecular and electrophysiological profiles of hESC-CMs moved toward more mature phenotypes, including ventricular, atrial, His and Purkinje-like cells (Boheler et al. 2002). However, the relatively depolarized resting membrane potential of hESC-CM is typical of the foetal phenotype although the membrane potential becomes more negative in developing atrial-like and ventricular-like hESC-CM (He et al. 2003).

Metabolism and Excitation-Contraction Coupling

The development of bio-energetic circuits operates a switch between ES cell glycolysis-dependent metabolism to a functional mitochondrial oxidative metabolism in differentiated ESC-CM (Chung et al. 2007) similarly to the embryonic heart (Razeghi et al. 2001). The switch in substrate preference is accompanied by metabolic-specific gene transcription (glycolysis, fatty acid oxidation, the Krebs cycle, and the electron transport chain) and the re-organization of mitochondrial network with the developing myofibrils. The maturation of bio-energetic circuits is essential for cardiogenic development of hESC-CM as well as for supporting forceful excitation-contraction coupling. However, hESC-CM still have an immature metabolism compared to foetal adult cardiac myocyte with less active cellular respiration, mitochondrial biogenesis and lipid metabolism (Cao et al. 2008). The full maturation of the myofibril patterning is not achieved in all hESC-CMs cultures and resembles foetal and dedifferentiated cardiomyocytes with disarrayed myofibril patterns (Cao et al. 2008), while some which are either stretched or matured in long term cultures possess highly organized sarcomeres (Snir et al. 2003). Contractile proteins organized in sarcomeres are present in hESC-CM with functional calcium stores in the sarcoplasmic reticulum (Liu et al. 2007). Human ESC-CM express calcium handling proteins SERCA, IP3R, RyR and the sodium/calcium exchanger channel but lack junctin, triadin, and calsequestrin (Liu et al. 2007). The absence of the calcium buffer calsequestrin and the absence of t-tubule contribute to the small and delayed Ca^{2+} transient and to the non-uniform Ca^{2+} dynamics in hESC-CM respectively (Lieu et al. 2009). The mechanism of excitation-contraction coupling in hESC-CM is controversial. According to Dolnikov et al. (2006), the calcium transient in hESC-CM results from trans-sarcolemmal entry through calcium channels and not from intracellular stores. In contrast, ryanodine-sensitive calcium store have been found in other studies but still in a restricted population of hESC-CM and are significantly less than those in adult (Liu et al. 2007). In another study which investigated the relation between I_{Ca} and the calcium transient in hESC-CM, it was concluded that synchronized calcium sparks that initiate whole

cell calcium transient in hESC-CM were mediated through the local activation of L-type calcium channels (Zhu et al. 2009). This study also showed that I_{Ca} density in hESC-CM is similar to foetal and to adult human cardiomyocytes. Together, these suggest that hESC-CM excitation-contraction coupling relies on a functional and mature calcium-induced calcium release mechanism but incomplete calcium handling protein profile and foetal-like morphology result in immature calcium transient generation.

Differences and Similarities Between Human Embryonic Stem Cell- and Human Pluripotent Stem Cell-Derived Cardiomyocytes

Differentiation of hiPSC into cardiomyocytes has been reported since 2009 (Zhang et al. 2009). Like the ESC, cardio-differentiation of iPSC consists of activation of myogenic genes similarly to ESC. The global transcriptional signatures of hiPSC-CM and hESC-CM (from beating clusters) are highly similar while sarcomeric organization, calcium handling proteins and electrophysiological properties are indistinguishable (Gupta et al. 2010). In this study only 1.9% of the analyzed transcripts differed between cell lines. Conversely, a recent paper comparing calcium handling properties in hiPSC-CM and hESC-CM suggested a more immature phenotype of hiPSC-CM (Lee et al. 2011). However, the significance in these differences between iPSC and ESC can be moderated by the fact that different cell lines of hESC and hiPSC-CM have different cardiomyogenic competence. Recent reports have demonstrated that reprogrammed cells show preferential lineage commitment for the tissue from which they come. This suggests that the epigenetic memory of induced pluripotent cells does not interfere with the pluripotent state but can influence further differentiation preference (Xu et al. 2012).

Pluripotent Stem Cells-Derived Cardiomyocytes for Toxicological Screens

Human Pluripotent Stem Cell-Derived Cardiomyocyte as a Cell Candidate for Preclinical Tests

The potential of new chemical entities to induce cardiac toxicity is a leading cause of withdrawal in preclinical and clinical tests, and impacts on the cost of developing candidate drugs. In order to eliminate unsafe drugs in an early stage of clinical development, laboratories and pharmaceutical companies develop cellular toxicological screens. Ideally, these cell assays have a physiological response similar to the target organ and will account for the genetic factors that may determine the cardiac outcome of developing compounds. In the perspective of developing early cell cardiotoxicity test and possibly to make way to personalized medicine, hPSC-CM and particularly hiPSC-CM appear as most attractive candidate. Conventional tests assess toxicity on animals and surrogate cells. They represent the most relevant model of cardiovascular physiology with associated cardiac output and electrical response using technologies similar to what prevails with patients (e.g., ECG, echocardiography). However, they do not account for the genetic differences between animal and human which may result in different toxico-kinetics and even different outcome, as seen by unexpected cardiotoxicity of drugs in patients that passed animal testing. In this perspective, there is a need for *in vitro* cardiac cell system with associated toxicity assays that mimic the human heart phenotype. Cardiac myocytes isolated from adult patients are limited in tissue availability, cell number, culture condition (they spontaneously dedifferentiate or die) and their phenotype is highly dependent on the disease state of the patient. Immortalized cardiomyocytes lines are karyotypically unstable so they may poorly represent the adult myocyte phenotype, in particular

in term of their survival and proliferative activities. Human PSC-CM can potentially serve as a new screening system for cardiac toxicity. They are phenotypically similar, although not identical, to the adult myocyte in term of excitation-contraction coupling, electrophysiology, metabolism and function and are superior to primary adult myocyte as they would survive and continue to beat in culture for months. Overall, hiPSC-CM responded in predictable manner to 40 different known cardiac compounds (Dick et al. 2010). Furthermore, isolation of particular phenotype, for instance ventricular subtype, may be possible by applying upgraded differentiation protocols or by using genetically modified lines. However, the signalling network of hPSC-CM, in particular the survival and death pathways, has not been fully elucidated in toxicological response. Also, whether the response to a stress signal can predict their adult counterpart is still largely unknown.

The *In Vitro* Clinical Trial to Assess Cardiotoxicity

Cardiac toxicity refers to contractile dysfunction and change in metabolic activity. At the extreme, metabolic changes can lead to cell death through the inhibition of cell survival pathways or exacerbation of pro-death signals. These depend on calcium signalling, redox status, and the intricate signalling network where proliferative/growth and survival pathways often converge. Depending on the pathway initiated, the strength and the duration of the signal, cardiomyocyte can either undergo necrotic, apoptotic or autophagic cell death. There is not a universal marker for cell death (except late necrotic-like state, but this poorly informs on the original cell death signal) but instead a combination of morphological and biochemical events that depends on the action of the drugs, the cell type, and may differ between species. These markers include fluorescent probes for mitochondrial function, lysosomal activity, calcium homeostasis, redox status, caspase activation, caspase substrates cleavage, protein translocation, nuclear remodelling, cell permeability and cell membrane remodelling. Whether monolayer culture where cells are attached to a synthetic matrix with no or little cell-cell contact mirrors the *in vivo* situation is debated. Cell contact-related signalling, cell-cell communication, paracrine regulation, proliferative activity and drug penetration in cell aggregate would likely be more physiologically relevant to the *in vivo* situation than isolated cells in monolayer. Because hPSC-CM grown in 3-dimensional EBs are particularly stable in culture, experiments involving their long term exposure to compounds can be designed, allowing the investigation of chronic drug effects, which may not be possible in monolayer culture. Recent progress in automated microscopy imaging has benefited the production of scalable toxicological screens using hPSC-CM, particularly well adapted for pharmaceutical companies interested in evaluating off target effects of developing compounds. Indeed, one advantage of hPSC-CM is their suitability for high-throughput methodologies, which will match functional cellular outputs to array-generated information. An exciting aspect of hESC-CM or hiPSC-CM is the ability to compare cells directly with individual patient responses for particular mutations or haplotypes.

Human Pluripotent Stem Cell-Derived Cardiomyocyte for Disease Modelling

The potential of stem cell-derived cardiomyocytes for disease modelling has been enhanced by the realisation that from hiPSC-CM and later from hESC-CM can be obtained also with disease-specific genotypes (Park et al. 2008). These phenotypes provide validation that disease pathogenesis can be reproduced *in vitro*. Indeed, the ability to restore pluripotency in somatic cells by forced expression of reprogramming factors has created potent novel opportunities for modelling human diseases with hiPSC (Park et al. 2008). Somatic cells can be readily obtained from

Table 7.1 Cardiovascular derivatives of human pluripotent stem cells with genetic disease

Disease	Molecular defect	Reference
LEOPARD syndrome	Heterozygous mutation in PTPN11	Carvajal-Vergara et al. (2010)
Type 8 long QT syndrome (Timothy syndrome)	R406W mutation in the L-type calcium channel CaV1.2	Yazawa et al. (2011)
Type 1 long QT syndrome	Dominant mutation in KCNQ1	Moretti et al. (2010)
Type 2 long QT syndrome	Missense mutation in KCNH2	Itzhaki et al. (2011) and Matsa et al. (2011)
Catecholaminergic polymorphic ventricular tachycardia	Autosomal dominant mutations in ryanodine receptor 2	Jung et al. (2012)

PTPN11 tyrosine-protein phosphatase non-receptor type 1; *KCNH2* potassium voltage-gated channel, subfamily H (eag-related), member 2; *KCNQ1* potassium voltage-gated channel, KQT-like subfamily, member 1

patients - from skin fibroblasts, whole blood or hair follicles - and derive cultured hiPSC lines that are genetically matched to the patients. The appropriate genetic background of a hiPSC line from a patient with the disease may therefore be more likely to display the relevant disease phenotype at the cellular level. This has already been used for a number of human diseases including diabetes mellitus type 1, muscular dystrophies, Parkinson's disease, Huntington's disease and Down's syndrome (Park et al. 2008). In cardiac disease, the initial focus for hESC-CM or hiPSC-CM was modelling acute cardiac responses, with the aim of producing models to investigate contractile impairment, modulated contractile frequency or arrhythmias, or for using cells as a screen to detect acute cardiotoxicity of experimental compounds. Two major aspects of heart function have been modelled by hPSC derivatives: growth and compensatory structural changes as well as disturbance in conduction and electrical activity.

Models for Hypertrophy

Development from the initial immature cardiomyocyte phenotype to a cell with aspects of mature ventricular myocytes has been observed over months in culture, and can be accelerated by treatments such as mechanical stretching or neurohormones (Foldes et al. 2011). Clearly, the more developed phenotype might reasonably be expected to model the adult myocyte more closely. However, immature phenotype may also have a useful role, since the failing human heart reverts to a protective foetal phenotype and gene program. Hypertrophy, a risk factor for cardiovascular disease, can be well modelled in hESC-CM. We have shown that hESC-CM display typical increases in cell size, protein content, sarcomere assembly, and hypertrophic markers when exposed to hypertrophic stimuli such as Gq agonists (Sartiani et al. 2007; Snir et al. 2003). In addition to pharmacological models, hiPSC-CM have recently been generated from patients with LEOPARD syndrome, an autosomal dominant multisystem disease which include hypertrophic cardiomyopathy (Carvajal-Vergara et al. 2010). *In vitro* these hiPSC-derived cardiomyocytes had a greater size, better organised sarcomere, and higher nuclear NFAT than controls (Table 7.1).

Inherited Arrhythmia Models

Researchers have now devised methods to generate patient-derived cells that can mimic the phenotype that causes inherited diseases of the heart's electrical system such as long QT syndrome, which may help in the development of new drug therapies for the disease (Itzhaki et al. 2011; Matsa et al. 2011; Moretti et al. 2010) (Table 7.1). In the first study to describe the physiological mechanisms of genetic variation in inherited arrhythmogenic diseases, hiPSC were differentiated from patients with type 1 long QT syndrome into cardiomyocytes. Human iPSC-derived cardiac

myocytes showed a prolonged action potential and an impairment of trafficking and localization of the mutant channel to the surface of the cell (Moretti et al. 2010). The hiPSC-CM with type 1 long QT syndrome showed a higher susceptibility to catecholamine-triggered tachyarrhythmia. As expected, treatment with β-adrenergic receptor inhibitors attenuated the long QT phenotype (Moretti et al. 2010). Type 2 long QT syndrome has also been modelled in hiPSC-CM to evaluate the effectiveness of pharmacological agents that may worsen or improve the condition. Their studies show that the long QT syndrome phenotype was aggravated by potassium-channel blockers, whereas calcium-channel blockers, K_{ATP}-channel openers and late sodium-channel blockers ameliorated the long QT syndrome phenotype. The next step could be to learn how hiPSC technology can be applied to more complex diseases with unknown genetic background. A further interesting example of stem cell derivatives such as hESC-derived cardiomyocytes is their use to model foetal arrhythmias in obstetric cholestasis by utilizing advanced biophysical methods such as the scanning ion conductance microscopy (Abdul Kadir et al. 2009).

Toward Therapeutic Application of Human Pluripotent Stem Cell-Derived Cardiomyocytes

Cell-based therapy has emerged as a future option for patients with advanced heart failure, one of the leading causes of morbidity and mortality in our industrial societies. Completed clinical trials using adult stem cells (Abdel-Latif et al. 2007) have demonstrated feasibility but inconsistent efficacy due to the limited repertoire of differentiation of somatic or adult stem cells. The use of highly plasticity cells that can recapitulate cardiomyogenesis is warranted to offset this limitation. To this end, cardiovascular derivatives of human pluripotent stem cells emerge as appealing alternatives, although their potential for therapy is hampered in part by the risk of dysregulated growth and differentiation, and potentially incomplete cardiac maturation. On the other hand, fully differentiated cardiomyocytes are particularly sensitive to hypoxia and oxidative stress, and are unable to provide sufficient replacement tissue. Thus, these early cardiovascular progenitors that are resistant to stress such as hypoxia and inflammation, could offer a superior cell type for use in regenerative cardiology.

Biological Pacemakers

Using hPSC-derived cells for generating biological pacemakers can be a fascinating new route, and it would also address the question of whether the complex structures of the sinus node are indeed a requirement for sustained pacemaker activity. One of the novel approaches to restoring cardiac electromechanical functions may be to transplant excitable myogenic cells into the dysfunctional zone of the heart. For this, spontaneously beating clusters of hESC-derived myocytes were tested first as pacemaker elements in proof-of-principle studies. When hESC-CM are implanted in hearts with slow beating rate, such as pigs, they formed pacemakers when the native one was dysfunctional or recipient's conduction system was blocked (Xue et al. 2005). Human ESC-derived pacemaker cells are now under investigation as a preferred cell source for a biological pacemaker in the atria of patients with nodal disorders such as sick sinus syndrome (Xue et al. 2005). Manipulating intracellular signalling to get an enriched preparation of hPSC-derived nodal-like cells would be of potential use in the formation of a biological pacemaker as well (Zhu et al. 2010).

Cardiomyocyte Transplantation in Myocardial Infarction

Efforts to optimize culture conditions for the enrichment of hPSC-CM and much higher number of competent cells with stable phenotype facilitated upscale for transplantation studies. In initial cardiac transplantation experiments, hESC-CM were transplanted into uninjured hearts of mice, rats, and pigs, and formed grafts of human myocardium (Kehat et al. 2001; Laflamme et al.

2007). The hESC-CM have also been shown to engraft in mouse, rat, guinea pig and pig hearts after myocardial infarction and functionally integrated to replace scar tissue and restore pump function (Caspi et al. 2007; Laflamme et al. 2007; van Laake et al. 2007). The hESC-CM continued to proliferate, mature, align, and form gap junctions with host cardiac tissue after transplantation, so the myocardial graft size increased significantly. These studies have demonstrated efficacy in improving cardiac function but it is consistently noted that improvement is in excess of that explained by cell engraftment and cardiomyocyte replacement. These bring into question the mechanism of their actions, with the majority settling for a favourable remodelling effect through paracrine mechanisms. Indeed, stem cells derivatives secrete factors beneficial for myocyte survival, contractility, decreased inflammation and fibrosis, and blood-vessel formation (Laframboise et al. 2010). However, beneficial effects on regional and global cardiac function from the transplanted cells may not be sustained at later time points (van Laake et al. 2007).

Production of Clinical-Grade Cardiomyocytes

Despite advances in production of competent cells and delivery method for transplantation, hPSC-CM therapy has not been authorized in patients yet because hESC still raises safety issues such as immunorejection and teratoma formation by undifferentiated residues and most methods of hiPSC production rely on insertion of retroviral vector carrying oncogenic genes, despite new generation of virus/oncogene free vectors. The most recent methods of production of hESC-CM utilize synthetic medium supplemented with a cocktail of proteins obtained from DNA recombinant technology or from human tissue extraction. However, current differentiation protocols generate too few myocytes to meet therapeutic needs. Retroviral vector carrying selectable cardiac specific genes have shown successful in increasing cardiomyocytes fraction up to 99%. Mechanical method can be used when using large amount of cells but this does not eliminate completely the non-myocyte fraction. Alternatively, cardiomyocytes can be sorted after staining with the mitochondrial-specific fluorescent probes Tetramethylrhodamine methyl ester (TMRM), cardiomyocytes being the cell type with the greater mitochondrial content. Furthermore TMRM spontaneously washes out without significantly affecting cell physiology (Hattori et al. 2010). This simple non-transgenic method of purification is compatible with scaling up and future therapeutic applications although reproducibility of the method still need to be proven. Finally, cardiac (or cardiac progenitor) specific surface antigens have been successfully used to purify cardiomyocytes produced from hPSC. These markers include VCAM1, SIRPA, ALCAM (CD166), EMILIN-2, SSEA-4 and cellular prion protein. Immune rejection is another issue in the practice of transplantation of hESC-CM as an allogeneic cell resource. Different strategies were carried out in prevention of potential immune rejections against transplanted hESC-CM including the use of stem cell bank to pre-select HLA homozygous hESC lines or to create a genetically modified universal donor cell line evading immune rejection. Generation of hiPSC offers a new intervention that is not compounded by immunosuppressive drugs to prevent tissue rejection, while harnessing targeted gene-repair strategies to repair genetic defects. It is unclear however whether using hiPSC would completely avoid an immune response, because a recent study has shown the immune rejection of teratomas formed from hiPSC even in syngeneic mice (Zhao et al. 2011).

Conclusions and Future Challenges

Human PSC represents an excellent *in vitro* cell system to investigate the molecular events that drive the formation of cardiac myocytes for future heart repair, disease modelling, drug screening and personalized medicine (Fig. 7.1). Human PSC-CM presents most of characteristics and respond to cardiac compounds similarly to their human *in vivo* counterparts which argue

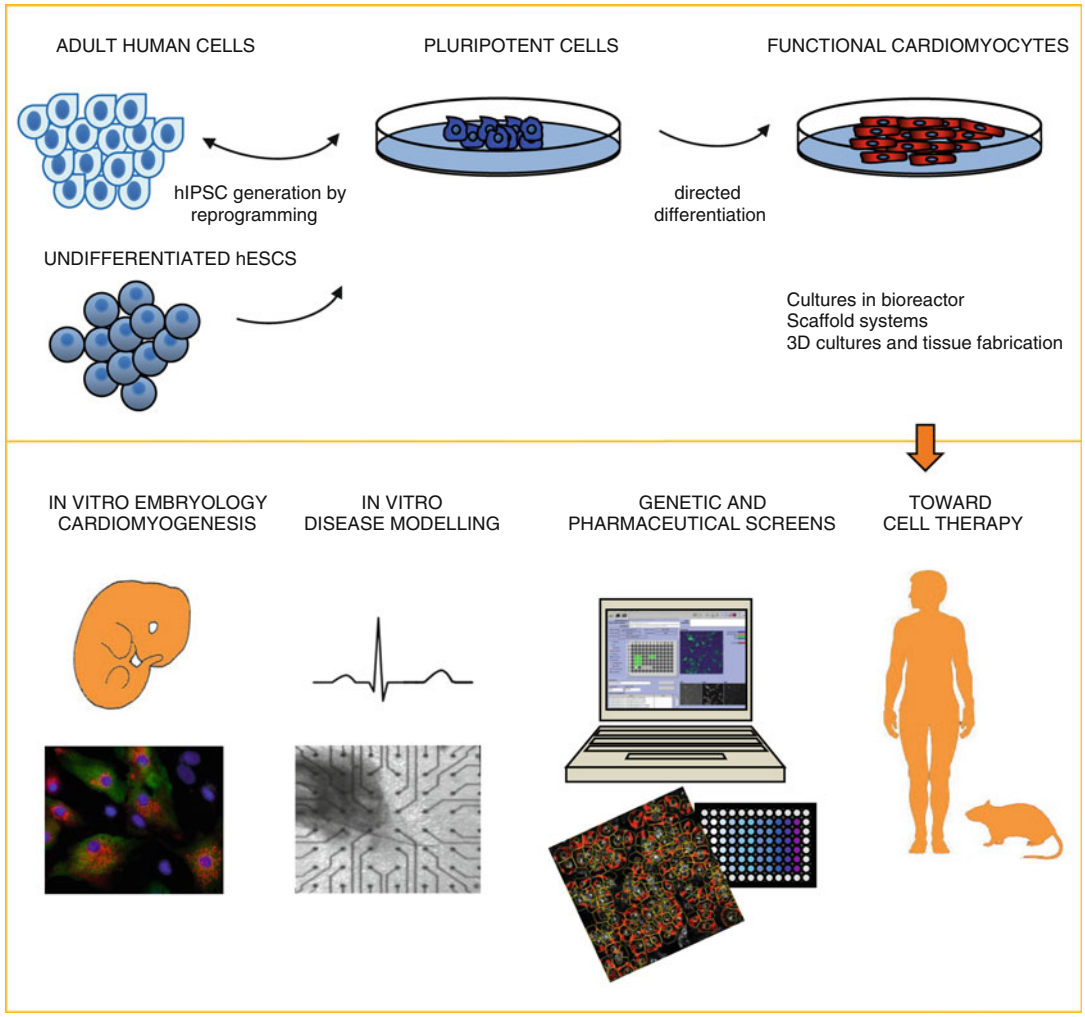

Fig. 7.1 Relevant cardiac cell type such as cardiomyocytes can now be achieved by purifying cells of the cardiac lineage from differentiating human embryonic stem cells or induced pluripotent stem cells. Assays based on electrophysiology, morphology, or gene or protein expression can be performed on these cells thereafter and cells can be used as disease and developmental model systems, drug or genetic screening platforms, or transplanting such cells to the heart

in favor their superiority to other current cell types. However, whether they reproduce pharmaco-kinetics similarly to the adult myocyte is still unknown. The high fidelity of hiPSC-CM to the diseased phenotype of patient with inherited heart disorders represent a promising application of hPSC-CM to model and study heart diseases *in vitro*. However, the lack of standardization and optimization in method of production of cardiac myocytes from different hPSC possibly account for heterogeneity in cardiac phenotypes and variable yield. In addition, this may be complicated by phenotypic changes occurring during maturation of hPSC-CM in culture. Industry and clinic are highly demanding in reproducible protocols and require reliable material. These cells are very complex therapeutic products and only further comparative animal studies and human trials will assess whether they will be suitable for transplantation or for predicting compounds that will be effective against cardiac disease.

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Metabolism in Cancer Cells and Pluripotent Stem Cells

8

Tiffany B. Satoorian and Nicole I. zur Nieden

Contents

Abstract

Both cancer cells and pluripotent stem cells have the ability to rapidly proliferate, but one major difference between them is the ability of pluripotent stem cells to control the cell cycle thus evading malignancy. Studying the mechanisms that both cell types have in common versus those that are differentially regulated could provide researchers with novel drug targets to "kill" malignant cancer cells.

For example, it has long been suggested that a cancer cell's metabolism sets it apart from more differentiated cells. In fact, cancer cells seem to share an increased anaerobic metabolism with pluripotent stem cells. Although both cell types share similar metabolic features, they have opposite responses to reactive oxygen species (ROS) removal and senescence. This review will highlight how cancer cells are unable to eliminate ROS due to misregulation of the transcription factors forkhead box O (FoxO) and p53, leading to an increase in DNA damage and loss of cell cycle control. Also, it will summarize how pluripotent stem cells activate different enzymes responsible for ROS removal, relieving the cells of damage due to oxidative stress. Furthermore, we will provide evidence that pluripotent stem cells also rely on a different mechanism of cell cycle regulation, namely not relying on signaling through cyclin D and the retinoblastoma (Rb) protein. Ultimately, this review aims to contrast the differences and to highlight the similarities in cancer and pluripotent stem cell metabolism.

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Introduction

The process of tumorigenesis consists of multiple stages: immortalization, transformation, invasion, and in some instances, metastasis. Immortalization requires the evasion of apoptosis and senescence by growth factor independent cell proliferation. In order to grow more aggressively following immortalization, cancer cells need to be anchorage independent, resistant to contact inhibition, angiogenic, and exhibit changes in catabolic, energy producing pathways. After decades of research, it is now common knowledge that cancer cells do not catabolize glucose via the TCA cycle nor rely on oxidative phosphorylation to produce adenosine triphosphate (ATP), but that their metabolism is rather dependent on anaerobic glycolysis with an increased reliance on glutamine.

Interestingly, pluripotent stem cells (PSCs) have a similar metabolic profile to cancer cells and can be used as a method for understanding cellular responses to oxidative stress and senescence in cells that proliferate indefinitely, but do not become transformed. The definition of PSCs include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which both share the quality of limitless proliferation and differentiation capacity, and this is also exemplified by studying their metabolic profile.

Like cancer cells, PSCs have a more "primitive" form of metabolism, breaking down glucose primarily through glycolysis alone. The process of glycolysis results in an increase in ROS formation, which can lead to oxidative stress and DNA damage. Because cancer cells acquire mutations that prevent proper response to DNA damage and cell cycle regulation, the ROS-induced DNA damage cannot be properly repaired and the cell continues to proliferate. Over time, this results in a pool of cells that have acquired many different types of mutations. Response to oxidative stress in PSCs is regulated by a different mechanism than in cancer cells, leading to the ability to respond to DNA damage and also be able to control the cell cycle.

The Warburg Effect

Both cancer cells and pluripotent stem cells rely mostly on generating energy by anaerobic glycolysis, a discovery made by Otto Warburg over seven decades ago. He found that cancerous tissues and cells display an increase in glycolysis and produce more lactic acid when compared with local tissue, giving cancer cells a selective advantage by generating energy faster and not depending on oxidative phosphorylation. This observation is clinically relevant and is used in practice for the detection of metastatic tumors and is necessary for adaptation to hypoxic conditions, an important characteristic of transformed cells (Kondoh 2008). Perhaps it is due to this decrease in available oxygen that has allowed for cancer cells to rely on anaerobic metabolism for energy generation.

One potential therapy for cancer is the induction of differentiation of the cancer cells. Intriguingly, a study by Schneider et al. (2011) has not only demonstrated that retinoic acid induced differentiation of the neuroblastoma cell line SH-SY5Y, but that it also resulted in a decrease in reliance on glycolysis and ATP production. This implies that targeting anaerobic glycolysis may serve as different mechanism for developing cancer therapies.

When considering mitochondrial mass and mitochondrial DNA (mtDNA) in pluripotent stem cells, it is not surprising that these cells rely mostly on anaerobic glycolysis. Both mitochondrial mass and mtDNA is low in undifferentiated pluripotent stem cells, and increases upon differentiation. This increase also corresponds to an increase in ATP production. During reprogramming of somatic cells to iPSCs, the amount of mitochondria, which is higher in the somatic differentiated cell, is decreased to a level comparable to ESCs (Prigione et al. 2010). Furthermore, a dependence on anaerobic glycolysis and high levels of lactate production in undifferentiated PSCs was observed when compared with mature cells, similar to the metabolic profile of cancer cells. In addition, several glycolytic proteins in ESCs are enzymatically more

activate in ESCs while mitochondrial oxygen consumption is reduced when compared with terminally differentiated fibroblast cells (Kondoh 2008). In stark contrast of what is observed in transformed cells, the genome of murine ESCs at least seems more resistant to oxidative damage. In conclusion, the increase in proliferation of ESCs appears to be related to enhanced glycolytic activity, and upon differentiation, the reliance on glycolysis decreases.

The Warburg Effect: Who Are the Downstream Players?

The observation of enhanced glycolysis in cancer cells and PSCs can result from a number of different adaptations. For example, increases in glucose transport into the cell could facilitate increases in overall metabolism. Indeed, ESCs express glucose transporter proteins GLUT1, 3 and 8, which allow them to take up glucose from the environment (Tonack et al. 2006). In fact, PSCs are routinely cultured in medium that contains higher than physiological glucose concentrations to meet their need for readily converting glucose into ATP. However, studies from our lab suggest that by lowering the glucose concentration to physiological levels those portions of the population that spontaneously differentiate are reduced (Dienelt and zur Nieden 2011). Therefore, increases in extracellular glucose seem to signal ESCs to differentiate and no longer contribute to the pool of cells that are self-renewing.

Not only is the intracellular glucose concentration important for enhanced glycolysis, but the activation of proteins involved in anaerobic metabolism must also increase. The question that remains is which signaling pathway would be likely to lead to this phenotype? One candidate pathway is the phosphoinositide 3-kinase (PI3K) pathway, whose activation results in an increase in glucose intake that accrues in glucose concentrations that saturate the enzymes used for oxidative phosphorylation. As a result, the remaining glucose is broken down by anaerobic metabolism and this leads increases in lactic acid formation. Aberrant PI3K signaling is observed in transformed cells, possibly explaining the high glycolytic rate even in the presence of oxygen. Also, it has been observed that there is an increase in expression of components in PI3K signaling in PSCs and this expression is decreased during differentiation (Armstrong et al. 2006). Hence, the activation of PI3K in cancer cells and pluripotent cells seems to allow an increase in glucose entry, but what mechanism results in increase anaerobic glycolysis?

One other mechanism by which anaerobic glycolysis could be favored over aerobic glycolysis is by an increase in transcription of glycolytic genes. Expression of the oncogene c-Myc is known to activate the transcription of several glycolytic enzymes and overexpression of c-Myc has been shown to immortalize epithelial cells *in vitro* (Gil et al. 2005), providing a link between a protein that is active in cancer cells and an increase in anaerobic metabolism. Interestingly, an increase in expression of glycolytic enzymes has been shown to increase lifespan in primary mouse embryonic fibroblasts (MEFs) (Kondoh 2008). This last finding demonstrates that enhanced glycolysis alone may be sufficient to immortalize cells. Also, c-Myc is one of the four factors used for reprogramming human fibroblasts and has been shown to promote mouse ESC self-renewal in the absence of leukemia inhibitory factor (LIF), an important additive for maintaining mouse ESCs in the undifferentiated state (Kidder et al. 2008). Active c-Myc in cancer cells and pluripotent stem cells results in an increase in enzymes involved in anaerobic metabolism, shifting the balance of glucose catabolism to glycolysis.

Another mechanism by which an increase in anaerobic glycolysis could occur in cells is by a loss of function mutation in a protein that negatively regulates the process. One example of this is the tumor suppressor protein p53, known as a "guardian of the genome" and is responsible for activating transcription of genes involved in cell-cycle arrest, senescence and apoptosis in response to cellular stress. Loss of function mutations in p53 occur in a wide variety of cancers and p53 is found to be present, but inactive in pluripotent stem cells. It is known that p53 is a negative regulator of anaerobic glycolysis and one example of this regulation

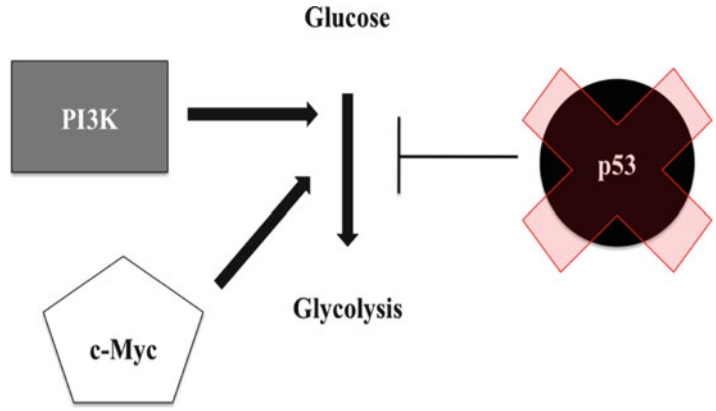

Fig. 8.1 Summary of glycolytic activity in cancer cells and pluripotent stem cells. In both cell types, activation of PI3K and c-Myc promotes increases in anaerobic metabolism. The tumor suppressor p53, an inhibitor of glycolysis, is generally mutated in cancer cells and inactive in pluripotent stem cells, relieving its inhibition on both cell types

is the ability of p53 to activate expression of the protein TP53-induced glycolysis and apoptosis regulator (TIGAR). Expression of TIGAR causes a decrease in glycolysis accompanied by an overall decrease in ROS. The ability of TIGAR to decrease ROS levels may aid in the ability of p53 to protect the cell from DNA damage (Bensaad et al. 2006). Because p53 is mutated in many cancers, it cannot properly regulate TIGAR expression and its ability to inhibit anaerobic glycolysis, resulting in increased anaerobic metabolism.

It has also been observed that ESCs express an abundance of p53 protein, but is found to be inactive (Solozobova et al. 2009). The protein is predominantly localized to the cytoplasm of undifferentiated cells, and this might account for the decrease in p53 activity. During differentiation, p53 activity is increased and the transcription of its target genes are also known to increase. This implies that the increase in glycolysis observed in pluripotent stem cells can be due to the inactivation of p53, mimicking what occurs in transformed cells.

In summary, there is an increase in glucose uptake in cancer and pluripotent cells as a result of aberrant PI3K activation. Activation of the oncogene c-Myc and the inhibition of the tumor suppressor p53 result in a molecular switch from oxidative phosphorylation to glycolysis and an increase in lactic acid production (Fig. 8.1).

Glycolysis and Reactive Oxygen Species (ROS)

As a result of normal cell metabolism, ROS are generated at low levels and are eliminated by a cellular antioxidant system that includes multiple enzymes responsible for ROS removal, including manganese-containing superoxide dismutase (MnSOD) and catalase. The amount of ROS in a cellular environment is a balance between ROS production and metabolic elimination. If this scale is imbalanced and results in an increase of ROS, the cell is said to be in oxidative stress. This is important because ROS are known to induce nucleic acid and amino acid damage, resulting in malformations in DNA, RNA, and proteins. In cancer cells and ESCs, there is an elevation in ROS levels as a result of increased glycolysis, leading to an observed increase in oxidative stress. However, these two cell types have different mechanisms for responding to this dilemma. In cancer cells, the increase in oxidative stress aids cancer progression through promotion of DNA damage and reduced mismatch repair (Singh 2006) while ESCs appear to be resistant to ROS induced DNA damage. Although both cell types exhibit increases in ROS, how is it that ESCs are able to be resistant to DNA damage?

In order to clarify this puzzle, it is important to study proteins known to be activated in response to

oxidative stress. The FoxO transcription factors play an important role in ROS removal, stress resistance, apoptosis, and cell proliferation. These proteins are very important in untransformed cells, as they can halt the cell cycle in response to oxidative stress and DNA damage. The FoxO proteins are regulated by two mechanisms in untransformed cells: stress response and PI3K signaling (Myatt et al. 2011). Following PI3K activation, protein kinase B (PKB/Akt) is activated and phosphorylates FoxO, leading to its nuclear exclusion. In addition, increases in ROS result in JNK and silencing information regulator 1 (SIRT1) activation, both of which positively regulate FoxO and its ability to activate transcription of genes involved in cell cycle arrest and resistance to oxidative stress (Dansen 2011). Additionally, FoxO proteins can directly sense ROS through the ability of ROS to induce the formation of disulfide bridges between cysteine residues on FoxO on the acetyltransferase p300/CBP. This results in crosslinking of the two proteins and the ability of p300/CBP to efficiently acetylate and activate FoxO transcription of ROS removal enzymes (Dansen 2011). On the other hand, hydrogen peroxide (a by-product of ROS removal) can activate the insulin receptor substrate-1 (IRS-1), resulting in PI3K activation (Heffetz et al. 1992). Following PI3K activation then, as mentioned above, Akt is activated and is responsible for FoxO removal from the nucleus. As mentioned previously, activation of the PI3K/Akt pathway is common in cancers, leading to inhibition of FoxO and its ability to regulate ROS removal, apoptosis, and senescence. In other words, the increases in ROS as a result of increased glycolysis are not removed by FoxO, due to the aberrantly active Akt. Additionally, PI3K activation results in the inhibition of the tumor suppressor gene product PTEN, which is responsible for cell cycle regulation. Taken together, an increase in glycolysis in cancer cells leads to an overproduction of ROS which cannot be properly removed due to aberrant PI3K activation that leads to FoxO inhibition. Because there are defects in ROS removal, there is an increase in DNA damage, further contributing to cancer progression.

How does this differ in pluripotent stem cells? In a paper published in 2010, it was reported that a FoxO orthologue, FoxO1, is essential for hESC pluripotency by mediating activation of Oct4 and Sox2, two genes necessary for hESC "stemness" (Zhang et al. 2011). The researchers also observe that although Akt phosphorylates FoxO1 in human ESCs, the phosphorylated FoxO1 remains in the nucleus and is active. This independence of Akt inhibition is a significant difference compared to what occurs in cancer cells. In cancer cells, phosphorylation of FoxO proteins by Akt results in nuclear export and an inability to activate gene expression. In ESCs, Akt phosphorylation of FoxO1 does not result in FoxO1 removal from the nucleus or a decrease in transcriptional activity. Although PSCs have a similar metabolic profile when compared with cancer cells, one major difference is the regulation of FoxO proteins. Since FoxO proteins are active in PSCs, ROS removal will occur, resulting in a decrease in DNA damage due to oxidative stress.

Levels of ROS are also influenced by p53 and this is important because p53 is often mutated in cancers and is inactive in PSCs. Paradoxically, p53 can activate transcription of both pro-oxidant and anti-oxidant genes and the role p53 will play depends on the cellular context. Following transcription of several pro-oxidant genes, ROS levels are increased and contribute to p53-induced cell death. At the same time, p53 is known to activate expression of glutathione peroxidase, an important antioxidant protein. Although this seems illogical, scientists were able to determine that the level and activity of p53 determines the course p53 will take regarding ROS removal. At physiological levels of p53, there is an increase in transcription of antioxidant genes, resulting in a decrease in ROS, the cell cycle being arrested and DNA being repaired. However, when p53 is upregulated due to cellular stress, there is an increase in pro-oxidant gene expression that is imbalanced compared to the antioxidant gene expression. This results in an increase in ROS levels and cell death (Sablina et al. 2005).

Not only is p53 nuclear activity important, but also p53 localization changes in response to oxidative stress. When there is an increase in cellular ROS, p53 is translocated to the mitochondria and binds directly to the antioxidant

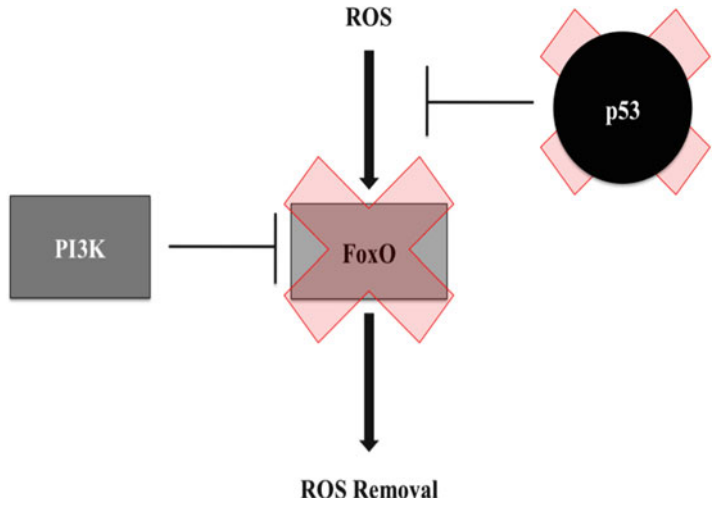

Fig. 8.2 Summary of ROS response in cancer cells. Due to mutations in p53 and inhibition of FoxO proteins, cancer cells cannot protect themselves from DNA damage induced by ROS

MnSOD, inhibiting its activity. This results in a decrease in mitochondrial membrane potential, an increase in ROS formation, and the cell undergoes apoptosis mediated by p53 (Zhao et al. 2005). Like FoxO proteins, p53 is also redox-sensitive, containing cysteine residues in the DNA-binding domain that can serve as redox sensors. Following exposure to ROS, the thiol (–SH) groups in cysteine form disulfide bonds, which can then directly affect the DNA binding and transcriptional activity, resulting in transcription of genes that will protect the cell against harmful ROS damage (Hainaut and Milner 1993). Since p53 is commonly mutated in cancer cells, the protein cannot protect the cells from this oxidative damage, leading to further DNA mutations and cancer progression.

Earlier, it was mentioned that p53 is inactive and mainly localized to the cytoplasm in ESCs even though ESCs are more resistant to oxidative damage due to increases in ROS. In a paper by Han et al. (2008), the researchers investigate the response of ESC to ROS. Following oxidative stress, SIRT1 is activated, resulting in ESC apoptosis and inhibition of the antioxidant role of p53. As previously noted, SIRT1 activation also results in FoxO activation, which can then protect the cell from DNA damage. Oxidative stress also results in repression of Nanog, a very important transcription factor involved in maintaining cells in the pluripotent state. Although p53 is inactive in ESCs and in most cancers, ESCs have a mechanism for responding to oxidative stress through SIRT1 independently of p53.

In summary, because FoxO and p53 function in ROS removal, DNA damage response, cell cycle arrest, and apoptosis, inhibition of these two proteins in cancer cells only leads to cancer progression (Fig. 8.2). Although ESCs also generate a lot of ROS as a result of increased glycolysis, they are able to respond to these increases through indirect activation of FoxO mediated by SIRT1 (Fig. 8.3).

Senescence

Senescence is an irreversible form of terminal differentiation and is thought to protect against neoplasia by inhibiting cellular growth. Both cancer cells and PSCs do not undergo senescence, as both cell types continuously proliferate. However, while cancer cells do not, PSCs maintain their ability to respond to signals that promote senescence.

It has been proposed that mitochondria serve as an important link between the production of ROS and physiological function. Increase in ROS

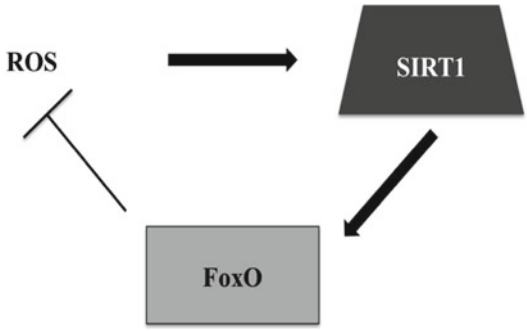

Fig. 8.3 Summary of ROS response in pluripotent stem cells. Pluripotent stem cells react to increases in oxidative stress by activating SIRT1, which then activates FoxO proteins that are responsible for ROS removal

production in mitochondria results in oxidative damage in cells and tissues and contributes to aging, with this affect being observed more in tissues with an intrinsically high amount of mitochondrial activity. The senescence of human dermal fibroblasts was used to study aging *in vitro* and the results showed that mitochondrial respiration and content is increased in senescent cells. In agreement with these findings, a 2-h exposure to H_2O_2 induced an abrupt, senescent-like arrest in human fibroblasts (Chen and Ames 1994). Therefore, an increase in oxidative metabolism results in an overproduction of ROS, which results in oxidative damage and is closely linked to senescence in normal cells.

One major player for inhibiting senescence is PI3K, a kinase that was previously described as being active in cancer cells and in PSCs (Ho et al. 2008). Repression of PI3K in cancer cells resulted in premature senescence and FoxO activation. Further implicating a role for PI3K in senescence, inhibition of PI3K using the chemical inhibitors wortmannin or LY294002 results in cell cycle arrest and cellular senescence in primary MEFs through FoxO-induced p27 expression. Overexpression of FoxO or p27 in MEFs recapitulates the same phenotype. It is therefore possible that senescence cannot be mediated by FoxO activation in cancer cells and PSCs due to the elevated PI3K signaling in both cell types.

In addition to PI3K, the tumor suppressor p53 also regulates senescence. It is well known that p53 activation results in transcription of genes involved in cell cycle regulation. However, this will lead to cell cycle arrest, but must be further maintained for the cell to become senescent. As discussed earlier, p53 has both pro-oxidant and anti-oxidant capabilities, depending on the cellular context. In order to promote cellular senescence, p53's ability to increase cellular ROS results in the activation of mammalian target of rapamycin (mTOR). Although mTOR is usually associated with cellular growth, its function changes to promote senescence under conditions promoting cell cycle arrest (Vigneron and Vousden 2010). Rapamycin, a small molecule inhibitor of mTOR, is known to inhibit proliferation of many cancers, signifying the active state of mTOR in cancer cells. Taken together, p53 inhibition and mTOR activity in transformed cells results in the ability of mTOR to promote cell proliferation and not to function in senescence. The role of mTOR is also similar in ESCs, promoting cell proliferation and inhibiting differentiation (Zhou et al. 2009). In summary, oxidative stress in normal cells results in p53 activation and the inhibition of the cell cycle. The interaction between p53 and mTOR in this circumstance leads to cellular senescence. In cancer cells and ESCs, p53 is inactive and cannot function together with mTOR to promote senescence. At the same time, mTOR will be free to regulate cellular proliferation, the exact opposite of the desired response.

The Rb protein is also involved in cell cycle regulation and senescence. It is commonly mutated in cancer cells and is expressed yet inactive in ESCs. Cyclin-dependent kinases (Cdks) phosphorylate and inactivate Rb, promoting entry into the S phase of the cell cycle. In rapidly dividing cells, Cdks are active, resulting in Rb inhibition and cell cycle progression. The Cdks are inhibited in senescent cells and Rb can effectively inhibit cell cycle progression. Because Rb is commonly mutated in cancer cells and inactive in pluripotent stem cells, Rb-mediated cell cycle inhibition does not occur. Upstream, the Rb proteins are positively regulated through FoxO. Activation of FoxO due to oxidative stress results in transcription of the genes p21 and p27, the protein products of which inhibit Cdks and result in Rb activation.

In human cells, once Rb is fully engaged, senescent growth arrest is irreversible and subsequent inactivation enables senescent cells to reinitiate DNA synthesis, but the cells fail to complete the cell cycle (Beausejour et al. 2003).

It appears that p53 works cooperatively with Rb to induce and maintain senescence. Following Rb and p53 inactivation in senescent MEFs, the cells re-enter the cell cycle, suggesting that both proteins are required for the onset and maintenance of senescence (Sage et al. 2003; Dirac and Bernards 2003). The link between Rb and p53 in controlling cellular senescence is hypothesized to be the p21 protein. The p21 gene is a p53 and FoxO transcriptional target and the protein product is involved in DNA damage-induced cell cycle arrest, cellular senescence, and terminal differentiation. As mentioned previously, the downstream activity of p21 is to inhibit Cdks, resulting in Rb activation. To illustrate how this pathway works in transformed cells, loss of function of p53 and FoxO proteins results in an inability to decrease ROS and increases in ROS induced DNA damage. Transformed cells also lose control of the cell cycle due to mutations in the above-mentioned genes and also in the Rb protein. This results in a population of cells that show increases in anaerobic metabolism and ROS levels, but are incapable of removing ROS and inhibiting the cell cycle.

How are these proteins interacting in PSCs? It appears that ESCs express very low levels of Cdk D-type cyclins, major regulators of the Rb protein. They are also resistant to the growth-inhibitory effect of the cyclin D inhibitor, p16. This implies that ESCs have a different mechanism of cell cycle control that does not rely on Rb inhibition. This decrease in Rb reliance can be compared to the high incidence of Rb loss of function mutations in cancer cells. As ESCs differentiate, the expression of the D-type cyclins is resumed, suggesting that differentiation induces a "normal" cell cycle control mechanism. As mentioned earlier, ESCs express high levels of p53, but keep it inactivated by sequestering the protein in the cytoplasm. Forced entry of p53 into the nucleus fails to induce cell cycle arrest, suggesting that it is not merely p53 location that prevents expression of target genes involved in cell cycle regulation. It has also been noted that the cell cycle regulatory function of p53 is restored upon differentiation (Savatier et al. 2002). So, by what mechanism do ESCs protect themselves from losing control of the cell cycle?

Perhaps a plausible explanation is the ability of ESCs to respond to oxidative stress by activating SIRT1, indirectly allowing FoxO proteins to become transcriptionally active and regulate the cell cycle. Also, we have already seen that Akt induced phosphorylation of FoxO1 proteins in ESCs does not affect the ability of FoxO1 to regulate genes. Therefore, one of the ways that ESCs could control the cell cycle is through activation of FoxO, a protein that is inactive in transformed cells due to Akt activation.

Cancer Cells and Glutamine

While cancer cells depend on aerobic glycolysis for their continued growth, they often secondarily also become addicted to glutamine (Eagle 1955). Illogically, glutamine is a nonessential amino acid that can be synthesized from glucose. To understand the reason for this glutamine addiction, it is important to note that in order for cells to proliferate, there is a requirement for production of nucleotides and amino acids for the synthesis of DNA, RNA and protein macromolecules. Based on its molecular structure, when catabolized glutamine specifically contributes nitrogen from its amide group for the biosynthesis of new macromolecules. However, the high rate of glutamine uptake exhibited by glutamine-dependent transformed cells does not appear to result solely from its role as a nitrogen donor. Instead, glutamine plays a required role in the uptake of essential amino acids and in maintaining activation of mTOR complex 1 (mTORc1). In response to PI3K/Akt activation, mTORc1 is activated and can activate the p70S6 Kinase 1 (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1). Both S6K1 and 4E-BP1 are key players in eukaryotic mRNA translation and play an important role in a rapidly dividing cell by aiding in rapid protein synthesis. As mentioned previously,

ROS-induced activation of p53 coupled with mTOR activation results in senescence. However, because of p53 inhibition and increases in glutamine concentration in cancer cells, mTOR is unable to promote senescence and instead promotes synthesis of biological molecules and cellular proliferation.

While cancer cells are termed "glutamine traps" because of their ability to deplete glutamine stores in the host, stem cells also rely on glutamine for continued proliferation, but react differently when in an environment containing an excess amount of glutamine. For instance, human mesenchymal stem cells, a type of adult stem cell, consumes glutamine preferably over other amino acids (Higuera et al. 2012). Furthermore, metabolism and cell proliferation of adipose derived stem cells from the rabbit slows down in the presence of very low glutamine concentrations (Follmar et al. 2006). Because these proliferative adult stem cells demand the synthesis of biological macromolecules, decreases in the glutamine supply seem to thus halt their ability to self-renew. However, adult stem cells only have a limited capacity to self-renew and senesce over time in culture. So, what happens when the indefinitely proliferating pluripotent stem cells are in an environment that contains an excess of glutamine? In fact, according to very recent study in 2011, PSCs appear to differentiate when they are in an environment that has a high glutamine content (McIntyre et al. 2011). Although human ESCs metabolize glutamine, this implies that excess extracellular glutamine concentrations could trigger human ESCs to no longer self-renew. This seems to be a fundamental difference between transformed cells and pluripotent stem cells.

In conclusion, cancer cells and pluripotent stem cells both exhibit an increase in anaerobic metabolism and glutamine utilization that must be beneficial for rapidly dividing cells. However, both cell types respond differently to increases in oxidative stress, cell cycle regulation and extracellular glutamine concentration. In this review, it has been demonstrated that FoxO, p53, and various other cell cycle regulators are the key players that are responsible this difference.

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Treatment of Patients with Long-QT Syndrome: Differentiation of Patient-Derived Induced Pluripotent Stem Cells into Functional Cardiac Myocytes

9

Daniel Sinnecker, Tatjana Dorn, Ralf J. Dirschinger, Alexander Goedel, Alessandra Moretti, and Karl-Ludwig Laugwitz

Contents

Abstract

The long-QT syndrome, which can be inherited (e.g. due to mutations in genes encoding cardiac ion channels) or acquired (e.g. due to the application of certain drugs), is characterized by a prolonged QT interval in the ECG which is caused by prolonged action potentials of single cardiomyocytes. Affected patients are susceptible to ventricular arrhythmias like torsades de pointes, which lead to palpitations, syncopes, or even sudden cardiac death. Induced pluripotent stem cell lines generated from skin fibroblasts of patients affected by congenital long-QT syndromes can be differentiated to cardiomyocytes, in which the pathophysiology of the disease can be studied *in vitro*. These patient-specific cardiomyocytes recapitulate typical features of the disease like a prolonged action potential duration, a defect of the physiological shortening of the action potential duration under conditions of increased heart rate, and a susceptibility to arrhythmogenic early afterdepolarizations. Moreover, the therapeutic effect of beta receptor antagonists can be recapitulated *in vitro* in these cells. Such patient-specific induced pluripotent stem cell models of the long-QT syndrome might be used in the future to screen for new drugs, to avoid unwanted drug side effects, and to individualize drug therapy.

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Long-QT Syndrome: Clinical Features and Pathophysiology

Long-QT syndrome is an etiologically heterogeneous group of disorders which leads to a prolonged QT interval in the ECG, occurring as the electrophysiological consequence of a prolongation of the action potential duration of single cardiomyocytes in the ventricle of the heart. Affected patients suffer from palpitations, syncopes, or even sudden cardiac death, which is typically caused by ventricular arrhythmias. The characteristic type of ventricular arrhythmia found in patients with long-QT syndrome has been termed "torsade de pointes" (Dessertenne 1966), meaning "twisting spikes" and referring to the typical ECG pattern of this arrhythmia in which the electrical axis (and, concomitantly, the vector of the spikes) changes gradually from beat to beat, apparently rotating around the electrical axis. Torsades de pointes are most often self-limiting, manifesting as palpitations or syncope; however, they can degenerate to ventricular fibrillation, which results in sudden cardiac death unless the patient is promptly subjected to cardiopulmonary resuscitation.

Long-QT syndrome can be a congenital disease, caused by mutations in several genes affecting key components of the machinery responsible for the generation and regulation of the cardiac action potential, or "acquired", that is, caused by exogenous factors like the administration of certain drugs. However, as detailed later, genetic factors also play a role in the acquired forms of the long-QT syndrome.

A prevalence of the congenital forms of the long-QT syndrome in the range of 1:2,000 has been estimated on the basis of ECG screening of neonates (Schwarz et al. 2009). Currently, mutations in 13 genes leading to 13 subtypes of congenital long-QT syndrome (LQT1-LQT13) have been described (Lehnart et al. 2007; Wu et al. 2008; Yang et al. 2010). The affected genes encode subunits of cardiac potassium (LQT1-2, LQT5-7, LQT13), sodium (LQT3, LQT10) or calcium channels (LQT8), as well as proteins involved in the regulation of or physically interacting with cardiac ion channels or transporters (LQT4, LQT9, LQT11, LQT12). While a prolongation of the QT interval and the susceptibility to ventricular arrhythmia are the common hallmark of the long-QT syndrome, the genetically-defined subtypes differ in several clinical aspects. For example, episodes of torsade de pointes are typically triggered by physical activity or emotional stress in LQT1, while in LQT3, they are precipitated by bradycardia and typically occur during sleep (Schwartz et al. 2001). Accordingly, the recommended therapies differ between long-QT syndrome subtypes.

The prevalence of the acquired long-QT syndrome exceeds that of the congenital forms. Several common conditions, such as serum electrolyte imbalance, hypothyreosis, advanced heart failure or, most importantly, the administration of several cardiac or non-cardiac drugs, may lead to a prolongation of the QT interval and an increased susceptibility to torsades de pointes. Acquired long-QT syndrome has become a major problem in drug development in the recent time. Since many new candidate drugs turn out to prolong the QT interval in some patients upon broad clinical application, and due to the potentially fatal consequence of such an effect, drug-induced QT interval prolongation has become the most important cause for use restrictions or the withdrawal of drugs from the US market (Lasser et al. 2002).

Two basic phenomena are responsible for the occurrence of ventricular arrhythmias in patients with a prolonged QT interval: early afterdepolarizations (EAD) and a phenomenon called the dispersion of repolarization (Eckardt et al. 1998; Antzelevitch 2005). EAD are plasma membrane depolarizations that occur during the second or third of the four phases of the cardiac action potential, that is, before complete repolarization is achieved. These EAD frequently develop under the conditions of increased action potential duration that are pathognomonic for the long-QT syndrome. EAD can give rise to single premature action potentials or to series of premature action potentials which are called triggered activity. The term dispersion of repolarization describes the observation that, across the ventricular myocardium, not

all cells share the same action potential duration. Specifically, the so-called M cells (the midmyocardial cells) have a longer action potential duration than the adjacent subepicardial or subendocardial cells. Moreover, the M cells are particularly sensitive to a prolongation of the action potential in response to drugs or physical stimuli. Thus, in long-QT syndrome, conditions that lead to a QT interval prolongation also lead to an increase in the dispersion of repolarization. This dispersion of repolarization serves as the substrate on which EAD can trigger reentrant arrhythmias like torsades de pointes.

Optimizing the Therapy for Long-QT Syndrome: The Need for Disease Models

In the age of molecular medicine (Strasser 1999), a precise understanding of the molecular pathophysiology of a disease is thought to provide the basis for the development of specific therapies. Since viable human cardiac tissue for physiological experiments is not easily obtainable, model systems are indispensable to study human heart diseases at the molecular level. Accordingly, most of our current understanding of the molecular pathophysiology of long-QT syndromes comes from animal models. Cardiomyocytes isolated from hearts of small animals, which have served cardiac electrophysiologists as standard tools for a long time, have been used to investigate the pathophysiology of the long-QT syndrome, for example by pharmacologically blocking the potassium channel that is known to be affected in LQT2 and LQT6 (Studenik et al. 2001). However, the properties of an ion channel blocked by a drug might very well differ from those of the same channel affected by a disease-causing mutation. Moreover, the specificity of pharmacological channel blockers is another issue relevant to the extrapolation of the results from such studies to human pathophysiology.

An important step towards molecular modeling of congenital long-QT syndromes has been the generation of several genetically-modified mouse models (Salama and London 2007). However, the findings in these mouse models do not always correspond to those in human patients. For example, LQT1 has been modeled by targeting the KCNQ1 locus in the mouse (encoding the α subunit of the ion channel responsible for the slow component of the delayed rectifier potassium current, I_{Ks}), which is mutated in human LQT1. Targeted disruption of this locus led to a mouse line displaying a prolonged QT interval (Casimiro et al. 2001). However, in another mouse line, disruption of the same locus did not lead to any cardiac phenotype (Lee et al. 2000). A mouse line characterized by cardiac-specific overexpression of a dominant-negative KCNQ1 ion channel subunit (Demolombe et al. 2001) showed a markedly prolonged QT interval, but also additive features not typically found in human patients affected by LQT1, such as sinus node dysfunction and atrio-ventricular block.

Besides the above-mentioned inconsistencies between mouse models and the corresponding human long-QT syndrome subtypes, some general aspects make small animals far-from-ideal models for arrhythmogenic human heart disease. Since the heart rate of a mouse is about ten times faster than that of a human, major differences exist between mice and men in the shape of their cardiac action potentials and the underlying ionic currents (London 2001; Nerbonne et al. 2001). An example relevant for the modeling of long-QT syndromes is that the major repolarizing currents in human myocardium are the fast and slow components of the delayed rectifier potassium current (I_{Kr} and I_{Ks}). Ion channel subunits responsible for these currents are affected in LQT1, LQT2, LQT5 and LQT6. However, the density of these currents in murine cardiomyocytes is low, while other currents like the transient outward potassium current $I_{to,f}$ are important for cardiac repolarization in the mouse (Nerbonne et al. 2001).

While the above-mentioned animal models have undoubtedly provided important insights into the pathophysiology of the long-QT syndrome, the physiological differences between human and rodent cardiomyocytes call for human cell-based disease models, as provided by the patient-specific induced pluripotent stem cell models described below.

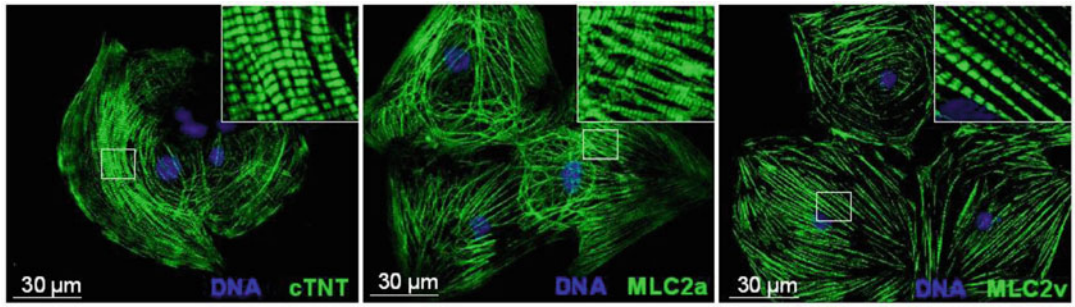

Fig. 9.1 Cardiomyocytes derived from human induced pluripotent stem cells. Immunofluorescence images of representative iPSC-derived cardiomyocytes stained for cardiac troponin T (cTnT) and the atrial (MLC2a) as well as the ventricular (MLC2v) isoform of myosin light chain as indicated are shown. The *insets* show magnifications of the areas indicated by the *white squares*

Modeling Human Heart Disease with Patient-Specific Induced Pluripotent Stem Cells

The first report of the reprogramming of somatic cells to induced pluripotent stem cells (iPSC) by overexpression of a specific set of transcription factors (Takahashi and Yamanaka 2006) has attracted a great deal of interest among scientists interested in modeling human heart disease. Induced pluripotent stem cell technology bears the potential to derive patient-specific stem cell lines from somatic cells taken from patients affected by genetically-caused diseases like congenital long-QT syndrome. These patient-specific iPSC lines could be then differentiated to the type of cells that are normally affected by the disease (e.g., cardiomyocytes), and the pathomechanisms of the disease could be studied *in vitro* in these cells. The first diseases for which such patient-specific human iPSC lines have been reported are neurodegenerative (Ebert et al. 2009; Lee et al. 2009) and hematological (Ye et al. 2009) diseases.

The human somatic cells that are reprogrammed to generate patient-specific iPSC lines can be fibroblasts (Takahashi et al. 2007), which may be isolated from a small skin biopsy performed under local anesthesia. Other methods that require even less-invasive cell harvesting procedures like reprogramming of keratinocytes from follicles of plucked hairs (Novak et al. 2010) or of blood lymphocytes (Brown et al. 2010) are being developed. Reprogramming is usually achieved by retroviral gene transfer of the transcription factors Oct3/4, Sox2, c-Myc and Klf-4. However, several variations of this basic technique have been developed (Sidhu 2011).

The protocols used to differentiate the iPSC to cardiomyocytes are usually based on differentiation in so-called "embryoid bodies", which form spontaneously when the cells are aggregated under the right culture conditions (Keller 1995). In these three-dimensional structures, spontaneous differentiation into different lineages occurs, eventually leading to differentiation to cardiomyocytes, which can be promoted by several cell culture supplements (Takahashi et al. 2003; Tran et al. 2009). After 10–20 days of differentiation, the cardiomyocytes, which are typically clustered, can be identified visually by spontaneous contractions. These contracting areas can be manually dissected from the rest of the embryoid bodies and kept in culture to allow further maturation of the cardiomyocytes. For experiments with single cardiomyocytes, these explants can be dissociated by collagenase digestion. Figure 9.1 shows immunofluorescence images of human iPSC-derived cardiomyocytes. The cells are characterized by expression of the cardiac isoform of troponin T (cTnT). A subpopulation of these cells stains positive for the atrial isoform of myosin light chain (MLC2a), while another subpopulation expresses the ventricular isoform (MLC2v). In a fraction of the cells, co-expression of both isoforms is observed.

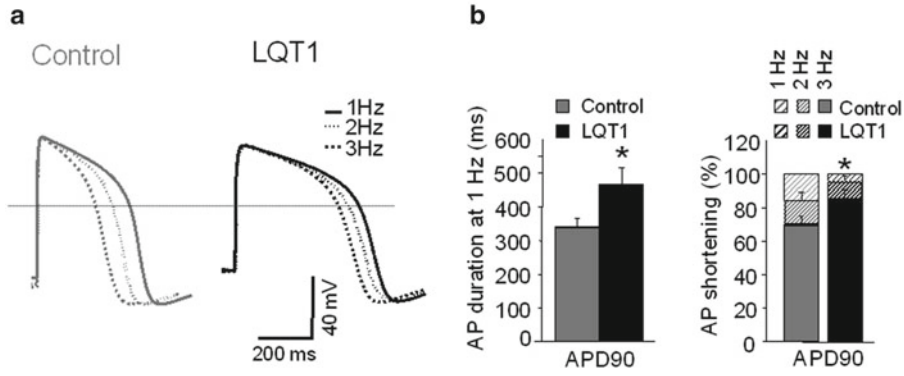

Fig. 9.2 Altered action potential characteristics in LQT1 cardiomyocytes (**a**) Representative tracings of action potentials (AP) recorded from cardiomyocytes derived from control and KCNQ1-R190Q (LQT1) iPSC lines at three different pacing rates (1, 2, and 3 Hz) are shown. (**b**) Statistics of action potential characteristics are shown. The *left bar graph* shows statistics for the absolute value of APD90 (the duration from the beginning of the action potential until repolarization is accomplished by 90%). The *right bar graph* shows the relative shortening of the APD90 upon increasing the pacing rate from 1 to 2 or 3 Hz as indicated (Adapted from data published in Moretti et al. 2010)

Induced Pluripotent Stem Cell Models for the Long-QT Syndrome

We have modeled the long-QT syndrome type 1 (LQT1) by reprogramming skin fibroblasts from two patients (father and son) affected by LQT1 caused by a heterozygous mutation of the KCNQ1 locus, leading to an amino acid exchange (R190Q) in the KCNQ1 protein which constitutes the α subunit of the ion channel responsible for the I_{Ks} current (Moretti et al. 2010). As a control, we have generated iPSC lines from a healthy individual unrelated to the patient. R190Q and control cardiomyocytes were generated from these iPSC lines by embryoid body differentiation and analyzed at the single-cell level by patch clamp electrophysiology.

When action potentials of ventricular cardiomyocytes were elicited by electrical pacing at 1 Hz in R190Q and control cells, it was obvious that the R190Q cardiomyocytes displayed prolonged action potentials as compared to control cells (Fig. 9.2). The same observation was made for spontaneously occurring action potentials in unstimulated ventricular cells. When the control cells were paced at different rates, it was observed in control cells that increasing the pacing rate led to a decrease of the action potential duration (APD, Fig. 9.2). This is consistent with normal cardiac physiology, where the QT interval (which reflects the action potential duration of ventricular cardiomyocytes) decreases with increasing heart rates. In the R190Q cardiomyocytes, the action potentials were already prolonged at a slow pacing rate (1 Hz) and the decrease of the APD at increased pacing rates was blunted (Fig. 9.2). Similarly, catecholamine stimulation led to a much lesser reduction of the APD in R190Q cells than in control cardiomyocytes. This points out that in the R190Q cardiomyocytes, the APD is not only prolonged under basal conditions, but the normal regulation mechanisms which lead to a shortening of the action potential at situations of increased catecholamine stimulation or heart rate (which are the situations in which LQT1 patients typically develop torsades de pointes) are dysfunctional. This was corroborated by an experiment in which spontaneously beating cardiomyocytes were subjected to stimulation with the catecholaminergic agonist isoproterenol (Fig. 9.3). In control cells, this led to an increased beating rate, but concomitantly to a decreased APD, resulting in a reduction of the ratio between the APD90 and the beat-to-beat interval. In the R190Q cells, however, the increased beating rate could not be compensated by a shortening of the action potentials, indicated by a increase in the APD90/beat-to-beat interval ratio. Moreover,

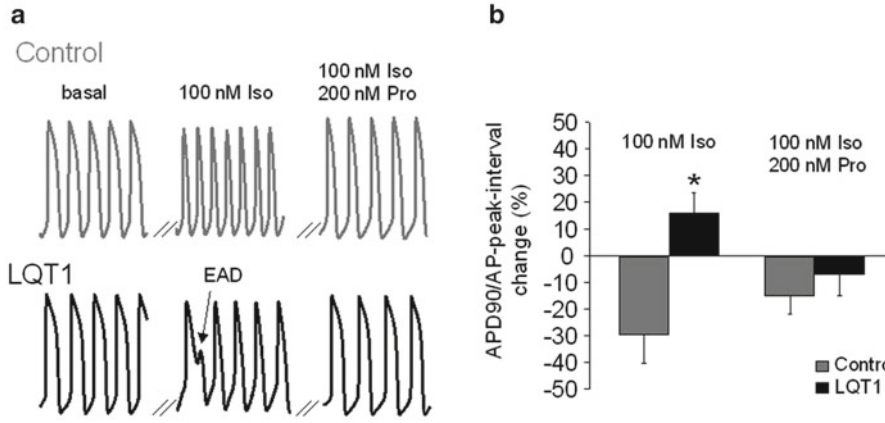

Fig. 9.3 Arrhythmogenicity and therapeutic effect of beta blocker in LQT1 cardiomyocytes. (**a**) Representative membrane potential recordings from spontaneously beating control and LQT1 cardiomyocytes before and after incubation with 100 nM isoproterenol (Iso), in the presence or in the absence of 200 nM propranolol (Pro), are shown. In the tracing from the LQT1 cell, an early afterdepolarization (EAD) is indicated by an *arrow*. (**b**) The *bar graph* shows the statistics for the ratio of the APD90 divided by the interval between two action potentials under isoproterenol stimulation in the absence and in the presence of propranolol (Adapted from data published in Moretti et al. 2010)

under conditions of catecholamine stimulation, the cells frequently displayed EAD. The β receptor antagonist propranolol (reflecting a class of medications that is typically beneficial for patients affected by LQT1) reversed both effects in the R190Q cells (Fig. 9.3).

The KCNQ1 gene mutated in the LQT1 patients encodes the α-subunit of the ion channel responsible for the I_{Ks} current. We therefore measured I_{Ks} in the R190Q cardiomyocytes and found that I_{Ks} was reduced to about 25% as compared to control cardiomyocytes. This reduction by more than 50% indicated that the mutation might exhibit a dominant-negative effect in the cells heterozygous for the R190Q mutation. Indeed, we could demonstrate that the R190Q mutation exerts a dominant-negative effect by forming multimers with wild type subunits and interfering with their trafficking to the plasma membrane (Moretti et al. 2010). Other groups have used patient-specific iPSC lines in a similar approach and successfully modeled other subtypes of the long-QT syndrome like LQT2 (Itzhaki et al. 2011; Matsa et al. 2011) and LQT8 (Yazawa et al. 2011).

Outlook

So far, most of the reports of induced pluripotent stem cell models for long-QT syndromes have been, at least to a large extent, proof-of-principal studies. However, the motivation behind the development of such models is the wish to broaden our understanding of the pathophysiology of these disorders and to improve the treatment of affected patients.

One way by which iPSC-based disease models could improve the treatment of patients would be the use of the patient-specific iPSC-derived cardiomyocytes as a platform for drug screening. For example, in a recent high-throughput drug screening approach based on a zebrafish long-QT model (Peal et al. 2011), new small molecule compounds were found that shortened the QT interval. However, due to the electrophysiological differences between human and zebrafish, the translatability of these results to human patients is questionable. The use of iPSC-derived human cardiomyocytes in similar screening experiments might provide a way to identify new drugs for the treatment of long-QT syndromes.

Human cardiomyocytes derived from iPSC might be also helpful to address the problem of

acquired long-QT syndrome in drug development. A test system consisting of human iPSC-derived cardiomyocytes that are used to screen possible drug candidates for action potential prolongation can be easily envisioned. However, several approaches might be pursued to select the best iPSC lines for such a test system. Since it is known that modulation of HERG channel activity is a frequent mechanism of drug-induced QT interval prolongation (Sanguinetti et al. 1995), iPSC derived from a LQT2 patient carrying a HERG mutation might be used. However, acquired long-QT syndrome is typically a problem of patients without mutations in their HERG locus. Since it is known that a genetic predisposition plays a role in acquired long-QT syndrome (although the involved genes are not known; Kannankeril et al. 2005), iPSC lines from patients that have already reacted to different drugs with a prolonged QT interval could be used. Finally, another strategy would be to use a panel of iPSC lines derived from genetically heterogeneous probands that are representative for the population in which the candidate drug will be used.

Finally, another area that could be brought forward by patient-specific iPSC lines is personalized drug therapy. It is well known that the response of different patients to a specific drug can vary considerably, both in terms of efficacy and in terms of unwanted side effects. It often has to be empirically tested which of several drugs that could be used to treat a certain condition fits best with the specific genetic background of a patient. In the case of long-QT syndrome, cardiomyocytes derived from a patient-specific iPSC line could be used as an *in vitro* system to test several antiarrhythmic drugs and to select the regimen that is most effective without leading to potentially dangerous adverse effects such as proarrhythmia.

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Part III

Embryonic Stem Cells

Protein Kinase C Signaling in Embryonic Stem Cell Self Renewal and Cardiac Differentiation

10

Deborah Schechtman and Denise Aparecida Berti

Contents

Abstract

Embryonic stem cells (ESC); proliferate while maintain the ability to differentiate into several cell types (self-renewal). For the efficient use of ESC in cell therapies it is necessary to characterize the specific signaling pathways that lead to ESC differentiation, proliferation and self-renewal. The Protein kinase C (PKC) family of serine/threonine kinases has been identified as key enzymes for the processes of proliferation and differentiation of ESCs to cardiomyocytes however, the exact function of each PKC isoenzyme remains unclear, and this is partially due to the conserved nature of these kinases and the fact that specific modulators have only recently become available. In the present chapter we discuss recent studies describing the function PKC isoenzymes in murine ESC proliferation, self renewal and cardiac-differentiation.

Introduction – The Protein Kinase C (PKC) Family of Kinases

The Protein kinase C (PKC) family of serine/threonine kinases is composed of at least ten isoenzymes subdivided into three families according to their activation requirements. In mammals different genes code for different isoenzymes except for βPKC in which βI and βII are alternatively spliced forms and differ only in the last 43 amino acids. PKCs are composed of multiple

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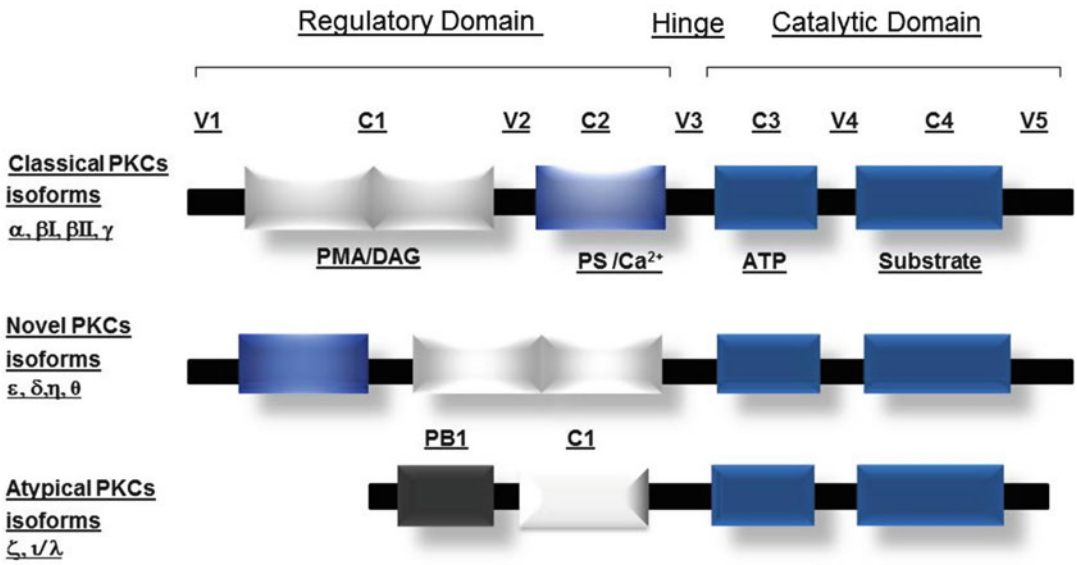

Fig. 10.1 Schematic representation of the, conserved (*c*) and variable domains (*V*) in the different PKC families of isoenzymes. Phorbol ester (PMA)/diacylglycerol (DAG), Ca^{2+}, ATP and substrate binding sites in a and cPKCs are indicated. The PB1 protein interaction domain of aPKC is also indicated (Adapted from Newton 2001)

domains and structurally can be subdivided into a regulatory N-terminal domain followed by a flexible region known as the hinge and a catalytic domain. The regulatory domain is responsible for binding of co-factors necessary for PKC activation and thus subfamily classification of PKCs depends on their regulatory domain responsible for activation requirements: (1) classical PKCs (cPKCs); PKCα, βI, βII, and γ, require calcium, phosphatidylserine and diacylglycerol (DAG) for their activation; (2) novel (nPKCs), δ, ε, η, and θ, are calcium-independent and require the same lipids as cPKCs for their activation; (3) atypical PKCs (aPKCs), ζ and λ/ι, are both calcium-independent and DAG-insensitive (Newton 2001) and can be activated by different lipids such as ceramide. PKCs are multi-domain proteins composed of more conserved (C) and variable (V) domains (Fig. 10.1). The double C1 domain confers binding to diacylgycerol and its analogue phorbol ester. The C2 domain binds anionic phospholipids such as phosphatidylserine and phosphatidylinositol-4,5-bisphosphate (PIP2) and is responsible for Ca^{2+} binding in cPKCs. Novel (nPKCs) lack Ca^{2+} binding residues in their C2 domain and have a greater affinity for diacylgycerol conferred by differences in their C1 domains. Atypical (aPKCs) possess a single C1 domain and are not capable of binding diacylglycerol and have specific protein-protein domains such as the PB1 domain, a dimerization/oligomerization domain that interacts with proteins containing an acidic OPCA (OPR/PC/AID) motif such as PAR-6 and p62/ZIP (Fig. 10.1), frequently found in scaffolds (Moscat et al. 2009).

PKC activation is accompanied by translocation, from the cytosol to membranes including plasma membrane and membranes of organelles. Activation involves conformational changes resulting in exposure of domains required for catalytic function and PKC anchoring to membranes and anchoring proteins (Schechtman and Mochly-Rosen 2001). Despite their similarities, individual PKCs mediate unique cellular functions phosphorylating select substrates and leading to specific physiological responses. Multiple PKC isoenzymes may contribute to specific signaling events and even exhibit opposing functions (Schechtman and Mochly-Rosen 2001). We and others observed in a variety of cell types that different PKC isoenzymes translocate to distinct subcellular locations (Costa-Junior et al. 2010).

Besides substrate interactions with PKCs at their catalytic domains other protein-protein interactions have been described. In c and nPKCs these interactions have been shown to occur in C1, C2 (also known as V1 domain in nPKCs) and V5 domains and in aPKCs in the PB1 domain (Fig. 10.1). Protein-protein interactions involving PKCs have been shown to be responsible mainly for: (1) enzyme maturation, (2) maintaining PKCs in its inactive conformation, (3) anchoring active kinase to specific subcellular locations (4) creating signaling complexes facilitating and determining specificity of interactions between PKC isoenzymes and their substrates (Mochly-Rosen 1995; Newton 2001; Schechtman and Mochly-Rosen 2001).

Due to the highly conserved nature of the catalytic domain of PKCs it has been difficult to develop PKC isoenzyme selective inhibitors. Taking advantage of PKC isoenzyme-specific protein-protein interactions PKC isoenzyme-specific modulator peptides that inhibit/enhance depict protein-protein interactions have been developed and used to elucidate and interfere with specific PKC isoenzymes. One example of such interactions is the interaction between PKCs and isoenzyme specific adaptor proteins that bind only active PKC, known as Receptor for activated C-kinase (RACK). RACKs exclusively bind to active PKCs and aid in establishing subcellular localization of PKC isoenzymes and interactions with their substrates (Schechtman and Mochly-Rosen 2001). PKC isoenzyme specific inhibitor peptides were derived from RACK binding sites in PKCs and compete for PKC binding to RACKs. PKC agonists are peptides that inhibit an intramolecular interaction between a site in PKC that binds to RACK (PKC-RACK binding site) and a sequence homologous to RACK named ψRACK, that helps maintain PKC in an inactive conformation (Mochly-Rosen 1995; Schechtman and Mochly-Rosen 2001).

Other intramolecular interactions also occur in inactive PKCs, amongst them the pseudosubstrate interaction with the catalytic domain is key in maintaining PKCs in an inactive conformation. Peptides derived from the pseudosubstrate act as inhibitor peptides competing for substrate binding. PKC autophosphorylation and phosphorylation by Phosphoinositide-dependent kinase 1 (PDK1) are responsible both for PKC maturation and activation (Newton 2001).

Protein Kinase C and Embryonic Stem Cell Self-Renewal

Self-renewal is the ability to proliferate maintaining pluripotency. Several known and yet unknown signaling pathways are responsible for the maintenance of self-renewing embryonic stem cells (ESC). These cells are maintained in culture in the presence of Leukemia inhibitory factor (LIF). LIF is a cytokine of the IL-6 family that binds directly to a heterodimeric receptor complex composed of two transmembrane proteins, gp130 and LIF receptor (LIFR), the activation of this heterodimer by LIF stimulates the Janus kinase (JAK) and Signal transducer and activator of transcription (STAT) signaling pathway. STAT3 is the principal STAT transcription factor activated in ESC involved with self-renewal. STAT3 activates the transcription of Inhibitor of differentiation (ID) genes (Ying et al. 2008). Inhibitors of specific signal transduction pathways, such as inhibitors of Fibroblast growth factor receptor (FGFR), Extracellular regulated kinase (ERK) and Glycogen synthase kinase 3 (GSK3) maintain ESC undifferentiated in culture (Ying et al. 2008).

Murine embryonic stem cells (Costa-Junior et al. 2010; Dutta et al. 2011; Zhou et al. 2003) have been shown to express mainly PKCs α, βI βII, δ, e and ζ/λ. A similar profile of expression was found in developing blastocysts (Dehghani and Hahnel 2005). We also determined the subcellular localization of these isoenzymes in undifferentiated murine ESC and observed that βIPKC is mainly expressed in the nucleus of undifferentiated ESC. PKC isoenzymes α, δ and ε are dispersed throughout the cytoplasm and ζ/λ PKCs are expressed throughout the cell and in cell contact regions (Costa-Junior et al. 2010). βIIPKC was localized at the perinuclear region and in a dot like structure within the nucleus. The different subcellular localizations of PKC isoenzymes in undifferentiated ESC suggest that each kinase has a different function in these cells.

To elucidate the function of different PKC isoenzymes in undifferentiated ESC we used a phosphoproteomics strategy and a βIPKC specific inhibitor peptide, βIV5-3, that prevents the interaction of βIPKC with its RACK, to detect βIPKC targets. We found that βIPKC regulates the phosphorylation of proteins involved in transcription of proteins that have a role in proliferation/differentiation (Costa-Junior et al. 2010). Amongst the βIPKC targets we found hnRNPC regulates protein expression at different stages of the cell cycle and Histone-binding protein RBBP4 (RbAp48), is involved in chromatin remodeling during the cell cycle, splicing factor (SRP20) and SUMO-activating enzyme subunit 1 (Sae1) are involved in self-renewal. The heterogenous ribonuclear protein K (hnRNPK) is involved in the transcriptional activation of the pro-oncogene, c-myc and the actin-like protein 6a (Baf53a), is critical for the oncogenic activity of c-Myc (Costa-Junior et al. 2010). The Myc family of transcription factors has been shown to regulate differentiation, self-renewal and proliferation. Recently Myc has also been shown to be involved in the regulation of ribosomal biogenesis (Van Riggelen et al. 2010). Translation elongation factor (eEF1A), another βIPKC target we detected, was also described as a βIPKC substrate in the mouse myoblast cell line C2C12. In these cells, βIPKC was also reported to be localized in the nucleus and to phosphorylate eEF1A upon insulin stimulation possibly having a role in ribosomal biogenesis (Piazzi et al. 2010). Ribosomal biogenesis has been suggested to occur during cell proliferation, differentiation and self-renewal (Van Riggelen et al. 2010). We observed that concomitant to a decrease in Oct3/4 expression some cells stopped expressing βIPKC and in others βIPKC was found in the cytoplasm (Costa-Junior et al. 2010). Taken together there is evidence to propose that βIPKC has a role in transcription processes that maintain ESC undifferentiated, or inhibit ESC differentiation (Costa-Junior et al. 2010). The isoenzyme, δPKC, has been shown to be involved in proliferation and apoptosis in different cell types. At low levels of oxygen, murine ESC undergoes differentiation induced by the expression of hypoxia inducible factor-1α (HIF-1α) and δPKC seems to stabililize HIF-1α. Inhibition of δPKC destabilized HIF-1α and maintained ESC pluripotency in hypoxic conditions (Lee et al. 2010), The aPKCs are essential for ESC differentiation and metabolism, once λPKC knockout mice are not viable and ESC derived from these mice have an impaired glucose stimulated insulin transport (Bandyopadhyay et al. 2004). Besides a role in metabolism, aPKCs have also been shown to control apicobasal polarity in early development (Dard et al. 2009).

Using pharmacological inhibitors and interference RNA for ζPKC it was demonstrated that ζPKC inhibition maintains the pluripotency of ESC through yet an alternative pathway that does not activate ID gene expression or inhibition of ERK/GSK3 (Dutta et al. 2011). The authors suggest that one of the mechanisms by which ζPKC could lead to ESC differentiation could be by activation of NF-κB and lineage commitment. Importantly these observations also suggest that there are alternative signaling pathways that maintain ESC undifferentiated (Dutta et al. 2011).

PKC and ESC Proliferation

There are few studies investigating the role of PKCs in ESC proliferation. One of the early studies suggesting a role for PKC signaling in ESC proliferation was carried out by Quilan et al. (2003). In this study using a phospholipase C inhibitor (U-73122), impairing the production of diacylglicerol and inositol phosphate (IP3), and consequently PKC activation led do a decrease in ESC proliferation. Furthermore, treatment with a general c and nPKC activator (diacylglycerol and phorbol ester) increased ESC proliferation. On the other hand the general PKC-inhibitor, bisindolylmaleimide II (Bis II), inhibited it (Quinlan et al. 2003).

Kapur et al. (2007) investigated Ca^{2+} signaling during cell cycle progression in undifferentiated murine ESC. They showed that spontaneous oscillations of Ca^{2+} in ESC cycle progression depends on the presence of IP3-sensitive intracellular Ca^{2+} storage and that these oscillations during ESC cycle were suppressed upon inhibition of phospholipase

C further confirming the involvement of PKC in ESC proliferation (Kapur et al. 2007).

Another study showed that ESC stimulated with epidermal growth factor (EGF) increased connexin 43 (Cx43) phosphorylation leading to an increase in ESC proliferation via Phosphoinositide-3-kinase (PI3K)/Protein kinase B (Akt), PKC, Mitogen activated protein kinase (MAPK) and Nuclear factor kappa B (NF-κB). Likewise, ESC stimulated with 5′-N-ethyllcarboxamidoadenosine (NECA), a non-specific adenosine agonist that also increased Cx43 phosphorylation via Ca^{2+}, also led to an increase in ESC proliferation via PKC and MAPK (Kim et al. 2010a; Park et al. 2008). Angiotensin II or TGF-β were also shown increase proliferation via PKC (Kim et al. 2010b). These studies using distinct stimuli clearly showed an involvement of PKC signaling in ESC proliferation, however, the question of which specific PKC isoenzyme is involved in ESC proliferation and under which conditions still remains.

A few studies have investigated the involvement of specific PKC isoenzymes in ESC proliferation upon stimulation with factors that are important for embryonic development (Heo and Han 2006; Heo et al. 2006, 2007; Kim et al. 2009, 2010a, b; Lee et al. 2009; Park et al. 2008; Yun et al. 2009). Among the proliferation stimuli analyzed, H2O2, ATP, Sonic Hedgehog and linoleic acid, induced mouse ESC proliferation through activation of α, δ and ζPKC isoenzymes (Heo and Han 2006; Heo et al. 2007; Kim et al. 2009; Lee et al. 2009). Murine ESC proliferation was stimulated by prostaglandin E2 (PGE2), and led to αPKC activation, but not to, ε nor ζPKCs activation (Yun et al. 2009). High glucose and angiotensin II activated α, ζ and εPKCs (Kim and Han 2008) and epidermal growth factor activated α, βI, γ, δ and ζPKCs (Heo et al. 2006). The identification of specific PKC substrates upon different stimuli that lead to ESC proliferation can further elucidate the specific role of each PKC isoenzyme in this process. The fact these studies were performed under serum free or low concentrations of serum, which could potentially change the characteristics of the undifferentiated state of these cells, should also be taken into consideration (Schratt et al. 2002).

A study analyzing the different kinases that are activated in ESC proliferation and differentiation upon stimulation with growth factors and extracellular matrix components combined with a computational systems biology analysis demonstrated that the combination of growth factors and extracellular matrix components are also essential to determine ESC self-renewal/differentiation. For example the combination of LIF, fibroblast growth factor-4 (FGF-4) and fibronectin led to differentiation, and εPKC activation induced differentiated cell proliferation but did not affect undifferentiated cell proliferation (Prudhomme et al. 2004).

PKC and ESC Differentiation to Cardiomyocytes

Despite the progress in treating ischemia-induced damage to the heart and heart failure, cardiovascular pathologies still are responsible for a large number of deaths. The use of cell replacement therapies to substitute the loss of cardiomyocytes or endothelial cells, are being considered as alternative therapies. However, we have to overcome several challenges to make cell replacement therapies for cardiovascular diseases a reality, amongst them generating a sufficient number of cardiomyocytes or endothelial cells, and retaining them at the damaged site (Burridge et al. 2012).

Cardiomyocytes were believed to be terminally differentiated non-replicating cells. Recent evidences suggest that these cells can proliferate under specific conditions (Burridge et al. 2012). An encouraging discovery was that the adult heart has few residing cardiac stem cells that upon damage to the heart, such as an ischemic event, are able to proliferate. These cells are heterogeneous, presenting different stem cell markers and are difficult to isolate and propagate, requiring several growth factors when maintained in culture. Efficient methods of differentiation of these progenitor cells both in vivo and in cultures are still under investigation (Burridge et al. 2012).

Takahashi and Yamanaka (2006) demonstrated that by the transfection of four transcription factors

(Oct-3/4, cMyc, SOX2, and Klf4), it is possible to reprogram adult somatic cells generating induced pluripotent stem cells (iPS). Since then several reprogramming protocols have been suggested envisioning the therapeutical use of IPS. One of the requirements to generate Germ-line competent iPS is not only the transfection of transcription factors required for pluripotency such as Oct-3/4, SOX2, c-Myc, and Klf4, but also the activation of endogenous transcription factors and epigenetic modifications (Okita et al. 2007). Therefore, activating specific endogenous signaling pathways may increase the efficiency of generating iPS.

When given appropriate signals in culture, murine (Boheler et al. 2002), human ESC (Zeevi-Levin et al. 2010) and iPS (Mauritz et al. 2008) differentiate into functional cardiomyocytes that have structural properties similar to cardiomyocytes isolated from hearts. However, the percentage of ESC/iPS differentiation to cardiomyocytes is still very low (Burridge et al. 2012).

Recently, it has been shown that direct reprograming of cells to cardiomyocytes, induced cardiomyocytes (iCM), is also possible using three key transcription factors that are activated during heart development (Gata4, Mef2c, and Tbx5). The iCM were epigenetically similar to cardiomyocytes but, presented some differences in gene expression (Ieda et al. 2010).

Cardiac development and differentiation involves transition from the induction of mesoderm progenitors to mesoderm which then differentiate to cardiac mesoderm and subsequently to cardiac progenitor cells that differentiate to immature cardiomyocytes and finally mature cardiomyocytes (Burridge et al. 2012). Different signaling pathways and the addition of defined growth factors and kinase inhibitors at specific stages of cardiomyocyte differentiation contribute to an increase in the number of cardiomyocytes (Burridge et al. 2012). For example, BMP4 and Fibroblast Growth Factor 2 (FGF-2) induce mesoderm, Granulocyte colony stimulating factor (G-CSF) and ascorbic acid induce cardiac progenitor cells and expression of the transcription factors, ISL1, NKX2.5, TBX5, TBX20, MEF2C and GATA4 (Burridge et al. 2012). Inhibition of NOTCH signaling induces mesoderm to cardiac mesoderm transition. These data support the idea that different signaling pathways are activated throughout cardiomyocyte differentiation and that manipulation of these pathways may help increase the number of cardiac progenitors and immature or mature cardiomyocytes.

PKC signaling has been shown to be involved in cardiac development and maturation and is important for physiological and pathological processes of the heart. Changes in expression, activation status and subcellular localization of the different PKC isoenzymes suggest that they will have distinct roles in these processes. Due to the conserved nature of these kinases single knockout mice have not produced significant phenotypes that could help elucidate the involvement of PKC signaling in heart development.

In other systems of differentiation different PKCs have been shown to have even opposite effects for example: αPKC expression enhanced Retinoic acid (RA) and cAMP-induced differentiation of F9 mouse embryonal carcinoma cells into parietal endoderm. Expression of βPKC in a parietal endoderm cell line led these cells to reacquire a "stem cell like" phenotype (Cho et al. 1998).

There is sufficient evidence to suggest that PKC isoenzymes also play an important role in ESC differentiation to cardiomyocytes, however, the exact role of these isoenzymes and specific roles for distinct isoenzymes in the different stages of cardiac differentiation have as of yet to be determined. In culture systems Pan PKC inhibitors, chelerythrine and calphostin C, inhibited ESC differentiation to cardiomyocytes (Ventura et al. 2003). Fibroblast growth factor receptor-1 (FGFR1) is essential for *in vitro* differentiation of ESC to cardiomyocytes, and FGFR1 knockout ESC have a delay and decrease in the number of cells that differentiate to cardiomyocytes. Nevertheless PKC activation of FGFR1 knockout ESC by phorbol ester partially restored cardiomyocyte differentiation (Dell'era et al. 2003), indicating that PKC signaling is downstream of FGFR1 activation.

Activation of opioid receptors was shown to activate PKC and nuclear translocation of (α, βI, and βIIPKCs) was accompanied by an increase in

the expression of the lineage promoting transcription factors (GATA-4 and Nkx-2.5). In this study δ and εPKC were found in the nucleus of both undifferentiated GTR1 and differentiated cardiomyocytes (Ventura et al. 2003). However, the subcellular localization of different PKC isoenzymes throughout differentiation and their role in specific stages of differentiation remains to be determined.

There are few studies characterizing the specific role of the different PKC isoenzymes throughout cardiomyocyte differentiation. Using PKC isoenzyme specific peptide modulators described above study embryonic stem cell differentiation, it was demonstrated that inhibition of both βPKC and ζPKC (Zhou et al. 2003) enhanced cardiomyocyte differentiation. Cells treated with an εPKC inhibitor peptide inhibited cardiomyocyte differentiation, as seen by a decrease in the number of beating foci. Interestingly, this inhibition was only observed when differentiating cells were treated at specific times throughout differentiation, suggesting that there may be different roles for εPKC throughout differentiation (Zhou et al. 2003). Combining inhibitors of PKG β and ζPKCs further increased cardiomyocyte differentiation (Mobley et al. 2010).

Another study by Bekhite et al. (2011) used small molecule PKC inhibitors and demonstrated that α, β and δPKCs are involved in vasculogenesis via Vascular endothelial growth factor (VEGF) activation. Inhibition of PKC had no effect on VEGF induced cardiomyogenesis. Taken together these data suggest that different stimuli that lead to cardiomyogenesis may activate different signaling pathways and distinct PKC isoenzymes.

Future Perspectives

There is sufficient evidence in the literature to support the fact that PKC is involved in ESC proliferation, self-renewal and cardiomyocyte differentiation. However, the specific targets and roles of different PKC isoenzymes remains as of yet to be determined. Elucidating the role of the distinct PKC isoenzymes will require a careful characterization of these enzymes, determining expression levels and subcellular localization as well as the identification of direct targets in self-renewing ESC and at different stages of cardiomyogenesis.

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Maintenance of Pluripotency in Mouse Embryonic Stem Cells with MicroRNAs

11

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Contents

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Abstract

miRNAs compose a class of short single-stranded RNA molecules that function by regulating the expression of their target genes. Recent evidence has shown that miRNAs play a critical role in the maintenance of stem cell pluripotency and differentiation. In this chapter, we will provide an overview about the biogenesis of the miRNAs and the principal of their mechanism of action. We will highlight the most common theories about the way they establish simple regulatory networks with their targets. We will also discuss, in more details, the role of ES cell-specific miRNAs in the maintenance of pluripotency of the mouse embryonic stem cells (ES cells), and their connection to epigenetic silencing and regulation of cell cycle.

Introduction

miRNAs are a class of endogenous, small RNA molecules that do not code for proteins, and regulate expression of their target molecules. miRNA target recognition is highly redundant and the outcome of miRNA expression or its alteration is always dependent on the target pool available in situ. Therefore miRNAs act in networks and integrate with the classical regulatory associations of proteins. This allows miRNAs to fine tune the spatial and temporal gene expression, making them uniquely suitable for the regulation of developmental processes. Indeed, the first miRNAs

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was discovered in a developmental timing study in C. elegans, than since miRNAs were proved to be essential for the regulation of pluripotency, differentiation, axis formation, cell cycle regulation, development and maturation of the nervous system, organogenesis as well as for pathological conditions such as skeletal and growth defects, cardiomyopathies and different cancers. Key experiments showed that the miRNA pathway is indispensable for stem cell maintenance and differentiation in mammalian systems and recent exciting work pointed out its importance in iPS cell generation. There is increasing evidence that cancer pathogenesis and progression shares some aspects of embryonic development (e.g., epithelial-mesenchymal transition), dedifferentiation and stem cell maintenance. Since miRNAs are crucial participants of these processes, stem cell biology and cancer molecular pathology will both benefit from the deeper understanding of miRNA regulation in embryonic stem cells.

Biogenesis of MircoRNAs

miRNAs are now recognized as an abundant class of genome-encoded, short, single-stranded RNAs that regulate the expression of their target genes. The biogenesis of miRNAs is a highly regulated, multistep process. As is the case with protein coding genes, the RNA II Polymerase complex is responsible for the transcription of the miRNA genes. The primary transcript (pri-miRNA) is usually a long RNA molecule, and is protected from the nucleases by a 5′CAP structure and a 3′ polyA tail. The miRNA coding part of the primary transcript (also called pri-miRNA) forms a hairpin secondary structure. This special structure recruits the microprocessor complex, which is comprised of the RNAse III-type nuclease Drosha, and its partner the Di Giorge Syndrome Critical Region 8 (DGCR8) protein. DGCR8 is responsible for directing the complex to the double-stranded RNA through two double-stranded RNA binding domains (dsRBD) (Winter et al. 2009). The Microprocessor complex recognizes the boundary between the double stranded hairpin structure and the adjacent single-stranded RNA, and specifically cleaves the long pri-miRNA at the base of the stem-loop, to produce the 60–70 nucleotide long pre-miRNA. The pre-miRNA is then transported to the cytoplasm by the Exportin 5 (XPO5) in the presence of the Ran-GTP cofactor (Yi et al. 2003). In the cytoplasm, the pre-miRNA is recognized by the double-stranded RNA-binding protein TRBP (the human immunodeficiency virus transactivating response RNA-binding protein), that recruits the miRNA loading complex (miRLC). miRLC has two members: the RNAse III-type nuclease Dicer, and the PAZ/PIWI protein Argonaute 2 (AGO2). Once the pre-miRNA is loaded into the TRBP-Dicer-AGO2 effector complex, Dicer further cleaves the pre-miRNA at about 22 nt away from the Drosha cleavage site, generating a miRNA duplex. The duplex is unwind with the contribution of the MID domain of AGO2, the miRLC disassembles and the RNA Induced Silencing Complex (RISC), containing the mature, single stranded miRNA, is released (Maniataki and Mourelatos 2005).

Mechanism of Action of miRNAs

The sequence at 2–7 position from the 5′ end of the mature miRNA is called the ‘seed’ motif, and is a critical region in the target recognition. The seed sequence usually hybridizes nearly perfectly to the target mRNA’s 3′UTR (also called the ‘miRNA recognition sequence’ or ‘miRNA response element’), to nucleate the direct miRNA:mRNA interaction. These 5′ dominant target sites require little support for pairing to the miRNA at the 3′ end. However, additional 3′ complementary sites are required for some targets which have insufficient 5′ pairing. Since the target recognition site is short, a particular miRNA may regulate several hundreds of different mRNAs, and the miRNA function is always dependent on the context of the current transcriptome, reflecting the given cell type, the cell’s age and its niche. Additionally, an individual mRNA may be simultaneously targeted by multiple different miRNAs, and in this case different miRNAs act synergistically, for a tighter repression of the target.

The complicated miRNA pool-transcriptome network is generally thought to fine-tune gene expression; though in some cases, miRNAs are suggested to act as a switch in developmental processes.

miRNAs are widely believed to repress gene expression via base paring to partially complementary sites located in the 3′UTR of their target mRNAs. However, it has also been suggested that miRNAs can enhance gene expression of their targets using a different effector complex, and that miRNAs use alternative recognition sites within the coding region of the mRNA or in the promoter. miRNA mediated repression primarily works by inhibiting the initiation step of the translation. It is widely believed that the mRNA transcripts form a loop by connecting the 5′ CAP structure to the polyA tail, thus the RISC complexes, sitting on the 3′UTR are in the vicinity of the translation initiation site and interfere with the assembly of the translation initiation complex. It is now also accepted that the miRNA mediated silencing is usually coupled to the destabilization and leads to degradation or/and deadenylation of the mRNA target (Djuranovic et al. 2011). Though somewhat controversial, it is generally accepted that the Ago2-miRNA-mRNA complexes accumulate in the P-bodies (also called 'decapping bodies' or 'GW bodies'), that is demonstrated to play fundamental roles in general mRNA decay, nonsense-mediated mRNA decay, AU-rich element mediated mRNA decay, and miRNA-induced mRNA silencing. Importantly, not all mRNAs which enter P-bodies are degraded, as it has been demonstrated that some mRNAs can exit P-bodies and re-initiate translation.

miRNA Function

miRNAs were originally described as regulators of the heterochronic development of Caenorhabditis elegans larvae. However, it didn't take long to realize their potential in other multicellular systems (Lagos-Quintana et al. 2001). miRNAs have a wide variety of expression pattern. Some miRNAs show strict differential expression in time and space, others seem to be more ubiquitously expressed. In the early discovery phase, miRNAs were identified by directional cloning from different tissues and organs. The cloning frequency from a particular tissue reflected the abundance of the miRNA. This revealed a very important feature of miRNA regulation: virtually all tissues and cell types have a specific miRNAs expression landscape (Wienholds and Plasterk 2005). The high number of tissue-specific miRNA expression patterns suggests that these miRNAs have a role in differentiation and in the maintenance of tissue identity. It was also demonstrated that miRNA expression profile could delineate the cell origin more efficiently than the mRNA pattern, creating extensive miRNA research in cancer pathology, cancer diagnostics, alternative prognostics and therapeutics.

Involvement of miRNAs was quickly proved in organogenesis, tissue morphogenesis, and cellular processes like apoptosis, proliferation, cell cycle regulation and major signaling pathways. Not surprisingly, emerging evidence suggests a direct link between miRNAs and disease and that the gain and loss of miRNA target sites appear to be causal to some genetic disorders (Kloosterman and Plasterk 2006). The most efficient way to modulate a biological process is to alter a step that has extensive downstream effects. Signaling pathways are highly interconnected, where the members form a network, creating plenty of possibilities for fine tuning. Signaling pathways are ideal candidates for miRNA-mediated regulation due to the dose-sensitive nature of their outcome. Here we summarize some of the theoretical models that predict the interaction between miRNAs and their targets at the network level, and are involved in the regulation of pluripotency-related pathways.

Context-Dependent Transcriptional Activation

The final outcome of miRNA action depends on the signaling pathway it regulates. For example, some proteins have cofactors, which are required for the protein to act on a subset of its targets.

In case a miRNA inhibits this cofactor, it restricts the effect of the protein, since it can only act on the targets that don't require the cofactor. Thus, the final effect of a miRNAs does not only depend on the transcriptome of the cell, but also on the behavior of the targets. Default repression ensures that the expression of a gene is turned off, and it can only be activated in the presence of an adequate signal. However, signaling noise may cause undesired release of default activation. miRNAs could be crucial for signal interpretation. By dampening positive mediators of signaling cascades, miRNAs can raise the threshold for pathway activation, restricting it only to appropriate zones of competence (Inui et al. 2010).

Although miRNAs repress gene expression, their function is not always repressive. Instead, their effect on signaling cascades depends on the pathway topology. Inhibition of a repressor of a pathway will lead to a default activation of that signaling pathway. Default activation of cell cycle by ESC-specific miRNAs is one of the best studied examples for that. miRNAs contribute to fast G1-S transition that is a characteristic of pluripotent ES cells.

miRNAs as Signal Modulators and Members of Signaling Networks

Once a miRNA targets an inhibitor of a signaling cascade, it serves as a positive regulator and its effect might be amplified through the downstream elements of the pathway. This can cause increased strength or duration of the miRNA action, and can empower cell responsiveness to otherwise sub-threshold stimuli. The intrinsic risk of this is that the launching of a potential chain reaction might lead to senescence or cancer promotion, depending on the targeted pathway. Therefore, multiple negative regulators are usually needed to prevent this risk.

Because of the redundancy of miRNA target recognition, a single miRNA may target both an agonist and an antagonist of a pathway. This kind of regulation can be used to reduce the absolute signaling level of the targeted pathway, or to create an optimal balance between the pathway activation and inhibition, or to set a limit for the fluctuating input signals (Inui et al. 2010).

A new extracellular signal does not always confer instruction that the cell has to follow. miRNAs may reinforce or dampen signals, differentiating between bona fide messages, that have to be answered and signaling noises, that have to be ignored. In a coherent feedback loop, the miRNA and its target are oppositely regulated by the same signal. Since the miRNA represses the target gene, the signal doubles its effect by both a direct action on the gene and an indirect opposite action on the inhibitory miRNA. Whether the signal enhances or restricts the action of the given miRNA, the coherent loop leads to signal stabilization. This mechanism is typical in fine tissue patterning, where miRNAs repress 'leaky' mRNAs that should be not expressed in the given cell.

In an incoherent loop, the signal simultaneously triggers or restricts the target gene and the inhibitor miRNA. A main advantage of such a design, that it prevents pathway activation upon stochastic signaling noise. This ensures that only a relevant stimulus can override the inhibition by the co-expressed miRNA. Moreover, an incoherent loop may also maintain steady-state levels of the target protein. Importantly, this ensures uniform responsiveness in equivalent groups of cells within a range of signal distribution (Inui et al. 2010).

miRNA Pathways in Pluripotency Maintenance of Mouse Embryonic Stem Cells

To address the biological function of miRNA and related Dicer-dependent small non-coding RNA molecules in the early mammalian development, Dicer1 gene, the only Dicer in mammals, has been knocked out in mice. Dicer1 loss proved to be lethal in early mouse embryo development. Dicer1-null embryos did not develop cells that express Oct4 protein, a canonical marker of embryonic stem cells. The first attempts to generate Dicer1-null embryonic stem cells have also failed. These data suggest a role for miRNAs in

maintaining the stem cell population during early mouse development (Bernstein et al. 2003).

To be able to analyze the function of miRNA machinery in pluripotent cell, different mouse embryonic stem cell (ESC) lines were created that carried a genetic ablation of essential components of the miRNA biogenesis machinery in an inducible system (Gangaraju and Lin 2009). The Rajewsky and the Hannon laboratories have independently generated mouse embryonic stem cell lines that were conditional knock out for Dicer. Kanellopoulou et al. (2005) deleted the exons 18–20, which encode a part of the PAZ domain and the first RNAse III domain, by Cre-loxP mediated recombination. This led to out-of-frame splicing of the primary transcript and abolished all downstream exons, inactivating the second RNAse III catalytic domain (Kanellopoulou et al. 2005). Murchison et al. (2005) created Dicer loxP sites around the 22–23 exons. The Cre-loxP mediated loss of these exons generated ES cells that were knock-out for the majority of both RNAse III domains of Dicer. In both cases, the Dicer knock-out ES cell lines were viable, displayed the typical colony morphology of mouse ES cells, and expressed Oct4 level that was undistinguishable from that of the wild type. Surprisingly, the Dicer-deficient ES cell lines displayed proliferation defect and did not form teratomas upon subcutaneous injection into nude mice. Dicer ablation also led to differentiation defects. Embryoid bodies from mutant cells ceased growing after 8–10 days and showed little evidence of differentiation. The cell aggregates continued to express high level of Oct4 and did not express endodermal or mesodermal differentiation markers.

In addition, both laboratories examined the possibility that Dicer may contribute to heterochromatin formation in the mouse genome, as it has been reported in fission yeast (Scizzosaccharomyces pombe) and in plants. Both groups described enrichment of transcripts from the centromeric and pericentric repeats, raising the possibility that these long RNA transcripts cannot be processed into siRNA-like species in the absence of Dicer. Kanellopoulou et al. (2005) also suggested that H3K9 methylation was reduced in Dicer –/– cells.

Since the knock-out of Dicer was thought to interfere with the processing of centromeric repeats, miRNA biogenesis has been disrupted at a different step, to create a system where miRNA function can be adequately studied. The DGCR8 protein is the DNA binding partner of Drosha and elimination of DGCR8 prevents the formation of pre-miRNA precursors. Disruption of DGCR8 led to early embryo lethality; therefore the function of DGCR8 was addressed in ES cells, in an inducible system (Wang et al. 2007). Similarly to the Dicer knock-out ES cells, the DGCR8 –/– mutant was reported to have low proliferation rate, but appeared morphologically normal and expressed the known stem cell markers. The initial description of DGCR8 –/– ES cell line emphasized on its altered cell cycle phase distribution. Increased G1 phase paralleled with decreased percentage of cells present in S phase, severely disrupting the characteristic cell cycle pattern of pluripotent, fast cycling cells. The differentiation capability of DGCR8 deficient cells was also compromised. Expression of endoderm, mesoderm, ectoderm, trophectoderm and germ line markers was absent or severely reduced in the DGCR8 knockout embryoid bodies. Teratoma differentiation experiments revealed that the expression of differentiation markers was abnormal, but was not completely lost in the DGCR8 knockout population (Wang et al. 2007).

Taken together, disruption of the miRNA biogenesis pathway by deletion of Dicer or DGCR8 had a pronounced effect on the population doubling time of the generated ES cells, as well as on their differentiation potential. These results indicate that miRNAs are essential for silencing ES cell self-renewal program.

Functional miRNAs in Mouse ES Cells

To understand how miRNAs regulate pluripotency and differentiation of a mouse ES cell, it is vital to know which miRNAs are expressed. In the early discovery phase of stem cell specific miRNAs, short RNA expression was profiled from mouse ES cell cDNA libraries. Surprisingly, the majority of the detected miRNAs (70–75%)

mapped to six genomic loci, four on which have been implicated in cell cycle control or oncogenesis. Cloning studies identified novel miRNA hairpins that showed little conservation among mammals and overlapped with annotated repetitive elements. The work of Calabrese et al. (2007) suggested that the most important and abundant miRNA clusters in ES cells include the miR-15b/miR-16 cluster, the miR-17–92 cluster, miR-21, the miR-290–295 cluster, a miRNA cluster in a repetitive region on chromosome 2 (also known as miR-669 cluster or C2MC cluster) and a repetitive cluster on chromosome 12 (Mouse Dlk1-Gtl2 Domain).

The C2M2 cluster spans a ~54 Kb region and is located in the intron 10 region of the Polycomb group gene Sfmbt2. This cluster is not conserved in other mammals except to some extent in the rat. The cluster encodes 57 members of miR-467 family, including miR-669, miR-297, miR-466 and miR-467. The seed region of miR-467 is UAAGUGC, very similar to the seed sequence of the miR-290-295 cluster or the miR-17 family (AAGUGC). Other cluster members, however, appear to contain unrelated, highly repetitive sequence instead (e.g.: mmu-miR-669c-3p: UACACACACACACACAAGUAA).

The cluster on chromosome 12 is composed of 49 experimentally detected or cloned miRNAs at the mouse Dlk1-Gtl2 domain. An IG-DMR (differentially methylated region) between the Dlk1 and Gtl2 genes, has been recently shown to play a key role in controlling imprinted expression within the whole locus. This locus is paternally imprinted and only expressed from the maternal copy. Interestingly, some of the miRNAs at this locus, like miR-342 and miR-345, are differentially regulated in some cancers, and miR-342 downregulation correlates with chemotherapy resistance. Furthermore, in an independent study, eight members of this cluster have been found to be potential tumor suppressors (Buffa et al. 2011). The cluster was linked to the initiation of cancer stem cell character in human.

Technical advances in sequencing made it possible to use alternative approaches for miRNA identification. Leung et al. (2011) combined photo-crosslinking immunoprecipitation using Ago2 antibodies and deep-sequencing of the precipitated RNAs, to find mES cell specific miRNAs and to deduce their targets at the same time. In this study, mES cells appeared to be dominated by a single miRNA seed family (AAGUGC) that is probably responsible for most of the miRNA regulation in this cell type. Sixty-eight percent of all sequenced miRNAs shared this seed sequence, including members of the miR-290-295 cluster, some members of the miR-467 family and the miR-302-367 cluster. Consequently, the Ago-2 precipitated mRNAs had high frequency of the complementary GCACUU motif in their 3′ untranslated and coding regions. The predicted targets were significantly enriched in genes that regulate G1/S transition of the cell cycle and the TGFβ-receptor signaling pathway. Intracellular TGFβ-pathway inhibitors, the cytoplasm-localized Smad7, the nucleus-localized Skil, and the extracellular inhibitors, Lefty1 and Lefty2 are among the experimentally validated targets of miRNAs with AAGUGC seed sequence. The TGFβ pathway has also been implicated in epithelial-mesenchymal transition (EMT), which is a naturally occurring step during embryogenesis. EMT is also a driving force of metastasis in cancer, and contributes to the establishment of cancer stem cells. The Lefty proteins function as antagonists of Nodal signaling and play a role in left-right asymmetry determination of organ systems during development.

The miR-290-295 Cluster/EEmiRC

The early embryonic miRNA cluster (EEmiRC) or miR-290-295 cluster is expressed in embryonic and some somatic stem cells, and is repressed during differentiation. It represents nearly 70% of all miRNAs of the mouse embryonic stem cell. The cluster encodes seven miRNA hairpins that mature to ten, highly similar miRNAs (Houbaviy et al. 2005). The promoter of the cluster contains binding sites for several well-known stem cell transcription factors: Nanog, Oct4, Sox2, Tcf3, c-Myc and n-Myc. The promoter also carries an ambivalent epigenetic signature, which is characteristic for pluripotent cells. This means that the promoter is simultaneously marked by the H3K4

trimethylated histones that are associated with active transcription; and by H3K27 trimethylated histones, that is linked to inactive transcription (Judson et al. 2009). This double epigenetic mark provides ES cells with the flexibility to express a gene set, but be able to switch it off quickly, when de novo expression is not required anymore. The H3K4 trimethylation of the miR-290-295 promoter is gradually lost during differentiation. Also, Tata et al. (2011) described an intragenic enhancer element within the miRNA coding region that acts synergistically with the promoter and is necessary to integrate miR-290-295 cluster expression with the ESC-specific regulatory circuit. The intragenic enhancer is dense in Oct4, Sox2 and CTCF binding sites, similar to the promoter. Both the promoter and the enhancer are hypomethylated in ES cells and hypermethylated in differentiated cells. The enhancer is also marked by ambivalent histone methylation, thus the hypomethylated enhancer is associated with H3K4me3 in ES cells and progressively changes to hypermethylated DNA and H3K27me3 trimethylated histones in the more differentiated progenitor cells.

To elucidate the role of this unique set of miRNAs, the Surani group studied the function of a representative and abundant member: miR-294 (Hanina et al. 2010). Restoring miR-294 expression in Dicer −/− mouse ES cells altered the transcriptome by causing primary (direct) and secondary (indirect) effects. mRNAs that co-immunoprecipitate with the endogenous Ago2 were compared between Dicer −/− and miR-294-expressing Dicer −/− cells. Consistent with the potential role of miR-294 in pluripotency maintenance, none of the stem cell factors were downregulated by miR-294, on the contrary, differentiation-associated genes were enriched among the downregulated genes. The stem cell factors Lin28 and c-Myc were highly upregulated upon miR-294 transfection. Importantly, c-Myc and miR-294 acted synergistically on a subset of genes. This result is consistent with the observation that miR-294 can partially substitute c-Myc in the generation of iPS cells. However, miR-294 also repressed some of the c-Myc targets. It is suggested that c-Myc directly activates a number of genes, and in parallel, also represses some of its targets via miR-294, creating an incoherent loop. In conclusion, miR-294 is not just a downstream effector of c-Myc, but a pluripotency factor by itself that can partially substitute c-Myc function induce pluripotency marker genes, such as Lin28.

Stem-Cell Specific miRNAs and Epigenetic Silencing

Induction of ES cell differentiation by LIF withdrawal or by retinoic acid treatment has been shown to increase the methylation of Oct4 promoter, which silences the expression of the pluripotency factor. Loss of Oct4 promoter methylation causes deficient differentiation upon LIF withdrawal, and maintenance of high Oct4 and Nanog levels.

Embryoid bodies of the Dicer1 deficient mouse ES cell lines show impaired differentiation, and the mutant cells do not form teratomas in nude mice. Notably, Dnmt3a and Dnmt3b double mutant ES cells, that lack de novo DNA methyltransferase activity, retain an undifferentiated morphology and are also defective in teratoma formation. The Filipowicz and the Blasco laboratories were pioneers in establishing a link between miRNAs and epigenetics (Benetti et al. 2008; Sinkkonen et al. 2008). A marked loss of DNA methylation was described in the Dicer1 deficient ES cells, which resembles the phenotype of Dnmt1 functional knock out cells that are deficient in DNA methylation maintenance. The heterochromatin domain of the subtelomeric repeats controls telomere recombination and telomere length. These subtelomeric regions of the Dicer1 deficient ES cells showed a significant decrease in DNA methylation compared to wild-type cells. It has also been proven that the decreased DNA methylation in the Dicer knock-out cells is a direct consequence of decreased Dnmt levels. This DNA methylation defect is likely to contribute to the increased telomere recombination and the aberrant telomere elongation that was observed in the Dicer knock-out cells.

Expression of the DNA methyltransferase genes that are responsible for de novo methylation

of CpG DNA motifs (Dnmt3a, Dnmt3b and Dnmt3L) was significantly downregulated at mRNA and protein levels in the Dicer deficient ES cell lines. In parallel, the expression of Rbl1, the Rb regulator Rb1cc1, Rbak and Rbbp1 (Arid4a) increased. Transient transfection of miR-290-295 miRNAs, the predominantly expressed miRNAs in mouse ES cells, rescued the expression of the Dnmt3 genes. Notably, miR-291a-3p alone was sufficient to restore both the mRNA and the protein levels of the DNA methyltransferases. This effect is clearly indirect and suggests that the miR-290 cluster represses an inhibitor of Dnmt3 genes. Target prediction of miR-290 family pointed out several transcriptional repressors that are upregulated during embryonic differentiation after the blastocyst stage, including Klf3, the nuclear receptor Nr2f2, the zinc-finger proteins Zmynd11 and Zbtb7 and the retinoblastoma-like 2 (Rbl2) gene. Interestingly, Rbl2 directly binds and represses Dnmt3a and Dnmt3b promoters in ES cells during neuronal differentiation and in human glioblastoma cells. When differentiation of ES cells was induced by RA treatment, the expression of GCNF, and early repressor of Oct 4 and Nanog, were upregulated both in Dicer +/− and Dicer −/− cells. This indicates that the very first steps of differentiation do not require a functional miRNA pathway. However, at the later stages of differentiation the Dicer −/− cells showed incomplete silencing of Oct4 and Nanog, and the Oct4 promoter fails to undergo DNA methylation. Re-introduction of miR-290 cluster to the Dicer deficient ES cells restored the de novo DNA methylation of Oct4 promoter, establishing a relevant link between miRNAs and epigenetic regulation.

miRNA Regulation of Cell Cycle

The cell cycle is tightly orchestrated during normal development. The proper switching between the phases is regulated by checkpoints at the G1/S and G2/M transitions. ES cells are highly proliferative and have an expedited cell cycle which is critical for fast growth of the inner cell mass. They bypass the cell cycle checkpoints, including the G1/S transition checkpoint, therefore ES cells exhibit very short G1 phase, while the percentage of S phase cells is high.

Unlike proper ES cells, Dicer1 and DGCR8 knock-out ES cells proliferate slowly and accumulate in G1 phase of cell cycle. The Blelloch laboratory reported that 12 miRNAs were able to rescue this proliferation and cell cycle defect, indicating a major contribution of the miRNA pathway to cell cycle regulation in mouse ES cells (Wang et al. 2008). Interestingly, all 12 miRNAs belong to or share similar seed region (‘AAAGUGC’ or ‘AAGUGCU/A’) with the miR-290 family, implying that they act on a common set of targets. Several cell cycle regulators are predicted to be directly regulated by the miR-290 family. Among them are the G1-S check point regulators Cdkn1a (p21), Cdkn1b (p27), Retinoblastoma protein 1 (Rb1), Rb-like 1 (Rbl1 or p107), Rbl2 (p130), Large tumor suppressor 2 (Lats2); and the G2/M check point regulator Wee1. Lats2 is also a confirmed target of the orthologous miRNA family in human testicular germ cell tumor.

Besides speeding up the cell cycle, the miR-290 family also targets pro-proliferative genes like cyclinD1 (CCND1) and E2F1, creating an incoherent loop that may fine-tune the speed of proliferation. mES cells exhibit elevated levels of cell cycle independent Cdk2-cyclin E activity that accounts for rapid cell division and a truncated G1 phase. The best understood target of the miR-290 family is the Cdk inhibitor p21. It is anticipated that p21 is a major factor contributing to the decrease in Cdk2 activity seen during differentiation. Data from Wang et al. (2008) indicates that decreased expression of the miR-290 cluster upon differentiation releases the translational block of the Cdkn1a transcript, leading to higher p21 protein levels (Wang et al. 2008). Assembly of Cdk2-cyclin E into inactive complexes with p21 delays the onset of G1-S transition. Thus, low miR-290 expression results in p21 upregulation and subsequent contribution to the G1-S transition check point, to engineer the cell cycle phase distribution that is typical for differentiated cells, exhibiting a higher proportion of cells in G1 and a proportion in the S phase.

Although the miR-290 family seems to dominate the mouse ES cell, other miRNAs are also proposed to regulate the cell cycle in pluripotent mouse cells. miR-125b, the vertebrate homologue of the C. elegans miRNA lin-4, has also been implicated in the regulation of neural and hematopoietic stem cell homeostasis. miR-125b has been proposed to regulate both apoptosis and proliferation in a cell-type specific manner. Several cell cycle regulators are among the predicted targets of miR-125b, including members of the p53 network: cyclin C, Cdc25c, Cdkn2c, Edn1, Ppp1ca and Sel1l. Importantly, the miR-125b regulation of the p53 pathway is conserved at the network level between C. elegans and human (Le et al. 2011).

Myc-Regulated miRNAsContribute to Stem-Cell Maintenance and Differentiation

The Myc protein family includes basic helix-loop-helix-zipper (bHLHZ) transcription factors. C-, N- and L-Myc can each form obligate heterodimers with the bHLHZ proteins and target the E-box sequences. Myc has been implicated in the transcriptional repression of many genes that normally limit cell cycle progression. Myc proteins recruit histone acetyltransferases (HATs) and thus, in addition to their role as classical transcription factors, they also function to regulate global chromatin structure by regulating histone acetylation in protein coding and intergenic regions. The Myc proteins' broad effect on normal and abnormal cell behavior have been assumed to relate to their regulation of RNA polymerases, required for translation and growth (Grandori et al. 2005). The demonstration that Myc proteins also control expression of a subset of miRNAs has added another class of critical Myc targets.

In an elegant experiment, Lin et al. (2009) overexpressed c-Myc in murine ES cell line (c-Myc+), then compared the miRNA landscape of the c-Myc+ESC line, the induced haematopoietic stem/progenitor cells (HSP) and tumors from the mice transplanted with c-Myc+cell-derived HSPs. Let-7, miR-29, miR-181 and miR-199 were downregulated by increased c-Myc expression, while the miR-200 family members (miR-141, miR-429, miR-200), miR-338 and miR-302 were upregulated. Interestingly, the miRNAs induced by c-Myc in ES cells were not increased or were significantly reduced in differentiated hematopoietic cells and in the tumors from transplanted mice. Similarly, the oncogenic miR-17-92 cluster was strongly induced in the c-Myc+ differentiated and tumor cells, while it was not substantially altered in the c-Myc+ES cells.

Importantly, the gene sets downregulated by the miR-200 family, miR-335 or miR-302 and the genes downregulated by c-Myc were significantly overlapping in mES cells. Target prediction analysis and experimental validation suggests that the targets of these miRNAs include a large number of genes that function as differentiation inhibitors. These include TGFβR3, the cadherin Cdh11, neuropilin (Nrp1) and Foxf2. TGFβR3 functions as a co-receptor with other TGF-β receptor superfamily members. It is involved in multiple developmental pathways, and its downregulation has been observed in various cancers. Cdh11 is an adhesion molecule that has an important role in developmental processes such as morphogenesis and induction of neurite outgrowth. Nrp1 has key roles in axonal guidance and is essential for neuronal and cardiovascular development. It is also a promising therapeutic target protein in the treatment for leukemia and lymphoma.

Therefore Myc-controlled miRNAs in ES cells may contribute to inhibition of broad aspects of cell-type differentiation during development. It should be noted that Myc proteins, on the other hand, can be regulated by miRNAs, as described below.

MicroRNAs and Differentiation

The let-7 family was among the first identified miRNAs. Originally it was known as a developmental regulator of progenitor cell maturation in C. elegans, and it was soon recognized that mammals express a large family of let-7 miRNAs. The mouse let-7 family is composed of 12 precursors

that code for ten distinct mature miRNAs, from let-7a to let-7i, and also include the highly similar miR-98. The family is not expressed in ES cells and cancer stem cells, but let-7 members are widely expressed across differentiated tissues and during ESC differentiation. The strict differentiation-related expression pattern and the abundance of this family made it an excellent candidate as a master regulator of early differentiation.

DGCR8−/− ES cells had two major defects: slow proliferation rate and impaired differentiation ability. Fast cycling could be restored by the miR-290 family. miR-290, however, could not silence pluripotency and induce a differentiation program. Melton et al. (2010) demonstrated that expression of let-7 members rescue the differentiation ability of DGCR8 −/− cells. let-7c overexpression led to downregulation of several ES markers, like the embryonic alkaline phosphatase, Oct4, Nanog and Sox2. Moreover, a range of let-7 members was able to effectively diminish the ability of ES cells to form colonies. Co-expression of the miR-290-295 cluster and let-7 family in DGCR8 −/− cells blocked differentiation. The functional antagonism between let-7 and miR-290 families on ES cell self-renewal suggests that they have opposing effect on the downstream mRNA targets. Similar to the miR-290 cluster, the let-7 family also regulates cell cycle and proliferation. let-7b was shown to target the cyclins D1, D2, D3, Cdk4, cyclin A and E2F2. D cyclins and the cyclin dependent kinase 4/6 form a complex that leads the cell from G1 to S phase. miRNA inhibition of any member of this complex blocks G1-S transition and slows down the cell cycle. Similarly, cyclinA complexes with Cdk2 to promote G1-S transition, and this step is negatively regulated by p21, p27 and p57. let-7 down-regulates both members of the CyclinA-Cdk2 complex, thus it synergizes with p21 to establish the G1/S checkpoint. This is probably the site of a very intensive communication between the miR-290 family and the let-7 cluster, since miR-290 inhibits p21and reinforces cell cycle. Over-expression of let-7 caused cell cycle arrest in G1 and a delay in G2/M transition.

The oncogenic RAS/RAF/MAPK pathway is among the validated let-7 targets in human cancers. Moreover, let-7 miRNAs are intimately related to an oncogenic downstream target of RAS/RAF/MAPK pathway, the Myc family. In ES cells, n-Myc and c-Myc are highly expressed. c-Myc has been identified as a let-7 target in cancer cells, and n-myc was shown to be directly downregulated by let-7 in mouse ESC (Melton et al. 2010). Disruption of c-myc and n-Myc in mouse ESC severely affects self-renewal, pluripotency, survival and accelerates lineage commitment. c-Myc was one of the four genes that were originally described to be able to reprogram terminally differentiated fibroblasts to pluripotent embryonic stem cells. Conditional deletion of Myc proteins in mouse ES cells prolongs G1 phase of cell cycle as well as accumulation of cells in M phase. It also enhances the expression of differentiation markers of all three germ layers. The loss of Myc leads to down-regulation of several epigenetic regulators (ex.:Chd1) suggesting a role for Myc in remodeling chromatin architecture in ESC.

Interestingly, c-Myc also contributes to the transcriptional regulation of let-7 and miR-290 family. The oncogenic c-Myc transcription factor broadly influences miRNA expression, leading to widespread miRNA down-regulation. miRNA transcripts repressed by Myc include tumor suppressor miRNAs, such as miR-15a/16–1, miR-34a, and let-7 family members. Myc operates through Lin-28A and Lin-28B RNA binding proteins to negatively regulate let-7 biogenesis, and Lin-28 is necessary and sufficient for Myc-mediated let-7 repression (Chang et al. 2009). Myc is directly associated with Lin-28 promoters and induces transcriptional transactivation of Lin-28 in multiple human and mouse tumor models. In turn, Lin-28 protein binds pre-miRNAs of the let-7 family at the loop sequence and inhibits further maturation of the miRNA. Therefore the Myc-Lin28 axis promotes pluripotency (and malignancy) by selectively blocking the biogenesis of the let-7 family.

The miRNAs that are predicted to target pluripotency factors and are expressed upon induction of differentiation expected to have a role in early differentiation. Several miRNAs were proposed to regulate pluripotency factors (e.g.: Oct4, Nanog, Sox2, Klf4, cMyc, Tbx3, Ezh1, Ezh2 and Eed),

and many were also validated experimentally. The let-7 family members target the Yamanaka-factor c-Myc and the PRC2 (Polycomb Repressor Complex) member Ezh2 (Pauli et al. 2011). miR-26a and miR-26b are also transcriptional regulators of Ezh2 and Tbx3 (Ma et al. 2011). Tbx3 has recently been shown to improve the germ cell competency of iPS cells. miR-29 is a validated regulator of Klf4, and is commonly linked to stem cell and cancer stem cell differentiation. miR-29 can also indirectly enhance p53 activity and this circuit is activated in age-related DNA damage (Ivey and Srivastava 2010).

In an elegant study, Chen et al. (2006) demonstrated that miR-1 is required for the smooth muscle cell differentiation of mouse ES cells. miR-1 directly targets Klf4 pluripotency factor, thus regulating indirectly the Nanog, Oct4, Sox2, c-Myc regulatory circuit. miR-1 also has a strong pro-myogenic effect on the undifferentiated and very aggressive rhabdomyosarcoma tumor cells (Xie et al. 2011).

Several other miRNAs have been pointed out to regulate the differentiation of mouse ES cells. Tarantino et al. (2010) carried out a systematic comparison of miRNA expression between undifferentiated ES cells, and cells where neural differentiation has been induced. miR-100 and miR-137 were undetectable in the pluripotent ES cells, but their expression rapidly increased upon differentiation. miR-34a was induced 2 days after differentiation, and its level increased further with prolonged differentiation (Tarantino et al. 2010).

miR-34 is also known to inhibit prostate cancer stem cells by direct repression of the cell adhesion marker CD44, and the of miR-34 has been linked to resistance to apoptosis. miR-34 is an immediate transcriptional target of p53, a central protein in cancer defense. The miR-34 coding locus is frequently deleted in neuroblastoma and hypermethylated in other cancers.

Decreased expression of miR-100 contributes to malignancy in oral cancers, moreover it was shown to block terminal differentiation and to contribute to cell survival in acute myeloid leukemia. miR-137 is generally believed to be a tumor suppressor miRNA. The epigenetic silencing of miR-137 is an early, cancer-specific event, and was frequently observed in renal carcinomas. Interestingly, inhibiting miR-34a, miR-100 and miR-137 delayed the differentiation of mouse ES cell in the absence of LIF and caused significant increase in the protein expression of some of their predicted targets: Jarid1B, Smarca5 and Sirt. These genes are involved in chromatin remodeling, and in pluripotency maintenance. It is speculated that miR-34, miR-100 and miR-137 contribute to the early differentiation events through modulating epigenetic changes in the ES cell. miR-34 and p53 were also shown to work synergistically to downregulate WNT pathway genes in other systems. Wnt1 is required for long term pluripotency maintenance; therefore, increased miR-34 expression might affect the differentiation of embryonic stem cells by controlling Wnt pathway in mice.

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Uni-directional Neuronal Differentiation of Embryonic Stem Cells by the Neural Stem Sphere Method

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Masahiro Otsu, Takashi Nakayama, and Nobuo Inoue

Contents

Abstract

Embryonic stem (ES) cells have two characteristics, pluripotency and self-renewal. ES cells can differentiate into all other cell types, including germ cells, indicating that they may be a limitless source of functional cells for stem cell applications. We have formulated a method, the neural stem sphere (NSS) method, to efficiently obtain functional cells from these sources. The NSS method is a simple method of quickly and efficiently generating numerous neural stem cells and neurons from mouse, monkey and human ES cells. Analysis of marker gene expression during the neurogenesis of mouse ES cells induced by the NSS method demonstrated that ES cells uni-directionally differentiate into neurons via epiblasts, neuroectodermal cells and neural stem cells. This process of neuronal differentiation resembles, in part, that of neurogenesis in early embryos, suggesting that the NSS method may provide a potentially powerful tool for elucidating the mechanism underlying the efficient neural differentiation of ES cells and for assessing drugs, chemical compounds and physical stimuli that may cause neurodevelopmental impairments.

Introduction

Research on stem cells may enhance the application of these cells in various systems, including for cell transplantation and drug screening tests.

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Human cells derived from pluripotent stem cells, including embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, may be particularly useful in these applications. Obstacles, however, must be overcome before performing these applications, with one of these obstacles being the efficient preparation of functional cells from unlimited sources. This review will focus on methods used for neural differentiation from these cells, in particular on our neuronal differentiation method, the neural stem sphere (NSS) method.

Embryonic Stem Cells Derived from the Inner Cell Mass of Blastocysts

Embryonic stem (ES) cells derived from the inner cell mass (ICM) are pluripotent and self-renewing in culture (O'Shea 1999). ES cells may be a limitless source of functional cells for therapeutic applications (Améen et al. 2008). To utilize this potential more fully, however, ES cells must be differentiated more efficiently into specific cell lineages that can supply functional cells capable of restoring the function lost by damaged tissues. Designing an efficient method for the differentiation of ES cells requires knowledge of the mechanism of normal cell development during embryogenesis. During the early stages of embryogenesis, the ICM in blastocysts can potentially generate epiblasts, from which all three germ layers of the actual embryo are derived (Gardner and Beddington 1988). During early gastrulation, the epiblasts organize into ectoderm, under the influence of factors in the surrounding environment, including adhesion molecules and secreted factors. This, in turn, gives rise to neuroectoderm in the anterior region of the ectoderm, defined as the neural plate (Tam 1989; Quinlan et al. 1995; Pfister et al. 2007; Glinka et al. 1998). At the cellular level, neuroepithelial cells in the neuroectoderm differentiate into neural stem/progenitor cells during neural tube development (Ericson et al. 1997; Pevny et al. 1998). Subsequently, these neural stem/progenitor cells proliferate and finally differentiate into neuronal cells and glial cells in a temporal- and spatial-specific manner. During early embryogenesis from implantation to the early gastrula stage, each cell populations expresses specific marker genes, thus enabling a determination of the efficiency and accuracy of ES cell differentiation *in vitro* (Pfister et al. 2007).

Neuronal Differentiation of Pluripotent Stem Cells

Recent advancements in stem cell research have included the establishment of human ES cells and the generation of iPS cells (Amit et al. 2000; Takahashi and Yamanaka 2006). Nevertheless, there are technical obstacles to the large-scale generation of highly homogeneous and functional cells, thus limiting the applications of stem cells on a large-scale. In addition, to ensure that these differentiated cells are normal and safe to use, it is necessary to confirm that the large-scale generation of these pluripotent stem cells yields cells that differentiate normally.

Embryoid body (EB) culture is the method used most frequently to test the pluripotency of ES cells and to prepare somatic cells from these sources. In this method, enzymatically digested ES cells are grown in hanging drop culture in serum-containing media without leukemia inhibitory factor (LIF) (Gokhan and Mehler 2001; Guan et al. 2001). These dissociated ES cells immediately form aggregates, resulting in EBs after several days. These EBs consist of endodermal, mesodermal and ectodermal cells, thus closely resembling early post-implantation embryos (O'Shea 1999). However, neural differentiation via EB formation is spatiotemporally unusual and not uni-directional, reducing the effective generation of neural cells. To overcome these limitations, retinoic acid, a well-known morphogenic factor, is added to culture media to promote neural differentiation (Bain et al. 1995). In addition, basic fibroblast growth factor (FGF-2) may be used to promote the selective proliferation of neural stem cells from EBs, increasing the total number of neural stem cells (Okabe et al. 1996). Unfortunately, even these optimized protocols involve elaborate and time-consuming procedures to generate homogeneous populations of neural cells.

The serum-free cell suspension (SFEB) method is based on EB formation and is a neural cell specific induction procedure using secreted factors similar to those utilized for neurogenesis in embryos (Watanabe et al. 2005). Treatment with Wnt and Nodal antagonists (Dkk1 and LeftyA) during the first 5 days of SFEB culture promotes the selective differentiation of dissociated ES cells into neural cells. After 5 days in culture, ~90% of these differentiated cells are Sox-1 positive, neuroepithelial cells. This method, in combination with cell sorting techniques, is capable of the efficient generation of CNS cells, including telencephalic progenitors, retinal progenitors, photoreceptor cells and hypothalamic neurons (Watanabe et al. 2005; Ikeda et al. 2005; Suga et al. 2011).

Uni-directional Neuronal Differentiation of ES Cells by the Neural Stem Sphere Method

We have developed a simple method to quickly and efficiently generate numerous neural stem cells and neurons from mouse and monkey ES cells (Nakayama et al. 2003). Briefly, ES cells were seeded at a clonal density and cultured for 7–9 days. The resulting ES cell colonies were detached from the mouse embryonic fibroblast (MEF) feeder layer using a glass capillary and transferred to non-adhesive bacteriological dishes in astrocyte-conditioned medium (ACM). While in culture, the ES cell colonies formed floating spheres and, within 4 days, gave rise to neural stem spheres (NSSs), containing a periphery of neural stem cells. Culturing these spheres on an adhesive substrate in ACM promotes neurogenesis, and cells in the spheres differentiate within 5 days into neurons, including dopaminergic (DA) neurons. By modifying culture conditions, the NSS method was able to generate a large number of neural stem cells from ES cells (Nakayama et al. 2004). When cultured in ACM with the mitogens FGF-2 and epidermal growth factor (EGF), colonies of ES cells gave rise to NSSs, composed of plentiful numbers of neural stem cells. Subsequent culture of these NSSs on an adhesive substrate in the presence of these mitogens resulted in the migration of neural stem cells onto the substrate. We also reported that these neural stem cells can be expanded, preserved by freezing, and differentiated into functional neurons (Nakayama et al. 2004) and astrocytes (Nakayama et al. 2006). Human ES cells could also be differentiated into neural stem cells and neurons by the NSS method, suggesting that the mechanism of neural induction from rodent and primate ES cells by our method is also applicable to human cells (Okuno et al. 2009). Transplantation of neural stem cells derived from cynomolgus monkey ES cells by the NSS method was able to restore DA function in a primate model of Parkinson's disease, suggesting that these neural stem cells can function normally *in vivo* (Muramatsu et al. 2009).

To address the mechanism by which the NSS method efficiently generates numerous neural stem cells and functional neurons, we analyzed changes in the expression of marker genes associated with early neurogenesis during the formation of NSSs from mouse ES cells (Otsu et al. 2011). Quantitative analysis of gene expression by real-time RT-PCR showed that the expression of mRNAs encoding the undifferentiated ES cell markers *Oct-3/4* and *Nanog* and the ES cell and epiblast marker *Cripto* in the ES cell colonies was down-regulated during culture of cell spheres, becoming very low on day 4 (Fig. 12.1a–c). Interestingly, *Fgf-5*, an epiblast marker, was transiently expressed in the cell spheres on day 2 (Fig. 12.1d). Although the expression of mRNA encoding the neuroectoderm marker of *Sox-1* (Fig. 12.1e) was gradually up-regulated, beginning on day 3, during culture of the cell spheres, expression of mRNAs encoding the neural stem/progenitor cell markers *Nestin* and *Musashi-1* was rapidly increased in the NSSs, beginning on day 4 (Fig. 12.1f, g). Moreover, expression of mRNAs encoding the neuronal markers *NF-M* and *MAP-2* was gradually increased from day 4 (Fig. 12.1h, i). In contrast, mRNAs encoding the astrocyte markers glial fibrillary acidic protein (*GFAP*) and *S100β* (Fig. 12.1j, k) and the oligodendrocyte marker *MBP* (Fig. 12.1l) were not up-regulated; and mRNAs encoding the primitive

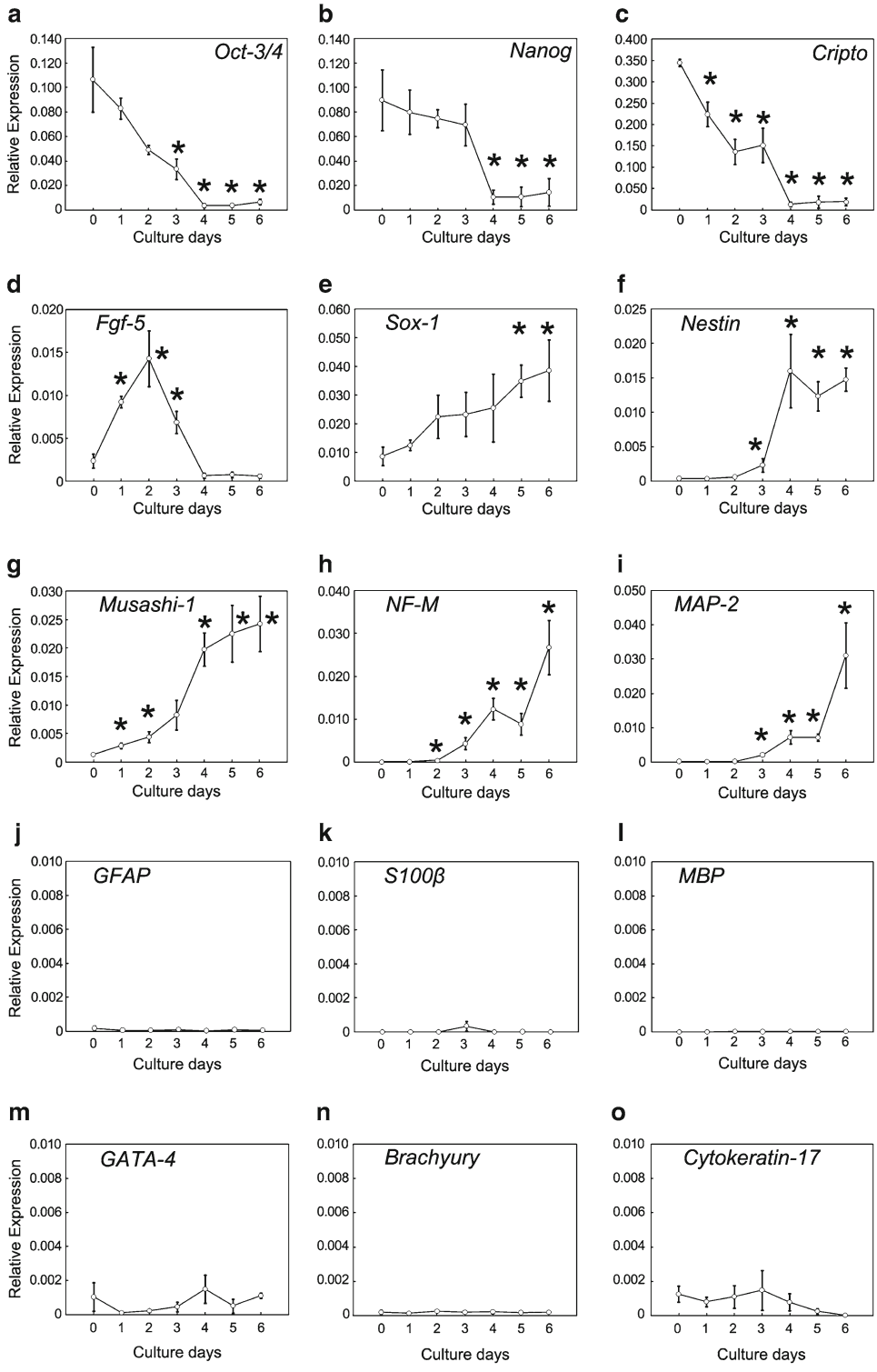

Fig. 12.1 Changes in gene expression during neuronal differentiation by the NSS method (From Otsu et al. 2011. © Elsevier) ES cells were cultured in non-adhesive dishes in ACM for 6 days (stage 1), giving rise to cell spheres. RNA was isolated from undifferentiated ES cell colonies (0 days) and from cell spheres grown for 1–6 days and analyzed by quantitative real-time RT-PCR. Expression of *Oct-3/4* (**a**), *Nanog* (**b**), *Cripto* (**c**), *Fgf-5* (**d**), *Sox-1* (**e**), *Nestin* (**f**), *Musashi-1* (**g**), *NF-M* (**h**), *MAP-2* (**i**), *GFAP* (**j**), *S100β* (**k**), *MBP* (**l**), *GATA-4* (**m**), *Brachyury* (**n**), and *Cytokeratin-17* (**o**) mRNAs was normalized relative to expression of *GAPDH* mRNA. Data are mean ± SEM (*bars*) of four determinations. *P < 0.05 compared with ES cell colonies

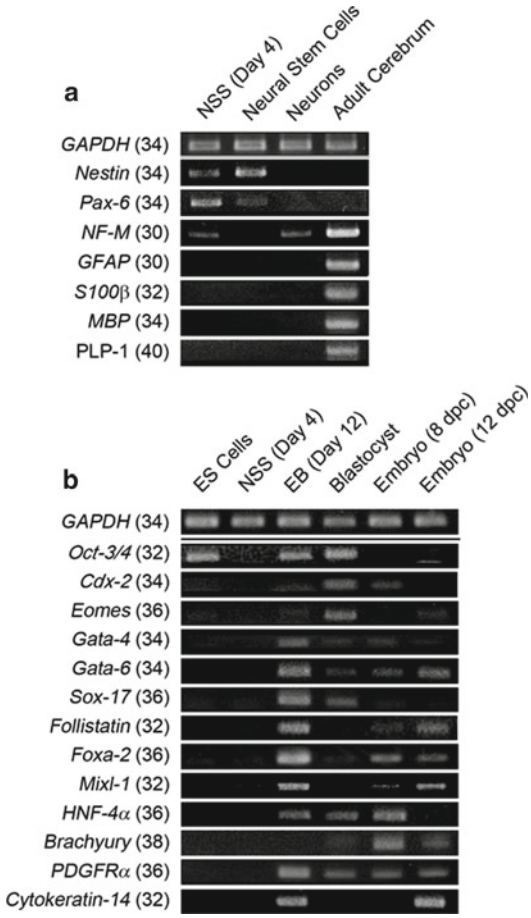

Fig. 12.2 Gene expression analysis of the NSSs (From Otsu et al. 2011. © Elsevier) RNA was isolated from undifferentiated ES cell colonies, NSSs (day 4 at stage 1) and positive controls; ES cell derived-neural stem cells and neurons, adult cerebrum, embryoid bodies (EBs), blastocysts, and whole embryos at 8 and 12 dpc, and the expression of marker genes was analyzed by semi-quantitative RT-PCR. The relative expression of each mRNA was normalized to that of *GAPDH* mRNA. Numbers of PCR cycles are shown in *parentheses*

endodermal marker *GATA-4* (Fig. 12.1m), the mesodermal marker *Brachyury* (Fig. 12.1n), and the epidermal marker *Cytokeratin-17* (Fig. 12.1o) were either completely absent or as low in cell spheres as in ES cell colonies.

To clarify the uni-directional differentiation of mouse ES cells by the NSS method, we examined this process more extensively to exclude the possibility that the mouse ES cells had differentiated into extraembryonic endoderm, mesendoderm, endoderm, mesoderm, epidermal cells or glia. Semi-quantitative RT-PCR analysis showed that the NSSs expressed mRNAs encoding the neural stem cell markers *Nestin* and *Pax-6* and the neuronal marker *NF-M*, but not mRNAs encoding the astrocyte markers *GFAP* and *S100β* and the oligodendrocyte markers *MBP* and *PLP-1* (Fig. 12.2a). Furthermore, these NSSs did not express mRNAs encoding the ES cell marker *Oct-3/4*; the trophectodermal markers *Cdx-2* and *Eomes*; the primitive endodermal markers *GATA-4* and *GATA-6*; the extraembryonic endodermal markers *GATA-4*, *GATA-6* and *Sox-17*; the parietal endodermal markers *GATA-4*, *GATA-6* and *Follistatin*; the visceral endodermal markers *Sox-17*, *Foxa-2*, *Mixl-1* and *HNF-4α*; the definitive endodermal markers *GATA-4*, *Sox-17*, *Foxa-2* and *Mixl-1*; the mesendodermal markers *Sox-17*, *Mixl-1*, *Brachyury* and *PDGFRα*; the mesodermal markers *Brachyury* and *PDGFRα*; and the epidermal marker *Cytokeratin-14* (Fig. 12.2b). In contrast, each of these marker genes was expressed, to a sufficiently high extent, by the appropriate positive controls, including neural stem cells, neurons, adult cerebrum, EBs, blastocysts, and whole embryos at 8 and 12 days postcoitum (dpc) (Fig. 12.2a, b). These results strongly suggest that mouse ES cells uni-directionally differentiate into neurons via epiblasts, neuroectoderm and neural stem cells.

A three-dimensional scatter graph of the expression of three representative genes, *Oct-3/4*, *Fgf-5* and *Nestin*, by single spheres at different culture stages (Fig. 12.3) also precisely showed their temporal pattern of distribution during neurogenesis by the NSS method, including the direction, progression and efficiency of neuronal differentiation. The distribution of expression of these three genes reflected the extent or proportion of three types of cell, ES cells, epiblasts and neural stem cells, in the spheres. Differentiation of ES cells into epiblasts on days 0–2 in culture was indicated by a coordinated decrease in *Oct-3/4* expression and an increase in *Fgf-5* expression, whereas *Nestin* expression was negligible throughout. During culture from days 2 to 6, the expression of *Oct-3/4* and *Fgf-5* was down-regulated, while that of *Nestin* was radically up-regulated. These results clearly demonstrated that, although the extent of differentiation differed among

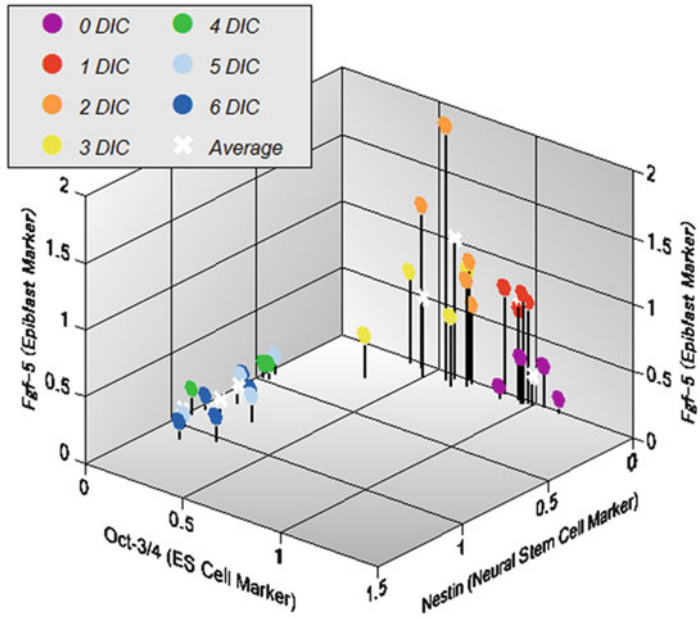

Fig. 12.3 Three-dimensional distribution of gene expression in single spheres during formation of neural stem spheres (From Otsu et al. 2011. © Elsevier) A three-dimensional scatter graph was derived from the expression of three representative cell specific marker genes, *Oct-3/4*, *Fgf-5* and *Nestin*, in each ES cell colony on 0 day (●) and in cell spheres grown for 1 (●), 2 (●), 3 (●), 4 (●), 5 (●) and 6 (●) days in stage 1, with the average (×) value calculated for each day. The normalized values are shown as relative ratios of the maximum value of the means. The values of the three genes are represented by the three Cartesian axes

spheres, the direction of differentiation of each sphere was the same.

To assess whether apoptotic cell death was induced during the formation of NSSs, we utilized the TUNEL assay to detect apoptotic cells on the cryosections of the spheres. We observed only a small number of apoptotic cells, localized at the surface of the spheres but not in the dense cores, with the percentages of TUNEL-positive cells being low throughout culture (Otsu et al. 2011). These results indicate that, using the NSS method, ES cells uni-directionally differentiate into neurons without selective apoptosis of non-neural cells. Although a default mechanism has been reported to randomly induce neuronal differentiation from mouse ES cells (Smukler et al. 2006), our NSS method initially induced the differentiation of ES cells into cells in the intermediate stage of early neurogenesis, followed by their rapid conversion into neural stem cells. The appearance of markers of epiblast cells, neural stem cells and neurons became noticeable on days 2, 4, and 6, respectively, of neuronal differentiation. This temporal stepwise progression of neuronal differentiation from ES cells resembles the progression of differentiation in embryos, from the ICM cells of the blastula to neuroectodermal cells via epiblast cells (Pevny et al. 1998; Loebel et al. 2003), suggesting that culture conditions may provide an environment similar to the physiological environment required for early neurogenesis in embryos, and also that this stepwise progression of differentiation results in the conversion of ES cells into homogeneous neural stem cells.

Maintenance of cell-cell communication is important in maintaining the homogeneity of neural cells derived from ES cells. Mouse ES cell colonies derived from a single ES cell at clonal density have a compact structure, reflecting the maintenance of cell-cell interaction. In culture, our method did not result in the dissociation of

colonies of undifferentiated ES cells; rather, it resulted in the differentiation of ES cells as whole colonies. This is similar to physiologic conditions, in that stem cells differentiate as a mass when ICM develop naturally *in vivo* and because cell-cell interactions facilitate cell differentiation and viability (Gurdon 1988). Unlike a selective culture method, accompanied by massive death of non-neural cells (Tropepe et al. 2001), the NSS method facilitates efficient neural differentiation without marked cell death (Otsu et al. 2011). Undifferentiated ES cells were reported to interact with each other through gap junctions, promoting the diffusion of second messengers such as calcium ion and cAMP (Wong et al. 2008). In addition, calcium gradients were shown to control the differentiation of Gap junction-coupled neural cells in Xenopus (Webb et al. 2005). Similar to the NSS method, the SDIA method gradually promotes the neural differentiation of mouse ES cells, which proliferate clonally on PA6 feeder layers (Kawasaki et al. 2000). Therefore, maintenance of cell-cell interaction may affect the efficiency of terminal differentiation and the homogeneity of differentiated cells *in vitro* as well as cell viability.

Conditioned medium has been found to promote the differentiation of ES cells (Morizane et al. 2006; Kato et al. 2006). Among the secreted proteins in ACM isolated by proteomic analysis (Lafon-Cazal et al. 2003) was cystatin C, a protein that promotes the efficiency of neurogenesis of mouse ES cells (Kato et al. 2006). Factors derived from striatum astrocyte were reported to promote the differentiation of human ES cells into tyrosine hydroxylase-positive neurons (Buytaert-Hoefen et al. 2004). The efficiency of neural differentiation was dependent not only on the promotion of neural differentiation and the selective cell death of non-neural cells, but on the prevention of neural cell death and their differentiation into non-neural cells. Thus, future research will focus on the identification of factors that may have different functional properties but synergistically promote the efficiency of neurogenesis by the NSS method.

Our previous findings have suggested that NSSs mimic the normal neurodevelopmental process and result in model clusters of CNS cells, and that the NSS method can provide a pluripotent stem cell-derived organ culture model of the brain. Therefore, using this culture model, our future research will focus on elucidating the mechanism of efficient neurogenesis and on assessing drugs, chemical compounds and physical stimuli that may impair neurodevelopment.

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Embryonic Stem Cell-Derived Motoneurons Develop Aberrant Axonal Sprouting

13

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Contents

Abstract

The organized neural cells form neural networks for receiving, transducing and integrating neural information. In developing embryo, asymmetrical distribution of specific molecules in neural progenitors contributes to neural polarization and initiates the formation of axons and dendrites on cell cycle exit. Axon is responsible for propagating action potential and triggering an electric or chemical output to the contacted cells. This chapter describes the recent findings about the cellular mechanisms of axon initiation and axon elongation *in vitro* by using isolated hippocampal neurons and embryonic stem cells-derived motoneurons. In addition, the *in vivo* studies related to the axon specification are emphasized. Environmental cues for stimulating the axon formation and axon guidance are also introduced.

Introduction

Developing neural progenitors in general are shaped with multineurites after existing cell cycle. These neurites consequently differentiate into dendrites and an axon, adapted to receive nerve impulses and propagate nerve signals to the connected cells, respectively. Some kinds of sensory neurons are featured with unipolar or bipolar morphology to convey external stimuli. Most interneurons possess multineurites and a short arborized axon to modulate neural signals and

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propagate the information to projection neurons, which have several branched dendrites and one long and thin axon. Receiving the signals from interneurons, projection neurons integrate the input information from the dendrites and initiate an action potential along the axon to activate or inhibit the activity of distal targeted cells.

The culture system of isolated hippocampal neurons is a classical experimental model for investigating axon initiation, elongation and dendrite formation (Fukata et al. 2002). Using this approach, scientists have extensively advanced the profiles of cellular and molecular interactions for the specification of axon and dendrites in the last decades. In addition, using isolated dorsal root ganglion cells and retina ganglion cells also provides substantial progresses for our understanding of the establishment of neural polarity. Besides of the genetic control in cultured neurons, extracellular environments also provide guiding cues for the controlling of axon extension and neural targeting. Recent investigations emphasize that intrinsic and extrinsic factors determine the early establishment of neural polarity and later axon guidance, respectively. Especially, parts of the *in vitro* findings of these determining factors are further consolidated by the genetic manipulation in experimental rodents.

Axon Formation *In Vitro*

The stereotype of morphological change of neurites makes the cultured hippocampal neurons a suitable approach for investigating axon formation. According to the morphological transition of isolated cortical neurons, the neurite specification in plated hippocampal neurons at embryo day 18 (E18) can be classified into five consecutive stages (Arimura and Kaibuchi 2007) (Fig. 13.1). At stage 1, the newly attached neurons on the culture plate extend lamellipodial and filopodial protrusion. These cell processes are dynamic and unstable on day 1. After cultured for 1–2 days, these cell processes extend and become more stable, called as immature neurites. On day 2–4 *in vitro* culture, one of the immature neurites will robustly grow and extend to be a distinguishable axon and the other neurites will become dendrites. This stage 3 is a critical step for the disrupting of cell symmetry and axon initiation. At stage 4, the axon is further extensively elongated and the dendrites become highly branched and further outgrowth. Finally, stage 5 neurons are fully maturated, featured with the emerging of numerous dendrite spines, functional synapses and the initiation segment of axon, where the action potential will be first initiated.

This observation in the isolated embryonic hippocampal neurons leads to the hypothesis that axon initiation can occur in any one of immature neurites at stage 3. The stochastic model of axon formation has been proposed, arguing that the axon specification is not predetermined but formed in a stochastic manner. Besides these morphological observations, artificially disrupting an axon can transform one of the existing dendrites to become the new axon, suggesting the existence of intrinsic inhibitory signal for preventing new axon formation and for maintaining existing one axon status (Goslin and Banker 1989).

Axon Formation *In Vivo*

While the *in vitro* culture advances our understandings on intracellular determinants of axon formation, single cortical neurons on culture plates face completely different environment as *in vivo* and the stochastic model requires further validation and examination *in vivo*. In utero and ex utero cortical electroporation give a new investigation system for modifying gene expression in neural progenitors before the occurrence of polarization in post-mitotic neurons (Hand et al. 2005). With these modern techniques, scientists can access to visualize the morphological changes and position transition for the labeled neural progenitors and explore the effects of cellular factors on the axon formation.

For cortical neurogenesis in vertebrates, embryonic neural stem cells, also named as radial glia cells on ventricular zone, extend and reach their neurites to the outer marginal zone of cortex (Fig. 13.2). On cell cycle exiting, the daughter cells usually migrate along the existing neurites

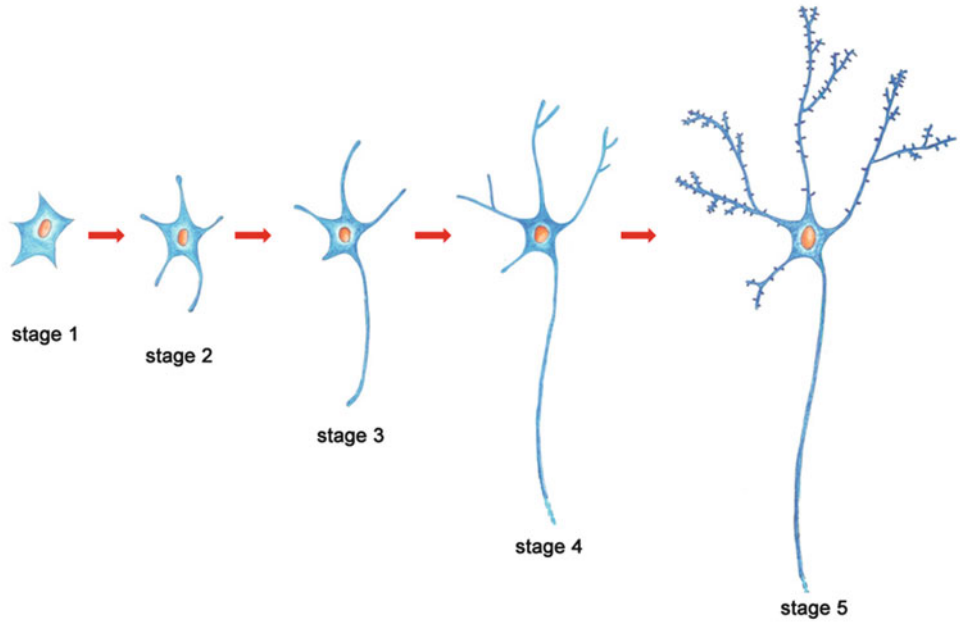

Fig. 13.1 The five stages of axon formation of isolated hippocampal neurons in vitro

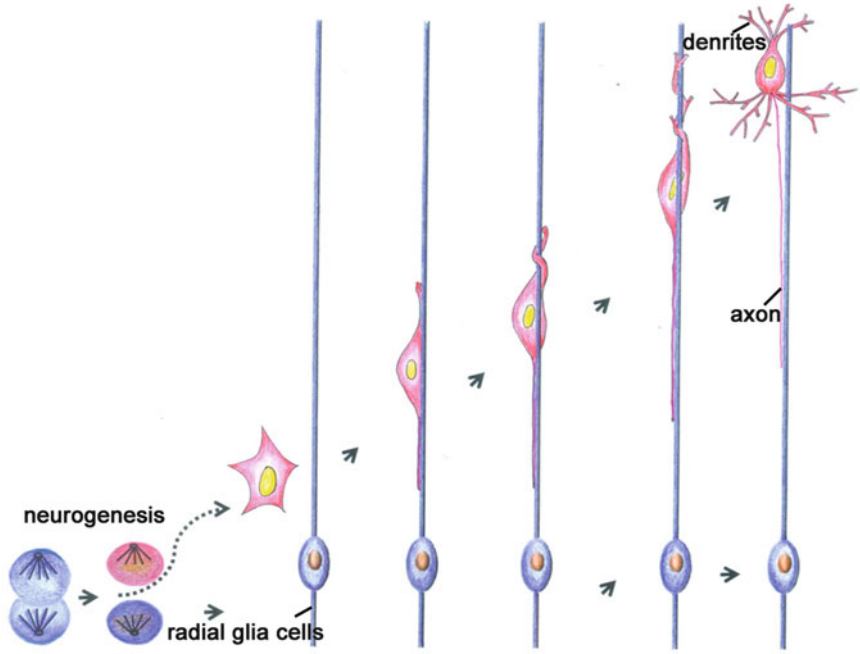

Fig. 13.2 The bipolar model of axon formation of cortical neurons in vivo

of radial glia cells toward the marginal zone before reaching their final destination. The migrating cortical neurons exhibit bipolar shape and present a leading process and a trailing process. The leading process is featured with thick growth cone but the trailing is usually thin and long. Accumulative evidences support that the leading process and the trailing process will differentiate

into the dendrite and the axon in developing brain, respectively.

Similar scenario of axon formation of migrating cortical pyramidal neurons is observed for retinal ganglion cells and bipolar cells in the developing vertebrate retina. In contrast to the stochastic model *in vitro*, this bipolar model of axon formation *in vivo* argues that the axon and dendrite polarity are inherited from the apico-basal bipolar configuration of a migratory neuron due to its stereotypical shape, but are not a stochastic determination (Barnes and Polleux 2009).

Signal Molecules Controlling Axon Formation

LKB1, SAD-A/B, and MARKs

The mammalian liver kinase B1 (LKB1), the counterpart of the *C. elegans* Par4, is an upstream serine/threonine kinase among six Par (partitioning-defective) proteins. Pars genes are first discovered from the screening of genetic candidates controlling epithelial cell polarity during the development and cell metastasis of *Caenorhabditis elegans* (*C. elegans*) (Kemphues et al. 1988). LKB1 is highly conserved and is critical for determining cell polarity in the nematodes and distant vertebrate species. Recent studies have indicated that LKB1 cooperates with SAD kinase (mammalian ortholog of Par1) to control neuronal polarization and axon development. Especially, genetic defect of LKB1 results in blockage of axon formation in developing pyramidal neurons *in vivo* (Shelly et al. 2007). LKB1 activity is regulated by brain-derived neurotrophic factor (BDNF) through the phosphorylation by protein kinase A.

It is suggested that the polarity-controlling activity of LKB1 kinase is dependent on the directly activated downstream effectors, including the SAD-A/B kinases, microtubule affinity regulating kinases (MARKs) and AMP-activated protein kinases (AMPKs). Expression knock-down by RNAi on SAD kinase abrogated the LKB1-triggered multiple axons formation. In addition, mice with double null for SAD-A and SAD-B kinase showed retardation of their axon initiation and elongation *in vivo* (Kishi et al. 2005). MARKs are the putative substrates of LKB1 and participate in the phosphorylation of microtubule-associated proteins, such as Tau protein, and axon elongation. Regarding to the AMPK, genetic and pharmacologic inhibitions of AMPK prevent the neurite growth in primary cells. Resveratrol, a polyphenol in plants, can activate AMPK and promote significant neurite growth in neuronal cell lines, primary neurons and neurons in brain (Dasgupta and Milbrandt 2007). Nevertheless, AMPKα1/α2 double null cortical neurons show normal cortical neurogenesis, neuronal migration, polarization and cell survival, suggesting that their genetic defect effects may be compromised by the redundant expression of other kinase members.

Par3-Par6-aPKC

Similar to the LKB1 (par4 ortholog), Par3 and Par6 were first discovered through genetic studies in *C. elegans* for their roles in cell polarity. Par3 serves as a scaffolding protein and associates with Par6, atypical protein kinase C (aPKC) and other polarity-regulating factors to set up an asymmetric intracellular signal center (Polleux and Snider 2010). In stage 3 cultured hippocampal neurons, Par3 and Par6 are accumulated in the nascent axon. Disturbance of Par3/6 location disrupts the established cell polarity and neurite specification. In addition, *in vivo* studies reveal that Par3-Par6-aPKC complexes are located in the apical region of ventricular cells of embryo and in the tip of nascent axon of migratory neuronal cells.

However, in *Drosophila*, disrupting the Par3, Par6, and aPKC orthologs genes did not result in abnormal axon or dendrite specification. Moreover, since the discovery of par3/Par6 in *C. elegans* and vertebrates, no genetic-modified mice were generated to illustrate the necessary role of Par3/6 in axon specification. While clear and strong evidences are validated in cultured cortical neurons, the importance of Par3/par6 for establishing neural polarity *in vivo* requires further sophisticated experiments to unveil.

Rac, Rho, Cdc42 Family

Rac, Rho and Cdc42 belong to the Ras superfamily and regulate cell adhesion, cell extension and cell migration through controlling the actin organization. Cellular morphological changes, like the filopodia, lamellipodia and stress fiber, are classical phenotypes of activated Cdc42, Rac and Rho protein, respectively. In addition, these small GTPase also influence the cell mortality and cell proliferation. For instance, the cell protrusion and migration are regulated by the Rac1 and the cell extension and lamellipodial are controlled by Cdc42 and Rac activity. Besides of the distinct morphology of cell process, all these three proteins have been shown to be involved in axon specification and axon growth (Arimura and Kaibuchi 2005).

A classical hierarchical cascade was observed in Swiss 3T3 fibroblast cells, where Cdc2 activates Rac and phosphorylated Rac further stimulates Rho. The activity of Rho-family GTPases is controlled by guanine nucleotide exchange factor (GEF), GTPase-activating protein (GAP), and guanine nucleotide dissociation inhibitor (GDI). GEF activates Rho-family GTPases by linking GTPases to their effectors and promoting the exchange of GDP with GTP. In contrast to GEF, GAP promotes the GTP hydrolytic activity of the Rho-family GTPases and inactivates these proteins into a GDP-bound status. The GDI regulators target to the prenylated carboxy terminus of Rho-family GTPases and sustain their inactive state in the cytoplasm.

AKT, PI-3 Kinase and PTEN

Besides the Rac and Rho small GTPase family, intracellular AKT, also called protein kinase B, are involved in the establishment of neural polarity and axon formation. The activated AKT is enriched in growth cones of polarized neurons (Shi et al. 2003). Expression of constitutive active AKT mutant leads to the formation of multiple axons. AKT proteins are positively regulated by phosphatidylinositol-3 kinase (PI3K) and negatively regulated by phosphastase and tensin homolog deleted on chromosome 10 (PTEN). Using LY294002 or Wortmannin inhibits PI3K activity and disrupts the axon formation. PTEN dephosphorylates phosphatidylinositol (3,4,5)-triphosphate (PIP3) and its overexpression also results in the blockage of neurite and axon formation in cultured hippocampal neurons.

Ras-MAPK Pathway

Similar to the AKT localization in plated cortical neurons, Ras downstream kinases, including c-jun N-terminal kinase (JNK) and extracellular related kinase (ERK), are restricted to the growth cones and the tip of growing axons. Pharmacological inactivation of the JNK and ERK kinases, such as PD98059 and U0126 respectively, also interrupted the axon growth and the neurite formation. The roles of these MAPK kinases may act on enhancing local transcription and translation, or regulating the cytoskeleton proteins by phosphorylation of microtubule-associated proteins.

Studies on DRG neurons revealed that ERK signal is essential for axon formation, elongation and further arborization. Here, the morphology and the development of DRG neurons are briefly introduced. Sensory neurons of the DRG are pseudo-unipolar neurons, which project their bifurcated axon to distinct peripheral targets, including skin, muscles and tendons. Receiving the sensory stimuli, the sensory neurons transduce the information through a short axon targeting into dorsal interneurons of spinal cord. Most sensory DRG neurons express the neurotrophin receptor TrkA and project thin and long axon fibers to innervate the epidermis. During the early development, nerve growth factor (NGF) is especially critical for controlling the cell survival, neurite maturation and target innervation. Disrupting the NGF expression in neonatal mice abrogated the epidermal innervation and axon branching in the skin tissue.

Recent evidences discovered that the mice lacking of an ERK upstream regulator, membranous Raf protein, and an ERK downstream effector, serum response factor (SRF) protein, showed impaired axon arborization in cutaneous skin but in companied with extensive axon elongation

(Wickramasinghe et al. 2008; Zhong et al. 2007). In addition, deletion of SRF in neonatal hippocampus caused disrupted configuration of the mossy fiber distribution (Knoll et al. 2006). Furthermore, the deficit of SRF in adult hippocampus resulted in impaired neural plasticity. These evidences demonstrate the importance of ERK and SRF on the neural elongation and arborization, especially when the growing axon meets the local growth factor cues, such as NGF, in the migratory tract to destined organs.

Wnt and GSK-3 Pathway

Providing synthetic Wnt3a can induce the neurite branching of DRG neurons, demonstrating that Wnt canonical pathway participates in the neurite formation. Glycogen synthase kinase 3 (GSK-3), a key molecule modulating the downstream activation of Wnt signal, is constitutively phosphorylated in the cytoplasm. Providing Wnt ligands steer the activation of Wnt receptor (Frizzle/LRP) and cause the dephosphorylation of GSK-3β, resulting in accumulated stabilized β-catenin in nucleus. Treating GSK-3β inhibitors with isolated cortical neurons results in multiple axon formation, similar to the Wnt ligands-treating results in DRG neurons.

Notably, administrating GSK-3β inhibitors, such as lithium chloride, to spinal cord-lesioned rats suppressed the activity of this kinase around lesion and induced a significant axon sprouting response in spinal cord (Dill et al. 2008). This result suggested that GSK-3 inhibitor is a potential drug for promoting functional recovery of damaged adult neural system. In a human trial, Dr. Wise Young first demonstrated the safety of lithium in chronic spinal cord injury patients (Young 2009). However, phase II test revealed that oral lithium carbonate administration did not achieve significant recovery for the functional outcomes of treated patients (Yang et al. 2012). These results illustrated that additional factors are required to gain function improvement in the human patients.

Immunocytochemical staining of Wnt-related proteins has demonstrated that adenomatous poyposis coli (APC) is selectively accumulated at the nascent axon tip and colocalized with Par3 in the stage 3 polarized hippocampal neurons (Arimura and Kaibuchi 2005; Shi et al. 2004). Cytoskeleton studies demonstrate that APC binds microtubule plus ends, enhances microtubule stability, and promote the elongation of cell process. In cultured hippocampal neurons, Par3 forms a complex with APC and kinesin family 3A proteins (KIF3A), a cargo transporter along microtubules to initiate neural polarity. Disturbing the APC function and APC expression leads to dislocalization of Par3 and defects in axon specification and neuronal polarity (Arimura and Kaibuchi 2005; Shi et al. 2004).

Interestingly, intracellular GSK-3β can directly regulate the microtubule-binding activity of APC. Dephosphorylated GSK-3β enhances the APC stability, leading to an accumulation of APC in neurites and stabilization of microtubules in the nascent axon. Conversely, sustained GSK-3β activity disrupts the spatial polarization of APC and Par3 accumulation at the nascent axon tip. These evidences strongly emphasize the importance of Wnt/GSK-3β on the axon specification, axon elongation and maintenance (Arimura and Kaibuchi 2005; Shi et al. 2004).

Protein Translation and Degradation

Local protein synthesis and degradation have been recognized as an important mechanism to control the neural polarity. The translocation of specific mRNA and the local synthesized molecules are necessary for the specification of axon and dendrites and further synapse formation. For instance, Par3 mRNA is highly enriched in the tip of axon process and its encoded protein is essential for the axon formation and initiation.

Besides the positive control of axon formation, dynamic polymerization of certain cytoskeletons and degradation of specific molecules in the nascent axon are also important for the axon specification, elongation and maintenance. Selective degradation of local protein content has been demonstrated to be a decisive event during neuronal development. For instance, local unphosphorylated AKT kinase in putative axonal process was shown to be more vulnerable than

the phosphorylated AKT kinase for proteosome degradation (Yan et al. 2006). Selective removal the inactive kinase increases the proportion of phosphorylated kinase and trigger spatial enrichment of the activated signals in the axon. Similar regulation of protein degradation in axon formation was observed for the GTPase Rap1b and Smurf2 proteins (Schwamborn et al. 2007).

In addition to the selective degradation of protein kinase or GTPase, specific ubiquitin proteosome system is also involved in the axon and dendrite specification. The E3 ubiquitin ligase Cdh1-anaphase-promoting complex (Cdh1-APC) has been shown to be involved in axonal morphogenesis of mammalian central nerve system. Distinct to their noted role in cell cycle progress, in post-mitotic neurons Cdh1-APC selectively degrades a transcription factor, SnoN, and consequently prevents SnoN to bind its downstream target gene, the scaffolding protein Cdc1 (Stegmuller et al. 2006). Reduced Cdc1 expression attenuated the local JNK activity and axon elongation. The importance of SnoN in axon formation is further validated by elegant experiments showing that gain or loss-of function analysis disturbs the axon morphology *in vivo*. Moreover, these intrinsic ubiquitin activities are regulated by the external TGFβ-Smad pathway. In cerebellar granule neurons, Smad2 expression is associated with SnoN and regulates the Cdh1-APC/SnoN activity in axonal growth. Inhibition of endogenous Smad signaling advances axonal growth. Conversely, Smad2 overexpression potentiates the SnoN degradation by activated Cdh1-APC and result in inhibition of axonal growth. These findings emphasize the role of upstream regulator of BMP/Smad pathway in axon growth control and indicate a possible therapeutic approach to enhance the neurite outgrowth in injured nerve tissues.

The Environment Cues on Axon Guidance

The aforementioned studies of environmental factors, such as Wnt, may determine the neural polarity and axon specification. In addition, these factors also contribute to the axon guidance, a later stage of axon elongation, supported by the genetic studies from several model animals, showing that external cues are essential for the direction guidance of axonal migration. These cues include extracellular matrix, axon guiding molecules, neurotrophic factors and neural patterning factors. The detail information of the axon guidance has been extensive reviewed (Bashaw and Klein 2010; O'Donnell et al. 2009). Several important concepts and related molecules are briefly illustrated here.

It is notable that migrating growth cones of axon are guided by both attractive and/or repulsive signals from the microenvironment. For instance, secreted extracellular netrin can attract the growth cone in a dose-dependent manner by the binding avidity to its membrane receptor, the deleted in colorectal cells (DCC) molecule. Other guiding molecules, including Ephs/Ephrins, Robos/Slits and Plexins/Semaphorins families are also critical mediators for modulating cell attraction or repulsion stereotypically to direct the migratory route of developing neurons.

In primitive nerve tissues, neural patterning factors, such as Wnt, sonic hedgehog (Shh) and BMP molecules, are also recognized as neural guiding factors for controlling the axon direction. For instance, the gradient concentration of Shh in ventral spinal cord provides an attractive cue for the dorsal neurons to migrate toward floor plate and extend the axon projection cross the midline of neural tube.

The initial axon guidance is associated with the asymmetrical distribution of guidance cue ligands on the cell membrane of growth cones where axon outgrowth will start. This specialized localization aims to sense and response the external cue gradients. In the next step, intracellular guiding-related molecules are polarized and enriched to the direction of environmental cues to orientate the tract of elongated axon.

The detail mechanisms about how the cell orchestrating the external stimuli signals and then integrating the intracellular cytoskeleton organization for directing elongation of axon are extensively elucidated in recent genetic studies of *C. elegans* (Quinn et al. 2006). In response to guidance cues, asymmetric occurrences of exocytosis and endocytosis, local accumulation of F-actin, microtubules, calcium signaling and

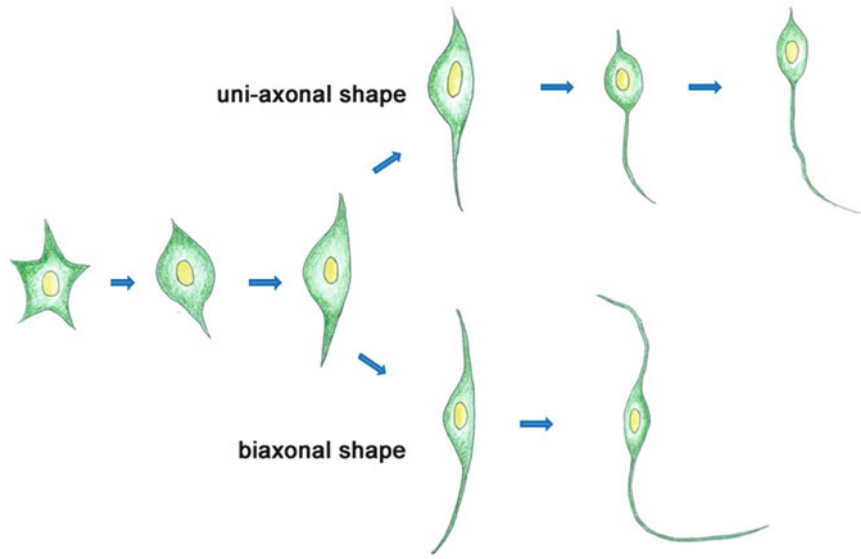

Fig. 13.3 The axon formation of ES-derived motoneurons in vitro

protein translation all participate in extending growth cone. Several membrane proteins, including Src, ezrin, radixin, moesin, cofilin and lamellipodin, are locally enriched in the growth cones and modulate the cytoskeleton organization and neurite extension. In the developing neurons of *C. elegans*, the direction of neural migration is mainly steered by the asymmetrical distribution of membrane DCC in order to response the higher gradient concentration of netrin. This signal of extracellular gradients are mediated and amplified by the intracellular Rac and PI3K pathway to the master molecule, lammelipodin, for coordinating actin and microtubules assembly and cell migration (Quinn and Wadsworth 2008).

Axon Formation of Embryonic Stem Cells-Derived Neurons

Most *in vitro* studies on axon formation are using primary hippocampal neurons of mouse embryo. However, the molecular mechanisms underlying axon formation in other types of projection neurons remains largely unexplored. Embryonic stem cells (ESCs)-derived neurons can be continually traced *in vitro* from the stages of post-mitosis to the axon initiation, elongation and maintenance, providing a new system for studying de novo axon formation and the effects of intrinsic neural polarization factors. In contrast, the isolated hippocampal neurons from neonatal rat are polarized prior to dissociation and re-initiate cellular polarization after being plating *in vitro* (Barnes and Polleux 2009). The new axon determination among immature neurites may be affected by the restricted distribution of pre-existing axon-regulating factors, which are already localized in the axon tip of hippocampal neuron before the cell isolation.

Neurons with different morphologies may utilize distinct molecular mechanisms to organize their cellular architecture. While the establishment of neuronal polarity has been extensively studied using rat hippocampal neurons, this process has been largely unexplored in mammalian motoneurons. When examining at axonogenesis in ES-derived HB9-GFP transgenic motoneurons, our study revealed that the GFP expression is faithfully recapitulated with the HB9 promoter activity, which are a well-established motoneurons-specific transcription factor (Pan et al. 2011) (Fig. 13.3). Immature cellular processes extended by the GFP^+ cells were highly dynamic and not preserved after the first 3 h of tracing. Later the GFP^+ cell retracted most of its initial cellular processes and developed a bipolar

organization. In most of the GFP$^+$ cells, one of the immature neurites underwent axonal specialization, which was accompanied by the compartmentalization of intrinsic polarizing factors into the axon, including the phosphorylated AKT, PI3K, and JNK. This mechanism for axon formation in the ES-derived motoneuron is similar to the model for bipolar definition, but distinct from the stochastic model, in the primary hippocampal neurons.

Interestingly, we found that two thirds of the total GFP$^+$ cells were precisely configured and correctly programmed to form the normal uni-axonal conformation, indicating that the appropriate intrinsic regulators were present and likely play a critical role in the morphogenesis of motoneurons.

In hippocampal neurons, an inhibitory feedback signal is necessary to prevent the re-specification of additional axons from dendrites and maintain the correct axon configuration. Especially, in the HB9-GFP$^+$ cells, one third of the nascent motoneurons extended bilateral cell processes that developed into aberrant bi-axonal configuration, suggesting the similar inhibitory mechanism may be absent in ES-derived motoneurons. However, regulatory factors contributing to appropriate bipolar motoneuron development are not well-understood, and we cannot exclude the possibility that environmental cues, such as an extracellular matrix or diffusible chemo-attractive factors, must be precisely integrated with intrinsic signals to orchestrate axon formation.

Engrafting the HB9-GFP$^+$ cells into the spinal cord did not rectify the aberrant bi-axon formation. The effects of bi-axon motoneuron after transplantation still require further exploration. The bi-axon motoneurons may serve as bridging cells to establish a stable connection of engrafted cells with host tissue and help the survival of engrafted neurons. However, we also did not rule out the possibility that the bi-axon motoneuron might be activated by adjacent neurons and consequently transmit inappropriate electric signals to sensory neurons or interneurons. The resulting misconnection might lead to allodynia-like neuropathy, thereby restricting the application of this engraftments for treating spinal cord injury (Hofstetter et al. 2005).

In conclusion, in this chapter the molecular mediators for controlling axon formation *in vitro* and *in vivo* are described in detail. Using ESC-derived motoneurons provides a new *in vitro* template for studying axon formation. Understanding the cellular mechanisms underlying axonal and dendritic specification will allow us to improve the function of injured neural tissues, direct the axon morphogenesis of transplanted neurons and thereby increase the safety and efficacy of stem cell-based therapy.

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Part IV

Hematopoietic Stem Cells

14 Crucial Role of the Polycomb Group Gene Product BMI-1 in the Maintenance of Self-Renewing Hematopoietic Stem Cells

George Wendt, Shunsuke Nakamura, and Atsushi Iwama

Contents

Abstract

Epigenetic regulation is critical for biological development and homeostasis. The polycomb group (PcG) proteins form the Polycomb Repressive Complexes (PRC) 1 and 2, and are responsible for reversibly silencing gene expression via histone modifications. BMI1, a component of PRC1, plays an important role in the maintenance of self-renewal of somatic stem cells as well as cancer cells, and is also involved in cellular differentiation and reactive oxygen species signaling. Loss of Bmi1 in mice results in the progressive depletion of HSCs, ultimately leading to lethal pancytopenia. This depletion is mediated primarily by the derepression of the *Ink4a/Arf* locus, resulting in cellular senescence and apoptosis. BMI1 appears to function in a similar capacity in most malignancies, where overexpression or ectopic expression of BMI1 silences tumor suppressor gene loci including the *INK4A/ARF* locus, enabling malignant growth of the tumor. Overexpression of Bmi1 is not sufficient to induce malignancies in mice, though it does accelerate tumorigenesis in certain model systems. *Bmi1*-overexpressing hematopoietic cells are significantly more resistant to stresses such as *ex vivo* culture and serial transplantation. BMI1 can function as both an oncogene and a tumor suppressor, depending on the cellular context. When functioning as an oncogene, BMI1 targets tumor suppressor gene loci including the *INK4A/ARF* locus, as is reported in numerous types of cancer. In line

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with recent reports that suggest PcG components can act as tumor suppressors, however, Bmi1 was recently found to act as a tumor suppressor in an *Ink4a/Arf*-deficient background. Future research on BMI1 will likely be focused on how to manipulate the PcG-mediated epigenetic machineries for regenerative medicine and cancer therapy.

Introduction

Epigenetic regulation is defined as the mechanisms that maintain cell-type specific gene expression patterns in daughter cells through cell division independently of the primary DNA sequence. Epigenetic mechanisms involve DNA methylation, histone modifications, and various types of RNA-mediated interactions (Cedar and Bergman 2011) and play a key role in cellular memory, the ability of cells to remember cell-lineage specific expression patterns induced by transiently expressed regulatory factors through subsequent cell divisions. The faithful retention of cellular memory is essential not only for normal development, but also for the maintenance of cellular homeostasis. This homeostasis is maintained in part by the controlled self-renewal and differentiation of adult stem cells. Various histone modifying entities are responsible for maintaining cellular memory, such as the transcriptional activator trithorax group (TrxG) proteins and the transcriptional repressor polycomb group (PcG) proteins (Simon and Kingston 2009). The PcG genes were initially discovered in *Drosophila* as sites of gene mutations that induced the homeotic mutations, or mutations that result in malformation of the body axis. Axial development in humans is also regulated by PcG-mediated repression of the *Homeobox* (*HOX*) genes. The PcG gene family is made up of several members, including the focus of this review, the BMI1 polycomb ring finger oncogene. Several recent studies have shown that PcG gene family members play critical roles in the function of hematopoietic stem cells, but this review will primarily focus on the role that the *BMI1* gene product plays in normal and malignant hematopoiesis. For a more complete review of other PcG proteins and hematopoiesis, see Konuma et al. (2010).

Polycomb Group (PcG) Complexes

The PcG proteins form multiprotein complexes that cooperate in order to silence the transcription of target genes. Two such complexes with conserved function across multiple species are termed Polycomb Repressive Complex (PRC)1 and PRC2 (Fig. 14.1). Canonical PRC2 is composed of the SUZ12, EED, EZH1/2, and RBAP48/46, and it catalyzes the di- and tri-methylation of histone H3 at Lysine 27 (H3K27me2/3) as well as the mono- and di-methylation of histone H1 at lysine 26 (H1K26me1/2) (Margueron and Reinberg 2011). Canonical PRC1 is composed of BMI1, RING1A/B, HPH, and CBX, and it catalyzes the mono-ubiquitylation of histone H2A at lysine 119 (H2AK119ub1). In *Drosophila*, the PRCs are recruited to their target sites through binding partners and genomic sequences known as Polycomb response elements (PREs). These PREs are not, however, functionally conserved in mammals. Instead, several proteins, such as YY1 and RYBP, and more recently long intergenic non-coding RNAs (lincRNAs) such as ANRIL have been shown to participate in the recruitment of PcG proteins (Aguilo et al. 2011; Simon and Kingston 2009).

According to the canonical model, PRC2 acts as an initiator complex, tri-methylating H3K27, a modification that recruits PRC1, which then acts as the functional gene silencing complex. Recently, however, several instances of PRC1 being recruited without PRC2 involvement have been demonstrated. For instance, recent studies have shown that murine PRC1 can be recruited to chromatin directly by the interaction between Bmi1 and the transcription factor Runx1 (Yu et al. 2012). In another study, it was demonstrated that a variant of PRC1, which includes Rybp instead of a Cbx protein, is sufficient for the maintenance of H2AK119ub1 in murine embryonic stem cells (ESCs) deficient in PRC2 (Tavares et al. 2012). Additionally, Gao et al. (2012) identified six

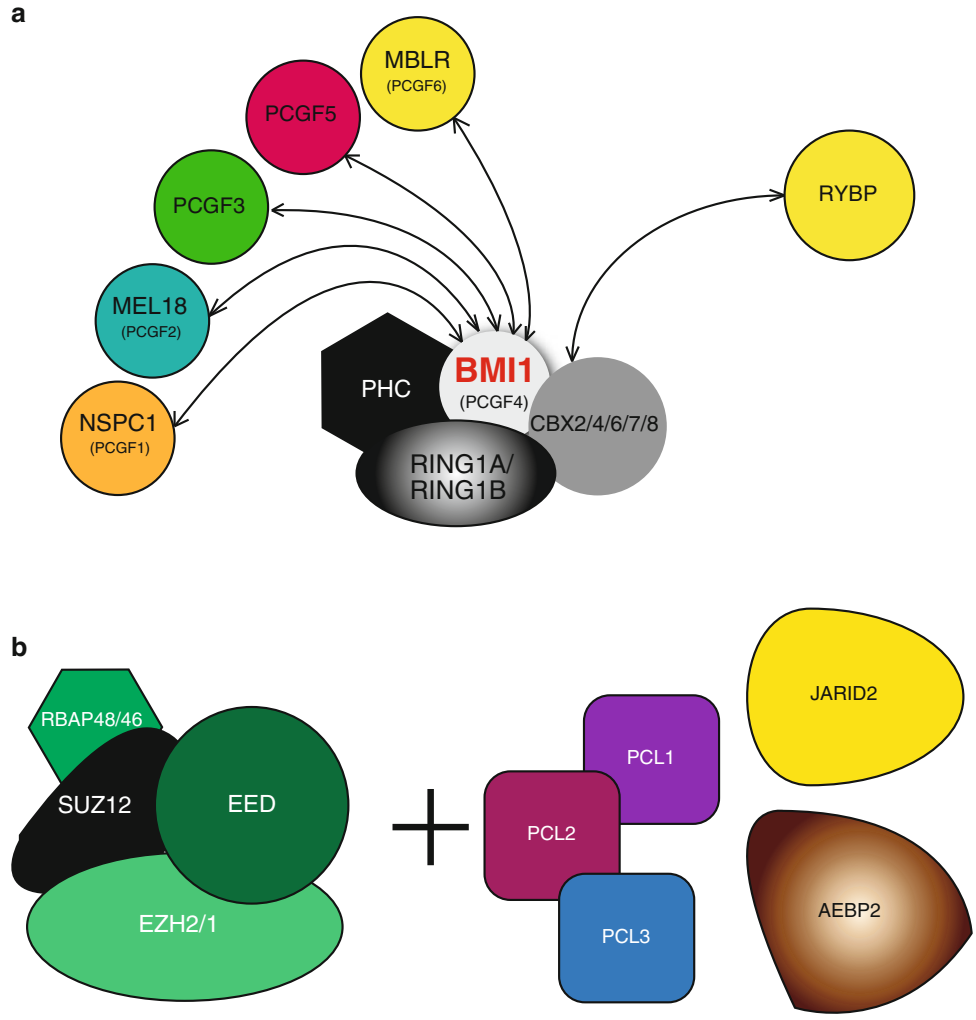

Fig. 14.1 The composition of the polycomb repressive complexes. (**a**) Canonical PRC1 composed of BMI1, a CBX protein, a PHC protein and the ubiquitin-protein ligase RING1B (or its homolog RING1A). Additionally, recent studies have reported that RYBP can also act as a part of the PRC1 complex, in an interaction that excludes CBX proteins. Another recent study highlighted the diversity of PRC1 complexes by showing that as many as six unique PCGF proteins (including BMI1) form six mutually exclusive PRC1 complexes, with non-canonical compositions having largely unknown functions. (**b**) PRC2 consists of SUZ12, EED, RBAP48/46, and the methyltransferase EZH2 (or its homolog EZH1), as well as other proteins of note known to associate with the complex. Together, PRC1 and 2 are responsible for silencing a variety of genomic loci via epigenetic mechanisms

distinct variants of PRC1 in human cells (Fig. 14.1a). Although all six variants contained RING1A/RING1B, each had a different Polycomb group RING finger protein (PCGF) and a different complement of polypeptide binding partners, RYBP or its homolog YAF2. The BMI1-(PCGF4)-RYBP/YAF2 containing variant also excludes CBX proteins and was also found to be essential for the differentiation of human ESCs.

Apart from their role in the regulation of development, PcG proteins are also well known regulators of somatic stem cell function. By silencing tumor suppressor loci and by keeping developmental genes poised for activation, PcG proteins are critical for the self-renewal and differentiation functions of somatic stem cells. Most famously, PRC1 and 2 cooperate to silence the *INK4B/ARF/INK4A* loci in numerous cell

types, preventing INK4A-mediated senescence and ARF-mediated activation of the p53 pathway. Silencing the *INK4B/ARF/INK4A* loci is essential for the self-renewal of stem cells. ANRIL, a lincRNA encoded in the *INK4B/ARF/INK4A* loci, has recently been reported to interact with the PRC1 component CBX7, promoting the silencing of the *INK4A* locus (Aguilo et al. 2011). These recent examples of regulation of PcG proteins by non-coding RNAs are a very exciting new avenue of investigation that will be discussed in greater detail later in this review.

BMI1: An Overview

As mentioned earlier, BMI1 (PCGF4) is a component of PRC1. Murine *Bmi1* was initially discovered as a frequent site of provirus insertion in Mo-MLV-induced B-cell lymphoma in *Eu-Myc* transgenic mice (van Lohuizen et al. 1991). Human *BMI1* was identified soon after and mapped to the short arm of chromosome 10 (10p13), a region known to be involved in translocations in various leukemias (Alkema et al. 1993). Shortly thereafter, *Bmi1*'s ability to act as an oncogene was verified by overexpressing *Bmi1* in murine lymphoid cells in order to induce lymphomas (Haupt et al. 1993). The development of a *Bmi1* knockout mouse yielded many insights into the physiological role that *Bmi1* plays *in vivo*, including its role in development and hematopoiesis (van der Lugt et al. 1994). The *Bmi1* knockout mouse will be discussed in greater detail later in this review.

BMI1 is a particularly interesting protein due to its apparent requirement by nearly all types of characterized somatic stem cells (Sauvageau and Sauvageau 2010). Examination of murine *Bmi1* has revealed that the gene product is required by or characteristic of hematopoietic stem cells, neural stem cells, hair follicle stem cells, intestinal stem cells, and pancreatic acinar stem-cell like cells. Mechanistically, Bmi1 appears to be essential for silencing the *Ink4a/Arf* locus, repression of which promotes self-renewal over senescence/apoptosis. Loss of Bmi1 leads to the derepression of the *Ink4a/Arf* locus and senescence/apoptosis in multiple tissues (Jacobs et al. 1999a). Bmi1 has also been shown to regulate somatic stem cells by keeping key differentiation genes poised for activation (Oguro et al. 2010) and by regulating the stem cell niche (Oguro et al. 2006) (Fig. 14.2a).

Of great clinical interest, *BMI1* expression has also been shown to be characteristic of or critical for the maintenance of many types of malignancies (Bracken and Helin 2009; Sauvageau and Sauvageau 2010), ranging from solid tumors such as medulloblastomas, neuroblastomas, squamous cell lung carcinomas, hepatocellular carcinoma, colorectal cancer and hematological malignancies such as acute myeloid leukemias (AML) and B-cell non-Hodgkin's lymphomas. The role of BMI1 in hematological malignancies will be discussed in depth later in the review. Mechanistically, murine *Bmi1* appears to maintain tumor cell self-renewal via repression of the *Ink4a/Arf* locus (Jacobs et al. 1999b). Similarly, expression levels of *BMI1* in human tumors tend to negatively correlate with expression of the *INK4a/ARF* locus (Fig. 14.2b). Interestingly, despite the vast amount of reports detailing *BMI1's* role as an oncogene, we have recently shown that Bmi1 can also act as a tumor suppressor under certain conditions (Oguro et al. 2012), a finding which will be discussed in greater detail later. These findings highlight the clinical importance of completely understanding the role that BMI1 and its targets play in regulating the many aspects of cancer.

BMI1 in Hematopoiesis

As mentioned previously, BMI1 is indispensible for the function of hematopoietic stem cells. In the following sections, we will discuss what has been learned about hematopoiesis from Bmi1 loss-of-function and gain-of-function tools, including the *Bmi1* knockout mouse, the *Bmi1* over-expressing mouse, and retroviral overexpression of *Bmi1*.

BMI1 Knockout Mouse

Although *Bmi1*$^{-/-}$ mice show normal embryonic hematopoiesis, they exhibit a progressive postnatal pancytopenia. The bone marrow becomes

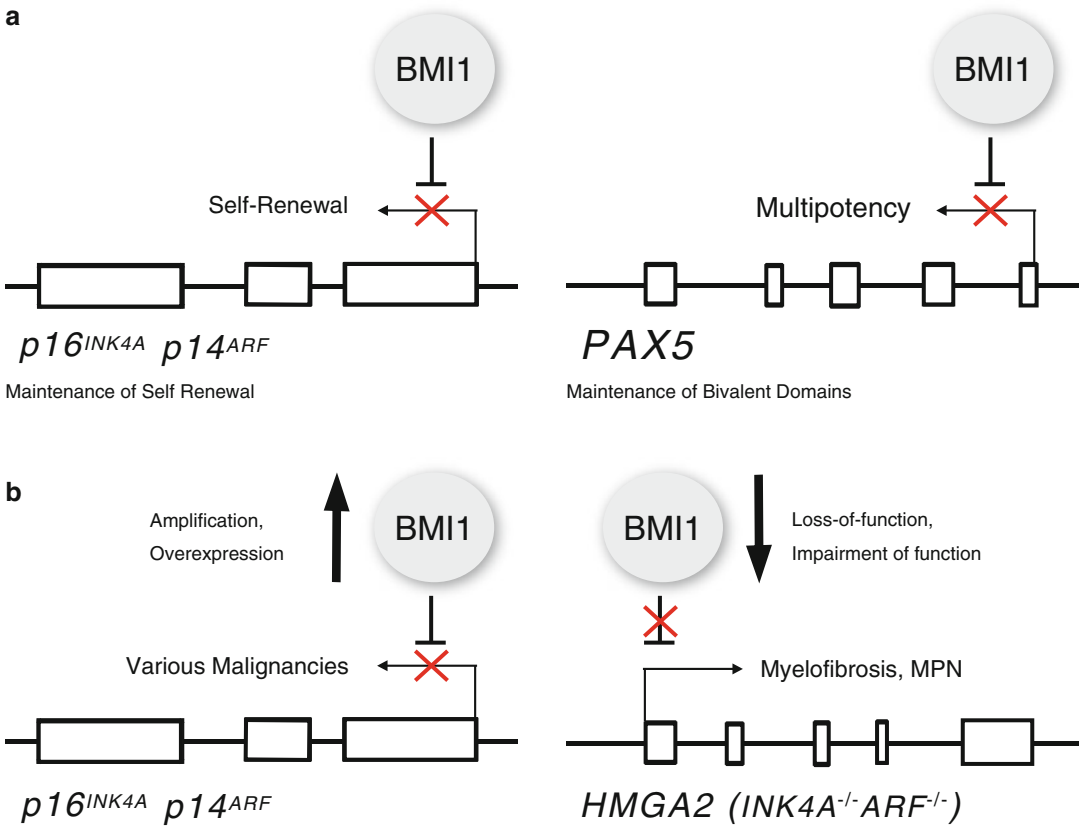

Fig. 14.2 BMI1 in normal and tumor cells. (**a**) BMI1 is required for the suppression of the $p16^{INK4A}$ and $p14^{ARF}$ and for the suppression of lineage commitment factors in somatic stem cells. (**b**) Amplification or overexpression of *BMI1* can repress the expression of the *INK4A/ARF* tumor suppressor gene locus in an oncogenic function, whereas loss of function of BMI1 can result in the derepression of oncogenes such as *HMGA2*, highlighting BMI1's simultaneous role as a tumor suppressor

severely hypoplastic and is gradually replaced by adipocytes with age (van der Lugt et al. 1994; Oguro et al. 2006). The hematopoietic defects in $Bmi1^{-/-}$ mice can be largely attributed to impaired HSC self-renewal. $Bmi1^{-/-}$ HSCs cannot maintain long-term hematopoiesis, although their short-term repopulating capacity is relatively preserved (Park et al. 2003; Iwama et al. 2004). This leads to a progressive postnatal depletion of HSCs in $Bmi1^{-/-}$ mice. The defect in HSC self-renewal is both cell-autonomous and non-autonomous, as the hematopoietic microenvironment in $Bmi1^{-/-}$ mice is also impaired (Iwama et al. 2004; Oguro et al. 2006). Of note, multipotent progenitor (MPP) cells in $Bmi1^{-/-}$ BM are grossly normal when compared to wild-type BM (Park et al. 2003).

Mechanistically, expression of *Ink4a* and *Arf* is markedly increased in the hematopoietic cells of *Bmi1*-deficient mice (Iwama et al. 2004; Park et al. 2003) and over expression of *Ink4a* and *Arf* in HSCs results in cell cycle arrest and p53-dependent apoptosis, respectively (Park et al. 2003). Self-renewal capacity of $Bmi1^{-/-}$ HSCs can be substantially rescued by the deletion of both *Ink4a* and *Arf* (Oguro et al. 2006). These findings define the *Ink4a/Arf* locus as a critical target of Bmi1 in the maintenance of HSC self-renewal (Fig. 14.2a). However, the BM microenvironment defects in *Bmi1*-deficient mice appears to occur independently of *Ink4a* and *Arf* derepression, as deletion of *Ink4a* and *Arf* in *Bmi1*-deficient mice does little to restore the microenvironment (Oguro et al. 2006).

Clonal analyses of HSCs *in vitro* revealed impaired proliferative potential accompanied by a reduction in multi-lineage differentiation capacity in *Bmi1*$^{-/-}$ cells. Paired daughter cell assays to monitor the behavior of HSCs *in vitro* demonstrated that Bmi1 is essential for CD34^{-}Lineage marker^{-}Sca-1^{+}c-Kit^{+} (LSK) HSCs to inherit multi-lineage differentiation potential through successive cell divisions. In most daughter cell pairs generated from wild-type CD34^{-}LSK HSCs, at least one of the cells inherits multi-lineage differentiation potential, whereas *Bmi1*$^{-/-}$ CD34^{-}LSK HSCs showed accelerated loss of multi-lineage differentiation potential, leading to inefficient expansion of their progeny (Iwama et al. 2004). Retroviral transduction of *Bmi1*$^{-/-}$ CD34^{-}LSK HSCs with *Bmi1*, however, completely rescued their defects in proliferation and multi-lineage differentiation potential *in vitro* and long-term repopulating capacity *in vivo*, suggesting that execution of stem cell activity is absolutely dependent on Bmi1 (Iwama et al. 2004).

Bmi1 is also critical for maintaining the multipotency of HSC/MPPs. Loss of Bmi1 results in the resolution of bivalent domains at the *Ebf1* and *Pax5* loci, leading to their premature expression in HSC/MPPs. *Ebf1* and *Pax5* are B cell specific master transcription factor genes that function in B lineage specification at the earliest stage of B cell differentiation. Premature activation of these transcription factors is accompanied by accelerated lymphoid specification and a marked reduction in HSC/MPPs (Oguro et al. 2010) (Fig. 14.2a).

Furthermore, Bmi1 appears to regulate a number of genes involved in mitochondrial function and reactive oxygen species (ROS) homeostasis, and *Bmi1*-deficient mice exhibit increased levels of ROS in HSCs and thymocytes when compared to wild-type mice. Treatment with the antioxidant *N*-acetyl-$_{L}$-cysteine (NAC) or interruption of the response to DNA damage by deletion of *Chk2* rescues multiple defects in *Bmi1*-deficient mice including the defect in the number of thymocytes and BM HSC/progenitor cells, but not the defect in self-renewal of *Bmi1*-deficient HSCs (Liu et al. 2009). Similarly, knockdown of *BMI1* using lentiviral RNA interference results in the accumulation of ROS in human cord blood CD34^{+} HSCs (Rizo et al. 2009). Recent studies have shown that an increased level of ROS primes *Drosophila* hematopoietic progenitors for differentiation, a process which involves down-regulation of PcG activity (Owusu-Ansah and Banerjee 2009), suggesting a novel functional interaction between ROS and Bmi1.

Overexpression of BMI1

A central role for *Bmi1* in HSC self-renewal has been demonstrated by the overexpression of *PcG* genes in HSCs (Iwama et al. 2004). Overexpression of *Bmi1* in HSCs induces a striking expansion of multipotent progenitor cells *ex vivo* and a marked augmentation of HSC repopulating capacity *in vivo*. Overexpression of other PRC1 components including *Ring1b*, *Mph1/Rae28*, or *M33* had no such effects. Only *Mel18* minimally expanded multipotential progenitor cells *ex vivo*. As mentioned earlier, overexpression of *Bmi1* significantly enhances the probability of HSCs giving rise to multipotent daughter cell pairs in paired daughter cell assays. This finding indicates that Bmi1 promotes symmetrical cell division of HSCs, shown by the preservation of multi-lineage differentiation potential. Unexpectedly, transduction of *Bmi1* into CD34^{+}LSK progenitor cells did not enhance their colony-forming efficiency or *in vivo* repopulating capacity at all. These findings suggest that Bmi1 promotes HSC self-renewal, but does not confer a growth advantage or long-term repopulating capacity on CD34^{+}LSK progenitor cells, a population with limited potential for proliferation and differentiation.

Recently, we analyzed the effect of overexpression of *Bmi1* with a new mouse line where *Bmi1* can be conditionally overexpressed in a hematopoietic cell-specific fashion. Overexpression of *Bmi1* in this mouse does not significantly affect steady state hematopoiesis, but it efficiently protects HSCs from stresses through *ex vivo* culture and serial transplantation, conditions in which HSCs are exposed to oxidative stress. However, our Bmi1-overexpressing mouse shows no difference in the levels of intracellular ROS compared to wild type

mice. This suggests that Bmi1 may be negatively regulated downstream of ROS signaling and an excess of Bmi1 may overcome this negative regulation (Nakamura et al. 2012). Further experiments to investigate this hypothesis are currently underway.

In human cord blood $CD34^+$ hematopoietic progenitor cells, retroviral transduction of BMI1 results in improved stem cell maintenance. *BMI1*-overexpressing cells retain HSC specific activity *in vivo* after *ex vivo* culture. It was also observed that *BMI1* overexpressing $CD34^+/CD38^-$ cells enriched in HSCs show less apoptosis and more quiescence (Rizo et al. 2008). Lentiviral knock down of *BMI1* in human cord blood $CD34^+$ cells impairs their long-term expansion and colony-forming capacity, and reduces stem cell frequencies. Knock down was also partially associated with increased expression of $p14^{ARF}$ and $p16^{INK4A}$ (Rizo et al. 2009).

Taken together, both loss-of-function and gain-of-function analyses have established a central role for Bmi1 in the maintenance and/or augmentation of HSC function and suggest that the level of Bmi1 is a critical determinant of the self-renewal capacity and multipotency of HSCs. In this regard, Bmi1 could be a useful target for *ex vivo* HSC expansion and other therapeutic manipulation of HSCs.

BMI1: Fetal vs. Adult Requirement

Bmi1 is necessary for efficient self-renewal and expansion of adult HSCs but is less critical for the generation of progenitor cells and is dispensable for fetal liver hematopoiesis. However, transplantation of $Bmi1^{-/-}$ fetal liver cells results in transient hematopoietic reconstitution, which suggests that $Bmi1^{-/-}$ fetal liver HSCs cannot self-renew in BM, but can give rise to MPPs that can sustain hematopoiesis for 4–8 weeks (Park et al. 2003). These findings underscore the requirement of Bmi1 in adult BM but not in fetal liver hematopoiesis. Conversely, *Ezh2*, a component of PRC2, is dispensable for adult hematopoiesis but required for fetal hematopoiesis, though *Ezh2*-deficient adult mice have defects in lymphopoiesis. Unlike *Bmi1*-deficient embryos, *Ezh2*-deficient embryos die prenatally due to anemia, and expansion of hematopoietic stem and progenitor cells is significantly impaired in *Ezh2*-deficient fetal livers (Mochizuki-Kashio et al. 2011). Of interest, however, *Ezh2*-deficient fetal liver cells can reconstitute hematopoiesis in recipient BM. Similarly, deletion of *Ezh2* in adult mice affects the differentiation (particularly of the lymphoid lineages), but not self-renewal, of HSCs. Collectively, these findings suggest that fetal and BM HSCs are differentially regulated by PcG proteins.

Additionally, the role that Bmi1 plays in leukemogenesis appears to differ between fetal hematopoietic progenitors and adult hematopoietic progenitors. In 2003, Lessard and Sauvageau showed that *Bmi1*-deficient fetal liver cells can be transformed into a primary leukemia, but that the leukemia is not transplantable. In contrast, *Bmi1*-deficient BM cells cannot be transformed into a primary leukemia (Yuan et al. 2011). This has interesting implications for cancer biology, suggesting that pediatric malignancies can be fundamentally different from adult malignancies despite having similar cytological profiles.

Oncogenic Function

Bmi1 is essential for maintenance of not only normal HSCs, but also leukemic stem cells (LSCs), a rare cell population in certain leukemias capable of giving rise to leukemia in recipients following single cell transplants. Studies indicate that overexpression of only *BMI1* is not sufficient to induce leukemia but BMI1 is an important collaborating factor in leukemic transformation. As mentioned previously, Bmi1 was first identified as a molecule that cooperates with Myc in the induction of B-cell lymphomagenesis (van Lohuizen et al. 1991). Consistent with these findings, *BMI1* expression levels in the $CD34^+$ cells correlate with the progression and prognosis of myelodysplastic syndrome (Mihara et al. 2006) and chronic and acute myelogenous leukemia, with high levels of BMI1 indicating a poor prognosis. In a $Bmi1^{-/-}$ background, granulocyte-macrophage progenitors (GMPs) transformed

with the leukemic fusion gene *MLL-AF9* show enhanced differentiation and retain less LSCs, suggesting a critical role for Bmi1 in leukemic maintenance (Yuan et al. 2011). Taken together, these findings show that BMI1 can act as a potent oncogene in hematological malignancies.

Tumor-Suppressor Function

Interestingly, recent findings have shown that PcG proteins are capable of acting not only as oncogenes but also as tumor suppressor genes. The identification of inactivating mutations in the PcG gene *EZH2*, a component of PRC2, unveiled a tumor suppressor function in myelodysplastic syndrome and myeloproliferative neoplasms including myelofibrosis (Ernst et al. 2010; Nikoloski et al. 2010), T cell acute lymphoblastic leukemia (T-ALL) (Ntziachristos et al. 2012), and early T-cell precursor acute lymphoblastic leukemia (ETP ALL) (Zhang et al. 2012). Recent findings also showed that PRC1 also has a tumor suppressor function in *Drosophila* through repression of genes related to Notch signaling (Classen et al. 2009) and JAK-STAT signaling (Martinez et al. 2009).

We have also recently shown that *Bmi1* acts as a tumor suppressor. Loss of *Bmi1* in an *Ink4a/Arf*-null background restores the long-term self-renewal capacity of HSCs, but results development of a myeloproliferative disease with progressive and lethal myelofibrosis. Further investigation revealed that loss of Bmi1 results in the derepression of the oncogene *Hmga2*, which is at least partially responsible for the development of myelofibrosis observed in the *Bmi1/Ink4a/Arf* triple knockout mice (Oguro et al. 2012) (Fig. 14.2a). This overexpression of *Hmga2* is also found in human primary myelofibrosis (PMF). In another study, 13% of enrolled PMF patients exhibited loss-of-function mutations of *EZH2* (Ernst et al. 2010). Although no mutation of *BMI1* has been indentified in PMF patients without *EZH2* mutations, it is possible that other mechanisms exists that compromise PcG function in human malignancies. Together, these results suggest that PcG proteins including BMI1 act not only oncogenes but also as tumor suppressors.

Human Disease

Given its central role in both hematopoiesis and cancer, it is not surprising that BMI1 is intricately linked with many human hematopoietic malignancies. As mentioned above, *BMI1* expression increases with disease progression and high levels of *BMI1* correlate with poor overall survival in myelodysplastic syndrome, acute and chronic myeloid leukemia, and non-Hodgkin B-cell lymphomas. In one study, *BMI1* gene amplifications were observed in 12 % of mantle cell lymphoma (MCL) cases, a type of malignancy in which *BMI1* expression is also elevated (Rubio-Moscardo et al. 2005).

There is still much to be discovered about the signaling pathways that regulate BMI1. SALL4, an oncogene that is expressed in AML, is capable of upregulating *BMI1* expression by directly binding to its promoter (Yang et al. 2007). Moreover, transgenic mice that constitutively express SALL4 develop leukemia and have increased levels of *Bmi1* expression, particularly upon progression from a preleukemic myelodysplastic stage toward a more acute myeloid leukemic phenotype (Yang et al. 2007).

Future Perspectives

Since the discovery of BMI1 over 20 years ago, much progress has been in understanding how this unique gene product maintains somatic stem cells and cancer. There is still much, however, that remains to be investigated about BMI1. Though its role in silencing the *INK4a/ARF* locus has been well demonstrated, recent studies have suggested that BMI1 also plays a role in the regulation of diverse cellular events. As such, it will now be critical to elucidate not only the loci that BMI1 is responsible for suppressing, but also the various proteins and noncoding RNAs that might play a role in regulating BMI1 function.

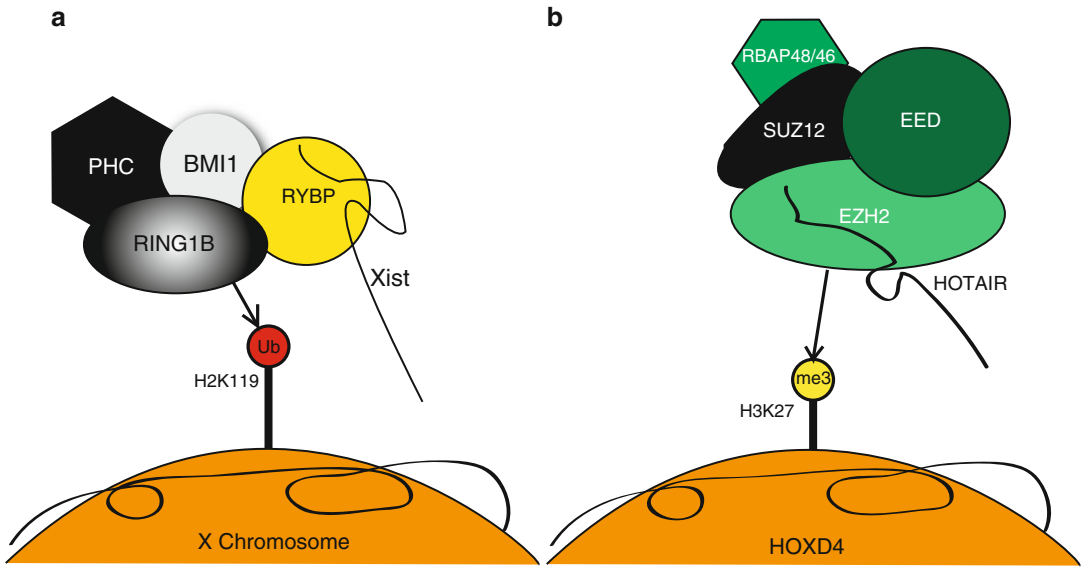

Fig. 14.3 lincRNAs act as binding partners to the polycomb complexes. (**a**) The lincRNA *Xist* is critical for recruiting the RYBP-PRC1 complex to loci on the X chromosome for dosage compensation during embryonic development. This interaction is independent of PRC2. (**b**) *HOTAIR* plays an important role in the recruitment of PRC2 to loci critical for development and tumorigenicity, including the *HOXD4* locus. Other lincRNAs may function in a similar fashion, acting as the initial step in recruiting PRC2 to its genomic binding sites

The importance of non-canonical PRC1 complexes has come into the spotlight recently. A report describing the role of RYBP1 in non-canonical PRC1 recruitment highlights the importance of discovering and characterizing BMI1's as-of-yet unknown binding partners (Tavares et al. 2012). Beyond protein-protein interactions, recent studies have suggested a novel role for non-coding RNAs in the regulation of PcG proteins (Fig. 14.3). Cao et al. (2011) identified a regulatory network in which microRNA targets of EZH2 regulate the transcription of BMI1. Post-transcriptional regulation by non-coding RNAs is also an exciting new avenue of investigation. The long non-coding RNA ANRIL has been implicated in the recruitment of PRC1 to the *INK4a/ARF* locus through interactions with CBX7 (Yap et al. 2010). Similarly, the long non-coding RNA HOTAIR has been implicated in the recruitment PRC2 to the HOXD locus (Rinn et al. 2007), and overexpression of HOTAIR in human cells has been shown to result in genome-wide retargeting of PRC2 (Gupta et al. 2010). Given the significant roles these non-coding RNAs play in the regulation of PcG proteins, searching for non-coding RNAs that interact with BMI1 promises to be an interesting avenue of investigation.

The biochemical regulation of BMI1 also remains largely uncharacterized. Voncken et al. (2005) showed that Bmi1 is phosphorylated by the MAPKAP kinase, 3pK, and that this phosphorylation results in a dissociation of Bmi1 from chromatin followed derepression of target genes. Since 3pK is downstream of the p38 signaling pathway, a pathway activated in response to ROS, this suggests a role for ROS in the regulation of BMI1 function. Of interest, overexpression of *Bmi1* confers resistance to oxidative stress on HSCs, but does not attenuate ROS levels in HSCs (Nakamura et al. 2012), suggesting that Bmi1 is located downstream of ROS signaling and negatively regulated by it. This is particularly interesting given the body of research into the role that ROS plays in the regulation of hematopoietic stem cells (Shao et al. 2011), potentially indicating a new pathway through which ROS restricts BMI1 function in stem cells.

Given its prominent role in the maintenance of both stem cells and cancer cells, advancing our

understanding of BMI1 will not only be fascinating from a perspective of stem cell biology, but may also lead to the development of new cancer therapies and regenerative medicine applications.

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Hematopoietic Stem Cell Function and Skeletal Formation: Positive Role of *Hemp* Gene

15

Hiroaki Honda

Contents

Abstract

Hematopoietic stem cells (HSCs) have a capacity to undergo self-renewal and differentiation into several distinct cell lineages, which maintain blood production and provide mature blood cells throughout life. To clarify molecular mechanisms governing HSC development, we performed a genome-wide gene expression analysis of fetal liver (FL) HSC cDNA library, and identified a novel gene, *hemp* (*hematopoietic expressed mammalian polycomb*), that encodes a protein with a zinc-finger domain and four malignant brain tumor (mbt) repeats. To elucidate its biological role(s), we generated *hemp*-deficient mice and found that Hemp plays pivotal roles in HSC function and skeletal formation. Mononuclear cell number in $hemp^{-/-}$ FLs was markedly reduced, and $hemp^{-/-}$ FL HSCs exhibited significant defects in colony-forming and competitive repopulation assays. In addition, $hemp^{-/-}$ embryos exhibited various skeletal malformations, such as a fusion of cervical vertebrae. Since osteoblasts and HSCs coexist in the bone marrow niche and physically and functionally interact with each other, it is suggested that Hemp-mediated signalling positively and coordinately regulates normal development of these types of cells. In addition, skeletal abnormalities detected in $hemp^{-/-}$ mice closely resemble to those observed in human Klippel-Feil anomaly, suggesting that Hemp might be involved in the pathogenesis of this disease.

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Introduction

Stem cells are characterized by their ability to balance self-renewal with differentiation into mature cell lineages. Among various types of stem cells, hematopoietic stem cells (HSCs) are most intensively studied and well characterized (Dzierzak and Speck 2008; Zon 2008). A number of genes that regulate HSC activity have been identified, but the overall mechanisms governing HSC function are not fully understood.

To address this issue, we performed a large-scale, gene-expression screen in mouse fetal liver (FL) HSC cDNA library and identified a number of genes preferentially expressed in HSCs (SCDb, http://stemcell.mssm.edu/v2/). Among them, we focused on one that encodes a protein containing a C2C2 zinc finger domain and four tandem mbt (malignant brain tumor) repeats. Mbt is a protein domain originally identified in the protein product of a *Drosophila polycomb group* (*PcG*) gene, *lethal(3)malignant brain tumor* (*D-l(3)mbt*), whose recessive mutations cause the malignant transformation of larval and adult brain tissues (Wismar et al. 1995). Since the mbt repeats are also found in another *Drosophila PcG* gene, *Scm* (*sex comb on midleg*), and are highly conserved in their human counterparts, *H-l(3)mbt* (Koga et al. 1999; Wismar 2001), *SCMH1* (*sex comb on midleg homolog* 1) (Berger et al. 1999) and *SCML2* (Montini et al. 1999), we named the novel gene as *hemp* (*hematopoietic expressed mammalian polycomb*, also deposited in the database as *mbtd1* [*mbt domain containing 1*]).

Recent studies revealed that mbt domain binds to methylated histone residue(s) and regulates gene expression patterns (Bonasio et al. 2010). Thus, it is strongly suggested that Hemp regulates HSC function in an epigenetic manner, but biological role(s) of Hemp remains unclear. To address this issue, we generated and analysed *hemp*-deficient mice. Interestingly, *hemp*-deficient mice exhibited significant defects not only in HSC function but also in skeletal development, indicating that Hemp plays essential roles in definitive hematopoiesis and osteogenesis.

Structure and Expression of Hematopoietic Expressed Mammalian Polycomb (Hemp)

The comparison of the schematic structures of Hemp and previously identified mbt-containing proteins are shown in Fig. 15.1a. The mbt-containing proteins can be divided into two groups, depending on whether or not they possess an SPM (Scm, Ph and MBT) domain, which mediates protein-protein interactions (Bornemann et al. 1996; Peterson et al. 1997). SPM-lacking mbt proteins include Hemp, h-L(3)mbt-like a (Wismar 2001), h-L(3)mbt-like b (Wismar 2001) and M4mbt (Markus et al. 2003) (shown as SPM(−) in Fig. 15.1a), while SPM-containing mbt proteins include D-L(3)mbt (Wismar et al. 1995), H-L(3)mbt (Koga et al. 1999), SCMH1 (Berger et al. 1999), SCML2 (Montini et al. 1999), Sfmbt (Scm-related gene containing four mbt domains) (Usui et al. 2000) and MBT-1 (Arai and Miyazaki 2005) (shown as SPM(+) in Fig. 15.1a). It is noteworthy that Hemp and other mbt-containing, SPM-lacking proteins share a structural similarity in that they contain four tandem mbt repeats preceded by a C2C2 zinc finger domain SPM(−) (Fig. 15.1c). Therefore, these proteins may constitute a new subfamily of mbt-containing proteins.

Because the expression of *hemp* mRNA in distinct hematopoietic lineages was already reported (Phillips et al. 2000), we examined the expression patterns of *hemp* in mouse tissues and in human hematopoietic and non-hematopoietic cell lines. The results of Northern blots are shown in Fig. 15.1b. In adult mouse tissues, *hemp* showed relatively restricted expression with high expression in the testis (top panel). In human cell lines, *hemp* was expressed abundantly in most of the hematopoietic cell lines (middle panel), while it exhibited weak expression in all of the non-hematopoietic cell lines, except HEPG2 (bottom panel). These results indicated that *hemp* is preferentially expressed in hematopoietic cell lines and suggested that Hemp functions largely in hematopoietic lineages. We then investigated *hemp* expression patterns during embryogenesis

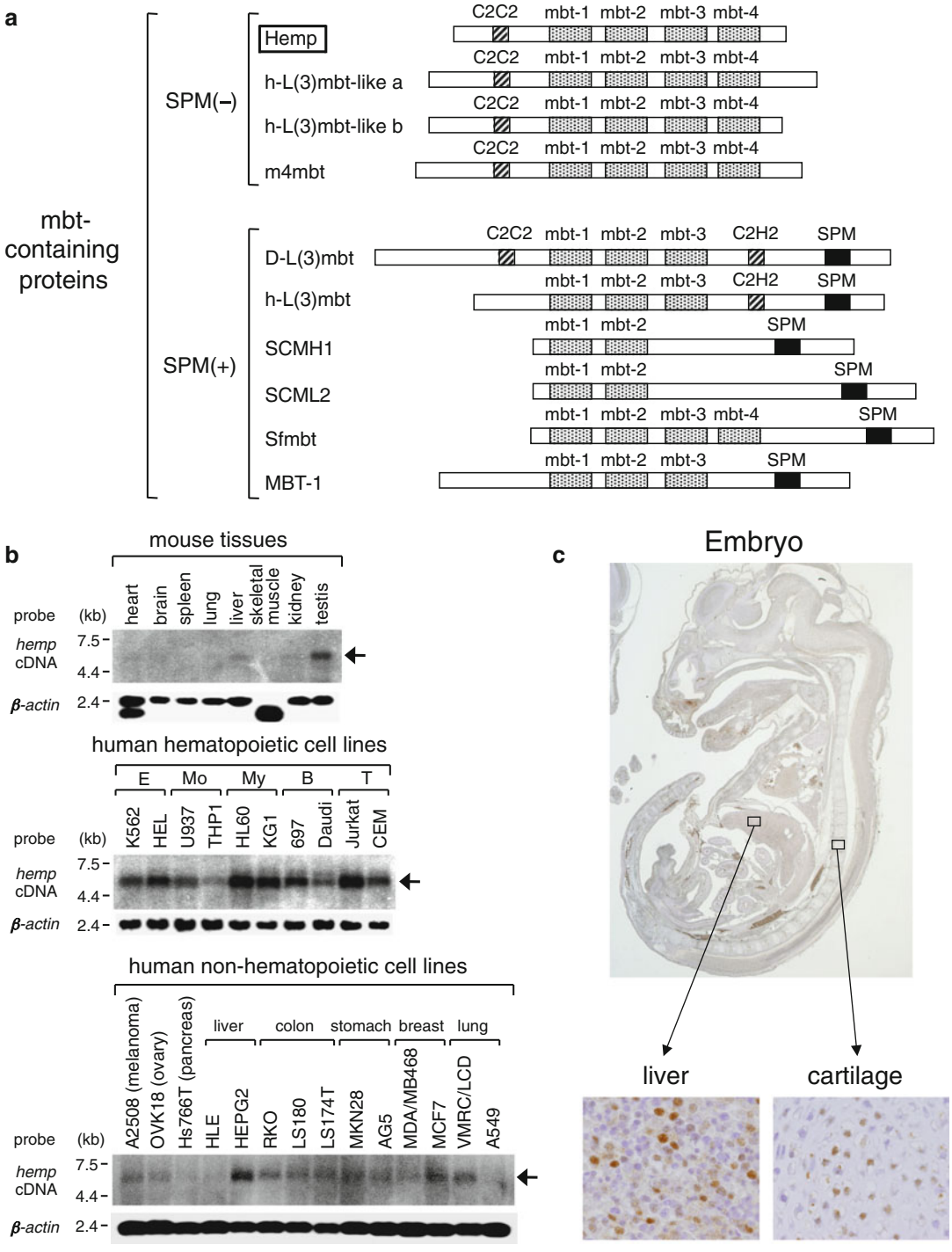

Fig. 15.1 (**a**) Comparison of the primary structures of Hemp (*boxed*) and previously reported mbt-containing proteins. The proteins are divided into two groups, SPM(−) and SPM(+), depending on whether, or not they possess the SPM domain (shown as a *black box*). The mbt domain and the C2C2/C2HC Zn finger domain are indicated by *gray* and *dotted boxes*, respectively. (**b**) Expression of *hemp* RNA in mouse tissues and in human hematopoietic and non-hematopoietic cell lines. The position of *hemp* is indicated by an *arrowhead*. Hybridization with *β-actin* is shown as an internal control. (**c**) Immunohistochemical staining for Hemp expression. Sagittal sections of an embryo at E14.5 were immunohistochemically stained with an anti-Hemp antibody (×50). Staining patterns of the whole embryo are shown in the *upper panel* and those in the *boxed areas* of the liver and the cartilage are magnified in the *lower panels*

by immunohistochemical staining using an anti-Hemp antibody. The representative result is shown in Fig. 15.1c. As shown in the upper panel of this figure, various tissues were positively stained, indicating that Hemp functions in a variety of cell types. As *hemp*$^{-/-}$ embryos exhibit phenotypical and functional abnormalities in the hematopoietic and skeletal tissues (see below), the liver and cartilage, where definitive hematopoiesis and osteogenesis occur, were examined in detail. In the liver, interstitial cells, including hematopoietic cells, showed positive staining, and in the cartilage, most of the chondrocytes and some undifferentiated mesenchymal cells exhibited positive signals (lower panels of Fig. 15.1c). These results strongly suggested that the defects in *hemp*$^{-/-}$ embryos (see below) were primarily attributed to Hemp deficiency. Positive staining was detected in the nucleus (lower panels of Fig. 15.1c), confirming that Hemp functions as a nuclear protein, as indicated by its primary structure (Fig. 15.1a).

Hemp-Deficient Mice Exhibit Skeletal Abnormalities Similar to Human Klippel-Feil Anomaly

We generated hemp-deficient heterozygous (*hemp*$^{+/-}$) mice by a conventional gene targeting method (Honda et al. 2011). Intercrossing of *hemp*$^{+/-}$ mice produced no live homozygous (*hemp*$^{-/-}$) mice at weaning (3 weeks after birth), indicating that that *hemp*$^{-/-}$ mice die perinatally. To determine the time of lethality, embryos and neonates, obtained from timed *hemp*$^{+/-}$ intercrosses, were genotyped. The results showed that *hemp*$^{-/-}$ die soon after birth (Honda et al. 2011).

To investigate the cause of *hemp*$^{-/-}$ postnatal lethality, prenatal and neonatal mice were subjected to pathological analysis. However, despite intensive analysis, no obvious changes were detected in the internal organs of *hemp*$^{-/-}$ mice (not shown). We then examined skeletal formation in *hemp*$^{-/-}$ mice, because previous studies showed that mice with deficiencies in several of the PcG proteins exhibited abnormal skeletal development, which would induce lethality in mice (van Lohuizen 1998).

Remarkable skeletal abnormalities were found in *hemp*$^{-/-}$ embryos. However, the phenotypes were different from those observed in mice lacking several of the *PcG* genes, such as *bmi-1*, *mel-18*, and *M33*, which exclusively displayed anterior/posterior homeotic transformations of vertebrae due to the ectopic or deregulated expression of *Hox* gene families (van Lohuizen 1998). Thus, Hemp appears to regulate skeletal development in a manner distinct from that of the PcG proteins. The most prominent and commonly observed one was a fusion of cervical vertebrae. The representative photographs of *hemp*$^{+/+}$ and *hemp*$^{-/-}$ embryos are shown in the left and middle panels of Fig. 15.2a. As shown in this figure, *hemp*$^{-/-}$ embryo exhibited C2/C3 and C4/C5 fusions (indicated by black arrows and a bracket). Interestingly, these features closely resemble to those observed in human Klippel-Feil anomaly (Klimo et al. 2007; Smoker and Khanna 2008). A radiograph of a patient with Klippel-Feil anomaly is shown in the right panel of Fig. 15.2a, which also shows fusions of cervical vertebrae (C2/C3 and C4/C5, indicated by white arrows, cited from http://www.klippel-feil.org/). In addition, rib anomalies, which were detected in *hemp*$^{-/-}$ embryos (Honda et al. 2011), were reported to be frequently associated with this disease (Baga et al. 1969). Therefore, it is suggested that Hemp might be involved in the pathogenesis of the disease.

The Klippel-Feil anomaly is etiologically heterogeneous, but several cases bear chromosomal aberrations and thus are considered to have a genetic component (Fukushima et al. 1995; Goto et al. 2006). Of note, one case with Klippel-Feil anomaly had a translocation involving chromosome band 17q23 (Fukushima et al. 1995), which is close to the human *hemp* orthologous locus at 17q21.3. In addition, several patients with unrelated skeletal abnormalities

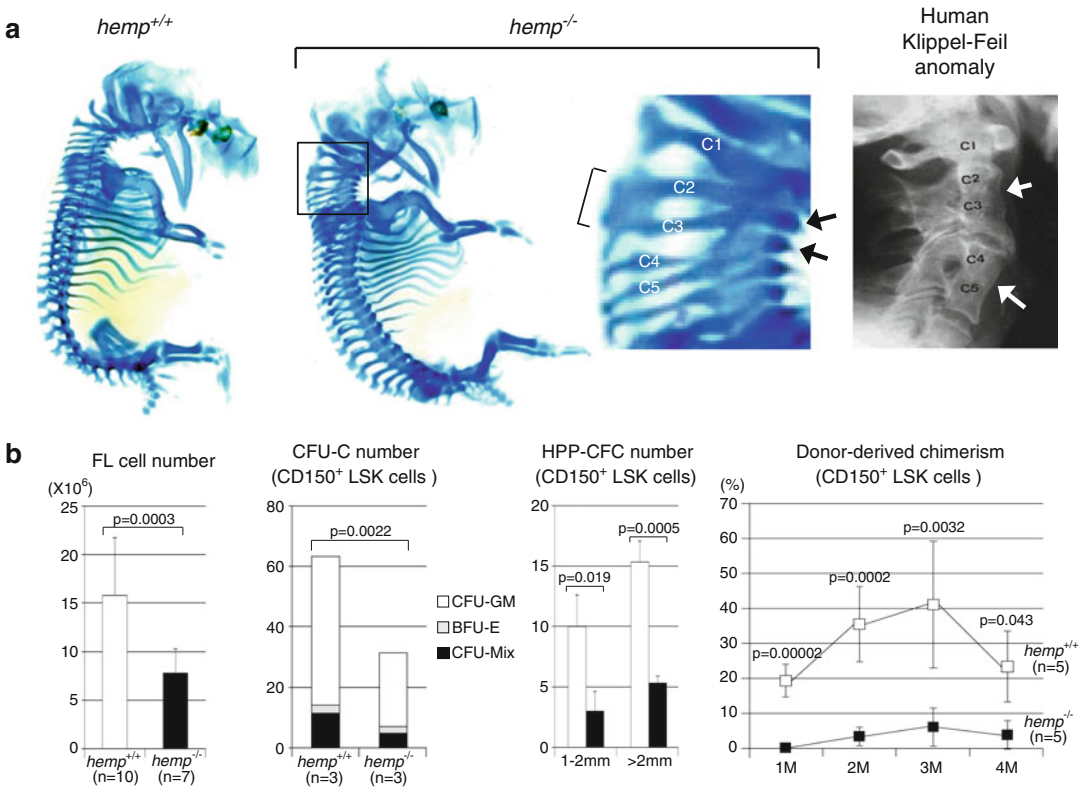

Fig. 15.2 (**a**) Skeletal photographs of *hemp*$^{+/+}$ and *hemp*$^{-/-}$embryos at E14.5 (*left and middle panels*, respectively) and a radiograph of a patient with Klippel-Feil anomaly (*right panel*). Abnormal cervical region in *hemp*$^{-/-}$ embryo are *boxed*, *magnified*, and fusions of cervical vertebrae are indicated by *black arrows* and *a bracket*. Fusions of cervical vertebrae in human Klippel-Feil anomaly are indicated by *white arrows*. Cervical bones are numbered as C1 ~ C5. (**b**) Phenotypic characterization and stem/progenitor assays of *hemp*$^{+/+}$ and *hemp*$^{-/-}$ FL HSCs. *Left panel*; comparison of cell numbers between *hemp*$^{+/+}$ (*open bar*) and *hemp*$^{-/-}$ (*closed bar*) FLs at E14.5 (data ± SD). *2nd left panel*; average numbers of colony forming units in culture (CFU-C) generated from *hemp*$^{+/+}$ and *hemp*$^{-/-}$ CD150^{+} LSK cells at E14.5. The colony types formed were classified into colony-forming unit-granulocyte, macrophage ([CFU-GM] *open bar*), burst-forming unit-erythoid ([BFU-E] *gray bar*), CFU-Mix (*closed bar*) and counted (data ± SD). *2nd right panel*; average numbers of high proliferative potential-colony forming cells (HPP-CFCs) generated from *hemp*$^{+/+}$ (*open bar*) and *hemp*$^{-/-}$ (*closed bar*) CD150^{+} LSK cells at E14.5. Colonies from 1 to 2 mm and >2 mm were classified and counted independently (data ± SD). *Right panel*; competitive repopulation assay of *hemp*$^{+/+}$ (*open box*) and *hemp*$^{-/-}$ (*closed box*) CD150^{+} LSK cells at E14.5. Donor cells (Ly5.2) were transplanted into recipient mice (Ly5.1) together with competitors (Ly5.1). Donor-derived chimerism in the peripheral blood (PB) at 1, 2, 3 and 4 months after transplantation are shown (data ± SD)

were shown to bear chromosomal aberrations involving 17q21.3 (Yue et al. 2007; Rooryck et al. 2008; Zahir et al. 2009). Therefore, it would be intriguing to investigate whether human *hemp* expression is affected in patients with Klippel-Feil anomaly and/or patients with skeletal abnormalities carrying chromosomal aberrations involving 17q21.3.

Impaired Proliferative and Repopulating Ability of *Hemp*-Deficient Hematopoietic Stem Cells

We then investigated whether Hemp deficiency affected embryonic hematopoiesis, since *hemp* was originally isolated from a mouse FL HSC

cDNA library (Phillips et al. 2000). We first examined fetal liver (FL) cell number of *hemp*$^{+/+}$ and *hemp*$^{-/-}$ embryos. Macroscopically, the FL sizes of *hemp*$^{-/-}$ embryos were significantly smaller than those in *hemp*$^{+/+}$ embryos (not shown). In accordance with this observation, as shown in the left panel of Fig. 15.2b, the mean cell number of *hemp*$^{-/-}$ FLs was reduced approximately twofold relative to that of controls.

To investigate the effect of Hemp deficiency on functional properties of HSCs, we then performed colony-forming units in culture (CFU-C) and high proliferative potential colony-forming cell (HPP-CFC) assays, which reflect proliferative potentials of stem/progenitor cells. CD150^{+}, lineage marker (Lin)-negative, Sca-1^{+} and c-Kit^{+} (LSK) cells, which represent HSCs with long-term reconstitution ability, were used for these studies. As shown in middle two panels of Fig. 15.2b, *hemp*$^{-/-}$ CD150^{+} LSK cells generated a significantly reduced number of colonies in both CFU-C and HPP-CFC assays as compared to *hemp*$^{+/+}$ CD150^{+} LSK cells. These results indicated that the proliferative ability of *hemp*$^{-/-}$ HSCs was markedly decreased.

Next, to assess the repopulating ability of HSCs, we performed a competitive repopulation assay. As shown in right panel of Fig. 15.2b, the repopulation capacity of *hemp*$^{-/-}$ CD150^{+} LSK cells was significantly reduced as compared to *hemp*$^{+/+}$ CD150^{+} LSK cells, as indicated by a marked decrease in the percentages of donor-derived cells in the peripheral blood (PB) in the recipient mice. The overall results demonstrated that Hemp deficiency induced severe functional defects in FL HSCs.

To gain insights into the molecular mechanisms underlying the impaired hematopoietic activities of *hemp*$^{-/-}$ HSCs, we performed microarray analysis to search for genes whose expression levels were altered in FL HSCs by the Hemp deficiency. Several genes were identified whose expression levels differed in enriched *hemp*$^{+/+}$ or *hemp*$^{-/-}$ hematopoietic stem and progenitor cell arrays (Honda et al. 2011). Interestingly, these genes included those that have been shown to play pivotal roles in HSC self-renewal capacity and differentiation ability, such as *Sox4*, *Erdr1*, *KLF6*, and *Stat* and *chemokine* family members (Deneault et al. 2009). Thus, it is strongly suggested that Hemp plays pivotal roles in HSC activity through regulating expression patterns of these genes.

Summary and Future Perspectives

In this study, we have demonstrated that Hemp, an mbt-containing protein, plays essential roles in HSC function and skeletal formation. An association between skeletal anomalies and impaired hematopoiesis has been reported in human diseases (Charles et al. 2008), and in particular, the Klippel-Feil anomaly can be associated with Fanconi anemia (McGaughran 2003) and Diamond-Blackfan syndrome (Lazarus and McCurdy 1984; Greenspan et al. 1991). Therefore, it will be interesting to determine whether human Hemp dysfunctions are involved in the pathogenesis of these diseases.

Studies have shown that the mbt domain binds to methylated histone residues, such as H3K4, H3K9, H3K27, H3K36, and H4K20 (Bonasio et al. 2010). In fact, a recent report demonstrated that MBTD1, a human homologue of Hemp, has a high binding affinity to mono- and di-methylated lysine residue of H3K20 (H3K20me1 and H3K20me2) (Eryilmaz et al. 2009). Therefore, it is likely that Hemp mediates signalling by binding to H3K20me1 and/or H3K20me2 through its mbt repeats. Since osteoblasts and HSCs coexist in the bone marrow niche and physically and functionally interact with each other, it is suggested that Hemp-mediated signalling positively and coordinately regulates these types of cells and contributes to normal HSC development and skeletal formation (Fig. 15.3).

Since *hemp*-deficient mice die neonatally, its role(s) in adult tissues remains unclear. To address this issue, we generated and analyzed *hemp* conditional knockout mice, in which Hemp was inducibly ablated mainly in hematopoietic tissues. Preliminary results showed that Hemp plays pivotal roles in adult hematopoiesis as well as in embryonic hematopoiesis. Further studies will clarify which genes are direct targets of Hemp, with what protein(s) Hemp forms a complex, and how Hemp exerts its biological activity.

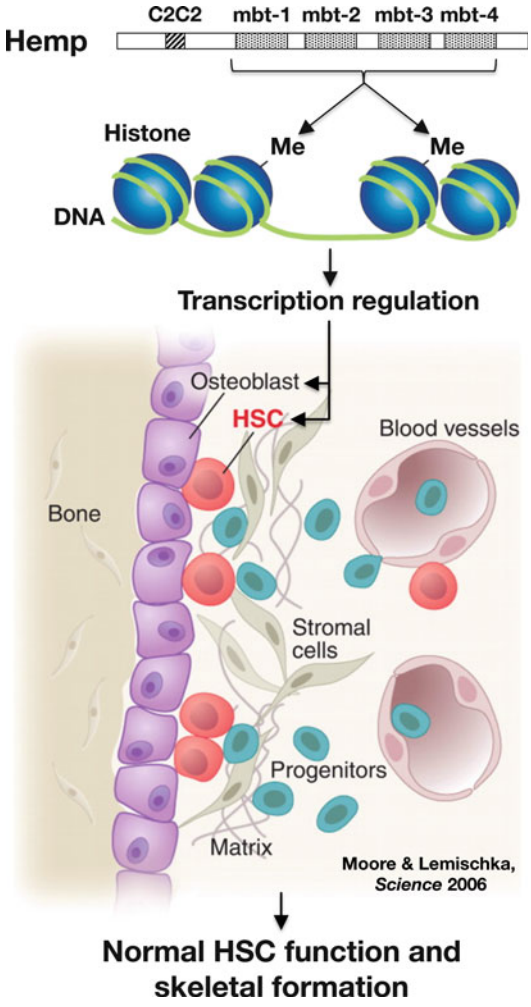

Fig. 15.3 Schematic illustration of Hemp function. Hemp binds to methylated histone residues through the mbt domains, which regulates gene transcription, defines proliferation and differentiation of osteoblasts and HSCs that interact with each other in the bone marrow niche, and finally contributes to normal HSC function and skeletal formation

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16 Relationship Between Radiosensitivity of Human Neonatal Hematopoietic Stem/Progenitor Cells and Individual Maternal/ Neonatal Obstetric Factors

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Abstract

Hematopoietic stem cells (HSCs) in placental/umbilical cord blood (CB), which is neonatal peripheral blood, have increasingly been used for hematopoietic stem cell transplantations. It is likely HSCs are sensitive to extracellular oxidative stresses, such as ionizing radiation and redox-directed chemotherapeutic agents. However, the radiosensitivity of HSPCs and neonatal hematopoietic system remains unclear. This study investigated the potential relationship between the radiosensitivity of hematopoietic stem/progenitor cells (HSPCs) in CB, which was obtained from singleton and full-term deliveries, and maternal/neonatal obstetric factors. Freshly prepared CB $CD34^+$ cells exposed to 2 Gy X-irradiation were assayed for hematopoietic progenitor cells such as colony-forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-Mix), and colony-forming unit-megakaryocyte (CFU-Meg). As a result, the neonatal weight, placental weight, CB volume, total low-density (LD) cells, and $CD34^+$ cells showed mutually significant positive correlations. The CB volume and total LD cells showed a significant reverse correlation with the surviving fraction of CFU-Meg. The surviving fraction of CFU-GM in spring (March–May) was significantly higher than that in autumn (September–November). The surviving fraction of CFU-Meg in the spring was significantly lower than that in the autumn. Male neonates

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showed a significantly higher surviving fraction of CFU-GM than female neonates. Contrarily, females showed a significantly higher surviving fraction of CFU-Meg than males. The present results suggest that the obstetric factors, such as the season of birth and neonatal gender, influence the radiosensitivity of neonatal hematopoiesis.

Introduction

Hematopoietic stem cells (HSCs) can self-renew and differentiate into all the hematopoietic lineages throughout the lifetime of an organism (Johnson 1980; Humphries et al. 1981). Due to their high proliferative potential, HSCs are extremely sensitive to extracellular oxidative stresses such as radiation and chemotherapeutic agents (Nagayama et al. 2002; Kashiwakura et al. 2006; Monzen et al. 2009). HSCs are abundantly contained in placental/umbilical cord blood (CB), which is similar to bone marrow and peripheral blood (Nakahata and Ogawa 1982). Therefore, CB has increasingly become an important source for hematopoietic stem cell transplantations in patients with diseases such as hematopoietic malignancies (Nagayama et al. 2002; Tse and Laughlin 2005; O'Brien et al. 2006). Meanwhile, because CB is neonatal peripheral blood, the CB volume, total number of nucleated cells, and $CD34^+$ cells are influenced by maternal/neonatal obstetric factors. Significantly positive correlations were observed among the neonatal weight, placental weight, CB volume, total number of nucleated cells, and $CD34^+$ cells (Ballen et al. 2001; Nakagawa et al. 2004; Omori et al. 2008, 2010). Furthermore, at birth, the cytokine responses of CB mononuclear cells to innate/adaptive stimuli tend to reflect the maternal/neonatal environment (Gold et al. 2009). Some previous studies have demonstrated relationships between parental/environmental factors and CB responses, including circulating cytokine concentrations and IgE levels, genetic/environmental factors, and month of birth (Kimpen et al. 1987; Malamitsi-Puchner et al. 2005). In addition, during normal pregnancy, the hemostatic balance changes in the direction of hypercoagulability to support a decrease in bleeding complications related to delivery, resulting in the consumption of platelets and blood coagulation factors (Hellgren 2003).

Despite a number of radiation researches conducted regarding tissues and organs, the influence of radiation on fetuses/neonates remains unclear. Therefore, it is highly possible that the evaluation of the radiosensitivity of HSPCs in CB may reveal the influence of radiation on the neonatal hematopoiesis. Although various associated factors, such as intrauterine influences, maternal exposures/environment, and neonatal factors, may influence the radiosensitivity and differentiation of hematopoietic stem/progenitor cells (HSPCs) in CB, the details of their specific mechanisms remain uncertain.

We have previously investigated the influence of maternal/neonatal obstetric factors on the CB volume, total number of nucleated cells, and $CD34^+$ cells, observing a wide-range of individual differences in the CB volume and total number of HSPCs (Omori et al. 2008, 2010). In the present chapter, a new approach to investigate whether maternal/neonatal obstetric factors are associated with the radiosensitivity of HSPCs in CB collected after birth is described based on the previous report (Omori et al. 2010). The results of the present study raise an important point regarding the influence of radiation on fetuses and neonates that details of which remain unclear.

Materials and Methods

Growth Factors and Fluorescent Antibodies

Recombinant human interleukin-3 (IL-3), human stem cell factor (SCF), and thrombopoietin (TPO) were purchased from BioSource (Tokyo, Japan). The recombinant human granulocyte colony-stimulating factor (G-CSF) and erythropoietin (EPO) were purchased from Sankyo Co. Ltd. (Tokyo, Japan). The recombinant human granulocyte/macrophage colony-stimulating factor

(GM-CSF) was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). These factors were administered at the following concentrations: IL-3 and SCF, 100 ng/ml; TPO, 50 ng/ml; G-CSF and GM-CSF, 10 ng/ml; and EPO, 4 U/ml medium. The fluorescence-labeled monoclonal antibodies, fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (gp105-120, FITC-CD34), and FITC-conjugated anti-human CD41 (gpIIbIIIa, FITC-CD41) were purchased from Beckman Coulter Immunotech (Marseille, France). Mouse IgG1-FITC and mouse IgG2a-FITC (Becton Dickinson Biosciences) were used as isotype controls.

Cord Blood Collection and Maternal/Neonatal Obstetric Factors

This study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). After obtaining informed consent from the mothers, CB was obtained at the birth center (Fukushi birth center, Goshogawara, Japan) or the hospital (Hirosaki National Hospital, Hirosaki, Japan). According to the guidelines of the Tokyo Cord Blood Bank, CB was collected from singleton, full-term, and vaginal deliveries before placental delivery (in utero collection) and immediately after neonatal delivery without any medical complications of pregnancy. Using sterile collection bags (CBC-20; Nipro, Osaka, Japan) containing the anticoagulant (28 ml) citrate phosphate dextrose, CB was collected, drained by gravity, and allowed to flow until the flow ceased. Extensive data regarding the medical and family histories were voluntarily collected and reported by the staff at both the birth center and hospital. According to our previous reports (Omori et al. 2008, 2010), the associated obstetric factors as parameters extracted from the clinical charts were as follows: the month of birth, maternal age, paternal age, residential area, number of pregnancies, number of parities, self-reported maternal weight and height, pre-pregnancy BMI, gestational weight gain, maternal smoking status, duration of labor, gestational age, neonatal gender, neonatal weight and height, placental weight, cord length, and meconium (+/−) in the amniotic fluids. According to the astronomical solar calendar, the month of birth was classified into four categories as follows: spring (March, April, May), summer (June, July, August), autumn (September, October, November), and winter (December, January, February) in the northern hemisphere sites (Sullivan Dillie et al. 2008). Samples with unknown or missing data with respect to the obstetric factors were excluded from this study. Finally, a total of 63 CB samples were characterized.

Separation of Mononuclear Low-Density (LD) Cells and Purification of CD34$^+$ Cells

Within 24 h after CB collection, LD cells were separated using Ficoll-Paque. Twenty milliliters of CB diluted twofold with PBS (−) containing 5 mM ethylenediaminetetraacetic acid (EDTA-PBS (−); Wako Pure Chemicals, Tokyo, Japan) were layered on 15 ml Ficoll-Paque (1.077 g/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). The samples were centrifuged for 30 min at 300 g at room temperature. The buffy coat was harvested and diluted with EDTA-PBS (−). To remove platelets, cells were washed twice with EDTA-PBS (−) by centrifugation for 10 min at 100 g and 4 °C. The cells were resuspended with 4 °C EDTA-PBS (−), and then counted using Türk's solution. According to the manufacturer's instructions, LD cells prepared from CB were processed for CD34$^+$ cell enrichment; magnetic cell sorting (Miltenyi Biotec, Germany) was used for the positive selection of CD34$^+$ cells. At the end of the procedure, the CD34$^+$ cell recovery from LD cells was approximately 0.1–0.6%, and the purity analyzed by flow cytometry was 80–95%. Viability was verified using the trypan blue dye exclusion method. Processing for CD34$^+$ cells was not performed when the number of total LD cells

was extremely low (fewer than 8×10^7 cells), which was due to poor recovery.

In Vitro Irradiation

Within 24 h after isolation, the $CD34^+$ cells were exposed to X-rays (2 Gy, 150 kVp, 20 mA, 0.5-mm aluminum and 0.3-mm copper filters) from an X-ray generator (MBR-1520R; Hitachi Medical Co., Tokyo, Japan) at a distance of 45 cm between the focus and target at a dose rate of approximately 80 cGy/min. The dose rate was monitored with an ionization chamber and evaluated.

Methylcellulose Culture

The colony-forming cell (CFC) was composed of colony-forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte-erythroid -macrophage-megakaryocyte (CFU-Mix). These cells were assayed using a methylcellulose culture, as described previously (Kashiwakura et al. 2000). Briefly, the $CD34^+$ cells (1×10^3) were suspended in 1 ml methylcellulose medium (Methocult H4230, Stem Cell Technologies Inc., Vancouver, BC, Canada) supplemented with SCF, IL-3, G-CSF, GM-CSF, and EPO. This mixture was transferred onto 24-well cell culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) at 0.3 ml/well and then incubated at 37 °C for 14 days in a humidified atmosphere containing 5% CO_2. The colonies consisting of more than 50 cells were counted using an inversion microscope (x40; Olympus, Tokyo, Japan). The identification of each colony was performed based on their morphology according to the methods described by Kaufman et al. (2001). Briefly, the CFU-GM-derived colony, which is composed of granulocytes and macrophages, shows no color. In contrast, the BFU-E-derived colony is composed of erythroid cells with hemoglobin red. Since the CFU-Mix multilineage colony contains many granulocytes, erythroids, macrophages, and megakaryocytes, the red parts appear in this colony. The generated mature cells contained in each colony have been checked using a cytospin smear technique, as described in previous studies (Liu et al. 2007).

Plasma Clot Culture

CFU-Meg was assayed by the plasma clot technique using platelet-poor human plasma. The culture medium contained 15–20% human platelet-poor AB plasma plus a combination of TPO, IL-3, and SCF, which supports maximum CFU-Meg growth, as the growth factors in IMDM. Further additives were penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), MEM vitamins (1%) and MEM nonessential amino acids (1%; Gibco BRL, Gland Island, NY, USA), thioglycerol (1×10^{-5} M; Sigma, St Louis, MO, USA), L-asparagine (2 μg/ml) and $CaCl_2$ (74 μg/ml; both from Wako Pure Chemicals, Tokyo, Japan), and 0.2% bovine serum albumin (BSA; Boehringer, Mannheim GmbH, Germany). The $CD34^+$ cells were added to the culture medium at a concentration of 1×10^3 cells/ml. This baseline culture was plated onto 24-well cell culture plates at 0.3 ml per well and incubated at 37 °C for 11–12 days in a humidified atmosphere containing 95% air and 5% CO_2.

Identification of Megakaryocyte Colonies by Immunofluorescence

Each well was fixed twice for 15 min with acetone and methanol (2:1). The plates were dried by airflow overnight and stored at −20 °C until the start of the staining procedure. Prior to staining, the plates were thawed to room temperature, and thereafter, PBS containing 0.5% BSA (PBS-B) was added to soften the clot. The solution was replaced with FITC-CD41 mAb diluted 1:100 in PBS-B; the plates were then incubated at room temperature for 1 h, followed by a single wash with PBS-B. The nuclei were counterstained with propidium iodine (0.3 ng/ml; Sigma, St Louis, MO, USA). After a final wash, the FITC-positive colonies were scored as CFU-Meg-

Table 16.1 Characteristics of CB and maternal/neonatal demographics

	Number of CB samples and percentage	Mean ± S.D.
Net weight of CB (g)	63	55.3 ± 18.9
Total LD cells ($\times 10^7$)	62	16.9 ± 11.1
Total $CD34^+$ cells ($\times 10^7$)	58	137.4 ± 86.6
Neonatal weight (g)	59	3135.4 ± 353.7
Neonatal height (cm)	59	49.6 ± 1.6
Placental weight (g)	59	562.0 ± 110.1
Neonatal gender		
Males	29 (49.2%)	
Females	30 (50.8%)	
The month of birth		
Spring	11 (18.6%)	
Summer	23 (39.0%)	
Autumn	14 (23.7%)	
Winter	11 (18.6%)	
Maternal age (years)	59	28.5 ± 5.8
Parity		
Nulliparous	26 (44.1%)	
Multiparous	33 (55.9%)	
The residential circumstances		
Inside the city	23 (39.7%)	
In the neighbor city, town and village	21 (36.2%)	
Temporarily return to hometown	14 (24.1%)	
Maternal smoking status		
Smoker	12 (24%)	
Nonsmoker	38 (76%)	

CB cord blood, *LD cells* low-density cells, *S.D.* standard deviation
Each value in the parentheses indicates the rate of the number of CB samples divided by the total CB samples

colonies using a fluorescence microscope at × 100 magnification (Olympus, Tokyo, Japan) (Takahashi et al. 2007). In contrast, the FITC-negative colonies stained by propidium iodine alone were determined as non-CFU-Meg-colonies.

Statistical Analysis

Multivariate linear regression analysis was performed to test for associations between the mutually adjusted obstetric factors (independent variables) and the characteristics of CB samples, including the CB volume, total LD cells, total $CD34^+$ cells, and surviving fraction of each HSPC (dependent variables). Then, univariate analyses were performed using the Student's *t*-test and Pearson's correlation coefficient, depending on the distribution pattern of the data. A multiple-comparison test was also conducted using the Bonferroni–Dunn test. The statistical analysis was performed using the SPSS software version 12 (SPSS Japan, Tokyo, Japan) and the software program Origin (Origin Lab, Northampton, MA, USA) for Windows. A value of $p < 0.05$ was considered to be statistically significant.

Results

Summary of the Characteristics of CB and Maternal/Neonatal Demographics

Table 16.1 summarizes the characteristics of the CB samples collected in this study and the maternal/neonatal demographics. The net weight of CB (CB volume), total LD cells, and total $CD34^+$ cells were 55.30 ± 18.87 (g), 16.90 ± 11.12 ($\times 10^7$), 137.38 ± 86.64 ($\times 10^4$), respectively. To assess the

radiosensitivity of HSPCs, the distribution of each progenitor cell including CFC, CFU-GM, BFU-E, CFU-Mix, and CFU-Meg was evaluated in nonirradiated and irradiated cells. As a result, the ratio of viable cell counts, the surviving fraction of CFC, CFU-GM, BFU-E, CFU-Mix, and CFU-Meg was 0.24 ± 0.09, 0.21 ± 0.08, 0.33 ± 0.22, 0.33 ± 0.27, and 0.20 ± 0.12, respectively.

Correlations Between the CB Volume, Total LD/CD34⁺ Cells, Maternal/Neonatal Obstetric Factors, and Surviving Fraction of HSPCs

The CB samples were analyzed to assess the correlations between the CB volume, total LD cells, total CD34⁺ cells, maternal/neonatal obstetric factors, and surviving fraction of each HSPC. Significant positive correlations were observed between the neonatal weight, placental weight, CB volume, total LD cells, and total CD34⁺ cells ($p < 0.05$; data not shown). Next, the correlations between the surviving fraction of each HSPC and the maternal/neonatal obstetric factors were estimated. The neonatal weight showed a significant positive correlation with the surviving fraction of CFU-GM ($p < 0.05$; Fig. 16.1a) but not with the surviving fraction of CFC, BFU-E, or CFU-Mix (data not shown). Neither the net weight of CB nor the total LD cells showed a significant correlation with the surviving fraction of CFU-GM (Fig. 16.1c, e). Although the neonatal weight showed a weak positive correlation (but not statistically) with the surviving fraction of CFU-Meg (Fig. 16.1b), both the net weight of CB and total LD cells showed a significant reverse correlation with the surviving fraction of CFU-Meg ($p < 0.05$; Fig. 16.1d, f).

Associations of the Maternal/Neonatal Obstetric Factors with the CB Volume, Total LD/CD34⁺ Cells, and the Surviving Fraction of HSPCs

The associations of the maternal/neonatal obstetric parameters including the month of birth, neonatal gender, parity, maternal smoking status, and meconium (+/−) in the amniotic fluids were evaluated. No significant difference was observed in the neonatal weight, placental weight, CB volume, total LD cells, total CD34⁺ cells, or surviving fraction of each HSPC among any of the parameters (data not shown). Meanwhile, after performing the multiple-comparison tests, the seasonal variation in the surviving fraction of each HSPC was determined. The surviving fraction of CFU-GM in the spring was significantly higher than that in the autumn and in the winter, respectively ($p < 0.05$; Fig. 16.2a). In contrast, the surviving fraction of CFU-Meg in the spring and in the summer was significantly lower than that in the autumn, respectively ($p < 0.01$, $p < 0.05$; Fig. 16.2b). In addition, male neonates showed a significantly higher surviving fraction of CFU-GM than female neonates ($p < 0.05$; Fig. 16.3a); however, females showed a significantly higher surviving fraction of CFU-Meg than males ($p < 0.05$; Fig. 16.3b). Interestingly, classifying gender into males and females (Fig. 16.4), and then focusing on the seasonal variations in the surviving fraction of each HSPC, females showed a significantly higher surviving fraction of CFU-GM in the summer than that in the autumn ($p < 0.05$; Fig. 16.4c). In contrast, the surviving fraction of CFU-Meg in the spring and in the summer was significantly lower than that in the autumn, respectively ($p < 0.01$, $p < 0.05$; Fig. 16.4d). Next, the correlations between the surviving fraction of each HSPC and the CB acid–base status and gas values were estimated. As a result, a significantly reverse correlation was observed between the surviving fraction of BFU-E and the umbilical artery base excess ($p < 0.05$; data not shown).

Discussion

The results of the present study showed the significantly positive correlations between neonatal weight, placental weight, net weight of CB, total LD cells, and total CD34⁺ cells. These findings were consistent with our previous reports (Omori et al. 2008, 2010) and those of other groups (Ballen et al. 2001; Nakagawa et al. 2004), confirming that the CB samples used in the present study were relevant subjects for investigation.

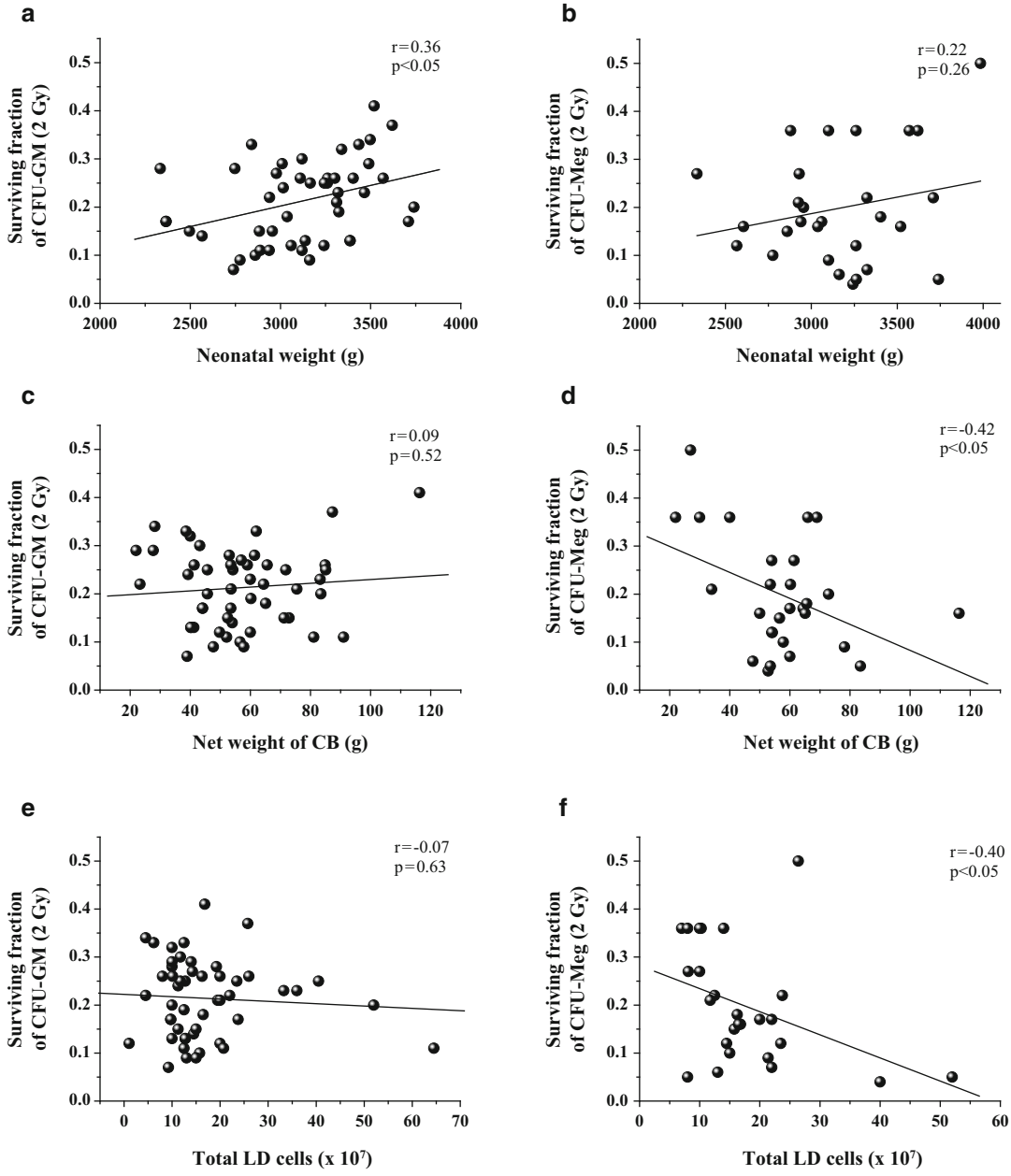

Fig. 16.1 Correlations between the surviving fraction of HSPCs and obstetric factors. A significant positive correlation was observed (**a**) between the neonatal weight and the surviving fraction of CFU-GM (n = 51). (**d, f**) Net weight of CB and total LD cells showed a significant reverse correlation with the surviving fraction of CFU-Meg (n = 28). Pearson's correlation coefficient: *p <0.05

A significant positive correlation was observed between the neonatal weight and the surviving fraction of CFU-GM (Fig. 16.1a), which is the major population (70%) of CFCs (Takahashi et al. 2010). On the other hand, despite the neonatal weight showing a weak positive (but not statistical) correlation with the surviving fraction of CFU-Meg, the net weight of CB and the total LD cells showed a significant reverse correlation with the surviving fraction of CFU-Meg

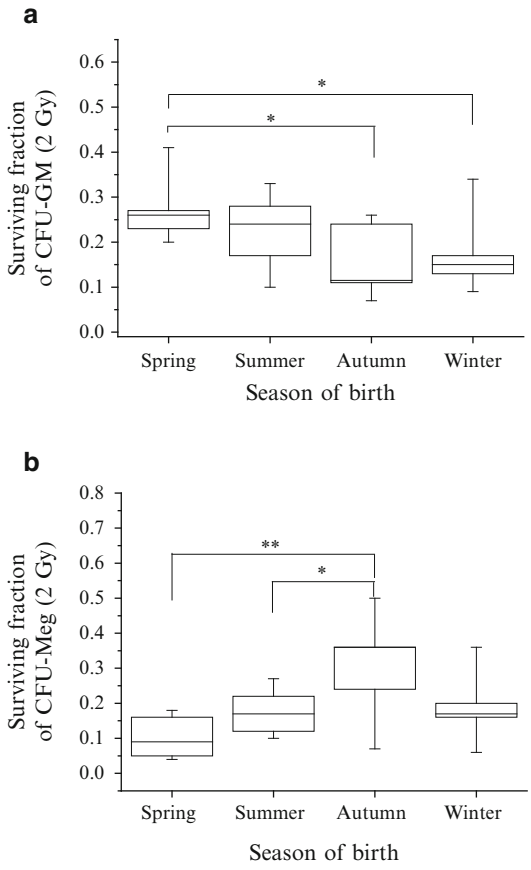

Fig. 16.2 Multiple-comparison test of seasonal variations in the surviving fraction of HSPCs. (**a**) The surviving fraction of CFU-GM in the spring was significantly higher than that in the autumn and in the winter (n = 51). (**b**) The surviving fraction of CFU-Meg in the spring and in the summer was significantly lower than that in the autumn (n = 28). Bonferroni–Dunn test: *p <0.05, **p <0.01

(Fig. 16.1d, f). The hierarchical development from hematopoietic stem cells (HSCs) to mature cells of the hematolymphoid system involves progressive loss of self-renewal capacity, proliferation ability, and lineage potentials (Manz et al. 2002; Edvardsson et al. 2006). That is, a small fraction of self-renewing multipotent HSCs differentiate into clonogenic common lymphoid progenitors and clonogenic common myeloid progenitors (CMPs) in early stage of human hematopoiesis. Furthermore, CMPs determine the downstream progeny, granulocyte/macrophage (GMPs) or megakaryocyte/erythrocyte progenitors (MEPs). Although no precise mechanism can be explained by the present results only, since the reverse correlations were observed between the surviving fraction of CFU-GM and CFU-Meg, there still exists the possibility that obstetric factors influence the radiosensitivity of neonatal CMPs and/or GMPs/MEPs.

Meanwhile, the most effective and profound parameter was the month of birth when the CB samples were collected. In particular, the significantly different seasonal variation in the surviving fractions was observed between in the spring and in the autumn (Fig. 16.2). Kimpen et al. (1987) have suggested that the concentration of CB IgE is higher in the spring and lower in the autumn. Also Sullivan Dillie et al. (2008) have suggested that the exposure to outdoor aeroallergens, such as grass and tree pollens, is high in the spring or early summer because IL-5 and IL-13 responses were the highest in the spring and summer. In addition, Mandel et al. (2008) have demonstrated that the ultraviolet, solar radiation exposure in the spring and early summer is much stronger than that in the autumn and winter. Although the details of the mechanisms associated with the radiosensitivity of neonatal HSPCs in CB are still unclear, the seasonal variation in the surviving fraction of HSPCs may be partly explained by the seasonal cytokine and IgE levels, which are sensitive to maternal environment and immune response.

Regarding the gender variation in the surviving fraction of HSPCs, male neonates showed a significantly higher surviving fraction of CFU-GM than female neonates but females showed a significantly higher surviving fraction of CFU-Meg than males (Fig. 16.3). Morrissey et al. (2008) have demonstrated that gender does not significantly affect the hypersensitivity response to ultraviolet radiation. However, it was reported by Hansen et al. (1992) that, considering the susceptibility to innate/adaptive stimuli, boys showed significantly higher levels of IgE and more often an elevated IgE value than girls. In addition, our previous report by Chiba et al. (2010) demonstrated that the CB cortisol concentration (the measurement of stress-associated hormone and oxidative stress markers) in male neonates was significantly higher than that in

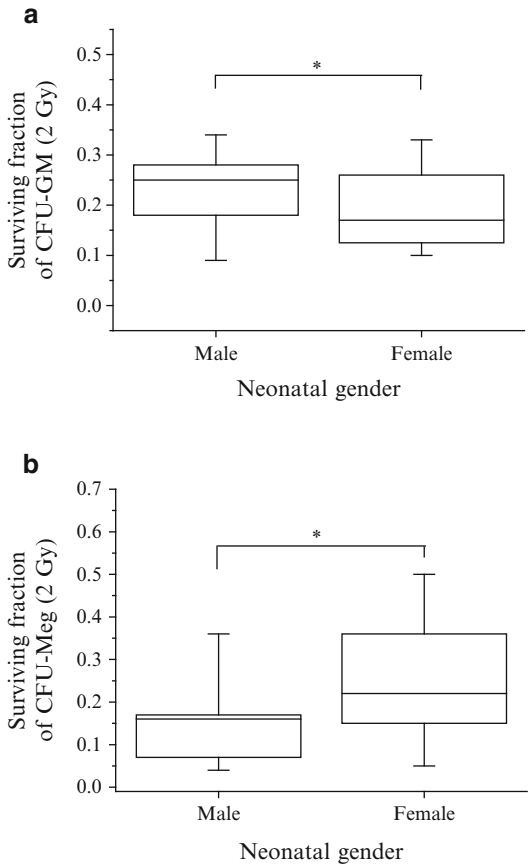

Fig. 16.3 Comparison of the surviving fraction of HSPCs by neonatal gender. (**a**) The male neonates showed a significantly higher surviving fraction of CFU-GM than the female neonates (n = 47; 23 males and 24 females). (**b**) the females showed a significantly higher surviving fraction of CFU-Meg than the males (n = 28; 14 males and 14 females). Student's *t*-test: *p <0.05

female neonates. Females showed a significantly higher surviving fraction of CFU-Meg in autumn than that in the summer and in the spring, respectively (Fig. 16.4d). Interestingly, it is similar to the results of all the samples (Fig. 16.2b). These findings may be reflecting the different susceptibility by gender. Therefore, the gender variation in the surviving fraction of HSPCs in CB may suggest that male neonates are more likely to be sensitive to the maternal exposures/environment, intrauterine, and hormonal influences. Although the number of CB samples collected in the present study was relatively small, there can be a possibility of some differences in neonatal hematopoiesis between males and females.

Moreover, the significant reverse correlation was observed between the surviving fraction of BFU-E and the umbilical artery base excess (data not shown). The higher LD cell yields in CB were observed in the vaginal deliveries (Omori et al. 2010) and the neutrophil granules were induced by the maternal stress factors such as a long, stressful labor (Lim et al. 2000; Yektaei-Karin et al. 2007; Juutistenaho et al. 2010). Therefore, it seems to be some relationships between the surviving fraction of BFU-E and the base excess due to metabolic acidosis caused by unanticipated complications during intrapartum. In general, the buffy-coat prepared from CB contains mainly lymphocytes and then monocytes, with small numbers of HSPCs. Askari et al. (2005) have reported that the total nucleated cell count appears to be more affected by different variables than the $CD34^+$ count. However, regarding the association between the behavior of these cells and stress, only limited information has so far been reported. In order to resolve these complicated individual differences in the radiosensitivity of neonatal HSPCs in CB, further researches using a large number of CB samples are required.

Collectively, the results of the present study suggest that the maternal/neonatal obstetric factors, such as the month of birth and neonatal gender, influence the radiosensitivity of neonatal hematopoiesis. Consequently, there is a possibility that the maternal/neonatal obstetric factors influence the balance or homeostasis in neonatal hematopoiesis during pregnancy. Therefore, it is important to consider the possibility that some interactions through the maternal external/internal intrauterine environments trigger cellular responses. Although the quality of HSPCs or the mechanism behind the radiation survival of HSPCs still remains unclear, the present study presumably provides a new insight into the relationship between the radiosensitivity of neonatal hematopoiesis and maternal/neonatal obstetric factors.

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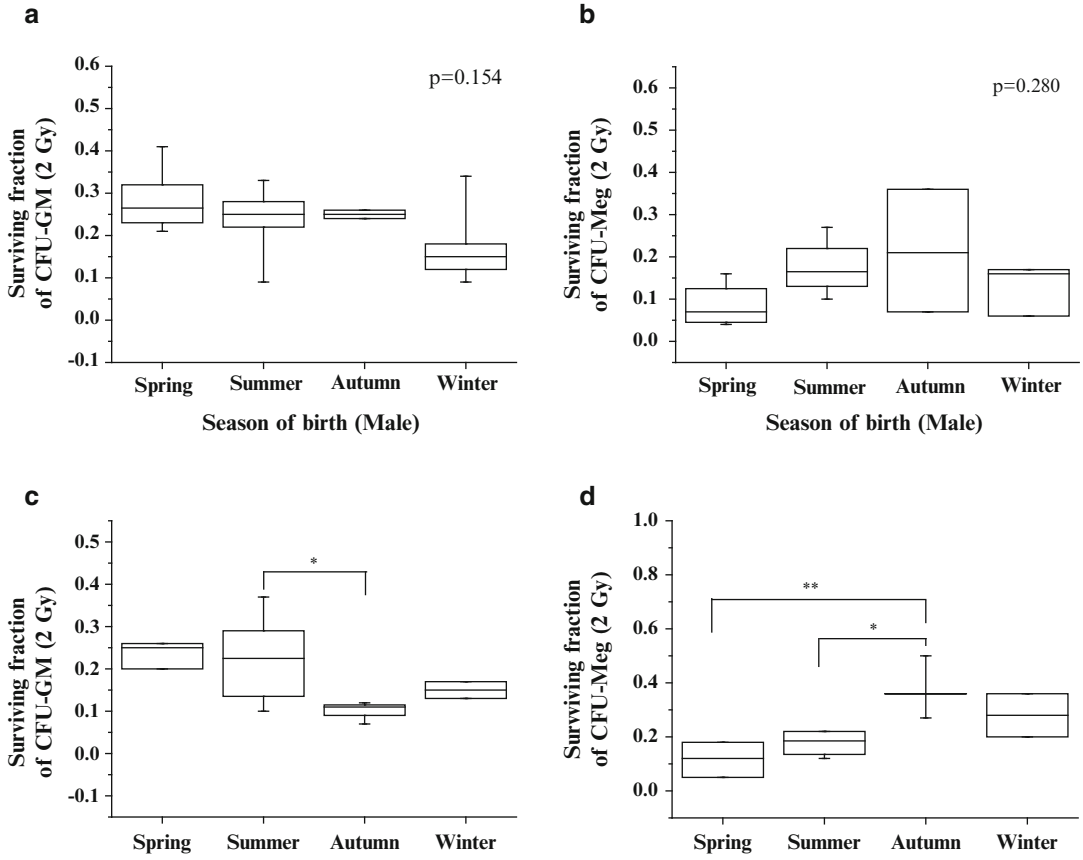

Fig. 16.4 Multiple-comparison test of seasonal variations in the surviving fraction of HSPCs among male neonates or among females. (**a, b**) The male neonates showed no significant difference between any seasonal variations (CFU-GM: n (male) =23; spring=6, summer=10, autumn=2, winter=5, CFU-Meg: n (male) =14; spring=4, summer=4, autumn=3, winter=3). (**c**) The females showed a significantly higher surviving fraction of CFU-GM in the summer than that in the autumn (n (female) =24; spring=3, summer=12, autumn=4, winter=5). (**d**) The surviving fraction of CFU-Meg in the summer and in the spring was significantly lower than that in the autumn, respectively (n (female) =14; spring=3, summer=4, autumn=5, winter=2). Bonferroni–Dunn test: *p <0.05, **p <0.01

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Part V

Stem Cells in Tumors and Cancer

Diagnosis of Primary and Metastatic Germ Cell Tumors Using Embryonic Stem Cell Transcription Factors

17

Lynette M. Sholl and Jason L. Hornick

Contents

Abstract

Germ cell tumors (GCTs) are the most common solid tumors that affect men in the third and fourth decades of life. The vast majority of GCTs arise in the testis, with a small percentage arising at extragonadal sites including the mediastinum, brain, and sacrococcygeum. These tumors frequently present at an advanced stage with distant metastases, but even advanced disease is highly amenable to therapy and can be cured. The original diagnosis may be based on limited biopsy material and without a clinical history of a testicular mass. As a result, it can be difficult to render a confident diagnosis of GCT. Historically, the available immunohistochemical markers for GCT were limited both in sensitivity and specificity. In the last decade, insights into the biology of embryonic stem (ES) cells have led to the identification of transcription factors that are critical to the maintenance of germ cell pluripotency. These ES cell transcription factors, in particular OCT4, NANOG, SOX2 and SALL4, are also highly expressed in GCTs. These markers can be used both to support a diagnosis of GCT, as well as to determine tumor subtype. This chapter will describe the clinical and pathologic features of germ cell tumors, discuss the role of ES cell transcription factors in maintaining pluripotency in the developmental and pathologic setting, and discuss how ES cell transcription factor expression patterns can be exploited in GCT diagnosis at both primary and metastatic sites.

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Introduction

The past decade has seen an explosion in our understanding of the genetic regulation of embryogenesis and stem cell biology. Embryonic stem (ES) cell transcription factors play a central role in maintaining pluripotency, driving cell proliferation, and blocking differentiation. Tumors that arise from gonadal primordial germ cells, termed germ cell tumors (GCTs), retain the expression of many of these ES cell transcription factors. Recognition of these expression patterns at the mRNA and protein level has led to the development of an array of new, more sensitive and specific diagnostic markers for GCTs.

Clinical Overview

GCTs represent a group of highly curable malignant neoplasms that, with some exceptions, involve the male gonad. According to the U.S. National Cancer Institute, >8,000 men are diagnosed with and ~350 die of GCTs annually (http://seer.cancer.gov/statfacts/html/testis.html#incidence-mortality). GCTs can affect men at any age, but more than half are diagnosed before the age of 35. These tumors are sixfold more common in men living in developed countries. The only clearly identified risk factor is cryptorchidism (Brooks et al. 2011).

Clinically, GCT typically presents as a painless testicular nodule or swelling; however, patients with advanced disease may have constitutional symptoms, back pain, or hemoptysis secondary to lung involvement (Brooks et al. 2011). High resolution trans-scrotal ultrasonography is recommended for resolving the differential diagnosis of testicular GCT versus a benign process such as hydrocele, torsion, or infection. The prognosis of GCT is best for patients with early stage disease, necessitating rapid surgical intervention (orchiectomy) for any lesions that are radiographically suspicious for malignancy.

GCTs spread in a predictable manner to the retroperitoneal (interaortocaval and peri-aortic) lymph nodes and distantly to lungs, liver, brain and bones. GCTs are associated with elevated serum human chorionic gonadotropin (HCG), alpha fetoprotein (AFP) and lactate dehydrogenase (LDH); of these, only a high level of serum HCG is specific to GCT and correlates with the presence of syncytiotrophoblasts. Stage I disease is confined to the testis, stage II involves the retroperitoneal nodes with low serum tumor markers, and stage III involves distant sites and/or is associated with high serum tumor markers (Edge et al. 2009). Stage I disease is associated with an excellent prognosis, with >95% cure rate.

The tumor histology impacts on the clinical course, including therapeutic management. Thus, it is critical that pathologists accurately and completely classify these tumors, which for clinical purposes are divided into seminomas and nonseminomatous GCTs; more than half of this latter category is mixed GCTs containing multiple tumor types.

Histopathology of Germ Cell Tumors

Seminomatous Germ Cell Tumors

Comprising approximately half of diagnosed testicular tumors, seminomas are the most common pure form of GCT (Ulbright 2005). Seminomas are associated with a better prognosis than nonseminomatous GCTs and are highly responsive to platinum-based chemotherapy. Histologically, seminomas are comprised of uniform, round cells with abundant, glycogen-containing cytoplasm and large, smooth nuclei containing prominent nucleoli (Fig. 17.1). The tumor cells are typically arranged in clusters or sheets traversed by fibrous bands and are often associated with dense lymphocytic and/or granulomatous inflammation. The correlates in the female gonad and central nervous system are dysgerminoma and germinoma, respectively. Ancillary immunohistochemical studies are rarely required to make the diagnosis in tumors with this characteristic histomorphology. However, the inflammatory infiltrate can occasionally obscure the tumor cells, possibly leading to a benign inflammatory misdiagnosis. In some cases,

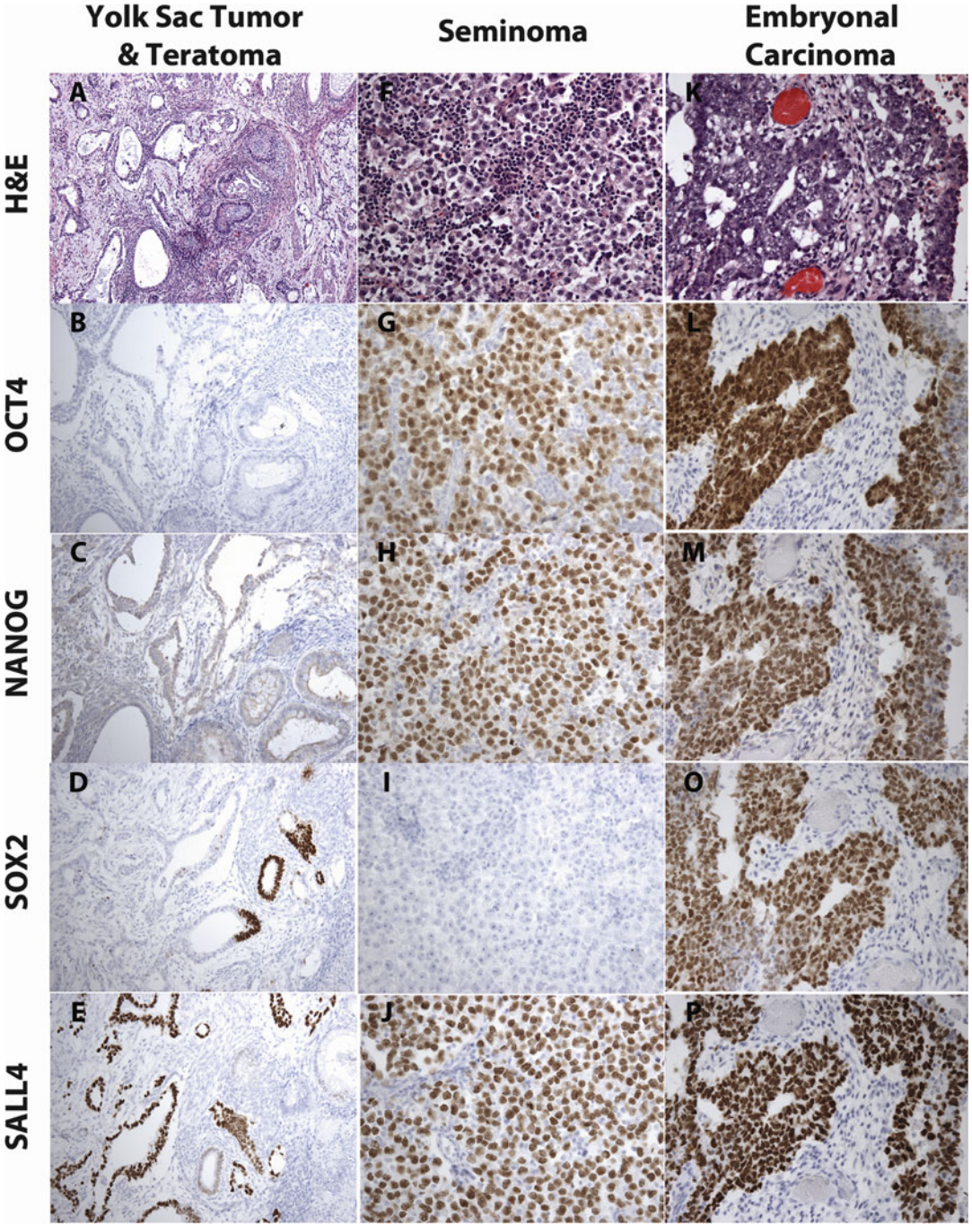

Fig. 17.1 Immunohistochemical stains demonstrating the protein expression patterns of ES cell transcription factors in germ cell tumors: yolk sac tumor (*YST*), teratoma (*panel A*), seminoma (*panel F*) and embryonal carcinoma (*EC*) (*panel K*). OCT4 and NANOG are negative in YST and teratoma (*B* and *C*) and positive in seminoma and EC (*G*, *H*, *L* and *M*). SOX2 is negative in YST and seminoma (*D* and *I*) and positive in teratoma and embryonal carcinoma (*D* and *O*). SALL4 is negative in teratoma (*E*) and positive in YST, seminoma, and EC (*E*, *J* and *P*)

particularly if the specimen is not well fixed, seminoma may resemble embryonal carcinoma (Ulbright 2005). For the purposes of treatment, this is a critical distinction that can be aided by immunohistochemical studies (see below).

Spermatocytic Seminoma

This tumor type more frequently affects older men and is associated with an absence of tumor marker elevation. Three different cells types are typically visible: small cells with scant cytoplasm and dark nuclei, variably-sized round cells with round nuclei and filamentous chromatin reminiscent of spermatocytes, and giant mononucleate cells with round to indented nuclei. The tumor cells can be present within an edematous stroma, and the lymphocytic/granulomatous inflammation common in conventional seminoma is rarely observed in this entity. The differential diagnosis is with seminoma; however, in contrast to seminoma, this tumor type is thought to arise from a more differentiated cell of origin and should be considered as a biologically distinct entity from other GCTs.

Nonseminomatous Germ Cell Tumors

A GCT containing any nonseminomatous component is considered a nonseminomatous (mixed) germ cell tumor. It is unusual for any of these tumor types to appear in their pure form in the adult population; thus, the majority of nonseminomatous GCTs is mixed and frequently contains a component of seminoma.

Embryonal Carcinoma

This tumor type is the most undifferentiated of the nonseminomatous GCTs and most closely reflects the primordial germ cell state. Embryonal carcinoma is comprised of large, polygonal to columnar undifferentiated cells with large, vesicular nuclei and prominent nucleoli (Fig. 17.1). The cytoplasm is clear to granular and can range from eosinophilic to basophilic. The cells tend to overlap and have indistinct cell-cell borders. The cells can grow in solid sheets or can form papillae or blastocyst-like structures. The differential diagnosis includes seminoma, yolk sac tumor, and choriocarcinoma.

Yolk Sac Tumor

Yolk sac tumor is the most common testicular tumor in young children and is a frequent component of mixed GCTs of adults. Approximately 90% of patients have elevated serum AFP (Eble et al. 2004). Yolk sac tumor can manifest with a wide variety of patterns including microcystic or macrocystic, solid, alveolar-glandular, papillary, hepatoid, and enteric patterns. The endodermal sinus pattern, which contains connective tissue stalks lined by cuboidal tumor cells (the so-called Schiller-Duval body) is considered pathognomonic of yolk sac tumor.

Choriocarcinoma

Choriocarcinoma is rare as a pure testicular GCT and is an uncommon component of mixed GCT. It is associated with a very high level of serum HCG (>100,000 mIU/mL). Histologically, is contains a combination of syncytiotrophoblastic, cytotrophoblastic, and intermediate trophoblastic cells arranged in a hemorrhagic and necrotic background. The syncytiotrophoblastic cells are large, multinucleate, hyperchromatic cells. Cytotrophoblastic cells are smaller with pale to clear cytoplasm and irregular nuclear contours. Intermediate trophoblastic cells are morphologically similar to cytotrophoblastic cells, and may only be discernible with immunohistochemical stains, although this distinction is of no diagnostic relevance in testicular GCTs.

Teratoma

More than half of teratomas occur in the pediatric population and are benign. In adults, they represent a common component of testicular mixed GCT and have metastatic potential. Teratomas may be comprised of one or all three germinal layers. Ectodermal components include squamous epithelium, glandular

and neural tissue; skeletal muscle is the most common mesodermal component, and virtually any endodermal structure (gastrointestinal tract, respiratory tract, thyroid) can be seen (Fig. 17.1). When fetal-type tissue is present the teratoma is considered "immature". Somatic malignant neoplasms (e.g., squamous cell carcinoma, sarcoma) can arise within the tissues of a teratoma.

Mixed Germ Cell Tumors

Mixed GCTs comprise up to half of all GCTs, but are rarely seen in pre-pubertal children. The histopathology of the component parts is identical to their appearance in pure form. Teratoma together with embryonal carcinoma and/or yolk sac tumor i s the most common combination. Accurate classification of the subtypes is important, with a large proportion of seminoma predicting better response to therapy, and the presence of embryonal carcinoma correlating with stage II disease (Eble et al. 2004).

Intratubular Germ Cell Neoplasia

Intratubular germ cell neoplasia, unspecified (IGCNU) is the accepted terminology for GCT precursors. IGCNU is invariably seen adjacent to the resected GCT within the tubules of the residual testis, and has been identified in ~5% of contralateral testes in men with testicular GCT and in 1% of testicular biopsies performed for infertility (Reuter 2005). Morphologically, IGCNU appears as enlarged germ cells, sitting atop a thickened basement membrane, and displacing Sertoli cells to the center of the tubule. These cells have abundant clear cytoplasm and atypical features, including irregular nuclear contours and prominent nucleoli.

Developmental and Pathologic Roles of Embryonic Stem Cell Transcription Factors

In the early 2000s, basic research into the biology of ES cells identified *OCT4, SOX2*, and *NANOG* as key regulators of the ES cell transcriptional machinery (Boyer et al. 2005). These three genes encode transcription factors that, because of the timing of their expression and their regulatory targets, are thought to be central to the transcriptional identity of ES cells. OCT4 and NANOG are both homeodomain transcription factors and SOX2 is an HMG-box transcription factor; these evolutionarily conserved proteins bind their gene targets in a cooperative manner.

OCT4 (*POU5F1*, *OCT3/4*) is considered a master regulator and is critical to maintaining ES cell identity and "stemness". During embryogenesis, OCT4 is expressed to the blastocyst stage, during which time it specifically blocks trophoblastic differentiation and promotes primitive endoderm and germ cell layer differentiation. During differentiation, it undergoes methylation-based silencing. OCT4 expression is evident in primordial germ cells in the human fetus, is entirely absent soon after birth under normal circumstances, and remains silenced in all adult tissues (Gaskell et al. 2004). Using a culture model in which the levels of OCT4 expression could be titrated, Gidekel et al. (2003) demonstrated that low levels of OCT4 drive ES cell differentiation towards trophoectoderm, while high levels drive differentiation toward primitive endoderm. When injected into mice, these OCT4-high cells form embryonal carcinoma/primitive neural tumors. The authors also demonstrated that OCT4 can drive malignant transformation in somatic cell systems; fibroblasts transfected with OCT4 demonstrate robust colony formation, anchorage-independent growth, and formation of malignant tumors when injected into a recipient mouse. Together, these observations suggest that *OCT4* is an oncogene that is expressed early in germ cell tumorigenesis (Gidekel et al. 2003).

OCT4, SOX2, c-MYC and KLF4 can induce reprogramming of adult fibroblasts to pluripotent stem cells (Takahashi and Yamanaka 2006). NANOG was initially thought to be dispensable in the process; however, subsequent studies demonstrated that NANOG is required for full reprogramming and that endogenous activation likely compensates for the absence of the transgene in experimental systems (Silva et al. 2009). NANOG specifically permits ES cell self-renewal, and when overexpressed can block ES

cell differentiation. Consistent with this function, NANOG mRNA is expressed only in undifferentiated cell types, including ES cells and primordial germ cells, as well as in embryonal carcinoma cell lines; it is undetectable in any adult tissue (Chambers et al. 2003). In the preimplantation mouse embryo, NANOG mRNA expression is confined to the inner cell mass, and is downregulated by the time of implantation. Expression of NANOG is restricted to the stem cell compartment in embryonic testes and is lost in all normal adult tissues. It is highly expressed in embryonal carcinoma and seminoma but not in yolk sac tumors. Quantitative PCR for *NANOG* gene copy number in testicular GCTs reveals that it does not undergo significant copy number gain, despite being present on chr12p, a region that is amplified in ~80% of GCTs (Hart et al. 2005). Rather, the methylation state of a regulatory element within the *NANOG* promoter appears to correlate with its level of expression, with hypomethylation predicting NANOG overexpression in GCTs (Nettersheim et al. 2011).

SOX2 or sex determining region Y-2 is a critical embryonic transcription factor that acts in tandem with OCT4 and other cofactors to drive an ES cell-specific gene expression program that includes NANOG upregulation (Fong et al. 2011). SOX2 is expressed in the blastocyst phase during embryonic development, but in contrast to OCT4 and NANOG, SOX2 is not expressed in primordial germ cells. In the adult testis, it is strongly expressed in Sertoli cells and shows variable expression in spermatogonia (Sonne et al. 2009). OCT4 and SOX2 have been shown to regulate the cell cycle via the miR302 family of microRNAs by increasing entry into the S phase and by inhibiting cyclin D1 regulatory activity (Card et al. 2008). In somatic tissues, SOX2 is critical to neural and lens development (Fantes et al. 2003) and plays a role in foregut differentiation. It has been shown to be amplified and/or overexpressed in a variety of tumor types, and in many cases its expression is associated with more aggressive tumor behavior (Bass et al. 2009; Sholl et al. 2010).

SALL4, a member of the spalt family of zinc finger transcription factors, is also required for ES cell pluripotency and for driving the fate of the inner cell mass away from trophoblastic differentiation. SALL4 is expressed at the two-cell stage of embryonic development; hence, it is expressed earlier than the other critical transcription factors discussed above and has been shown to exert direct transcriptional regulatory control over OCT4 and cooperates with NANOG in regulating embryonic transcription (Zhang et al. 2006). It has genome-wide regulatory effects and notably drives epigenetic modifications via polycomb repressive complexes and DNA methyltransferases (Yang et al. 2011). SALL4 is highly expressed in leukemic cells and appears to play a role in tumorigenesis via epigenetic regulation of the tumor suppressor gene *PTEN* and other important apoptotic pathway genes. During fetal development, SALL4 expression is largely restricted to the gastric epithelium, with limited to no expression at other sites (Ushiku et al. 2010). After birth, SALL4 is highly expressed in normal oocytes (Cao et al. 2009a). Aside from weak expression in a subset of gastrointestinal adenocarcinomas, among solid malignant tumors, SALL4 is specifically overexpressed in GCTs (Cao et al. 2009b).

The Role for Immunohistochemistry in Germ Cell Tumor Diagnosis

Resected primary testicular GCTs are readily classified based on histomorphology, as the tumor typically is well-preserved within the orchiectomy specimen and is present in large quantities. In contrast, extragonadal GCTs arising in the mediastinum or central nervous system and retroperitoneal testicular GCT metastases are often diagnosed on small biopsy specimens that suffer crush artifact. In this context, immunohistochemistry may be an invaluable adjunct to morphologic examination. The traditional markers of GCT are either nonspecific, such as CD30 or placental-like alkaline phosphatase (PLAP), or insufficiently sensitive, such as AFP. The discovery of KIT overexpression in germinomas has established it

Table 17.1 Embryonic stem cell transcription factor expression in germ cell neoplasia

Marker	IGCNU	Seminoma/ Germinoma	Embryonal carcinoma	Yolk sac tumor	Teratoma (mature)	Teratoma (immature)	Choriocarcinoma
Oct4	++	++++	++++	–	–	–	–
Nanog	++	++ to ++++	++++	–	–	–	–[a]
Sox2	++	–	++++	–	++	++	–[b]
SALL4	++	++++	++++	++++	– to +	–	+ to +++

[a]Nanog expression has been reported in choriocarcinoma arising in association with gestational trophoblastic tumors
[b]Limited data
"+" indicates intensity of expression from weak (+) to strong (++++)

as a marker of seminoma as well; however, KIT is expressed in a variety of other tumor types and is therefore of limited specificity.

OCT4

OCT4 was the first ES cell marker demonstrated to be of utility in the diagnosis of GCT. Looijenga et al. (2003) performed an exhaustive immunohistochemical screen of human tumors and found that OCT4 protein expression is specific to IGCNU, seminoma/dysgerminoma/germinoma and embryonal carcinoma (Table 17.1). These authors found that <0.01% of differentiated nonseminomatous GCTs and extragonadal malignant neoplasms expressed OCT4 protein, thus describing the first highly specific antibody for the diagnosis of GCTs (Looijenga et al. 2003). Subsequent studies have confirmed the specificity of OCT4 protein expression for embryonal carcinoma and seminoma, both in pure form and as part of mixed GCTs at both primary and metastatic sites (Santagata et al. 2007). Jones (2004) demonstrated the superior sensitivity and specificity of OCT4 protein expression in the diagnosis of metastatic embryonal carcinoma and seminoma, as compared to traditional markers of GCTs, including PLAP and CD30. In the female gonad, OCT4 expression is specific for both primary and metastatic dysgerminoma and gonadoblastoma associated with dysgerminoma (Cheng et al. 2004).

NANOG

Gene expression studies demonstrated that NANOG is highly expressed in testicular carcinoma in situ (IGCNU) and GCTs, at levels similar to those seen in ES cells (Almstrup et al. 2004). Not surprisingly, the NANOG protein is expressed at high levels in seminoma and embryonal carcinoma, as well as in IGCNU. NANOG is the most sensitive marker for central nervous system germinoma compared to other ES cell transcription factors and to the traditional diagnostic marker PLAP (Santagata et al. 2006). A study of primary and metastatic testicular GCTs demonstrated that NANOG is highly sensitive and specific for seminoma and embryonal carcinoma (Santagata et al. 2007) (Table 17.1). NANOG is not expressed in yolk sac tumors, teratomas, or testicular choriocarcinomas; however, NANOG expression has been reported in choriocarcinomas arising in the context of gestational trophoblastic disease, where it appears to be critical for invasion and proliferation of tumor cells (Siu et al. 2008). NANOG protein expression otherwise appears to be restricted to GCTs, with only a few cases of somatic tumors showing rare cells staining (Liu et al. 2010; Santagata et al. 2007).

SOX2

Of the ES cell transcription factors, Sox2 appears to be the least specific for GCTs, as it is highly expressed in squamous cell carcinomas arising at multiple sites, as well as in a subset of pulmonary adenocarcinomas and neuroendocrine tumors (Long and Hornick 2009; Sholl et al. 2009). However, within the GCT differential diagnosis, it is highly expressed in embryonal carcinoma but not in seminoma, thus proving useful in making this sometimes morphologically difficult distinction (Santagata et al. 2007) (Table 17.1). In contrast to OCT4, NANOG, and SALL4, SOX2 is expressed weakly to moderately in components

of mature and immature teratomas (Liu et al. 2010).

SALL4

SALL4 has more recently been adopted into clinical diagnosis of GCTs. Its use in pathologic diagnosis was introduced in 2009, with an emphasis on its utility in distinguishing yolk sac tumors from other GCTs and non-GCT mimics (Cao et al. 2009a). Similar to OCT4, it is highly expressed in seminoma/germinoma and embryonal carcinoma; however, in contrast to the other ES cell transcription factors discussed above, it is uniquely expressed in yolk sac tumors (Table 17.1). Numerous studies examining SALL4 have confirmed its utility in the diagnosis of yolk sac tumors primary to the testis, ovary, mediastinum, and sacrococcygeum, as well as metastatic sites (Liu et al. 2010). Variable SALL4 expression has been reported in malignant rhabdoid tumors (Venneti et al. 2011); otherwise it is not known to be expressed in other solid tumors. Among hematopoietic malignant neoplasms, it is expressed in precursor B-lymphoblastic lymphoma (Cui et al. 2006).

Expression of Other Regulatory Factors in Germ Cell Tumors

UTF1 (Liu et al. 2010) is another pluripotency marker expressed in normal and neoplastic testis that is regulated by OCT4 and SOX2. In contrast to OCT4 (and NANOG), UTF1 is expressed in spermatogonia of the adult testis, where it is thought to play a role in self-renewal (Kristensen et al. 2008). It appears to be slightly less specific than the other ES cell transcription factors for GCTs, as it is weakly expressed in approximately 4% of hematopoietic and other solid tumors (Wang et al. 2010). In the spectrum of GCTs, UTF1 shows strong expression in IGCNU, seminoma and embryonal carcinoma, and variable expression in yolk sac tumors, but is negative in teratomas and choriocarcinoma. There are conflicting reports on the expression profile of UTF1 in spermatocytic seminoma (Kristensen et al. 2008; Wang et al. 2010). TCL1 (Liu et al. 2010), the T-cell lymphoma/leukemia 1 oncogene, is expressed in T and B-cell lymphomas and is thought to mediate AKT function. Of the GCTs, it is expressed only in seminoma and IGCNU, at a sensitivity of up to 91% (Lau et al. 2010).

The miRNA LIN28 has also been implicated in maintenance of pluripotency, with expression of LIN28 in tandem with OCT4, SOX2 and NANOG demonstrated to reprogram adult human fibroblasts to inducible pluripotent stem cells (Yu et al. 2007). LIN28 is highly expressed in stem cells and regulates the let7 family of miRNAs by blocking their processing to the mature state. LIN28 is down-regulated during differentiation, permitting a rise in let7 expression, and leading to repression of Lin28, Myc, Sall4, and downstream effectors of NANOG, OCT4 and SOX2 (Melton et al. 2010). In contrast to the transcription factors, Lin28 expression localizes to the cytoplasm, consistent with its role in mediating extranuclear RNA to protein processing. It is both sensitive and specific for IGCNU, as well as for gonadal and extragonadal seminoma, embryonal carcinoma and yolk sac tumor, with one study demonstrating equivalent performance to Sall4 and increased sensitivity for yolk sac tumor as compared to Oct4 (Cao et al. 2011).

In conclusion, over the past decade, insights into embryonic stem cells established in basic science laboratories were rapidly translated into clinical diagnostic tools. Now, the ES cell transcription factors OCT4, NANOG, SOX2, and SALL4 are in widespread use in pathology immunohistochemistry laboratories for the diagnosis of GCTs, particularly in metastatic sites. As the field of ES cell biology moves forward, additional factors that are critical to the regulation of pluripotency continue to be discovered. In addition to potentially serving as diagnostic, prognostic, and predictive markers of GCTs, these discoveries promise to shed light on the regulatory factors responsible for maintenance of stemness in health and disease. Our increasingly sophisticated understanding of epigenetic regulatory networks, including histone modification, CpG-island methylation, and microRNAs will likely have implications for our understanding of

germ cell tumorigenesis and open new avenues for treatment.

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Novel Internalizing Human Antibodies Targeting Brain Tumor Sphere Cells

18

Scott Bidlingmaier, Xiaodong Zhu, Yue Liu, Yang Su, and Bin Liu

Contents

Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive form of primary brain tumor current treatments are hampered by resistance and recurrence. A subpopulation of GBM tumor cells can grow as spheres when cultured in serum-free medium and these sphere cells exhibit enhanced tumor-initiating ability and drug resistance. Human monoclonal antibodies targeting brain tumor sphere cells have been identified by phage antibody display technology and could be useful for the development of novel therapies that target subpopulations of GBM cells to combat recurrence and resistance to treatment.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive form of brain cancer. Treatment typically involves a combination of surgery, radiotherapy, and chemotherapy, but only a modest increase in overall survival has been accomplished (Nicholas 2007; Benitez et al. 2008; Chang et al. 2007; Reardon et al. 2008) and the median survival time for a newly diagnosed patient is only ≈1 year (Krex et al. 2007). Even when complete surgical resection of the tumor is possible, recurrence has been nearly impossible to prevent with subsequent treatment.

Tumors are known to be heterogeneous, and it has been hypothesized that a fraction of cells within the tumor (frequently referred to as cancer

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stem cells (CSCs) or more accurately defined operationally as tumor initiating cells (TICs)) may be responsible for initiating and sustaining tumor development (Reya et al. 2001; Pardal et al. 2003; Dalerba et al. 2007). Subpopulations with TIC-like properties were initially identified in hematologic malignancies and later in many types of solid tumors, including brain tumors (Singh et al. 2003, 2004a, b; Bidlingmaier et al. 2008). A subpopulation of malignant glioma cells that are positive for the glycosylation-dependent CD133 epitopes (AC133 and AC141) has been shown to have significantly enhanced ability to initiate tumors in immunocompromised mice (Singh et al. 2003, 2004b; Yuan et al. 2004; Bidlingmaier et al. 2008). For example, one study found that injection of as few as 100 CD133 epitope-positive human glioma cells was sufficient to initiate tumors in immunocompromised mice, whereas 100,000 CD133 epitope-negative cells isolated from the same tumor mass were unable to initiate tumors (Singh et al. 2004b). It has been speculated that while current therapies may be successful in eliminating the vast majority of the bulk tumor, they may not completely eliminate tumor-initiating cell subpopulations, leading to tumor recurrence and treatment failure (Eyler and Rich 2008; Jiang et al. 2007). Therapies optimized for eliminating theses subpopulations would therefore be more effective in preventing recurrence than those targeting the bulk tumor (Eyler and Rich 2008; Sims et al. 2007).

The TIC subpopulation in primary glioma tumors can be enriched by culturing the cells in serum-free medium supplemented with epidermal growth factor and basic fibroblast growth factor (Yuan et al. 2004). Under these conditions, GBM cells proliferate as detached spheres that retain the characteristics of the original tumor (Lee et al. 2006). GBM tumor sphere cells have an enhanced capacity for self-renewal and are able to initiate tumors after serial transplantation. In addition, the ability of primary GBM tumors to form spheres *in vitro* has been shown to be a significant prognostic factor (Laks et al. 2009; Pallini et al. 2008). Thus, GBM tumor spheres provide an attractive target for the development of therapies targeting brain tumor-TICs.

Human Monoclonal Antibodies and Phage Antibody Display

Tumor targeting monoclonal antibodies are currently in use and in development as effective cancer therapies (Adams and Weiner 2005; Nelson et al. 2010). Antibody phage display has proven to be an efficient approach for the identification and development of human monoclonal antibodies against both known and novel tumor antigens (Liu et al. 2004; Ruan et al. 2006; An et al. 2008). Very large phage display libraries containing billions of unique antibodies can be efficiently constructed from naïve human B cells (Sheets et al. 1988), resulting in much smaller bias against self-antigens. Selection of a phage antibody library has lead to the identification of lead compounds for therapeutics, diagnostics and prognostics as well as affinity agents for target discovery (Roth et al. 2007; He et al. 2010; Bidlingmaier et al. 2009).

Selection of Glioblastoma Multiforme (GBM) Sphere Targeting Antibodies by Phage Display

To identify antibodies that bind to glioblastoma TICs, we employed a FACS-based scFv phage antibody library selection procedure (Zhu et al. 2010). Tumor spheres were grown from five fresh human GBM brain tumors and nine previously established GBM xenografts by seeding monodispersed GBM cells in serum free medium containing basic fibroblast growth factor and epidermal growth factor. After 2 weeks of culture, tumor spheres of sizes ranging from a few hundred to a few thousand cells formed. The GBM tumor sphere cells were found to have a higher colony forming capacity than the bulk tumor cells, suggesting that the spheres are potentially enriched for cancer initiating cells. CD133 expression was also analyzed in the spheres and CD133 expression was observed in 11 of 14 GBM tumor sphere cultures. A 500 million member naïve phage antibody library was selected on GBM tumor spheres under internalizing conditions to generate

a polyclonal population of antibodies that target internalizing epitopes (Zhu et al. 2010). In the second and third rounds of selection, the polyclonal populations were incubated with monodispersed tumor sphere cells and cell subpopulations expressing high levels of CD133 (along with bound phage) were sorted by FACS and recovered (Zhu et al. 2010). After the third round, the polyclonal phage antibody selection output was analyzed for binding to GBM sphere cells and about 45% of phage antibodies were found to bind GBM sphere cells but not control cells. In total, 20 unique scFvs that bind to GBM spheres were recovered. The phage antibodies bind to varying fractions of the GBM sphere cell population (3–99%) (Zhu et al. 2010). One of the GBM sphere binding scFvs had been previously isolated and shown to bind ALCAM/166, while the other 19 target unknown surface antigens.

Characterization of Anti-GBM Sphere Antibodies

Costaining experiments with anti-CD133 antibody were done and two phage antibodies, GB8 and GH9, were found to bind primarily to the CD133+ subpopulations of GBM tumor spheres (Zhu et al. 2010). Neither GB8 or GH9 was found to compete with the anti-CD133 antibody, and FACS and co-IP experiments confirmed that GB8 and GH9 do not bind to CD133. The surface expression of the antigens targeted by all of the phage antibodies is concurrently downregulated with CD133 when GBM cells are switched from sphere selective medium to serum-containing medium. The phage antibodies were selected for the ability to internalize and several were tested and confirmed to undergo significant internalization into GBM sphere cells in as little as 1 h (Zhu et al. 2010), demonstrating that the selection strategy was effective.

The phage antibodies were converted to the scFv form, purified, and tested for their ability to inhibit tumor sphere self-renewal. One of the scFvs, GC4, significantly inhibited GBM sphere growth but not cell lines that are not bound by GC4 (Zhu et al. 2010). GC4 also inhibited the growth of established GBM tumor spheres. A GC4 IgG1 was constructed from the scFv sequence and was found to inhibit GBM tumor sphere self-renewal and GBM cell colony formation in soft agar assays (Zhu et al. 2010). Inhibition of proliferation by GC4 scFv and IgG1 was observed in GBM cells derived from four out of five independent cases (Zhu et al. 2010).

GBM Sphere Targeting Antibodies – Therapeutic Potential

We have identified novel internalizing human scFvs that target GBM spheres, which are potentially enriched in TICs. In addition, we have identified a scFv and corresponding IgG that inhibits self-renewal of GBM tumor sphere cells. This antibody will be tested in appropriate *in vivo* models. Since the anti-GBM sphere antibodies possess internalizing function, they can also be used as delivery agents for molecules such as drugs or drug-loaded nanoparticles, toxins, or radionuclides. These TIC-targeted therapies could be used after traditional therapies such as surgery, chemotherapy, or radiotherapy that reduce the bulk tumor load to reduce the potential for tumor recurrence and improve patient outcome.

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Use of Bone Marrow Mesenchymal Stem Cells as Tumor Specific Delivery Vehicles

19

Ting Zhang and Gang Li

Contents

Abstract

Eradication of cancer, especially when it has metastasized is extremely difficult and conventional cancer therapies are simply unable to specifically target tumors/cancers, thus causing unwanted side effects and complications. Recently, it has been shown that bone marrow mesenchymal stem cells (MSCs) are able to migrate specifically to tumors and contribute to the formation of tumor-associated stroma. These properties make MSCs good candidates as anti-tumor agent delivery vehicles and lead to a great deal of interest in the possibility of genetically modifying MSCs to express anticancer molecules and using them as specific targeted anticancer agents. In this chapter, we will review the biological properties of MSCs and the contribution of MSCs in tumor establishment. The potential therapeutic applications of MSCs in the treatment of tumor using different MSCs-delivered anticancer agents will also be reviewed.

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Introduction

Cancer is a major public health problem throughout the world and remains one of the leading causes of mortality and morbidity. A major obstacle for the effective treatment of cancer is the invasive capacity of the tumor cells. Due to the absence of sufficient specificity to the tumor tissue, current conventional cancer therapies (surgery, chemotherapy and radiotherapy) are frequently unsuccessful

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and cause severe side effects and results in low therapeutic efficiency. Therefore, it is critical to develop remedial tumor-targeting approaches for improved efficiency and minimizing systemic toxicity. In many cases, using a gene promoter to drive specific gene expression selectively in a cancer context is the strategy for avoiding toxicity to normal cells and tissues. A gene delivery vehicle that will deliver the tumor killing factors into a majority of the cancer cell population, with the potential to be administered to patients multiple times without eliciting an immune response, represents an essential requirement for successful cancer gene therapy. Mesenchymal stem cells (MSCs) are multi-potent adult stem cells that are easy to acquire and expand *in vitro*; MSCs also have higher migration potential to the injury or tumor sites (Studeny et al. 2002). The tropism of MSCs for tumors makes MSCs uniquely destined to function as cellular delivery vehicles for antitumor agents. It is possible to genetically modify MSCs for the purpose of expressing therapeutic proteins and secreting these proteins into the tumor, such as interferon IFN -β, cytosine deaminase, tumornecrosis factor-related apoptosis-inducing ligand, and oncolyticviruses (Ren et al. 2008; Kucerova et al. 2008; Duan et al. 2009; Sasportas et al. 2009). These approaches have been demonstrated in various pre-clinical models and yielded potent antitumor effects. In the present chapter, a general description of MSCs will be described and we will also review the interactions of MSCs with cancers and therapeutic potential of genetic engineered MSCs.

Mesenchymal Stem Cells (MSCs)

MSCs are multipotent adult stem cells of mesodermal germ layer origin which were first identified in the stromal compartment of bone marrow by Friedenstein et al. in 1960s (Friedenstein et al 1968). In addition to the primary source of bone marrow, MSCs can be isolated from different tissues including adipose tissue, tendons, synovial membrane, skeletal muscle, dermis, pericytes, trabecular bone, human umbilical cord, lung, dental pulp, amniotic fluid, fetal liver, and even peripheral blood, suggesting that MSCs are diversely distributed *in vivo*. Although there is not a single cell surface marker that uniquely characterizes MSCs, a panel of specific cell surface antigens for MSCs has been identified, including expression of CD105, CD73, and CD90 in greater than 95% of the culture, and lack expression of CD45, CD34, CD14 or CD11b, CD79a, or CD19 and HLA-DR surface molecules. The immune phenotype of MSCs is widely described as MHC I+, MHC II-, CD40-, CD80- and CD86- (Loebinger and James 2010). Therefore, transplantation of MSCs into an allogenic host may not require immunosuppression, and thus, MSCs are regarded as non-immunogenic through bypassing host immune surveillance. MSCs are considered non-hematopoietic multipotent stem-like cells that possess an innate ability for self-renewal and capable of differentiating into a variety of mesodermal lineage, including chondrocytes, osteoblasts, adipocytes, muscle cells, pericytes, reticular fibroblasts under proper experimental conditions *in vitro* and *in vivo*. A growing body of evidence suggests that MSCs also have endodermic and neuroectodermic differentiation potential (Loebinger and James 2010). The multilineage potential of MSCs plays an important role in wound healing and tissue regeneration through differentiation and the release of important growth factors and cytokines (Hall et al. 2007).

Their multilineage potential as well as easy acquisition, fast *ex vivo* expansion, and the feasibility of autologous transplantation makes MSCs the ideal choice for cell therapy application in regenerative medicine. Moreover, once MSCs are intravenously or systematically delivered, they are endogenously recruited and selectively homed to sites of inflammation and injury, and improved recovery in animal models of skin wounds, stroke and myocardial infarction (Cheng et al. 2008). Tumor/cancer resemble chronic wounds and is considered as “a wound that never heals”, also tumor microenvironments share many similarities with the tissue repair processes that attract specific homing of MSCs (Hall et al. 2007). The specific tumor-oriented migration and incorporation of MSCs have been demonstrated in various preclinical models, including malignant glioma (Sasportas et al. 2009), melanoma pancreatic and

breast carcinoma (Zischek et al. 2009; Kidd et al. 2009), colon carcinoma (Mueller et al. 2011) and neuroblastoma in immuno-compromised mouse models, regardless the location of the tumors, which demonstrated the potential for MSCs to be used as ideal delivery vehicles for anticancer agents.

However, some studies reported that bone marrow-derived MSCs could increase the growth of human breast cancer, colon cancer, lymphoma, and melanoma cells *in vivo*. Once they are incorporated into the tumor mass, MSCs contribute with other cells like myofibroblasts, endothelial cells, pericytes, and inflammatory cells to create a microenvironment and influence the morphology and proliferation of cells within their vicinity through a combination of cell-to-cell interactions and the produce of tumorigenic chemokines (Direkze et al. 2004; Cogle et al. 2007). Due to the potential effects of MSCs on tumor growth, understanding the interaction between MSCs and tumor cells has become fundamental to determine whether the homing ability of MSCs can be harnessed for tumor-targeted delivery of therapeutic agents. Therefore MSCs were described as a "double-edged sword" in their interaction with tumors. However, if MSCs are suitably engineered with anticancer genes they could be employed as a valuable "single-edged sword" against cancers.

Tumor Microenvironment and Involvement of MSCs in Tumor Establishment

Malignant cells cannot survive alone but live within a complex microenvironment termed as tumor "stroma". Tumor stroma are composed of four main elements: (1) tumor vasculature, (2) cells of the immune system, (3) extra cellular matrix (ECM), and (4) fibroblastic stromal cells-also known as tumor-associated fibroblasts (TAF), carcinoma-associated fibroblasts (CAF), and reactive stroma. Most tumors have some degree of tumor stroma and the tumor viability is dependent on the nonmalignant cells of the tumor microenvironment. These stromal elements respond to signals and factors produced by the tumor cells and provide components necessary for tumor survival, including structural support, cytokines, growth factors, and removal of metabolic and biological wastes, vasculature and extracellular matrices (Hall et al. 2007).

Tumor cells need the necessary oxygen and nutrient supplies for growth and survival. Tumors can switch on angiogenesis by recruiting surrounding mature host blood vessels to sprout new capillaries which grow toward, and eventually infiltrate the tumor mass. The pro-angiogenic molecules such as basic fibroblast growth factor (bFGF) could function as a tumor-derived capillary growth factor and stimulate angiogenesis in various models and vascular endothelial growth factor (VEGF) functions as a potent pro-survival (anti-apoptotic) factor for endothelial cells in newly formed vessels. Tumor cells, tumor-associated macrophages, and TAF could produce VEGF and are involved in tumor-induced angiogenesis. Both tumor-associated macrophages and TAF could support tumor growth and are abundantly detected in tumors (Hall et al. 2007). The cellular origin of TAF remains unclear. Many studies have showed that in addition to their involvement in haematopoiesis, bone marrow is a source of myofibroblasts for many tissues including the gut, lung, and kidney and this phenomenon has been demonstrated particularly in areas of damage and repair, including simple wound healing. Actually, tumors have been compared to unresolved wounds that produce a continuous source of inflammatory mediators. Direkze et al. (2004) showed that bone marrow can contribute to myofibroblast and fibroblast populations in the stromal tissue of tumors in a mouse model of pancreatic insulinoma; they also provided much of the evidence for the participation of BMSCs in tumor tissue and demonstrated with up to 40 % of the myofibroblasts were bone marrow-derived in mouse model of bone marrow transplantation (Direkze et al. 2004). Another study by Cogle et al. (2007), showed that and up to 20 % of the neoplastic cells are bone marrow derived in a patient with lung cancer after sex-mismatched bone marrow transplantation. Tumor neovasculature is critical for tumor growth. Bexell et al. (2009), indicated that MSCs could efficiently migrate to and integrate into tumor vessels and act as pericyte-like cells for delivery

of antiangiogenic substances to vascularized tumors following intra-tumoral grafting. In addition, another research group demonstrated that MSCs are precursors of TAF-like cells in tumors and act as a component of tumor associated fibrovascular networks, including the pericytic population that contributes to the microvessels involved in the neovacularization as well as the fibroblastic population that contributes to matrix remodeling and tumor growth (Spaeth et al. 2009). However, our understanding of the fate and function of MSCs inside the tumor microenvironment is still limited, better understanding of the nature of MSCs-tumor cell interaction is needed to ensure high efficacy of MSCs-based anticancer strategies. New approaches targeting both malignant cells and tumor stromal elements may lead to advancement of anticancer therapy.

Tumor-Tropic Characteristics of MSCs

Numerous studies of various pathological conditions have demonstrated that after systemic or local infusion, MSCs could selectively migrate to sites of injury, or inflammation and to stimulate proliferation and differentiation of resident progenitor cells through growth factor secretion and matrix remodeling. Moreover, their immunomodulatory and anti-inflammatory effects may benefit wound healing. Recent studies have demonstrated that treatment of brain injury with MSCs accelerates wound healing and tissue regeneration (Hall et al. 2007). Tumors can be characterized as sites of tissue damage, or "wounds that never heal", as well as sites of potential inflammatory mediators, cytokine and chemokine production. These signals are capable of recruiting responding cell types including MSCs. The first report of the homing of MSCs to tumors was demonstrated by implantation of rat MSCs into rats bearing syngeneic gliomas, and showed that genetically modified with interleukin-2 (IL-2) had antitumor effect and prolonged the survival time of tumor-bearing rats (Nakamura et al. 2004). Since then, the innate tropism of MSCs for tumors and incorporation of MSCs into tumors have been confirmed in a number of pre-clinical studies in animal tumor models. In these animal models, intravenously delivered MSCs have been shown to selectively migrate to and survive in cancer tissues, such as breast and melanoma lung metastases, Kaposi's sarcoma, colorectal cancer, pancreatic cancer, ovarian cancer and malignant gliomas (Loebinger and James 2010). MSCs administrated by other routes have shown consistent results as intravenous administration, including intraperitoneal delivery for ovarian cancer and intracerebrally in a glioma model.

While many factors have been implicated, the exact mechanisms underlying the migration of MSCs to tumors have not been fully characterized. Secretion of chemokines/cytokines from tumor tissues stimulates the migration of MSCs. MSCs have a variety of chemokine and cytokine receptors on their surface and could respond to the ligands. Cytokines and growth factors such as VEGFs, TGFs, FGFs, PDGFs and IL-8 released from the neoplasm or inflammatory tissues are the possible factors that mediate MSCs migration toward tumors. It is known that these factors released from cancer cells promote the migration of endothelial cell and stromal progenitors from the bone marrow towards the cancer bed and participate in the formation of tumor stroma (Direkze et al. 2004). Adhesion molecules, such as integrins and L-selectin may also play roles in the mobilization and homing of MSCs to tumors. The engraftment of MSCs in tissues is likely triggered by tissue damage or tumor growth. Among the chemokines, stromal cell-derived factor-1 (SDF-1) is of particular interest. The receptor for SDF-1 is CXCR4, is expressed on MSCs. CXCR4 and SDF-1 are found to play very important roles in inflammation and tumor tropism of MSCs. The migration of BMSCs to fibrotic lung is reduced with SDF-1 neutralization. When retrovirally transduced MSCs constitutively express CXCR4, more MSCs were homed toward the infarct region of the myocardium (Cheng et al. 2008). Overexpression CXCR4 in human umbilical cord blood derived MSCs (hUCB-MSCs) also enhanced the migratory capacity of MSCs toward gliomas (Loebinger and James 2010). A better understanding of the signaling pathways associated with tropism of MSCs to tumors may permit more targeted MSCs delivery to desired sites for therapeutic purposes.

In Vivo Imaging Demonstrating MSCs Tumor-Homing Potentials

Multimodal imaging methods have been developed to monitor MSCs tracking in the past decade. *In vivo* imaging techniques help to understand the cell fate *in vivo* and design effective strategies for MSCs administration route and timing. There are optical and non-optical techniques for tracking MSCs fate *in vivo*, including fluorescent in situ hybridization (FISH), flow cytometry methods, histology, immunohistochemistry, immunofluorescence etc. FISH has been used to identify male MSCs within gliomas of female mice (Bexell et al. 2009). Flow cytometry allows for quantification of MSCs within a tumor after digesting the tumor into a single cell suspension. Histology and immunofluorescence can detect MSCs *in vivo* using specific antibodies to detect the labeled cells, such as anti-green fluorescent protein (GFP), anti-firefly luciferase, or anti-human antibodies. Histology is often used in combination with bioluminescent imaging (BLI) to validate MSCs engraftment and their spatial orientation, morphology, differentiation, and function within tumors.

BLI technology is noninvasive, nondestructive, quantitative, and commonly used in cancer studies. Real-time MSC tracking is often done in animal models employing fluorescent dyes and enzymes as cell labels. Reporter genes, such as GFP and *luciferase* are considered indirect labels and produce a signal undiminished following cell proliferation and differentiation that can be detected using *in vivo* optical imaging devices. Different cell types may be distinguished by indirect labeling with different reporter genes. Wang and colleagues have conducted study to track the distribution and differentiation of MSCs in tumor-bearing mice; the 4 T1 murine breast cancer cells were labeled with renilla luciferase-monomeric RFP reporter gene and the MSCs were labeled with firefly luciferase-enhanced GFP reporter gene; and results showed that the MSCs can selectively localize, survive, and proliferate in both subcutaneous tumors and lung metastastic tumors by bioluminescence imaging (Wang et al. 2009). BLI is ideal for longitudinal studies that will significantly reduce the number of animal used. But the BLT imaging cannot be used clinically due to the absence of inherent MSC-specific markers and FDA approved genetically modified MSCs or agents for optical imaging purposes. Non-optical methods such as magnetic resonance imaging (MRI), positron emission tomography (PET), and single photon emission computed tomographies (SPECT), which are already used clinically for cell tracking, but their sensitivity and accuracy need further important.

Evidence for Use of MSCs as Anti-tumor Agents Delivery Vehicles

Upon homing to the tumor mass, MSCs may promote tumor progression through immune response suppression, inhibition of tumor cells apoptosis, and stimulation of epithelial-to-mesenchymal transition, angiogenesis, cell proliferation and metastasis. It has been found that co-injection of mouse melanoma cells, Lewis lung carcinoma cells or colon cancer cells and MSCs into syngeneic mice led to increased tumor size compared with injection of cancer cells alone through enhanced tumor angiogenesis (Suzuki et al. 2011). In the other hand, some studies demonstrated that MSCs also display their intrinsic anticancer activities. In an *in vivo* model of Kaposi's sarcoma, intravenously injected human MSCs homed to tumor sites and potently inhibited tumor growth (Loebinger and James 2010). When mixed with tumor cells, adherent bone marrow cells inhibit primary tumor growth and metastases formation in mice transplanted with Lewis lung carcinoma or B16 melanoma due to some soluble factor(s) released by marrow stromal cells (Loebinger and James 2010). Human skin-derived MSCs also exhibited tumor targeting characteristics and significantly inhibited tumor growth when systemically injected into tumor bearing animals (Loebinger and James 2010). Therefore, the effects of MSCs on tumor growth may vary in different tumor types or at different stages of tumor development. Because MSCs can be easily acquired, culture expanded, and manipulated by viral transduction, as (Fig. 19.1) showed, genetically modified MSCs could be

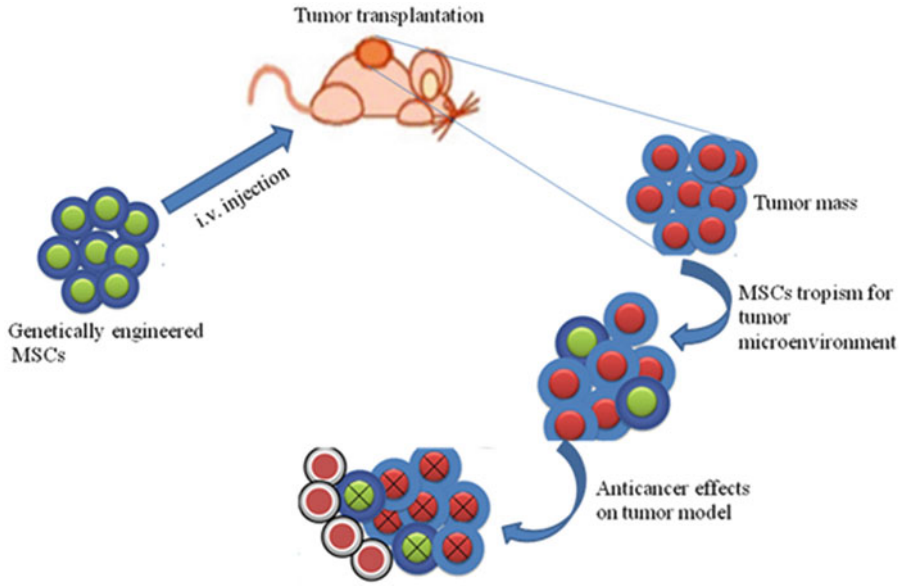

Fig. 19.1 Engraftment of MSCs within tumor xenografts. Subcutaneous tumor xenografts were established prior to intravenous injection of MSCs, which were subsequently found within the tumor stroma. The therapeutic potential of delivering agents to tumors via genetically engineered MSCs was effective in inhibiting the tumor growth

promising tools for delivery therapeutic agents such as small chemical molecules, modulators of the immune system, oncolytic and antiangiogenetic factors, as well as enzymes that can be activated by pro-drugs. As indicated in Table 19.1, a number of anticancer genes have been engineered into MSCs, resulting in anticancer effects on various carcinoma models.

We have showed that when MSCs were infected with herpes simplex virus thymidine kinase (HSV-TK) gene by lentiviral transduction, TK-BMSCs maintained their tumor tropism capabilities and significantly inhibited the growth of subcutaneous PC3 prostate cancer xenografts in nude mice, in the presence of pro-drug Ganciclovir (GCV) (Fig. 19.2a, b). Xenogenic TK-BMSCs also survived and exerted a significant anti-tumor effect in an animal model bearing metastastic RIF-1 (fibrosarcoma) tumor in the presence of pro-drug GCV. Using the TK-BMSCs alone did not cause any harmful side effects *in vivo* (Song et al. 2011). Another research group reported that engineered MSCs expressing the suicide gene cytosine deaminase::uracilphosphoribosyltransferase (CD::UPRT), which convert the relatively nontoxic 5-fluorocytosine (5-FC) into the highly toxic antitumor 5-fluorouracil (5-FU), significantly inhibited prostate cancer tumor growth following intravenous administration (Cavarretta et al. 2010). MSCs as cellular vehicles delivering prodrug-activating enzymes together with their ability to engraft into tumors make them an attractive form of tumor gene therapy. MSCs derived from adipose tissue expressing fusion yeast CD::UPRT gene in combination with prodrug 5-FC also showed potent cytotoxic effect over human colon adenocarcinoma cells HT-29 *in vitro* and *in vivo* (Kucerova et al. 2008).

Using viral-mediated transfection approach, MSCs could be genetically modified to express different kinds of interleukins and mediate efficient cytotoxic effect on target tumor cells *in vivo*. For instance, when MSCs were transfected with IL-12 gene and intravenously delivered, they induced IL-12 production in the tumors and inhibited tumor growth (Duan et al. 2009).

Table 19.1 Anticancer agents delivered by mesenchymal stem cell

Anticancer agents	Anticancer mechanism	Tumor model	Administration route	Species: MSC/ tumor hosts	Reference
CX3CL1	Activates cytotoxic lymphocytes and NK cells	Lung	iv	Mouse/mouse	Xin et al. (2007)
NK4	Inhibits angiogenesis and lymphogenesis and promote apoptosis	Colon	iv	Mouse/mouse	Kanehira et al. (2007)
CD	Pro-drug conversion	Prostate	sc/iv	Human/mouse	Cavarretta et al. (2010)
		Colon	sc/iv	Human/mouse	Kucerova et al. (2008)
HSV-tk		Pancreas	iv	Mouse/mouse	Zischek et al. (2009)
		Prostate	iv	Rat/mouse	Song et al. (2011)
		Fibrosarcoma	iv	Rat/mouse	Xiang et al. (2009)
IFNα	Immunostimulatory, apoptosis inducing and anti-angiogenic	Melanoma	iv	Mouse/mouse	Ren et al. (2008)
		Glioma	it/ic	Mouse/mouse	Sato et al. (2005)
		Glioma	it/iv	Human/mouse	Nakamizo et al. (2005)
IFNβ		Breast	sc/iv	Human/mouse	Studeny et al. (2004)
		Pancreas	ip	Human/mouse	Kidd et al. (2009)
		Melanoma	sc/iv	Human/mouse	Studeny et al. (2002)
IL2	Immunomodulatory cytokine	Glioma	it/ic	Rat/rat	Nakamura et al. (2004)
IL7	Immunostimulatory	Glioma	it	Rat/rat	Gunnarsson et al. (2010)
IL12	Activates cytotoxic lymphocyte and NK cells and produce IFNγ	Melanoma	iv	Mouse/mouse	Chen et al. (2008)
		Hepatoma	iv	Mouse/mouse	Chen et al. (2008)
		Breast	iv	Mouse/mouse	Chen et al. (2008)
		Ewing sarcoma	iv	Mouse/mouse	Duan et al. (2009)
IL18	Immunostimulatory	Glioma	it	Rat/rat	Xu et al. (2009)
TRAIL	Induce apoptosis	Glioma	it	Human/mouse	Sasportas et al. (2009)
		Glioma	ic	Human/mouse	Menon et al. (2009)
		Glioma	iv	Human/mouse	Kim et al. (2008)
		Lung	iv	Human/mouse	Loebinger et al. (2009)
		Breast, lung	sc/iv	Human/mouse	Grisendi et al. (2010)
		Colon	sc	Human/mouse	Mueller et al. (2011)
		Pancreas	iv	Human/mouse	Mohr et al. (2010)

CD Cytosine deaminase, *CX3CL1* Chemokine fractalkine, *HSV-tk* Herpes simplex virus-thymidine kinase, *ic* Intracerebral, *IFN* Interferon, *IL* Interleukin, *ip* Intraperitoneal, *it* Intratumoral, *iv* Intravenous, *NK* Natural killer, *sc* Subcutaneous, *TRAIL* Tumor necrosis factor-related apoptosis inducing ligand

In a mouse melanoma model, application of MSCs expressing IL-12 strongly reduced the formation of lung metastases through activating CD8+ T cells (Chen et al. 2008). In a mouse xenograft model of hepatocellular carcinoma, MSCs overexpressing IL-12 homed to tumors after intravenous injection and significantly reduced the tumor growth and prolonged mouse survival time (Chen et al. 2008).

Type I interferon (IFN) also displays multiple antitumor effects including inhibition of tumor cell proliferation, tumor angiogenesis, induction of tumor cell apoptosis, and activation of host immune defense against tumors. Experiments

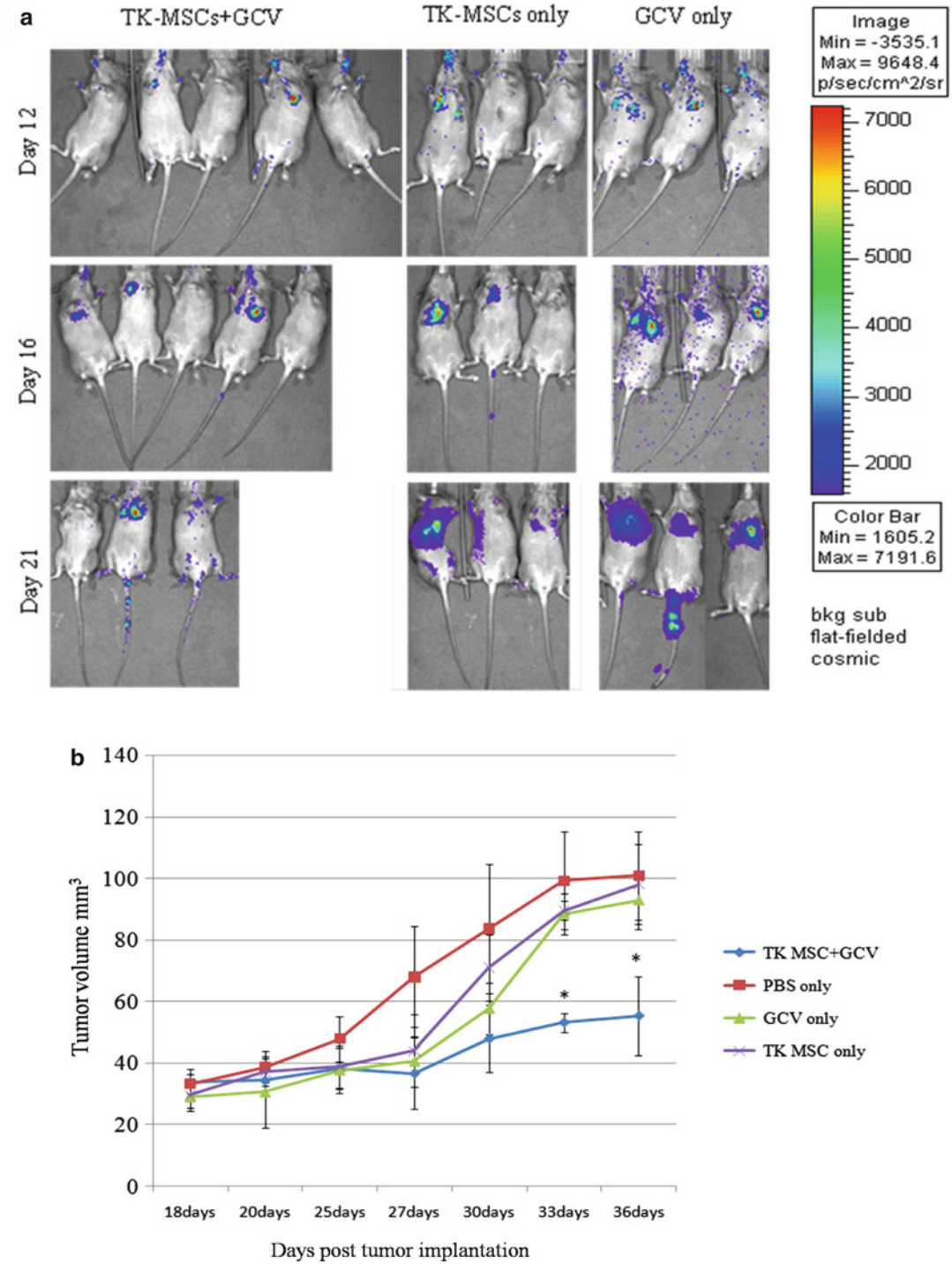

Fig. 19.2 (**a**) In the RIF-1 lung metastasis model, significant inhibition of tumor growth was found only in the TK-BMSCs + GCV group. (**b**) Anti-tumor effect of systemically administered TK-BMSCs in the presence of GCV in PC3 tumor transplant mice was confirmed. At day 33 and day 36 post tumor implantation, tumor volume was significantly smaller in the TK-BMSCs + GCV group (*p <0.05)

showed that treatment of human intracranial glioma xenografts with hMSC-IFN-β significantly increase animal survival time (Nakamizo et al. 2005). In a mouse prostate cancer lung metastasis model, MSCs producing IFN-β at tumor sites in the lungs was found to mediate natural killer (NK) cell activity and anti-tumor effects; and MSCs constitutively producing IFN-α could reduce the growth of melanoma lung metastasis (Ren et al. 2008).

Induction of tumor apoptosis by necrosis factor-related apoptosis inducing ligand (TRAIL) has also been reported. TRAIL induces apoptosis in tumor cells, but has no or minimal toxicity in normal cells. TRAIL has a short pharmacokinetic half-life *in vivo*. When TRAIL gene is stably transduced to MSCs, MSCs serve as a continuous source of TRAIL production that overcomes the problem. In a recent study, when injected i.v. or s.c. into the tumor bearing mice, MSCs overexpressing TRAIL localized into tumors and mediated tumor apoptosis without apparent toxicities to surrounding normal tissues. MSCs expressing TRAIL have also shown to target a variety of tumor cell lines *in vitro*, including human cervical carcinoma, lung cancer, breast cancer, pancreatic cancer and colon cancer (Loebinger et al. 2009; Grisendi et al. 2010). Menon et al.(2009),-demonstrated that after transduced with a lentivirus expressing secretable TRAIL (S-TRAIL), the tumor tropic ability of human bone marrow MSCs are retained and hMSC S-TRAIL together with ipsilateral significantly inhibited tumor growth in an established intracranial glioma tumor model (Menon et al. 2009).

In conclusion, in this chapter we have described the rationale for using MSCs as delivery vehicles for therapeutic agents in anti-tumor therapy. We have also discussed the mechanisms underlying the MSCs homing and killing. Because of their better accessibility, higher expansion potential, low immunogenicity and suitability for genetic manipulation, MSCs become an ideal source for transgene or drugs delivery and for anti-tumor therapy. At present it is unclear whether MSCs have positive or negative effects on tumor progression. Studies supported using genetically engineered MSCs as anticancer vehicles and efficiency of MSCs mediated anticancer therapy has been demonstrated. When genetically modified MSCs were administrated systemically, they significantly inhibited tumor growth in multiple animal models, supporting their clinical application. Issues like how long the engineered MSCs will survive *in vivo*, how specific will the MSCs target tumors as well as the optimal does and timing of MSCs delivery needed further careful investigations.

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CD34+/AC133+ Endothelial Progenitor Cells as Imaging Probes for Neovascularization of Tumors

20

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Contents

Abstract

It was generally considered that blood vessel formation during postnatal life is restricted to angiogenesis only, and for decades tumor vascularization was thought to be the exclusive result of the sprouting of new vessels from the preexisting ones. However, recent studies demonstrated the existence of additional angiogenic and vasculogenic mechanisms associated with tumor growth. Tumor growth and metastasis strongly depends on neovascularization. Endothelial cells that contribute to tumor neovasculatures can originate from sprouting and co-option of neighboring preexisting vessels. However, there is emerging evidence indicating that bone-marrow derived endothelial progenitor cells (EPCs) also contribute to the vasculogenesis and growth of certain tumors. A subpopulation of CD34+ human hematopoietic stem cells (HSCs) identified by the cell-surface molecule CD133 (AC133), have been shown to be more specific for endothelial differentiation and angiogenesis. We have published the reports showing involvement of peripheral blood, cord blood and bone marrow derived EPCs into tumor neovascularization, and the EPCs' involvement in tumor neovascularization was determined by *in vivo* magnetic resonance and nuclear medicine imaging. In this communication different imaging modalities for *in vivo* tracking of EPCs to the sites of active angiogenesis/vasculogenesis are discussed, where EPCs are used as imaging probes.

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Introduction

Tumor growth and metastasis strongly depends on neovascularization. The formation of blood vessels occurs by two mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is the process where blood vessels are formed *de novo* by *in situ* differentiation of the primitive progenitors – i.e. angioblasts – into mature endothelial cells, which was thought to only take place during embryonic development (Risau and Flamme 1995). In contrast, angiogenesis occurs both during the embryonic development and the postnatal life, and is defined as a process that gives rise to new blood vessels by proliferation and migration of preexisting, differentiated endothelial cells (Folkman and Shing 1992). Angiogenic factors, such as vascular endothelial growth factor (VEGF), that are released by tumor cells stimulate angiogenesis by promoting activation, proliferation, sprouting and migration of endothelial cells to the tumor tissue and therefore allow for a rapid formation of new blood vessels (Ellis et al. 2001). Endothelial cells that contribute to tumor neovasculatures can originate from sprouting and co-option of neighboring pre-existing vessels (Zhang et al. 2002). However, there is emerging evidence indicating that bone-marrow derived endothelial progenitor cells (EPCs) also contribute to the vasculogenesis and growth of certain tumors (Asahara et al. 1997). A subpopulation of CD34+ human hematopoietic stem cells (HSCs) identified by the cell-surface molecule CD133 (AC133), have been shown to be more specific for endothelial differentiation and angiogenesis (Gehling et al. 2000; Janic et al. 2010). Higher expression of genes regulating angiogenesis was also observed in AC133+ cells. Additionally, our published results also showed migration and incorporation of intravenously administered CD34+/AC133+ cells in the xenoplanted tumors' neovasculatures (Arbab et al. 2006). EPCs collected from bone marrow, peripheral or cord blood have been used in different animal tumor or ischemia models to determine if these cells have the capacity to become part of the neovasculatures in tissues. When infused into immune compromised mice, EPCs were incorporated into the vasculature of xenotransplanted tumors and were correlated to tumor volume and production of vascular endothelial growth factor (VEGF) (Mancuso et al. 2003). As reported by our group and other investigators, hypoxia induced stromal cell derived factor 1α (SDF-1α) has been detected as one of the potent chemo-attractants for the migration and incorporation of bone marrow derived EPCs due to the presence of CXCR4 receptors in these cells (Ceradini et al. 2004; Arbab et al. 2008). Moreover, we have also reported the role of inflammatory cytokine RANTES, which also act as chemo attractant for these EPCs (Janic et al. 2010).

Capacity of EPCs or CD34+/AC133+ stem cells to migrate and incorporate into the tumor neovasculatures is usually demonstrated by histopathology or immunohistochemistry. Recently it was demonstrated that magnetic resonance imaging (MRI) can also monitor the migration of systemically administered magnetically (iron labeled) labeled mammalian cells to different organs, tumors, and at sites of active angiogenesis (Arbab and Frank 2008). Nuclear medicine and optical imaging techniques are also being used to determine the migration and accumulation of administered cells to the sites of lesion or sites of interest. *Ex vivo* manipulation of EPCs will allow the *in vivo* detection of the sites of homing and active neovascularization in tumors.

Characteristics of CD34+/AC133+ Endothelial Progenitor Cells (EPCs)

Characterization and phenotypical expression of EPCs is still controversial and there is still no consensus on the EPC's definition. Some investigators pointed out that EPCs are CD45 marker negative and should express CD31, KDR (VEGFR2), VE-cadherin and vWF (Ingram et al. 2004). These investigators usually collect cells from peripheral or cord blood mononuclear cells as an adherent cell population and propagate them in differentiating media containing high amount of fetal bovine serum (FBS). There are reports showing peripheral blood CD45 positive

angiogenic cells that show all the hallmarks of EPCs as well as CD45 surface marker (Duda et al. 2007).

Previous studies demonstrated the existence of circulating endothelial cells (ECs) in peripheral blood in various vascular diseases (Hladovec et al. 1978). However, for a while it was unclear whether these cells or their precursors play a role in postnatal vascular growth. The breakthrough came from the work by Asahara et al. (1997) who demonstrated the presence of CD34+/vascular endothelial growth factor receptor-2 (VEGFR2)+EPCs in human peripheral blood. These cells gave rise to mature ECs in culture and were capable of incorporating into the sites of active neovascularization in animal models. This landmark work opened the possibility that in adults, endothelial stem or precursor cells may contribute to the formation of new blood vessels by vasculogenesis. Since then, researchers have been gaining significant insights into the postnatal neovascularization and the EPCs' origin, phenotype and function. However, the main factor hindering the EPCs research is the controversy on the identity of EPCs. Earlier studies defined EPCs as the cells co-expressing hematopoietic stem cell marker CD34 and endothelial marker VEGFR2. Since subsequent work showed that some mature endothelial cells also co-express CD34 and VEGFR2 and that CD34 was not an exclusive marker for hematopoietic cells, a novel CD133 glycoprotein was accepted as a more appropriate marker for immature progenitor cells (Gehling et al. 2000; Peichev et al. 2000). Glycosilated form of CD133 protein is expressed on hematopoietic stem cells but not on mature endothelial cells, and it is recognized by AC133 monoclonal antibody. Peichev et al. (2000) suggested that a subset of circulating CD34+ cells that are positive for both VEGFR-2 and AC133 represent a functional EPC population that plays a role in postnatal angiogenesis or vasculogenesis. EPCs also share many cell surface markers with ECs and with stem/progenitor cells of different tissues. However, currently it is customary to define EPCs as cells that are positive for AC133, CD34 and VEGFR2 markers, with the following distinction: AC133+/CD34+/VEGFR2+ cells represent an immature, highly proliferative EPC population localized mainly in the bone marrow, while AC133-/CD34+/VEGFR2+ cells are considered circulatory, more mature cells that are limited in their proliferative capacity (Khakoo and Finkel 2005). In addition, these more mature cells also express some of the endothelial specific antigens such as platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31), E-selectin (CD62E) and VE-cadherin (CD144), chemokine receptor CXCR-4 (CD184) and have the ability to migrate in response to the CXCR-4 ligand, SDF-1α and vascular endothelial growth factor (VEGF). It is now generally accepted that new vessels can also be formed *via* recruitment of circulating EPCs. Indeed, studies in recent years demonstrated that intravenously administered progenitors isolated from bone marrow, peripheral blood or cord blood can home to ischemic sites, including tumor sites, emphasizing the significance of the paracrine effect of tumor secreted factors. Despite of the significant amount of data available, controversy still remains on the identity and function of the putative EPC and its' functional significance and contribution to tumor vasculature and growth. Gradually, the consensus on the putative EPC phenotype is arising, nevertheless further identification and characterization of novel, more specific EPC markers is warranted.

To show the endothelial potential of cord blood CD34+/AC133+ cells, we have performed extensive *in vitro* and *in vivo* studies. Our recent publications showed the potential of long- and short-term cultured cord blood derived CD34+/AC133+ EPCs to make tube like structured both *in vitro* and *in vivo* matrigel angiogenesis studies (Janic et al. 2010). Some of our *in vivo* animals studies have already been published (Arbab et al. 2006, 2008), where we showed the incorporation of administered CD34+/AC133+ EPCs in the tumor neovasculatures.

The Role of EPCs in Tumor Neovascularization

It was generally considered that blood vessel formation during postnatal life is restricted to angiogenesis only, and for decades tumor vascularization

was thought to be the exclusive result of the sprouting of new vessels from the preexisting ones. However, recent studies demonstrated the existence of additional angiogenic and vasculogenic mechanisms associated with tumor growth, such as intussusceptive angiogenesis, vessel cooption, vasculogenic mimicry, lymphangiogenesis, and the recruitment of endothelial progenitor cells (El Hallani et al. 2010). In most cases, these mechanisms take place concomitantly and are the potential targets for novel anti-angiogenic/antitumor therapeutic strategies.

Mobilization of EPCs from the bone marrow into the circulation and their homing to the sites of neovascularization, and subsequent differentiation into mature ECs are tightly regulated processes. Investigators have tried to identify the paracrine signals associated with tissue ischemia and tumor growth that create favorable environment that attracts EPCs. The recruitment of EPCs from bone marrow (BM) is initiated by increased circulatory levels of factors such as VEGF, fibroblast growth factor (FGF), SDF-1α, granulocyte-monocytes colony stimulating factor (GM-CSF), osteopontin, etc. that are released by growing tumors. These factors activate BM microenvironment to switch from a dormant to a pro-angiogenic state and the process involves the activation of matrix metalloproteinase-9 (MMP-9) that releases bone marrow stromal cells' membrane bound c-Kit (CD117) ligand. Generated soluble form of c-Kit ligand stimulates c-Kit positive EPCs to move from BM niche to the BM vascular zone and translocate to the circulation (Heissig et al. 2002). Tissue hypoxia present in tumors and ischemic vascular diseases is considered to be central to this paracrine mechanism and this ischemic effect was shown to be mediated by marked increase in VEGF and SDF-1α circulating levels (Ceradini et al. 2004). VEGF and SDF-1α expressions are transcriptionally upregulated by tissue hypoxia induced expression and/or activation of hypoxia inducible factor-1α (HIF-1α) (Ceradini et al. 2004). In addition to the increase in their circulatory levels, VEGF and SDF-1α expressions are increased locally, within the hypoxic tissue itself that in turn stimulate recruitment of progenitor cells to the hypoxic site as well. Our recent work also indicated that the homing of EPCs into the neovessels of implanted tumor was related to HIF-1α induced SDF-1α expression (Arbab et al. 2008). Recent studies also demonstrated the correlation between VEGF and SDF-1α expression at the transcriptional and functional/effector level. VEGF was shown to up-regulate SDF-1α and CXCR-4 molecules. In addition, it was demonstrated the possible synergistic effect between two cytokines by showing that without a concurrent VEGF signal; SDF-1α was insufficient in recruiting EPCs to tumor sites (Kollet et al. 2001). In addition to VEGF and SDF-1α, numerous other factors produced by tumor and surrounding cells have been implicated to play a role in EPC recruitment to tumor sites. Tumor produced CCL2 and CCL5 were reported to mobilize EPCs from the circulation. Neurotrophins induced angiogenesis was implicated in breast tumor growth and in ischemic animal models. Adiponectin, a peptide hormone secreted by adipocytes, was also shown to promote EPC numbers, migration, and mammary tumor growth in animal model. In addition, factors that regulate physiological angiogenesis can also play a role in EPC recruitment and mobilization (Janic and Arbab 2010).

The relationship between the temporal and spatial distribution of EPCs recruited from the bone marrow and tumor growth is not completely delineated. Moreover, migration and incorporation of EPCs in relation to the expression of different angiogenic or chemo-attractant factors (such as VEGF, MMPs, PDGF, SDF-1α, HIF-1α, bFGF, $\alpha_v\beta_3$ integrin, etc.) at different stages of tumor development have not been elucidated. Noninvasive imaging methods may provide for the localization of EPCs and HSCs within the tumor, thus furthering the understanding of extracellular and stromal components required for incorporation of these cells into the neovasculatures.

It is hypothesized that once recruited to tumor sites, EPCs can have a dual role in tumor angiogenesis, i.e. they can provide structural function by incorporating into the vessels, and a supporting paracrine role where they secrete angiogenic

factors. Our initial results with cord blood derived EPCs also showed both synergistic as well as paracrine effects in the formation of tube-like structures in matrigel plate by microvascular endothelial cells (MEC) (Janic et al. 2010). Many attempts have been made in answering what is the actual contribution of EPCs on tumor vessel growth and more importantly, do EPCs incorporate into the newly form vasculature. Lyden et al. (2001) first demonstrated the contribution of EPCs to tumor neovascularization, where they showed that transplantation of circulatory EPCs restored tumor angiogenesis and growth in angiogenesis defective, Id1+/-Id3-/- host mouse and donor-derived cells were detected throughout the tumor neovessels, with 90% percent of contribution. Since then, many similar studies were done, however, the reported percentages of EPCs contributing to the tumor neovascularization varied significantly, depending on the tumor model used. On the other hand, Purhonen et al. (2008) reported that in animal model, BM derived or other EC precursors did not contribute to tumor vascular endothelium at all, and that cancer growth does not require BM derived endothelial progenitors. Since demonstrating the significant luminal incorporation of EPC within the tumor neovasculatures proved very challenging, the biological role of EPCs in tumor angiogenesis was very often questioned. On the other hand, more recent reports showed that specific ablation of BM-derived EPCs with anti-VE-cadherin antibody markedly impaired tumor growth associated with reduced vascularization (Nolan et al. 2007). This also support the idea that even with the low vessel incorporation rate, the pararcrine EPC function may be the one that is critical for tumor angiogenesis. Nolan et al. (2007) also indicated that the percentage of incorporated BM progenitors and the density of BM progenitors-derived vessels might depend on the stage and size of the tumor. Therefore, the differences in EPC incorporation in previously published reports may not only be due the diversity of tumor models/types studied, but also due to the temporal differences in tumor development at the time of study.

CD34+/AC133+ Endothelial Progenitor Cells as Imaging Probes

With more understanding of the neovascularization processes and the involvement of bone marrow derived or circulating endothelial cells in tumor neovascularization, it is now the demand of the scientific community to *in vivo* monitor the neovascularization process and to understand the involvement of EPCs. However, there has been no *in vivo* technique invented to determine the involvement of endogenous or exogenegous EPCs in tumor neovascularization unless EPCs are from transgenic source carrying reporter such as green fluorescent protein/red fluorescent protein (GFP/RFP) or luciferase or EPCs are exogenously manipulated to carry reporter gene/s or to carry contrast agents for specific imaging modalities.

Converting EPCs as Optical and Fluorescent Imaging Probes

There is growing interest in the field of optical imaging for establishing the efficacy of engineered stem cells for therapeutic applications. Organic fluorophores such as rhodamine, fluorescein, DAPI, PKH26 and alexa488, are among the most commercially available, inexpensive, widely and easily used for a variety of straightforward shorter term labeling applications in cell and developmental biology. However, organic dyes hold little promise for the current calling of long term labeling for cell tracking strategies. One of the major ways to *ex vivo* label cells is the use of reporter gene systems. The gene of interest is chosen based on the imaging modality to be used, physiological events that are to be monitored or therapeutic goals to be achieved. Even genetically encoded fluorescent proteins like GFP are subject to the limitations of generally broad emission spectra capable of generating false positive results and an overlap of emission spectra with tissue autofluorescence as well as absorption and limited resolution to a few millimeters.

Enhanced red-shifted versions of fluorescent proteins such as DsRed proved capable of giving several orders of magnitude higher signal intensity *in vivo*, as compared to bioluminescence, however the large background autofluorescence severely reduces signal-to-noise ratio. Potential improvements in brightness and photostability of *in vivo* fluorescence imaging are underway with subsequent generations of monomeric red fluorescent protein (mRFP1), of the 'mFruits' such as tdTomato and TagRFP-T. In addition methods for optimizing expression of individual components of multimodality fusion vectors are in progress, such as a thermostable variant of firefly luciferase joined with mRFP and herpes simplex virus 1 thymidine kinase gene (tk) that demonstrated superior expression from all three reporter proteins. Similarly cells can be transduced to carry bioluminescent probe such as luciferase (Arbab et al. 2009).

Involvement of exogenously administered bone marrow or peripheral blood derived or endogenous bone marrow derived EPCs in tumor neovascularization has been determined mostly by invasive or *ex vivo* methods such as immunohistochemistry from biopsy materials or by fluorescent microscope following the administration of genetically altered or organofluorophore tagged EPCs. Alternatively investigators have used transgenic animal model (usually carrying reporter protein, such as GFP or RFP) to determine the involvement of endogenous cells in tumor neovascularization (Hillen et al. 2008). Two types of models have been used; (1) animals carrying reporter protein positive cells (such as GFP+), which is universally present in all cells of the animals, (2) animals carrying promoter driven GFP+cells that can only be present in endothelial cells. The later model has been used to determine tumor angiogenesis (Hillen et al. 2008). Animals with universally GFP+cells can be used to monitor the migration and involvement of GFP+cells in implanted tumors but cannot differentiate involvement of surrounding (sprouting and co-opting) cells from bone marrow cells. Making of animal model that will allow *in vivo* tracking the involvement of endogenous bone marrow cells to tumor development and neovascularization is challenging. The following criteria should be present to make an ideal model; (1) the animal should have reporter (such as GFP or RFP) only in bone marrow cells if the target is to determine the effect of bone marrow cells, (2) all other tissues of the body except bone marrow cells should not have any reporter positive cells, (3) if vasculogenesis/angiogenesis is the target, then reporter gene should be expressed under the guidance of specific promoter, (such as Tie2 or ICAM), (4) tumors or lesion should be produced with cells that should not have similar reporter gene or protein. However, to be able to track the migration of reporter positive endogenous bone marrow cells by *in vivo* imaging, the number of promoter driven reporter positive cells should be sufficient enough or all migrated bone marrow cells should be positive for the reporter. Optical imaging (such as fluorescent or bioluminescent) and nuclear medicine imaging can be utilized to track the reporter gene positive endogenous cells to the sites of tumor or other lesions (Arbab et al. 2009). Recently a chimeric animal model has been developed in our laboratory to determine the involvement of BMPC in the tumor neovascularization (Fig. 20.1). Sub-lethally irradiated athymic mice received bone marrow cells from green fluorescent protein positive (GFP+) transgenic mice. GFP+bone marrow cells were transplanted in athymic mice 24 h following sub-lethal irradiation and the tumors were implanted after 28 days when flowcytometric analysis showed more than 70% engraftment of GFP+cells. Migration and accumulation of transplanted bone marrow cells in the implanted breast cancer and glioma were determined by optical imaging (Kodak, Carestream multi-spectral system, Carestream, USA) with proper excitation and emission profiles. Optical imaging showed gradual increase in GFP intensity in the tumors. Immunohistochemical analyses under fluorescent microscope showed multiple GFP+cells lining the blood vessels and other infrastructures of the tumors. Staining for different angiogenic factors indicated that the GFP+cell accumulated to the sites of higher expression of PDGF, SDF-1 and VEGF in the tumors. Analysis also showed that GFP+cells not only incorporated into the

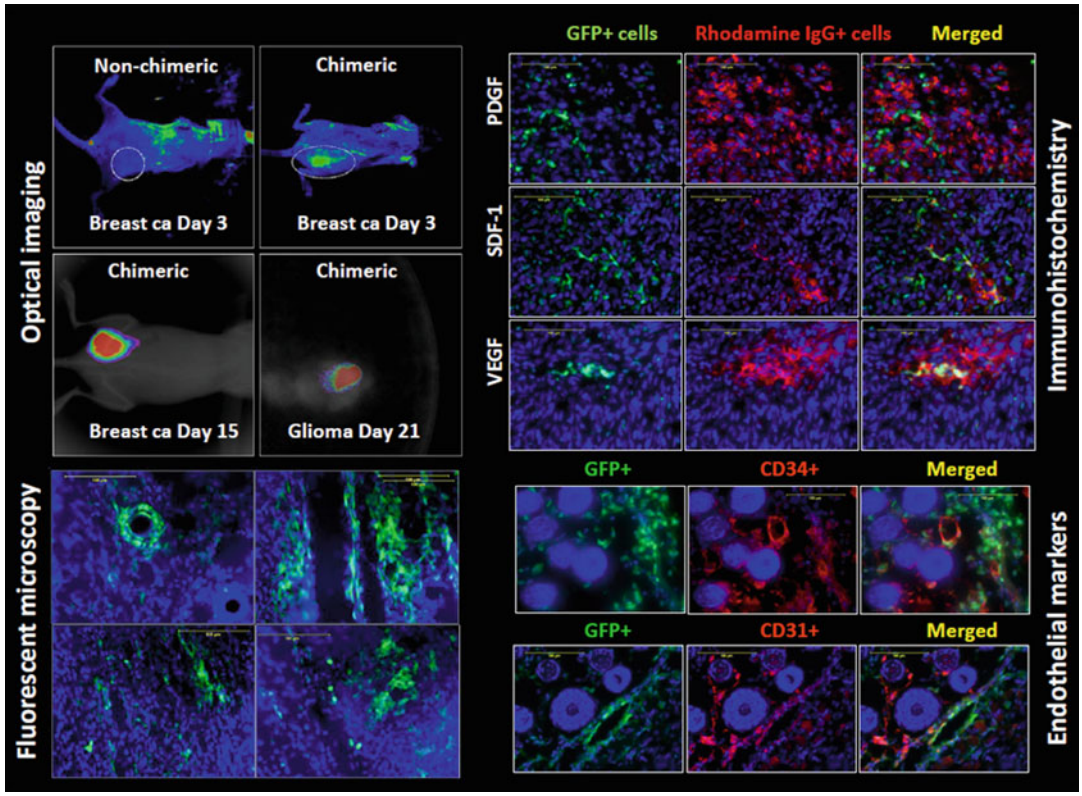

Fig. 20.1 Tracking of endogenous bone marrow progenitor cells (BMPC) to tumors. Chimeric mouse was developed by transplanting GFP+bone marrow cells from donor mouse in an athymic sub-lethally irradiated mouse. Tumors (breast cancer or glioma) were implanted when 70–80% engraftment efficiency achieved. Optical imaging shows (*left upper panel*) accumulation of GFP+cells (fluorescent signals) in the tumors. Accumulation of BMPC was observed as early as 3 days following implantation of tumors. Non-chimeric athymic mouse does not show any fluorescent intensity in the tumor. Fluorescent microscopy (*left lower panel*) shows accumulation of GFP+cells all over the tumor tissues including blood vessels. Immunohistochemistry shows higher expression of PDGF, SDF-1α and VEGF (*right upper panel*) at the sites of accumulation of GFP+cells in tumors. Some of the GFP+cells that are incorporated into tumor blood vessels also show endothelial cell markers, CD31 and CD34 (*right lower panel*)

neovasculatures but majority of the accumulated GFP+cells also remained at the peri-vascular regions. The GFP+cells that incorporated into neo-vessels also showed endothelial cell markers (CD34 and CD31). However, there were also GFP+cells in the tumors which were not positive for endothelial cell markers. These findings are in line of our previously reported results where we showed that cord blood derived EPCs not only incorporated into *in vitro* angiogenesis (matrigel studies) but also helped human dermal microvascular endothelial cells (HDMEC) to form tube like structures (Janic et al. 2010) indicating their paracrine effects. One of the disadvantages of making chimeric animal using universally GFP+bone marrow cells is that there is no way to determine the precise involvement of bone marrow derived EPCs in tumor neovascularization. To know the precise involvement of EPCs in the formation of tumor neovessels we need to make chimeric animal where reporter gene will be expressed only by conditional promoter such as Tie2 in endothelial cells.

Making EPCs as Nuclear Medicine Imaging Probes

Different radioisotopes and radiopharmaceuticals have been used to label cells *in vitro* and track the

administered cells *in vivo* by gamma camera and or single photon emission computerized tomography (SPECT) imaging. Indium-111 (In-111) oxine is one of the Foods and Drug Administration (FDA) approved agents that is readily available and used for cell labeling (Read et al. 1990). The early biodistribution and homing of free/dissociated In-111 in the bone marrow may not reflect the location of labeled cells due to presence of unbound free In-111. Therefore, to determine the homing of In-111 oxine labeled cells it is necessary to wait until the clearance of unlabeled radioactivity from the circulation. Post-administration scanning is usually performed 24–72 h after injection of labeled cells to determine the specific distribution at the sites of interests. Due to short half-life, In-111 labeled stem cells cannot be tracked for more than 7 days (Read et al. 1990). However, it is also important to balance between the added radioactivity and number of cells during labeling. Added radioactivity more 4–5 Bq per cells showed impairment of functions (Yoon et al. 2010). Mesenchymal stem cells (MSC) have been labeled with In-111-oxine to track the migration of the cells in myocardial infarction. However, high resolution computed tomography (CT) images were used to co-localize the migrated cells in the heart. SPECT images with limited spatial resolution are commonly superimposed on high-resolution imaging such as MRI or CT for tracking cell-based therapies and/or cellular probes. We have used In-111-oxine labeled cord blood EPCs to monitor the migration and accumulation to the sites of glioma and confirmed that these cells incorporated in tumor neovessels (Fig. 20.2a). Cord blood derived EPCs were labeled with In-111-oxine on the day of IV administration in human glioma U251 bearing rats. SPECT images were obtained on days 0, 1 and 3. The whole body biodistribution and accumulation of administered EPCs in glioma was also calculated. It was determined that about 2–2.25% administered EPCs accumulated in the tumors in 24 h. Concomitant injection of magnetically labeled EPCs confirmed the accumulation and incorporation into glioma neovessels on histochemistry. *Ex vivo* labeling of cells with radioactive agents is very advantageous when whole body biodistribution and number of accumulated cells to the sites of interests need to be calculated. Precise calculation of the distribution of administered cells is possible by determining the doses of administered cells (both the number and the radioactivity at the time of administration). The easiest way to calculate the number of accumulated cells is to normalize it with the whole body activity on day zero (considered as administered dose).

SPECT can also be used to monitor the migration and homing of genetically modified stem cells (Arbab et al. 2009). The most effective reporter gene that can be inserted into stem cells is sodium iodide symporter (NIS) using viral vectors. Injection of radioactive iodine (such as I-123 or I-131) or technetium-99m-pertechnetate (Tc-99m) will result in the uptake by stem cells carrying NIS and these cells can subsequently be tracked by gamma camera or SPECT scanners. Tracking of these genetically tagged cells can be performed repeatedly as long as the cells express the gene, however, the long term effects of radiation to these cells following repeated exposures needs to be determined. Herpes simplex virus thymidine kinase gene carrying cells can be tracked by using iodinated 2′-fluoro-2′-deoxy-1-ß-D-arabinofuranosyl-5-iodouracil (FIAU) or 1-(2-Deoxy-2-fluoro-ß-L-arabinofuranosyl)-5-methyluracil (FMAU). Most favorable isotope is I-123, which would be expensive for repeated scanning procedures. We have successfully transduced cord blood derived EPCs by both adeno and lenti viral vectors and showed the migration and incorporation of the IV administered transgenic EPCs carrying NIS to the sites of tumor neovessels both in breast cancer and glioma studies (Rad et al. 2009; Varma et al. 2012) (Fig. 20.2b). Both locally or systemically administered transgenic EPCs showed incorporation of EPCs into tumor neovessels and expression of transgene products, which was detected by Tc-99 m SPECT. To determine the number of administered cells that are migrated and incorporated into tumor neovessels, one has to confirm that 100% administered cells should be traduced and should express gene products, and the expression should be sufficient enough to accumulate administered radioactive agent to be detected by SPECT. Based on our In-111-labeled EPCs studies in glioma, we concluded that at least 100,000 NIS positive EPCs need to be accumulated in the tumors to be detected by Tc-99 m-SPECT (Varma et al. 2012).

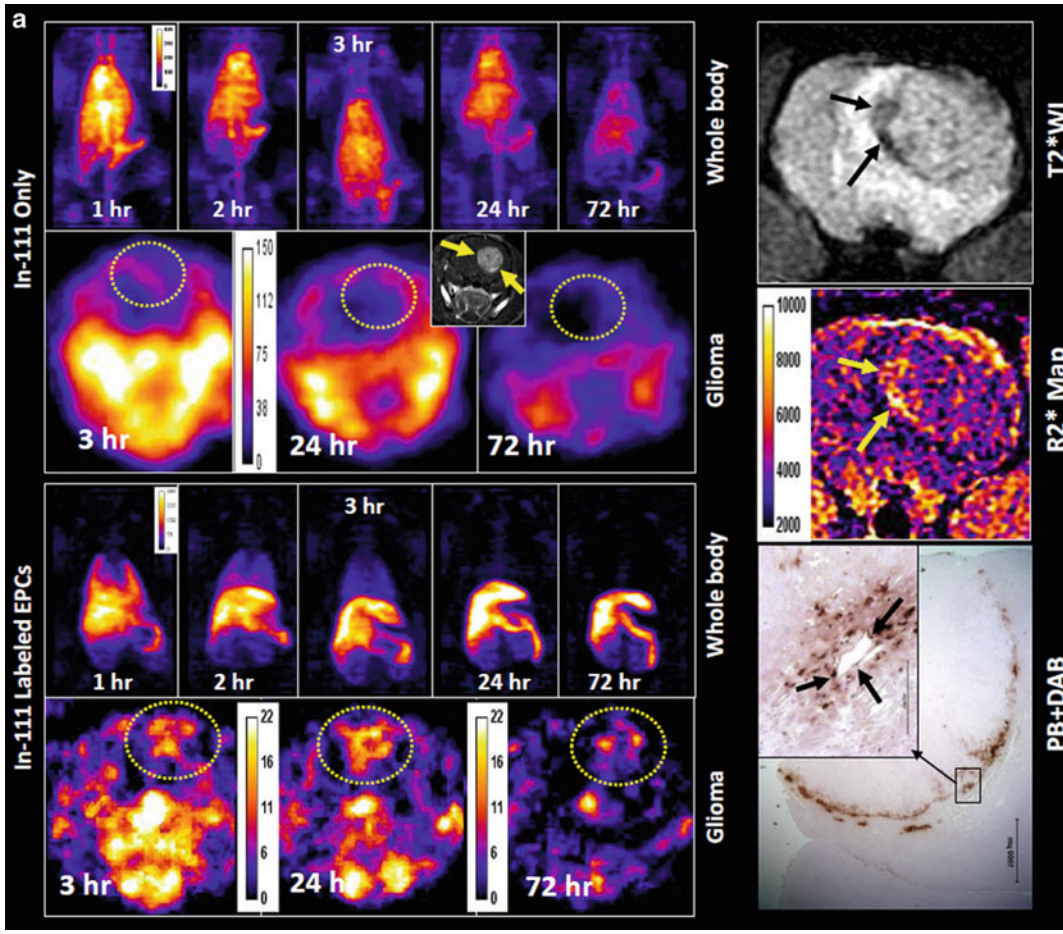

Fig. 20.2 Tracking of EPCs by SPECT and MRI. (**a**) Tracking of labeled EPCs: EPCs were labeled either with In-111-oxine or iron oxides and the migration and accumulation of administered cells were determined by SPECT or MRI, respectively. SPECT images show the biodistribution of administered cells in different organs as well as in the tumor (*left lower panel*, *yellow circles*). The accumulation of In-111 labeled cells was highest after 24 h. Biodistribution of cell associated radioactivity is different from that of administered In-111 only (*left upper panel*). Note that injected In-111 does not accumulate in the tumor (*yellow circle left upper panel*). MR images detect the iron oxides labeled EPCs in the tumor (*right panel*) which is also confirmed by DAB enhanced Prussian blue staining. Both In-111 and iron oxides labeled EPCs were injected into the same animals. Note the incorporation of administered EPCs in tumor vessels. (**b**) Tracking of transgenic EPCs: SPECT and MRI have detected the transgenic and iron oxides labeled EPCs to the sites of glioma (*upper panel*) and breast cancer (*lower panel*). EPCs were genetically transformed to carry NIS and then magnetically labeled with ironoxides. Post EPC administration MR images show low signal intensity in the tumor (circle in glioma and arrows in breast cancer). Tc-99m-SPECT show increased activity of Tc-99m in the tumors indicating functional expression of transgene (NIS). Immunohistochemistry show the presence of iron positive cells in the tumors as well as endothelial cells which are also positive for NIS

There has been limited success in the *in vitro* labeling of stem cells with positron emitting radioisotopes because of acute radiation injury causing death of the labeled cells (Arbab et al. 2009). Moreover, the half lives of available positron emitters are short, making this technique impractical for long-term follow up beyond 2–3 days. Transfecting cells with the reporter genes such as thymidine kinase has allowed for the tracking of cells in experimental and clinical studies by PET when combined with intravenous injection of positron emitters (i.e., 18 F-FHPG) or SPECT agents FIAU. PET reporter gene approaches assume that the primary stem cells would be stably transfected

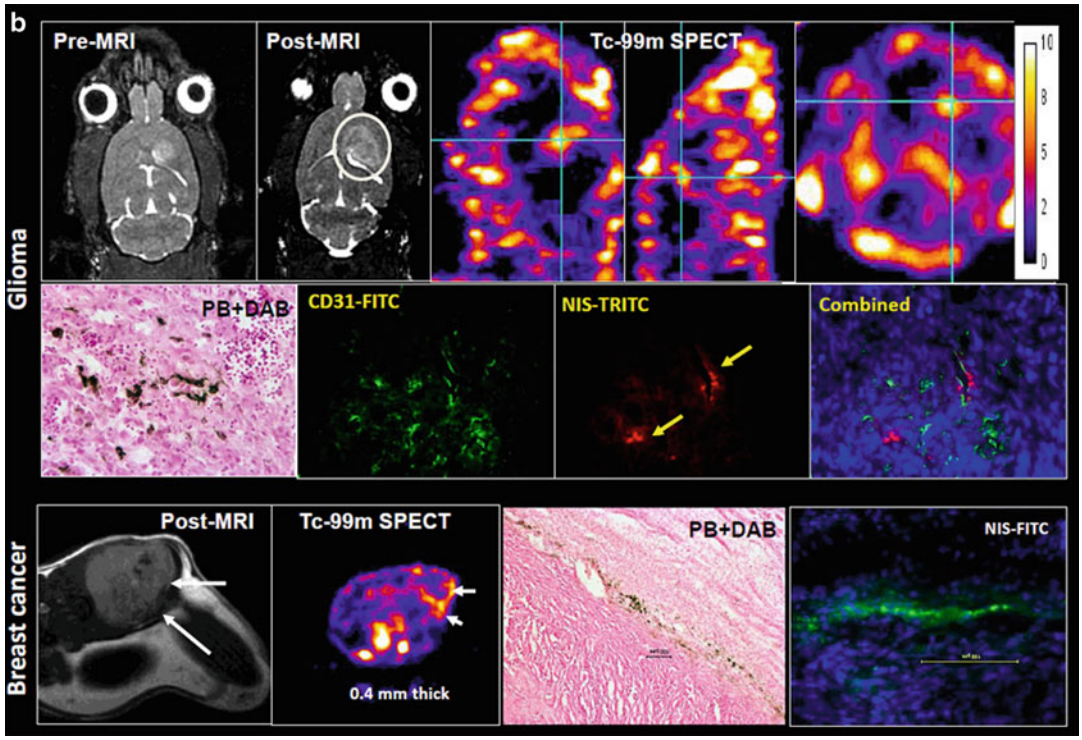

Fig. 20.2 (continued)

and these cells can be tracked following repeated injection of positron emitters. Although PET scans have higher sensitivity (i.e., contrast to noise because of lack of inherent background signals) they have an inherent low spatial resolution as compared to MRI and CT. Therefore similar to SPECT, PET scans usually require high resolution anatomical image to locate the activity *in vivo*. The potential loss of expression of reporter genes over time makes PET impractical for longitudinal imaging studies. Investigators have also used genetically engineered cells for multimodality scanning, however, image resolution and localization of the signal from cells is limited. Tamura et al. (2004) reported the recruitment of bone marrow EPCs to implanted tumor by PET scanning where EPCs were *ex vivo* labeled with [2-(18)F] 2-fluoro-2-deoxy-D-glucose (FDG).

Making EPCs as MR Imaging Probes

Superparamagnetic iron oxides nanoparticles (SPIO) are a class of MRI contrast agents that are being used to magnetically label to track these cell using T2W or T2*W images (Arbab and Frank 2008). Although several approaches for labeling cells with SPIO nanoparticles have been explored, most of the agents used were proprietary compounds, involved unique or complex synthesis, or biochemical modification of the dextran coat of the SPIO nanoparticles to stimulate endocytosis by cells. In 2002, a straightforward approach was developed combining (U)SPIO nanoparticles (e.g., ferumoxtran and ferumoxides) with commonly available polycationic transfection agents to effectively label cells (Frank et al. 2003). Arbab et al. (2004) showed that by mixing two FDA approved agents, ferumoxides (Feridex IV, Berlex, NJ) and protamine sulfate together to form a complex efficiently and effectively labeling stem cells. Ferumoxides is a dextran-coated colloidal SPIO nanoparticles that magnetically saturates at low fields and has an extremely high NMR T2 relativity. Changes in R2 (R2 = 1/T2) are linear with respect to iron concentration. Protamine sulfate is a drug that contains >60% arginine and is used for treatment of heparin anticoagulation

overdose. Both agents are being used off-labeled for magnetic labeling of cells. Cells are labeled with the ferumoxides-protamine sulfate (FePro) complex via macropinocytosis and can be imaged at clinically relevant MRI fields using standard imaging techniques. Unlabeled stem cells usually contain less that 0.1 picograms of iron per cell whereas labeled cells grown in suspension (i.e., HSC, T-cells) contain 1–5 picograms iron per cells and cells that adhere to culture dish (i.e., MSC, human cervical cancer cells, macrophages) can take up from 5 to >20 picograms iron per cell. Labeling embryonic or adult stem cells with ferumoxides-protamine sulfate does not alter the viability and functional capability of cells or the differential capacity of the stem cells (Arbab et al. 2004). Recent studies have shown that cells can safely handle the iron load following FePro labeling (Pawelczyk et al. 2006). In the cell, SPIO nanoparticles are in endosomes that with time fuse with lysosomes and start to dissolve over 3–5 days. Cellular metabolism of the increased iron load results in a transient decrease in transferrin receptor mRNA and protein levels and a corresponding increase in ferritin gene and protein expression that was maintained for at least 1 month in MSCs (Pawelczyk et al. 2006). Previously we have shown that retention of iron in the labeled cells depends on the rate of division and metabolic activity of cells (Arbab et al. 2003). In rapidly growing cells the intracellular iron completely disappeared by 5–8 divisions. On the other hand, the intracellular iron was observed after 6 weeks in cells, where cell division was almost inhibited. Janic et al. (2009) has further improved the labeling procedures using ferumoxides-protamine sulfate and used extensively to label EPCs and track them in neovascularization both in the tumor and stroke.

Anderson et al.(2005) first reported the tracking of IV administered mouse EPCs to the sites of implanted glioma (orthotopic) and tumor neovascularization by *in vivo* MRI and confirmed it with *ex vivo* very high resolution MRI followed by immunohistochemistry. Endothelial lining of new blood vessels showed positive markers for endothelial cells (CD31) and some of the CD31+ cells also positive for iron (Prussian blue staining). In this proof of principal study the authors have not investigated the relation of tumor size and timing of injection of EPCs for incorporation into tumor neovessels. In the next report Arbab et al. (2006) from the same group showed that the migration and incorporation of IV administered human peripheral blood derived AC133+ EPCs were related to the size of the tumor (subcutaneously implanted glioma). The investigators had administered magnetically labeled EPCs in groups of animals on the day of tumor implantation and on day 3 (when tumor was at 0.2 cm size) and the MRI were obtained when the tumor grew to 0.5, 1.0, and 1.5 cm in size. MRI was able to detect the migration and accumulation of iron positive EPCs to the sites of tumors as early as 3 days following IV administration in tumors that received EPCs on day 3 of implantation. However, MRI showed higher amount of low signal intensities on T2*-weighted images (T2*WI) in tumors when it grew to 1.0 cm in size in both groups of animals (IV injected on day 0 or day 3 of tumor implantation) indicating tumor size dependent accumulation and incorporation of EPCs into neovessels. The goal of this study was to detect the migration of labeled cells into growing tumors. Once the tumors were approximately 1 cm in size, there was no apparent difference on MRI or histopathology between group that received IV EPCs on the day of tumor implantation and group that received IV EPCs on day 3 of tumor implantation (i.e., the timing of labeled cell administration did not matter). At the early stages of tumor growth (0.5–1 cm), most of the labeled cells were found along the tumor margins and in between tumor and surrounding muscles or connective tissues. When tumors grew to 1.5 cm, the areas of low signal intensity detected on MRI as well as iron positive cells on histology were no longer at the periphery of the tumor. This might be a result of the lack of availability of magnetically labeled AC133 cells for migration and homing from the bone marrow, lung, liver, or spleen to the tumor due to apoptosis. Alternatively, the labeled cells may have undergone several cell divisions, thereby diluting the intracellular iron label to levels that could not be visualized

on MRI or histopathology. The importance of the size of the tumor for the accumulation and incorporation of administered EPCs might be related to activation and release of angiogenic growth factors or cytokines. It has been proven that release of VEGF, a strong angiogenic factor, is related to tumor hypoxia, which is related to tumor size and distance from existing vessels. This study also clearly showed the involvement of exogenously administered EPCs in tumor neovascularization. There is an argument that host macrophage will take up the dead iron-labeled cells after homing and incorporation into target tissues, and these macrophages along with dead cells will produce misleading low signal intensity on MRI or can show iron positive cells on Prussian blue staining. With this report and with subsequent publications Arbab et al. have shown that host macrophages (mouse) did not show any iron on Prussian blue staining, where either rat glioma or human tumors were implanted subcutaneously in mouse and magnetically labeled human EPCs were administered intravenously. Mouse macrophages were seen mostly at the site of necrosis in the tumor whereas iron positive human EPCs were seen along the peripheral part of the tumors (Arbab et al. 2006, 2008).

In a subsequent investigation Arbab et al. (2008) showed the involvement of magnetically labeled peripheral and cord blood derived EPCs in the tumor neovascularization following local administration within the tumors. Using rat glioma and melanoma models the migration of locally implanted, magnetically labeled peripheral and cord blood derived CD34+/AC133+ EPCs was monitored by MRI. After completely mixing two populations of cells (CD34+/AC133+ and tumor cells) and implanting them as a mixture, one would expect a homogenous distribution of human CD34+/AC133+ cells throughout the growing tumor mass. The objectives of this study were to determine (1) whether cellular MRI can detect the migration of magnetically labeled AC133+ cells in relation to tumor growth when co-implanted with tumor cells, and (2) whether the migration of AC133+ cells is related to the expression of angiogenic and other tumor or surrounding tissue micro environmental factors. It was hypothesized that if hypoxia is the sole factor for stimulating angiogenesis, the co-implanted CD34+/AC133+ EPCs will remain at the center of the tumor to support neovascularization. Alternatively, if angiogenic factors from within the growing tumor or at the tumor-tissue interface are expressed in sufficient amount, EPCs will migrate towards those areas. Visualizing implanted EPCs' differential migration by MRI at different stages of tumor growth or development can provide insight into some of molecular mechanisms involved in *in vivo* tumor angiogenesis. However, after implantation, CD34+/AC133+ cells re-distributed, and when tumor grew to 1–1.5 cm in size MRI and histological analysis revealed that most of the cells migrated towards the peripheral parts of the tumor that co-localized with the strong expression of HIF-1α and SDF-1α that indicated the more hypoxic microenvironment. The same areas also expressed high levels of PDGF and MMP-2 that usually reflects high angiogenic activity. Western blot analysis also indicated higher expression of SDF-1α, PDGF, and MMP-2 at the peripheral part of the tumors. It was also shown by immunofluorescent staining that human CD34+/AC133+ cells that were detected at the periphery incorporated into the tumor neovasculature (Arbab et al. 2008). In addition to shedding more lights into the biological mechanisms of EPC migration within the tumor environment, this work also introduced a novel non-invasive MRI method for *in vivo* stem cell tracking that may facilitate the development of novel diagnostic and treatment strategies (Fig. 20.3). This study also showed the tumor size dependent migration of locally implanted EPCs towards the periphery of the tumor and incorporation into tumor neovascularization. The phenomenon was unrelated to tumor types. Both glioma and melanoma showed similar migratory patterns of locally implanted EPCs and the migration was clearly observed by MRI. Moreover, iron labeled EPCs also worked as histological marker, which was easily detected by Prussian blue. Surprisingly, host macrophage did not show any iron positivity even with local implantation of magnetically labeled EPCs.

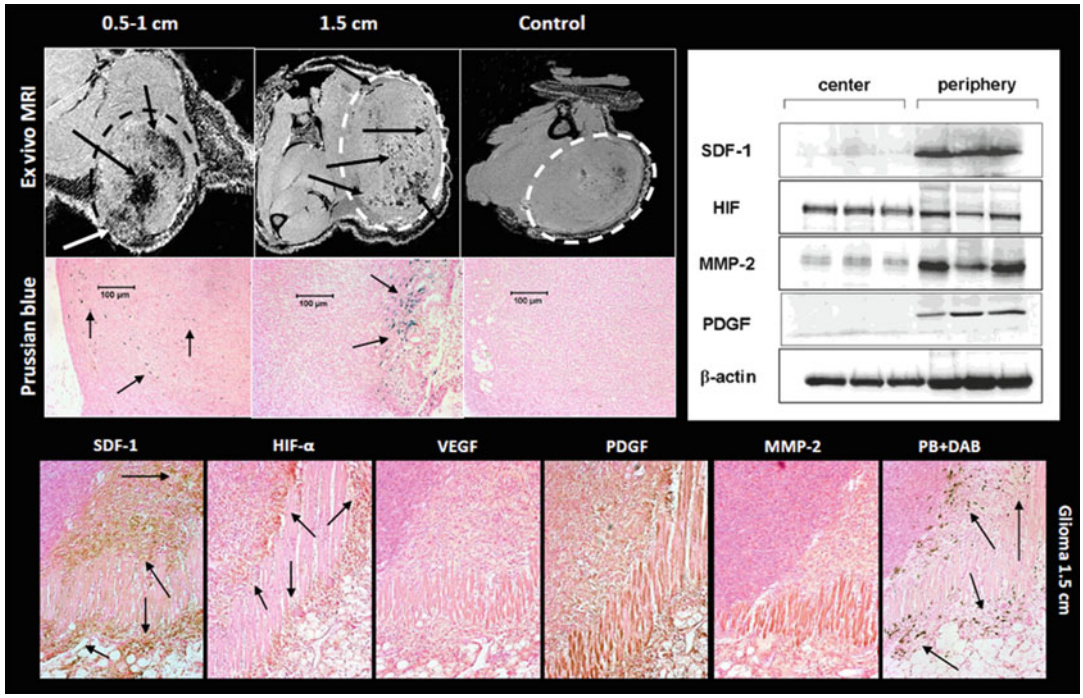

Fig. 20.3 Relation of EPCs' migration and expression of angiogenic factors. MRI images show the migration of locally implanted magnetically labeled EPCs towards the periphery of the tumors (*left upper panel*). The low signal intensity areas seen on MRI due to iron oxides labeled EPCs are different from the low signal intensity areas seen in control tumor (due to necrosis). Presence of iron positive cells is seen at the periphery of the tumors that received EPCs. Control tumor does not show any iron positive cell. Immunohistochemistry (*lower panel*) show the expression of different angiogenic factors at the peripheral part of the tumor, where abundant iron positive cells are observed. Western blot analysis of the tumor lysate show higher amount of SDF-1, MMP-2 and PDGF at the peripheral part of the tumor (*right upper panel*)

Using EPCs as Multimodal Imaging Probes

Very recently our group also reported the use of EPCs as gene carrier and MRI imaging probes to target tumor neovascularization (Varma et al. 2012). The gene that was used also acted as imaging probes for nuclear medicines techniques, such as SPECT. The purposes of this study were to determine whether (1) intravenously administered genetically transformed cord blood derived EPC can carry hNIS to the sites of tumors in rat orthotopic model of human glioma and express transgene products, and (2) whether accumulation of these administered EPC can be tracked by *in vivo* MRI and the expression of hNIS can be determined by *in vivo* Tc-99m SPECT. Ultimate goal was to determine whether in future, EPC could be used as gene carrier/delivery system for glioma therapy. This was the first study to report the use of cord blood derived EPCs to carry a gene (hNIS) to the sites of glioma, and the migration and the expression of gene products were determined by *in vivo* MRI and SPECT studies, respectively. These EPCs were used both as gene carrier and imaging probes. EPCs can be used to deliver therapeutic genes to the sites of lesions. The use of EPCs to carry therapeutic gene to the sites of neovascularization in different lesions are underway in our laboratory. Ultimate goal is to use EPCs as therapeutic agent either to enhance or reduce neovascularization.

In conclusion EPCs can effectively separate from peripheral blood, bone marrow and cord blood. With the established culture technique developed by our group, one can propagate the

separated EPCs to many folds, which then can be manipulated *ex vivo* to carry contrast agents or reporter gene. Following systemic administration these cells can be tracked by different imaging modalities to the sites of active angiogenesis/vasculogenesis in tumors. EPCs can be used as probes for real time detection of tumor neovascularization.

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Intestinal Stem Cells: From Homeostasis to Cancer

21

Julie Pannequin, Dominique Joubert, and Frédéric Hollande

Contents

Abstract

The gastrointestinal epithelium is a highly organised tissue that is constantly renewed. For this reason, it is one of the most rapidly proliferating organs in the body. A complete turnover of the epithelium occurs every 3–5 days in the mouse, a process that is maintained by a small population of intestinal stem cells (ISCs) that reside in the crypt bases. In order to maintain homeostasis, the balance between intestinal stem cell (ISC) renewal and differentiation must be carefully monitored. In this review, we first summarize 50 years of research in this field starting with the work of Christopher Leblond in the late 1950s. We then debate on the precise localization of intestinal stem cells underlining the existing battle between researchers supporting the columnar base crypt theory or the +4 position (+4LRCs). Some of the proposed specific markers of intestinal stem cells are detailed. Herein, we finish by describing colon cancer stem cells and giving some of the consequences of their existence.

Introduction

Because of its role as a protective barrier between the internal organs and the luminal content, the intestinal epithelium is constantly faced with multiple toxic substances susceptible to generate mutations or other cellular alterations. To minimise the potential impact of such alterations

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while maintaining its complex function as an absorptive and secretory organ, it undergoes a complete renewal within less than a week, thanks to a highly organised system: proliferative cells, located at the bottom of intestinal crypts, generate differentiated cells that migrate along the crypt and villi until they undergo anoikis and shed from the surface into the lumen.

Following the discovery of this renewal mechanism, it wasn't long before scientists hypothesized that such system could only function throughout life if at least one cell type was capable of self-renewal, thus permanently feeding the crypt base with new cells. In addition, it became apparent that this or these cell type(s) would have the potential to generate all lineages present in the intestinal epithelium, including cells with such different phenotypes as enterocytes, enteroendocrine, goblet or Paneth cells (Fig. 21.1). Pioneer studies progressively demonstrated that these two properties were most likely restricted to a very small number of cells located in the bottom of intestinal crypts, suggesting that these cells, combining self-renewal and capacity to generate all the differentiated cell types, could be considered as ***intestinal stem cells***.

From the description of the pioneers studies, through the search for specific stem cell markers and their precise localisation, the present chapter will summarize the evolutions in our understanding of this so important "cell minority" for the largest organ in mammals Finally, we will discuss the genotypic and phenotypic link between intestinal stem cells and the recently identified "colorectal cancer stem cells", the discovery of which will without doubt influence future therapeutic approaches against this type of cancer.

Intestinal Stem Cells: The "Pioneer" Studies

The long story of intestinal stem cells started in the late 1950s with Leblond et al.'s (1959) work, who characterized for the first time intestinal stem cells, firstly named crypt base columnar cells, by using the then recent technique of autoradiography. Authors found that crypt base columnar (CBC) cells were radiosensitive and could be killed with relatively low doses of [^{3}H] thymidine. Within a few hours of [^{3}H]thymidine injection, some CBC cells died and were phagocytosed by neighboring viable CBC cells, generating a large radiolabeled phagosome in some CBC cells but not in any other cell type. Later, radiolabeled but more digested phagosomes appear in other cell types. Because labeled phagosomes initially appeared in CBC cells and only later in the four main differentiated cell types, they concluded that CBC cells had differentiated into each of the four cell lines. This was the first demonstration that the population of CBC cells includes cells capable of generating each of the lineages. The "Unitarian theory of the origin of the four epithelial cell types" was based on this work, but it remained for later studies to formally prove the existence of stem cells within the crypt.

Later, Bjerknes and Cheng (1999) provided additional information on these cells using elegant clonal marking techniques using the Dlb-1 locus which, in mice, generates an intestinal binding site for the lectin Dolichos biflorus agglutinin. SWR mice are Dlb-1$^{-/-}$, whereas C57BL/6 mice are Dlb-1$^{+/+}$, and F1 crosses of these two strains yield heterozygous Dlb-1$^{+/-}$ mice. Mutagens such as N-nitroso-N-ethylurea (NEU) induce somatic mutations in one Dlb-1 allele only in random F1 intestinal epithelial cells. Provided this mutation occurs on the "C57BL/6 allele", it should generate, if the affected cell is a progenitor, a clone of cells left unstained by the Dolichos biflorus lectin (Bjerknes and Cheng 1999). Using this approach, Winton and Ponder (1990) first showed that a single mutated cell can give rise to all cells in an adult mouse crypt. Although this study said little about the nature and the location of these cells, it supported the conclusion that there are multipotent stem cells among CBC cells, and the authors also estimated the necessary time for entire crypt epithelium renewal at approximately 12 weeks.

In the meantime, Potten (1990) also performed a number of breakthrough experiments concerning intestinal stem cells. They used different doses of irradiation in order to reduce the crypt

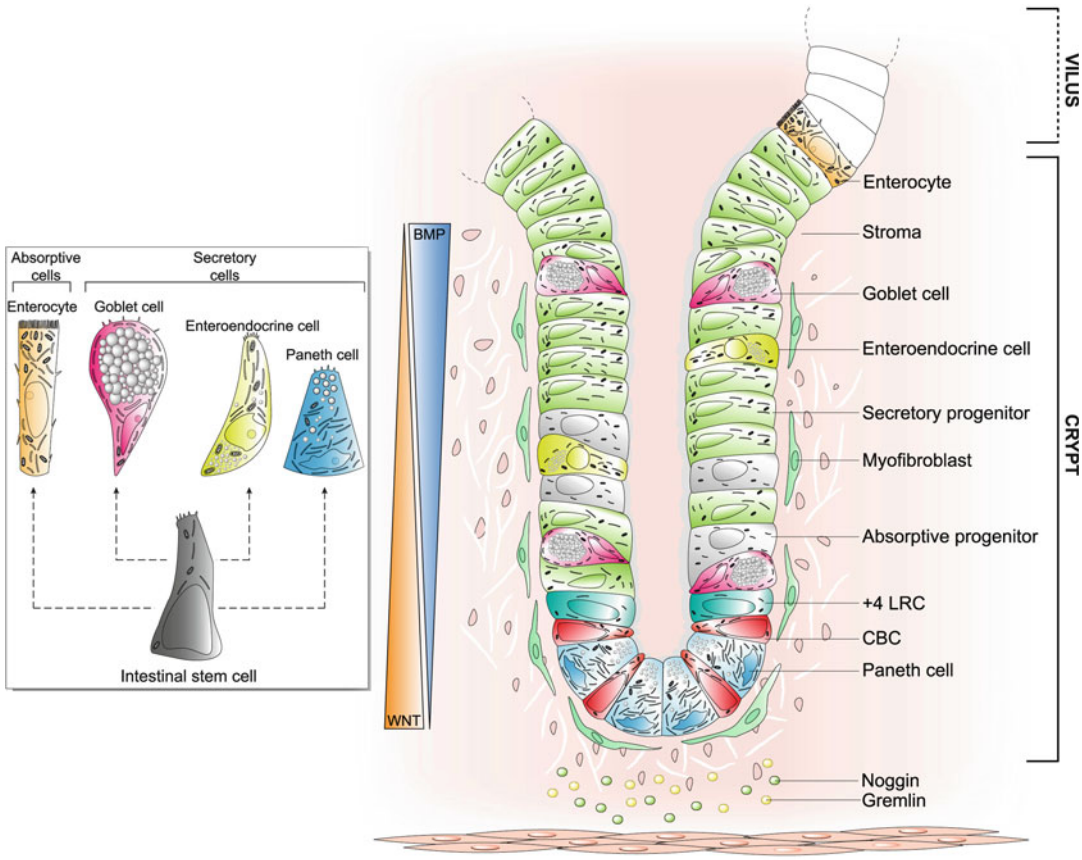

Fig. 21.1 Distribution of epithelial cell types in the adult small intestinal crypt. Structure of adult small intestine depicting the two suspected stem cells zone, the +4LRC situated immediately above the paneth cells and the base columnar cells intermingled between the paneth cells. Opposite gradients of Wnt and BMP signaling activity are represented *on the left*. In the large intestine (*colon*) paneth cells are absent and there are no villi but a flat surface *on the top* of the crypt (*Inset*). There are two main families of terminally differentiated cells: Absorptive and secretory cells. Secretory cells family includes goblet cells secreting mucus, enteroendocrine cells secreting various peptides, Tuft cells, and Paneth cells secreting antibacterial proteins such as lysozymes, defensins and cryptidins

stem cell compartment to different extents, so as to measure the ability of the crypt to survive and regenerate and to estimate the number of potential stem cells per crypt (Potten 1990). Such assays led to the proposal of a hierarchical stem-cell organization, with 4–6 stem cells per crypt upon which the entire crypt renewal ultimately depends. Asymmetric division was also well investigated by Potten's team, who suggested that stem cells initiate apoptosis if their number increases or trigger regenerative division if their number drops. Although this concept is very attractive, it remains controversial, and a number of recent studies argue against the existence of such mechanism in the gut (Escobar et al. 2011; Schepers et al. 2011).

Using a cell signaling perspective, Hans Clevers's team generated numerous important data concerning the essential role of Wnt signals for intestinal stem cells. Thus, Korinek et al. (1998) disrupted the gene encoding Tcf-4 by homologous recombination and found that it was lethal for newborn mice. In addition, the neonatal epithelium was composed entirely of differentiated, non-dividing villus cells. They concluded that the genetic program controlled by Tcf-4 maintains

the crypt stem cells of the small intestine. Van der Flier et al. (2007), by microarray data, generated a list of Tcf-4 target genes, and this group eventually demonstrated that crypt base columnar (CBC) cells (Fig. 21.1), expressing one of these target genes (Lgr5), are able to generate mouse intestinal crypts containing all main cell types (Barker et al. 2007). The same team was the first to show that single Lgr5 (+) cells can initiate crypt-villus organoids *in vitro* for a **short term**, with all differentiated cell types, confirming that these cells are highly likely to be intestinal stem cells (Sato et al. 2009). More recently they expanded their knowledge to successfully establish **long-term** culture of murine and human colon samples, notably through the addition of hormones, vitamins and other growth factors (Sato et al. 2011).

Where Do Intestinal Stem Cells Reside?

To date, there is still a clear discrepancy between results obtained by different teams concerning the exact nature and location of intestinal stem cells. Are these cells located at position +4 (label-retaining cells, or LRCs +4) in the crypt or interspersed between paneth cells (crypt base columnar cells, or CBCs) (Fig. 21.1)?

Three hypotheses have been brought forward to explain these seemingly controversial observations. The first states that CBCs represent the true intestinal stem cells, whereas the +4 LRC are an artefact of the long-term retention assay. An alternative would be that the +4 LRCs actually represent intestinal stem cells whereas CBCs do not. However, recent results from Barker et al. (2007) described above do not support this model9. Third, both CBCs and +4 LRCs could represent different types of stem cells. Evidence indicates that stem cells within the stomach and hair follicle are located in areas distinct from the base of the epithelial invaginations, such as the neck and bulge regions (Tumbar et al. 2004). Therefore, these locations, initially identified using long-term label retaining assays, could identify stem cells that exist in a prolonged quiescent state, reflecting the presence of an inhibitory microenvironment, whereas CBC cells represent a population of stem cells more readily responsive to stimulatory signals generated by adjacent mesenchymal cells, such as BMP antagonists like Noggin or Gremlin (He et al. 2004; Kosinski et al. 2007). Thus, it may be that in rapidly renewing adult tissues, two stem cell compartments coexist and work in a coordinated manner. The more active stem cell type would serve to maintain the regenerative capacities of these tissues under homeostatic conditions, whereas the other, less affected by environmental stress because of its quiescent state, would be held in reserve for "special occasions". In agreement with this theory, recent results by F. de Sauvage's group (Tian et al. 2011) demonstrate that complete loss of Lgr5-expressing cells does not alter homeostasis in the adult mouse intestine. This work suggests that Bmi1-expressing cells give rise to Lgr5 (+) cells and to all other cell lineages, and that they may represent a reserve stem cell pool in case of injury to the epithelium. Taken together, these results strongly suggest that at least two types of stem cells reside in the intestinal crypts and that they most likely regulate each other's behaviour to maintain the homeostasis of this tissue.

In Search of Specific Intestinal Stem Cell Markers

The formal identification of intestinal stem cells has proven a long and arduous task for researchers over the years, starting from candidates detected with radio-labelled isotopes during functional studies, to the recent identification of membrane markers that can be used for immunohistochemical or flow cytometry staining. While functional studies have been described in earlier paragraphs, the search for selective markers was originally influenced by reports characterizing stem cell markers in other tissues.

Thus, **CD133** (known as prominin-1 in the mouse) was among the first proteins to be considered as a putative marker of intestinal stem cells, similar to what had been suggested before in other organs. Despite conflicting results in earlier reports, generation of a knock-in (KI) reporter mouse strain enabling the visualization and tracing

of Prominin-1-positive cells (Snippert et al. 2009) conclusively demonstrated that Prominin-1 is also expressed in progenitors and can therefore not be considered as a marker of CSCs.

Another, more recently identified putative marker for stem cells is aldehyde dehydrogenase 1 (**ALDH1**). ALDH is a detoxifying enzyme that oxidizes intracellular aldehydes. Isolation of stem cells and precursors through the enzymatic activity of ALDH1 was first performed by Jones et al. (1995) in hematopoietic cells. Huang et al (2009) performed similar experiments on colon tumour samples. However, localization of ALDH-positive cells shown by immunohistochemistry is either at position +4 in the crypt or at the CBC position, which means that high ALDH expression is not restricted to one type of intestinal stem cells.

EphB receptors are important regulators of cell positioning and migration in the intestinal epithelium (Batlle et al. 2002). Although EphB receptors were originally considered as putative stem cell biomarkers, decreasing levels of EphB2 expression are found in intestinal progenitor as they migrate upwards and EphB3 is also detected in Paneth cells and early progenitors, indicating that they are not specific for intestinal stem cells. Nevertheless, using the high expression of EphB2, Jung et al. (2011) recently isolated and expanded human colonic stem cells.

Recently, the then orphan G-protein-coupled receptor **Lgr5** was proposed as a marker of intestinal stem cells (Barker et al. 2007). Its expression was observed at the base of both colonic and small intestinal crypts, in the so-called CBC cells. Lineage tracing experiments demonstrated that Lgr5-expressing cells could differentiate into all the expected lineages of the small intestinal and colonic epithelium, suggesting that they behaved as intestinal stem cells. In addition, the recent identification of R-spondins as probable ligands for this receptor and the description of their role as essential for the maintenance of stem cells adds some weight to the hypothesis that Lgr5-marks at least one stem cell subpopulation. However, the poor quality of biological tools to reliably detect this protein has so far hampered its use as a routine stem cell marker.

Bmi1, a potent negative regulator of p16INK4, was first identified as a protein selectively located in hematopoietic and neural stem cell compartments (Lessard and Sauvageau 2003). To further understand the role of Bmi-1 in adult stem cells, Sangiorgi and Capecchi (2008) generated a mouse expressingatamoxifen-inducibleCre-recombinase from the Bmi1 locus. They found that Bmi1 was expressed in LRC cells located four cells above the base of the crypt (+4 position). These cells are undifferentiated, proliferate, give rise to differentiated cells and self-renew over time. These properties being strictly required to identify any cell population as stem cells, they concluded that Bmi1-expressing cells represent at least one of the stem cell populations of the small intestine. However, controversy still persist as to whether this protein is only expressed in cells located at the +4 position, with some groups considering that Lgr5-positive CBCs also express Bmi1.

Colon Tumour-Initiating Cells

The concept of cancer stem cells originated more than 20 years ago when researchers hypothesized that the observed cellular heterogeneity within human tumours might come, similar to what was found in their organ of origin, from an ancestor cell type. Thus, in contrast to the "stochastic model" of tumorigenesis, which supposes that every cancerous cell has the capacity to proliferate extensively and regenerate the tumour, the "cancer stem cell" model assumes that only a subset of cells within the tumour population has the ability to initiate and sustain tumour growth and to generate more differentiated offspring. Yet, the experimental identification of "cancer stem cells" or "tumour-initiating cells" only occurred in the late 1990s and was initially performed in haematopoietic tumours, before being reproduced in many solid tumours including colon cancer (Zhang and Rosen 2006). Similar to what was described in other tumour types, their existence in colorectal tumours was originally hypothesized following the observation that, during their growth, these tumours were steadily able to generate both poorly and highly differentiated cells.

The "cancer stem cell" theory proposes that these cells are rare, difficult to detect, and that they display high tumorigenicity in immunosuppressed mice. Using the controversial stem cell marker CD133, two teams showed that an enrichment of tumour cells expressing CD133 from colorectal cancer led to a strong increase of tumour growth following xenografting in nude mice (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). Importantly they showed that colonic cells obtained from dissociation of cancer specimens can be propagated and expanded in a serum-free medium containing epidermal growth factor and fibroblastic growth factors. In these conditions, tumour stem cells and progenitors float and give rise to colon spheres. Later on, Dalerba et al. (2007) used the combination of CD144, EpCAM and CD166 as markers to select potential colorectal cancer stem cells. EpCAM and CD44 were selected on the basis of their previously described expression in human breast cancer, whereas CD166 was known as a mesenchymal stem cell marker. Interestingly, Shmelkov et al. (2008) recently reported that all epithelial cells within primary colon cancer samples from humans and mice express CD133, raising the question of whether CD133 should be considered as a marker of colon CSCs.

The identification of colorectal cancer stem cells raised multiple **new questions**, notably concerning their origin, their role in metastasis development, and the consequence of their identification for therapeutic approaches.

Where do these cancer stem cells originate? Since their discovery, healthy intestinal stem cells have often been suspected as the cell of origin for colorectal cancer initiation. Indeed, in view of their prolonged lifespan, these cells were always considered as having an increased likelihood to accumulate the documented sequence of mutations leading to human colorectal carcinoma development, in contrast with differentiated intestinal cells, which are short-lived. Yet, another possibility would be that initiating mutations could also appear in progenitors or differentiated cells and confer "stem-like" characteristics to these cells. Another major breakthrough in our understanding of this issue was obtained by Nick Barker and Hans Clevers, who clearly demonstrated that Apc mutations initiate the rapid growth of carcinomas in the mouse only when they occur in Lgr5-positive intestinal stem cells (Barker et al. 2009). However, whether human tumours are actually initiated by mutations in this cell type remains an open question to date.

Beyond the initiation phase, tumour-initiating cells have also been implicated in the development of metastases that, as observed in a number of other cancers, are at the source of most patients' death from colorectal cancer. Since epithelial to mesenchyme transition (EMT) is an essential feature of tumour invasion, and because Wnt signaling is highly active in the invasive front of colon tumor (Fodde and Brabletz 2007), Brabletz et al. (2005) have proposed an integrated model named "the migrating cancer stem cell concept". These migrating stem cells would have the plasticity to adapt to different environments and efficiently form metastasis at distant sites from the primary tumor (Brabletz et al. 2005). Moreover, a study by Joan Massagué's group on several cancers including colon, suggests that circulating cells having undergone EMT can not only colonise distant metastatic sites, but also return to their primary tumor of origin through a process called "tumor self-seeding" (Kim et al. 2009). Whether or not some of these cells at least are cancer stem cells, this process could provide a biological explanation for increased tumor aggressiveness and dysplasia, as well as for local tumor recurrences. More recently, a stream of results obtained on various cancers suggested that the cancer stem cell and EMT phenotype are tightly linked (Monteiro and Fodde 2010). Furthermore, the recent work by Pang et al. (2010) suggested the existence of different subsets of cancer cells with stem cell properties in tumor development and progression: stationary CSCs that are tumorigenic and responsible for growth of the primary tumors, and another subset of CSCs with metastatic capacity, expressing CD26, that are also involved in treatment resistance.

Innate or acquired **treatment resistance** remains a major issue in colon cancer therapy, leading to poor treatment response or to later relapse, and this notion is now clearly associated

with the presence of "cancer stem cells". Indeed, tumor relapse after chemotherapy could partly be explained by the enrichment of the chemoresistant population of cancer stem cells. Thus, Dylla et al. (2008) was one of the first to show that colon tumour-initiating cells are enriched in colon tumours following chemotherapy and remain capable of rapidly regenerating tumors from which they originated. More recently, Emmink et al. (2011) demonstrated that differentiated human colorectal cancer cells protect tumor-initiating cells from treatment with irinotecan, suggesting that specific tissue organization within tumours could also be a reason for increased treatment resistance.

In conclusion, a huge amount of knowledge has been gathered on intestinal stem cells since the very first hypothesis laid by Leblond et al. (1959) more than 50 years ago. However, many questions remained unanswered in this exciting field of research. For example, we are still to unravel the complexity of signals that maintain the constant size of stem cell compartments in intestinal crypts, to understand the degree of "programmation vs epigenetic regulation" in the transition between stem, progenitor and differentiated cells, and to identify whether mesenchymal stem cells have a role to play in intestinal physiology. The broad array of molecular and cellular tools available today will allow researchers to make further inroads in our understanding of intestinal stem cell origin and regulation, as well as of their functional relationship to colorectal cancer stem cells. Such inroads should hopefully benefit cancer patients by guiding the design of compounds in order to minimize tumor relapse.

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Cancer Stem Cells and Modeling Cancer in the Mouse

22

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Contents

Abstract

The complexity of cancer biology cannot be understood in all its depth solely with the study of human patients and the samples derived from them. These types of studies are undeniably essential, but the heterogeneity among human patients, together with the long latency of the disease and its usually delayed diagnosis, make it difficult to recapitulate all the phases of the disease from human studies. In this context, genetically engineered mouse models (GEMMs) of human cancer are essential tools for our understanding of the processes leading to the disease. The sophistication of the techniques allowing us to model cancer in mice has increased enormously over the last years, to the extent that we can now induce, study and manipulate the disease, its evolution and its response to treatment in a way that is not possible in humans. The identification of cancer stem cells (CSCs) as the only cells within the tumor with the capacity of propagating and maintaining the disease has added a new layer of complexity to our understanding of cancer. However, most of GEMMs generated and characterized to date have not being designed to take into account the existence of CSCs and their role in the disease generation, evolution and response to treatment. In this chapter we briefly revise the major milestones in the history of the generation of mouse models of cancer, and propose new strategies for the future, taking into consideration what we nowadays know about the hierarchical nature of tumors.

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Introduction

In spite of our constantly increasing knowledge about the molecular biology of tumors, cancer is already the second cause of death in developed countries, and its incidence keeps rising. Until very recently, cancer has been considered, and treated accordingly, mainly as a disease resulting from abnormal cellular proliferation. Most of the drugs currently used in the clinic are aimed at interfering with actively replicating cells. As such, they have very serious side effects since all other, non-tumoral, dividing cells are affected, therefore leading to the dramatic effects of chemotherapy in other tissues (hematopoietic, intestinal mucosa, hair, etc.). It is true that, in spite of these side effects, most patients show an initial response to the currently used therapies. However, after a remission period of variable length, most cancer patients relapse and only a few achieve a definitive cure that is, in any case, impossible to certify with current methodologies. This fact clearly indicates that our understanding of the disease is still incomplete, and that a new paradigm is required if we want to find new approaches to treat and, hopefully, cure cancer. Relapse occurs because current therapeutic strategies (surgery, radiotherapy and most chemotherapy) are aimed at eliminating only the most evident part of the tumor, i.e., the tumor mass, but they fail to kill the rare cells that are at the root of cancer (Sanchez-Garcia et al. 2007). The cancer stem cell (CSC) theory has already proven, for most human cancer types, that tumors are hierarchically organized, stem cell-based tissues in which only a subset, usually small, of the cancerous population has the capacity to maintain and regenerate the tumor (Sanchez-Garcia 2010). This is, therefore, the tumoral population that must be targeted too, if we want to achieve complete relapse and cancer cure. However, this implies that we must accept moving our focus in cancer research and treatment from proliferation to differentiation and stem cell biology. To be able to study and understand the intricate host-tumor interactions taking place during the development of the neoplasias, it is indispensable to carry out the experiments in an *in vivo* setting in which the cancer can emerge in the presence of the correct microenvironment. Research in mice allows us to integrate the complexity of the different organs and systems, with all their different cell types, within the framework of the global physiological status of the organism. Our final objective would be to recapitulate in the mouse all the molecular, cellular, tissular and organic characteristics of human cancers, including their initiation, progression, evolution, response to therapy and eventual cure or relapse. Since it was discovered that human tumors contain activated oncogenes, many research efforts have been dedicated to the creation of mouse models of cancer in which organ-specific tumors are generated from normal cells that reside in their physiological tissue microenvironments in the context of an otherwise healthy organism, with all its other systems (immune, endocrine, etc.) intact. The hundreds of genetically engineered mouse models (GEMMs) of cancer generated in the last 15 years have greatly increased our knowledge of the biology of cancer and have provided new tools where to test therapeutic strategies. However, in most cases, their capacity to fully recapitulate all the characteristics of human pathology is still incomplete (Cespedes et al. 2006; Cheon and Orsulic 2011). This is a direct consequence of our lack of knowledge of many of the cellular and molecular features behind cancer development and evolution. Of course, this might sound like a vicious circle: if there are so many things that we still ignore about human cancer, how can we possibly try to mimic the disease in GEMMs? However, we believe that the key to answer many of the unsolved questions about cancer lies precisely in this iterative loop of trying to progressively improve animal models and afterwards applying the obtained knowledge to the study of human disease, and then again refining the models under the light of the human data, and so forth. Clearly, at this point it is obvious that the proliferation-based model of understanding cancer has proved insufficient to efficiently tackle the disease, and that GEMMs based just on deregulated oncogene expression, although they still have a lot to offer in certain areas (e.g., pharmacological testing),

they cannot fully mimic the complexity of the human disease. Therefore, the most recent findings about the stem-cell based nature of human tumors, as postulated by the CSC theory, must be taken into account, from a bottom-up perspective, when trying to reproduce the disease in mice. In this chapter we will revise the approaches most commonly used until now to induce cancer in the mouse, and we will propose new ways of generating improved GEMMs based on the CSC theory.

The Intuitive Approach: Constitutive Transgenesis

The discovery that human tumors contain activated oncogenes in the early 1980s led to the logical speculation proposing that the introduction of these mutated forms into the mouse genomic DNA from the germline onwards would lead to the reproduction of the human pathology. Quickly it became clear that indeed, transgenic oncogene expression lead to the development of tumors in the mouse (Adams et al. 1985; Hanahan 1985; Leder et al. 1986; Stewart et al. 1984). Furthermore, more sophisticated approaches of controlling oncogene expression allowed to show that the expression of the tumor-inducing protein was not only required for cancer initiation, but also for disease maintenance, since the withdrawal of oncogene expression caused the reversion of the tumoral condition (Boxer et al. 2004; Chin et al. 1999; Huettner et al. 2000; Perez-Caro et al. 2009). These findings reinforced the concept of oncogenes as the therapeutic targets to be aimed at. However, in these initial transgenic approaches, the final tumoral phenotype was highly conditioned by the expression cassette chosen to drive and direct the expression of the oncogene to a given cellular compartment. The underlying hypothesis was, at that time, that the characteristics of cells forming the main bulk of the tumor mass were directly related to the cellular types that originated the tumor. Hence, the way to mimic human tumors would be to target oncogene expression to those specific differentiated cell types that seem to be the responsibles for the tumoral phenotype. However, this apparently intuitive reasoning might be flawed: for example, the most abundant cells in the blood are the erythrocytes, but they cannot contribute at all to their own generation, and they do not possess any information that can help us understand the process of their own development. So it might be a mistake to assume that targeting oncogenes to differentiated cell types is going to mimic human cancer just because the tumors are mainly composed by cells with these characteristics. Another potentially interfering experimental artifact stems from the fact that in human patients, oncogene activation takes place in a sporadic manner in individual cells during prenatal or postnatal life. However, in classical transgenesis, in general, the oncogene is expressed in all cells in which the chosen expression cassette is active. Finally, classical transgenes present the well-known problems of copy-number and integration effects, and both problems affect the reproducibility and control of oncogene expression.

A Step Forward: Knock-In Cancer Models

To reduce these artifacts and refine the control of the ectopic expression of the oncogenes, while at the same time expressing them in the right cellular context, one possibility was to specifically alter the endogenous copy of the mouse proto-oncogene or tumor suppressor by homologous recombination, replacing it with the mutated (deleted, chimeric, etc.) oncogenic form. The embryonic stem (ES) cells modified in this way were afterwards injected into mouse blastocysts to create chimeric mice. In this way, a single copy of the oncogene is expressed under the full control of its normal regulatory sequences. Also, in the chimeric animal, only a certain percentage of the cells carry the oncogenic alteration, and they can belong to any cellular type in the organism, provided that the cells carrying the alteration are viable. The fact that the alteration might be incompatible with the development of certain cellular types can also answer important questions on the molecular mechanisms of action of

the oncogene. Studies with chimeras have been very informative in shedding light on determining if the initial oncogenic mutation is, by itself, enough to lead to full-blown tumor development (Castellanos et al. 1997; Castilla et al. 1996, 1999; Dobson et al. 1999; Okuda et al. 1998; Yergeau et al. 1997). Two relevant examples of this approach are those of the *Bcr-ABLp190* and *Mll-AF9* oncogenes, associated with different types of leukemias in humans (Castellanos et al. 1997; Corral et al. 1996). Chimeric mice bearing the translocation developed only leukemias, in spite of the wider pattern of expression of the endogenous loci used. Therefore, these results show the lineage specificity of the oncogenes and the specific association with the corresponding tumors (leukemias, in this case). Furthermore, this indicates that the oncogenes are sufficient to give rise to the tumors when expressed from the right promoters. For other chimeric fusions, like *Aml1-ETO* and *Cbfb-MYH11*, associated with myeloid leukemia, however, the oncogenic proteins interfered with cell viability and the targeted ES cells could not contribute to hematopoietic development (Castilla et al. 1996, 1999; Okuda et al. 1998; Yergeau et al. 1997). Therefore, leukemia could not develop. Another problem of this approach is that, although the chimeras are viable, it was not possible to obtain germline transmission and give rise to heterozygous animals, furthermore showing the toxicity of the oncogenic proteins and demonstrating that the tumorigenicity of these oncogenes can only be revealed in the context of sporadic mutations, and not through inherited germline transmission.

Refining the Tools: Conditional Knock-In Mouse Models

The above-mentioned studies already pointed towards the idea that the cancer-initiating events might take place at the stem cell/progenitor level, independently of the final phenotype of the main bulk of tumoral cells. As pointed out before, this could be explained simply by accepting that the phenotype of the mature tumor cells is not just a reflection of the phenotype of the cells in which the oncogenic defect first appears, but rather that it is the oncogene itself the one determining the differentiation program of the targeted cell. We have seen that the introduction of the genomic alteration in a constitutively active form in the germline is not an effective approach to mimic human tumors. Therefore, the next alternative would be to temporally or spatially restrict the expression of the oncogenic alteration. This can be achieved by the use of inducible promoters and lineage-specific recombinases. The P1 bacteriophage Cre recombinase and the yeast FLP recombinase are the current tools of choice for mammalian systems, although new technological approaches are constantly being developed. With the combination of these systems, complex genetic recombination events can be induced, leading to the generation of inter- or intra- chromosomal rearrangements in a regulated, controlled manner. Therefore, mice can be created carrying recombinase-activatable, but silent, oncogenic alterations, under the control of the desired regulatory regions. These mice will develop and behave normally, but when crossed with transgenic tissue-specific recombinases, rearrangement will take place and the cancer-inducing alteration will become activated. The limitation of these conditional knock-in mouse models is that, once more, the final tumoral phenotype of these models is, to a large extent, determined by the temporal and spatial windows of expression of the recombinase (Coste et al. 2007; Forster et al. 2003; Grippo et al. 2003; Guerra et al. 2007; Johnson et al. 2001).

The Cancer Cell-of-Origin at the Center of the Scene

From what we have seen, until now, all the approaches trying to model cancer in the mouse have been centered on the oncogene's role, without really considering the role of the cell of origin in the generation and evolution of the tumor, always taking for granted that the differentiation status of the tumor cells was a caricature of the closest normal cellular counterparts. This point of view is in line with the conception of cancer as a disease of cellular proliferation, driven by the oncogene, and without taking much into account

the aspects related to cellular differentiation. It is well accepted that cancer is a clonal disease that arises in a single cell whose descendants generate the tumor. However, the nature of this cell has received little attention. In the last years, evidences have accumulated suggesting that the cells-of-origin of several types of cancer are stem cells (Ailles and Weissman 2007; Al-Hajj et al. 2003; Bonnet and Dick 1997; Sanchez-Garcia 2010; Sanchez-Garcia et al. 2007). This was already known to be the case for many years in some specific cases, like in chronic myelogenous leukemia (CML), characterized by an abnormal granulocytic expansion. In spite of this cell type-specific mature tumoral phenotype, the translocation t(9;22) causing the disease can be found in all the cellular types of the patient's hematopoietic system, indicating that it has taken place in the hematopoietic stem cells (Jamieson et al. 2004). If this happens to be the case for many tumors, as all these new evidences suggest, then all the data coming from mouse models that do not take this fact into account should be interpreted with caution. But even in tumors that do not have a stem cell origin, the nature of the cell suffering the initial oncogenic alteration is of relevance for correctly recapitulating the cancer phenotype in mice. Indeed, there are cancers in which the cell-of-origin is a differentiated cell (Cobaleda et al. 2007). But also here, it is the interaction between the reprogramming capacity of the oncogenic lesion and the plasticity of the target cell (that is, its capacity for being reprogrammed by the oncogene) what determines the outcome and the nature of the cancer-maintaining cell. Since not all the cells have the same propensity to being reprogrammed, and not all the oncogenes have the same reprogramming power, targeting an oncogenic alteration to the wrong cellular type will very likely fail in reproducing the phenotype of a given human tumor in the mouse.

Accommodating all the Variables: Stem Cell-Activated Knock-Ins

From a developmental point of view, it would make sense that, whatever reprogramming capacities the oncogenes have, they should be more effective when acting in plastic cells, like multipotent stem cells or early progenitors, since these cells are, according to their biological function, more prone to adopt new identities. Along these lines, it has been shown in both the haematopoietic (Eminli et al. 2009) and nervous (Kim et al. 2009) systems that, the more differentiated the cells are, the more difficult it is to induce them to change their fate. Indeed, it is 300 times easier to reprogram HSCs to pluripotency that to do it with B or T cells (Eminli et al. 2009). According to all these facts, in the last years some examples are already starting to appear in the literature showing that targeting oncogene expression to the stem cell compartment in model mice might be the correct way of reproducing the genotype-phenotype correlations found in human cancer. One case is that of CML. The t(9;22) translocation gives rise to the chimeric oncogene *BCR-ABL*, which is expressed, as we have mentioned, from the hematopoietic stem cell onwards in human patients, but causes a phenotype that, during the chronic phase, is restricted to the granulocytic compartment. It has recently been proven that restricting the expression of BCR-ABL to the stem cell compartment is enough to produce CML in the mouse (Perez-Caro et al. 2009). In this study, the promoter and locus-control region of the Sca-1 gene were used to drive a stem cell-restricted pattern of oncogene expression. The development of a full-blown CML in this model, in which mature differentiated cells constituting the tumor mass do not express the oncogene, shows that CSCs can indeed arise by reprogramming, and suggest that the oncogenes can initiate tumors but then be dispensable for posterior cancer progression (Perez-Caro et al. 2009). This model also identifies a main problem of our understanding (and treatment) of cancer: the fact that the oncogenic mutations (even pathognomonic ones like the t(9;22) translocation in CML) might have different roles in the CSCs that in differentiated tumoral cells. This would explain why targeted therapies such as imatinib can successfully eliminate differentiated CML cells without eradicating the CSCs (i.e., without eradicating the disease). Therefore, these improved mouse models could also be an essential tool for the

development of new, anti-cancer stem cells therapies. It could be argued that this kind of cancer-modelling approach might work from well-know stem cell-based cancers, as CML, but not for other tumor types, postulated as arising from differentiated cells. However, as we have seen, more and more evidences are accumulating supporting the idea that human cancers are not originated in differentiated cells, as the current dogma proposes, but rather in stem/progenitor cells. Therefore, these models should be valid for a wide range of tumor types. One example is provided by our results in the study of human mucosa-associated lymphoid tissue (MALT) lymphomas (Vicente-Dueñas et al. 2012). These are tumors composed of mature differentiated B cells, but current attempts at modelling the disease by targeting MALT-lymphoma associated oncogenes to differentiated cells have failed to reproduce the tumor in the mice. However, targeting the expression of the human MALT1 oncogene to the stem cell compartment using the Sca-1 regulatory sequences has recently allowed us to faithfully recapitulate the main features of the human disease in the mouse, showing that oncogene-mediated reprogramming might also be a mechanism for tumoral development in this type of tumors, and suggesting that maybe a similar process might be taking place in human disease (Vicente-Dueñas et al. 2012). In fact, very recently it has been shown for a paradigmatic differentiated B cell tumor as chronic lymphocytic leukemia (CLL), that the propensity to generate clonal B cells has been acquired already at the hematopoietic stem cell (HSC) stage (Kikushige et al. 2011). HSCs purified from CLL patients displayed lymphoid-lineage gene priming and produced a high number of polyclonal B cell progenitors. Strikingly, in xenogeneic recipients their maturation into B cells was restricted always to mono- or oligo-clones with CLL-like phenotype. These data suggest that HSCs can be involved in leukemogenesis even in mature lymphoid tumors. It appears that HSC in CLL serve as aberrant preleukemic cells that produce an increased number of polyclonal pro-B cells. The finding of aberrant HSCs in CLL raises the question of what mechanisms drive this process. Presumably, genetic and/or epigenetic mutations are present in these cells and determine their aberrant behavior, but this still remains to be determined. Without doubt, animal models of CLL that take into account these new findings will be indispensable for the elucidation of these molecular mechanisms.

As we have seen, the study of hematopoietic tumors has provided great insight into the nature of tumor development, but there are also mouse models of solid tumors of epithelial origin showing that directing the expression of the oncogene to the stem cell compartment is the right way of reproducing the genotype-phenotype associations seen in human cancer. In intestinal cancers, activation of the oncogenic Wnt signalling pathway specifically in the crypt stem cell compartment gives rise to hierarchically organized adenomas very similar to the human ones. On the contrary, inducing the same lesion in more differentiated intestinal cells only originates short-lived small lesions, therefore proving that the tumors are of stem cell origin (Barker et al. 2009; Zhu et al. 2009). Similarly, in the nervous system, when oncogenic lesions associated with astrocytomas are expressed in the progenitor cell compartment, they give rise to tumor development. However, when they are targeted to the more differentiated adult parenchyma, only local astrogliosis takes place (Alcantara Llaguno et al. 2009).

These and other results exemplify how the oncogenic initiating events require, for the development of the correct tumoral phenotype, an appropriate cellular context that can only be provided by a stem/progenitor cell target, even if the macroscopic tumor lesion will finally be formed by cells more similar to normal tissue differentiated cells. However, in spite of their improvement, also these models are different form the situation seen in human tumors. Indeed, in humans, given the natural clonal evolution of cancer, all the tumoral cells carry the initial tumor-inducing alteration, independently of what are the functions that this alteration is playing at the different stages of differentiation of the tumoral cells. Therefore, further improvements in the models are required. The objective would

be to initiate the expression of the cancer-initiating genetic defects in stem/progenitors, but then to maintain its expression in a similar manner as how it happens in human tumours. For this, conditional gene targeting should be used but, in this case, in combination with Cre recombinases driven by stem/progenitor-specific promoters. This would take us very close to reproducing, at least at the cellular and molecular level, what the current knowledge suggests is happening in the development of human tumors.

Future Prospects

From all the aforementioned evidences, we can conclude that the CSC theory should be the starting point for the design of new GEMMs of cancer. We cannot continue using the same old models and the same experimental approaches and just pretend that they are equally valid under the light of the CSC theory. We need to revise our strategies for generating mouse models of cancer and adopt a bottom-up approach in which we apply what we currently know about human cancer development to the generation of mouse models capable of recapitulating the genotype/phenotype correlations that characterize human tumors. These new models will be essential for exploring new therapies and predicting their effects in patients in a clinically relevant way.

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Is CD44 a Marker for Cancer Stem Cells?

23

Thalia Blacking

Contents

Abstract

CD44 is a cell surface transmembrane glycoprotein involved in cell adhesion and migration, and also the regulation of numerous intracellular signalling events. As well as the ubiquitously expressed standard form, alternative splicing and post-translational modification give rise to numerous variant isoforms. Altered expression of CD44 occurs in cancer, particularly in association with invasion and metastasis.

More recently the molecule has received considerable attention as a potential marker of cancer stem cells (CSC). The CD44+ fraction of numerous tumours has been reported to demonstrate enhanced tumourigenic potential and the ability to recapitulate the parental tumour. Blockade of CD44 may therefore represent a means of eliminating the putative tumour-propagating CSC fraction of some tumours.

However, CD44 is not a universal marker of CSC. More fundamentally, some evidence suggests that the CD44+ CSC phenotype could be unstable, or even acquired by cells within the CD44– fraction, questioning the concept of CD44+ cancer cells existing at the apex of a unidirectional developmental hierarchy. Moreover, any notion of targetting CD44 will be complicated by its widespread expression on normal cells. Thus, although many features of CD44 make it an attractive candidate as a CSC marker, there is need for a greater understanding of the significance of expression, and in particular the expression of variant isoforms, in the context of putative cellular hierarchies in cancer.

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Introduction

Stem cells and cancer stem cells (CSC) are defined by their functional capabilities, the cardinal properties - features essential for their definition as CSC - being those of unlimited self-renewal capacity and tissue- (or tumour-) specific multipotency. Although functional assays for CSC properties exist, a defined surface marker phenotype, which could reliably identify cells with these characteristics, would be of immense value. Firstly, it would permit rapid, accurate and quantitative assessment using flow cytometry – the technique is readily applicable to bloodborne neoplasias, and may also may be used to examine the cellular composition of disaggregated solid tumours. Additionally, histological techniques allow the identification of the distribution of marker-positive cells in the context of the overall architecture of solid tissues. This, unlike other means of identifying CSC from solid tumours requiring disruption of tissue structure (such as proliferation as spheroids in low-density serum-free culture) could demonstrate the relationship between any putative population and the cellular and stromal environment, or niche, in which it resides *in vivo*.

Cell surface antigens identifying stem cell populations in normal tissues have been reported to identify putative CSC populations in tumours. Among the most prominent of these are CD133 (a marker of haematopoietic stem cells (HSC), neural stem cells and endothelial progenitors) and the HSC marker CD34. One marker which has received considerable attention, despite not being exclusively associated with normal tissue stem cell populations, is the widely expressed glycoprotein CD44. Altered expression of CD44 in cancer has been recognised for many years, but it has more recently been associated with the tumourigenic, putative CSC fraction of numerous tumours. Here we will examine some of the evidence for CD44 as a CSC marker, particularly in the context of breast cancer (for which the molecule was originally reported to identify a specialised tumourigenic subpopulation), and its potential application as a therapeutic target.

CD44

CD44 is a cell surface glycoprotein made up of a cytoplasmic domain, a single-pass transmembrane domain and an n-terminal extracellular region. This extracellular region comprises several domains, including a motif that binds hyaluronan (HA), a polysaccharide molecule ubiquitously expressed on the extracellular matrix (ECM), which is the major ligand of CD44. Between the transmembrane and HA-binding domains is a stem-like region which, for the standard form of the molecule (CD44s), is composed of the gene products of exons 5, 6, and 7. Into this region may be inserted combinations of the gene products of an additional ten variant exons (v1–v10), through alternative splicing (Fig. 23.1a). Through this and other post-translational modification such as glycosylation, numerous variant forms exist (collectively CD44v) such that the size of the molecule may vary from 80 to 200 kDa (Fig. 23.1b, c).

Expression of CD44 is almost ubiquitous, with alternative splicing mechanisms being regulated by tissue-specific factors. The standard form – initially described as a marker of T-cells and other haematopoietic subsets – predominates. Variant isoforms show a more restricted distribution, associated particularly with certain epithelial tissues, though again specific expression patterns are recognised on cells of the haematopoietic lineages such as activated lymphocytes and dendritic cells. HA is ubiquitously expressed as a component of the extracellular matrix (ECM) and may also be expressed on the cell surface – CD44 is therefore involved in both cell-cell and cell-matrix adhesion. CD44 may also bind other extracellular ligands including selectins, fibronectin and osteopontin. The cytoplasmic domain interacts with the actin cytoskeleton through adaptor proteins such as ezrin, radixin and moiesin (ERM proteins) and ankyrin. This linkage facilitates both cell adhesion and also the cytoskeletal rearrangements and changes in morphology required for processes such as migration and extravasation (Marhaba and Zoller 2004).

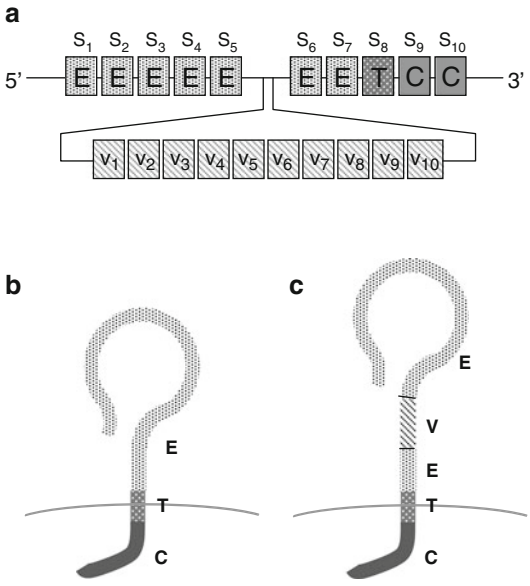

Fig. 23.1 Structure of CD44. (**a**) CD44 gene – Standard form encoded by ten exons, with combinations of a further ten variant exons incorporated through alternative splicing. *S* standard form, *E* extracellular, *T* transmembrane, *C* cytoplasmic, *V* variant. (**b**) CD44 standard form (CD44s) extracellular (*E*), transmembrane (*T*) and cytoplasmic (*C*) domains. (**c**) CD44 variant forms incorporate combinations of variant exon products (*V*) into stem region of extracellular domain

As well as its function in tissue structure and organisation, CD44 plays a critical role in many processes within the haematopoietic system, including lymphocyte activation and trafficking, homing of progenitors to the bone marrow, and antigen presentation. Blockade of CD44 using monoclonal antibodies inhibits normal proliferation, differentiation and homing *in vitro* and *in vivo* (Miyake et al. 1990; Khaldoyanidi et al. 1996). Interestingly, although it is also important in the adhesion and migration of cells during embryogenesis (Haegel et al. 1994), it is not an absolute requisite for normal development. CD44-knockout mice develop normally and survive to adulthood, albeit with impairments in immune cell function (Protin et al. 1999).

CD44, like many adhesion molecules, has no intrinsic catalytic activity. However, consensus sequences within its extracellular domain allow it to act as a co-receptor or modulator for other molecules involved in signal transduction and catalytic processes (Marhaba and Zoller 2004; Zoller 2011). These include receptor tyrosine kinases (RTKs) such as c-MET, VEGFR-2 and ErbB2, growth factors such as hepatocyte growth factor and scatter factor, and matrix metalloproteinases. CD44-ligand interaction may promote anti-apoptotic effects through mediators such as PI3k-AKT, BCL-xL and NFkB. Some interactions are specific to particular isoforms – for example, an epitope encoded by variant exon 3 provides a consensus sequence for interaction with fibroblastic growth factor (FGF), vital in the proliferation and outgrowth of cells in the developing limb (Sherman et al. 1998). Thus, beyond its structural role as a cell adhesion molecule, CD44 plays important costimulatory roles in many cellular processes.

CD44 in Cancer

There are many parallels between the processes of cell adhesion and migration as they occur in physiological (e.g., activation and homing of haematopoietic cells) and pathological (e.g., invasion and metastasis in neoplasia) situations. At around the same time as the CD44 molecule and its variants were being characterised, investigations revealed that it may play an important role in the pathogenesis and progression of cancer.

Gunthert et al. (1991) reported that a novel form of CD44, whose cDNA was homologous to the standard form at 3′ and 5′ ends but which encoded an additional extracellular domain, was associated with metastatic capability in a rat pancreatic carcinoma cell line. The molecule – which represented an alternatively spliced form of CD44 incorporating exons v6 and v7 – could be overexpressed in the nonmetastatic parental cell line, conferring metastatic ability (Gunthert et al. 1991). Moreover, using the same model, a monoclonal antibody to this variant could retard or prevent metastasis formation (Seiter et al. 1993). These investigations suggested a critical role in for CD44v expression in the metastatic process.

In other tumour types, it was the standard form of CD44 which was implicated as a mediator of invasive and metastatic behaviour. Although

many of these studies preceded the precise definition of the plethora of CD44 variants, it had been recognised that there was a larger, "epithelial" isoform ("CD44E"), functionally distinct from the standard, "haematopoietic" form ("CD44H") in terms of its hyaluronan-binding capacity (Stamenkovic et al. 1991). Increased expression of CD44, along with integrins (particularly $\alpha_4\beta_1$ and $\alpha_3\beta_3$), had been reported to correlate with progression in human melanomas; both human and murine melanoma cells expressing low levels of CD44 were less efficient in producing pulmonary tumour nodules upon xenotransplantation than were those expressing high levels (Hart et al. 1991). *In vitro*, Thomas et al. showed that transfection of CD44H (i.e., the standard form) into human melanoma cells could enhance their capacity to migrate on HA-coated substrate, whereas this was not the case for CD44E (Thomas et al. 1992). Similarly, transfection of human B-cell lymphoma cells with CD44H, but not CD44E, promoted local growth and metastatic behaviour in nude mice (Sy et al. 1991).

With improved understanding of the complex alternative splicing and post-translational modifications that may be undergone by CD44 transcripts, a multitude of variant isoforms has now been described. Multiple isoforms may be detected within a single tissue or tumour (or tumour cell line), and each may be associated with disease progression mechanisms. The significance of different expression patterns varies between tumour types. For example, in a panel of surgical samples from lung tumours, CD44s was expressed by 100% of non-small cell lung carcinomas (NSCLC) but only 57% of small cell lung carcinomas (SCLC). Moreover, whilst 69% of NSCLC expressed CD44v, this was not detected in any surgical samples from SCLC, suggesting that CD44 expression is enhanced in NSCLC and repressed in SCLC (Kondo et al. 1998).

Expression of variant exon 6 (v6) in particular has been associated with indices of more aggressive disease – higher grade tumours, a tendency towards invasion, metastasis or local recurrence, and a poor prognosis – in diverse malignancies. However, CD44 is by no means a universal marker of progression. Expression of CD44 or its variant isoforms does not necessarily correlate with tumour grade or metastatic propensity (Fox et al. 1994). For example, whilst early bladder carcinomas show increased expression of both standard and variant isoforms, this is progressively lost as they acquire a deeply invasive, aggressive phenotype (Sugino et al. 1996).

Whilst CD44 appears to have a causative role in metastasis in some experimental systems, it is not indispensable. Driessens et al generated CD44-double knockout (DKO) cells from a highly metastatic murine lymphoma cell line. Whilst DKO cells lost their capacity to bind HA *in vitro*, they showed no deficiency in their ability to form subcutaneous tumours, invade surrounding tissues, or metastasise to liver, spleen or bone marrow *in vivo* (Driessens et al. 1995). Importantly, any potential role for CD44 as a biomarker or prognostic indicator is likely to be complicated by the widespread expression of the standard and, to a lesser extent, variant forms in normal tissues. Thus, it appears that the implications of CD44s or CD44v expression in malignancy must be examined carefully, for each tumour type under scrutiny.

CD44, Cancer Stem Cells and Stem Cells

Many candidate CSC markers are investigated owing to their potential to discriminate stem cells within the corresponding normal tissue – these include the haematopoietic stem cell (HSC) marker CD34 and the HSC, neural stem cell and endothelial progenitor cell marker CD133. CD44, by contrast, initially received more attention as a potential CSC marker than as a means of identifying normal stem cells. Expression of CD44 had been described on some normal stem and progenitor cell populations including ESC and haematopoietic precursors (Haegel et al. 1994; Dimitroff et al. 2001). Immunohistochemical studies had identified greater levels of CD44 expression within zones of cellular proliferation, such as the basal layer of epithelia, where self-renewing cells are located (Alho and Underhill

1989), and expression on epithelial stem and progenitor populations had been described (Hebbard et al. 2000). However, prior to its investigation as a CSC marker, its role in normal stem cell isolation had not been widely explored. This is perhaps because its relatively widespread expression (even for variant forms) might suggest that it would have limited discriminatory utility.

The first report suggesting a role for CD44 in the prospective identification of CSC was published in 2003, and remains one of the most frequently cited in the field. Using flow cytometry, Al-Hajj et al. (2003) fractionated cells derived from human primary and metastatic breast tumours according to their expression of cell surface markers. For all but one of nine tumours, cells in the $CD44^{+}CD24^{-/low}Lineage^{-}$ fraction required much lower inoculums (10–50 times fewer cells) than the unsorted population to produce tumours in immunosuppressed mice. Tumourigenicity was further enhanced by additionally selecting for cells expressing high levels of ESA (epithelial surface antigen, also known as epCAM). $CD24^{+}Lin^{-}$ cells were unable to form tumours except in one subject (Al-Hajj et al. 2003).

The $CD44^{+}CD24^{-/low}Lin^{-}$ cells could be serially passaged in mice, forming tumours from which further $CD44^{+}CD24^{-/low}Lin^{-}$ cells could be isolated (i.e., self-renewal capacity), as could the other nontumourigenic cell populations found in the original tumour (i.e., multilineage differentiation). The ability to demonstrate these two cardinal CSC properties *in vivo* was considered a breakthrough in the demonstration of the CSC hypothesis within solid tumours. Critical also was the fact that these cells had been *prospectively* identified – confirming the concept that it might be possible to predict with accuracy the identity of CSC, making them relevant clinical targets (Al-Hajj et al. 2003).

CD44 has since been widely reported as a phenotypic marker of putative CSC in both solid and haematopoietic malignancies. When assessed as a single marker or in combination with other cell surface antigens, $CD44^{+}$ populations have shown enhanced tumourigenicity in "gold standard" xenotransplantation assays in immunosuppressed hosts. Associations have also been demonstrated between CD44 expression and other methods of CSC isolation, such as proliferation as "tumourspheres" in serum-free culture or the presence of a Hoechst 33342 dye-effluxing "side population".

In cell lines representing malignancies such as lung, colorectal and breast cancer, cells with a $CD44^{+}$ CSC phenotype demonstrate enhanced resistance to cytotoxic chemotherapy. Consistent with this, Lee et al. (2011) used immunohistochemistry to examine clinical samples taken from breast cancer patients before and after administration of combination chemotherapy (doxorubicin docetaxel/cyclophosphamide). Not only was a higher proportion of pre-treatment $CD44^{+}CD24^{-}$ putative CSC associated with more aggressive disease, but these cells also appeared more resistant to chemotherapy, suggested by an increased proportion in post-treatment samples. This increase in $CD44^{+}CD24^{-}$ cells post-chemotherapy was associated with shorter disease-free survival times (Lee et al. 2011). Conversely, (and crucial to its potential clinical relevance), direct targetting of CD44-expressing putative CSC, using gene knockout strategies or CD44-specific monoclonal antibodies, reduces tumour formation in some murine models of cancer (Jin et al. 2006; Zeilstra et al. 2008).

Prospective Isolation – Why Should CD44 Identify Cancer Stem Cells?

One now widely accepted proviso to the CSC hypothesis is that tumourigenic cancer stem cells do not necessarily arise through transformation of a normal tissue stem cell, but rather through the acquisition, by *any* cell, of functional stem cell characteristics. Thus, the existence of normal $CD44^{+}$ stem cells within a tissue does not necessarily dictate that the CSC of tumours arising in that tissue will be $CD44^{+}$.

However, expression of CD44 is associated with many mechanisms involved in normal stem cell homeostasis and implicated in the persistence, proliferative capacity and apoptotic resistance shown by CSC, supporting the case for its role as

a marker. These include Wnt-β-catenin signalling, which regulates self-renewal, and the prosurvival and antiapoptotic Pi3K-AKT and NF-κB pathways (Wielenga et al. 1999; Misra et al. 2005; Zeilstra et al. 2008; Bourguignon et al. 2009). Also, normal stem cell populations express membrane pump transporter proteins such as MDR-1 (P-glycoprotein) and ABCG2 (BCRP), allowing them to efflux xenotoxic substances. The expression of these multidrug transporters in cancer results in resistance to chemotherapeutic agents, and this is proposed as another mechanism by which CSC may persist in tissues after cancer therapy. CD44-HA interaction is reported to upregulate and stabilise expression of the membrane pump transporter P-glycoprotein (Misra et al. 2005; Bourguignon et al. 2009).

In experimental models where tumour suppressor function is impaired, CD44 may promote a growth advantage through promotion of premalignant cell growth and survival – this could represent an early event in cancer and would be consistent with a role for CD44 in tumour-initiating CSC. The tumour suppressor p53 interacts with the CD44 promoter, inhibiting its expression – Godar et al. (2008) demonstrated using RNA interference techniques that loss of p53 function may produce growth-stimulatory and antiapoptotic effects, which can be abrogated by concurrent blockade of CD44. Similarly, in a tumour-prone murine model of colon cancer with a point mutation in the APC tumour suppressor, knockout of the CD44 gene results in increased apoptosis at the crypt base, and reduced formation of aberrant crypts (Zeilstra et al. 2008). This suggests that the expression of CD44 might enhance cellular survival where apoptosis would otherwise occur in response to altered growth signals.

CD44+ Cancer Stem Cells, Metastasis and the Epithelial Mesenchymal Transition

One aspect of tumour biology for which the CSC hypothesis, where applicable, may provide critical insights is that most lethal feature of cancer, the process of metastasis. According to the hypothesis, only CSC may propagate the tumour – so active metastases will form only where CSC successfully migrate to other sites, whereas if a non-CSC / daughter cell is disseminated from the primary tumour, this will not be capable of proliferation – thus, analysis of metastases might provide important information about the identity of CSC. As discussed, considerable evidence implicates CD44 in the secondary spread of many tumours, with disruption preventing metastasis formation in *in vivo* models (Seiter et al. 1993; Yu et al. 1997; Weber et al. 2002; Draffin et al. 2004).

Eight of the nine breast cancers investigated by Al-Hajj et al. (2003) had been derived from metastatic samples (pleural effusion), suggesting the $CD44^{+}CD24^{-/low}$ CSC were capable of successfully establishing distant metastasis. In another study, although the prevalence of the putative $CD44^{+}CD24^{-/low}$ tumour stem cell phenotype in primary breast tumours did not correlate with tumour progression or prognosis, a greater prevalence *was* associated with a tendency for distant metastasis upon recurrence (Abraham et al. 2005). The presence of disseminated tumour cells in breast cancer can be detected with immunohistochemistry for cytokeratins (CK). Balic et al. (2006) assessed CK + bone marrow samples from 50 early breast cancer patients and found that all specimens had detectable $CD44^{+}CD24^{-/low}$ cells, with prevalence (33–100%) much greater than that seen in primary tumour masses, again associating the $CD44^{+}$ putative breast CSC population with a tendency toward metastasis.

However, it is important to recognise that any role of CD44 as a facilitator of metastasis does not necessarily imply a role within a putative CSC fraction. For example, in murine models of sarcoma formation, where CD44 gene knockouts were superimposed upon mutations in the tumour suppressors APC or p53, tumours formed and survival times were equivalent but metastases did not form. Thus, although CD44 was involved in the metastatic process, it was not essential for tumour incidence or growth at the primary site. Thus, in this model, CD44 expression could not define any putative CSC fraction (Weber et al. 2002).

Recently, attention has fallen upon the process of "Epithelial-Mesenchymal Transition" (EMT), and also the mechanisms by which this might relate to CSC-associated cellular phenotypes and behaviour. EMT is a normal process during normal embryonic development, whereby epithelial cells adopt a motile phenotype, allowing migration in the extracellular environment. There appear to be multiple parallels between this conversion between cellular states as it occurs in embryogenesis, and that observed during the progression of carcinomas. It is proposed that EMT enables carcinoma cells to escape the confines of the normally structured epithelial environment, facilitating local invasion and also breach of the basement membrane, leading to intravasation and distant dissemination. Many signalling pathways dysregulated in cancer and which may be mediated by CD44, including Wnt-βcatenin, TGFβ and those initiated by receptor tyrosine kinases EGFR and FGFR, influence EMT (Thiery 2002).

Some recent evidence has suggested that an EMT-like process may generate CSC. Mani et al. (2008) demonstrated upregulation of mesenchymal markers in normal and neoplastic mammary cells with the putative CSC phenotype $CD44^{high}CD24^{low/-}$. Immortalised normal (HMLE) or transformed (HMLER) mammary epithelial cells were exposed to TGFβ, or the EMT-inducing transcription factors Snail or Twist overexpressed. This resulted in a predominantly $CD44^{high}CD24^{low/-}$ phenotype, the ability to form tumourspheres, and (for HMLER) increased tumourigenicity (Mani et al. 2008). Whilst suggesting a mechanism which may result in the generation of $CD44^{+}$ cells with enhanced metastatic capability in epithelial tumours, less clear is why or if EMT should confer the cardinal CSC properties of self renewal or multilineage potential *per se*. Further work may shed more light on a role for EMT in the origin of CSC.

CD44 and the Cancer Stem Cell Niche

The potent effects imparted by the environment or "niche" in which a cell exists cannot be disregarded when considering tumour evolution and progression. In normal tissues, the stem cell niche provides important extracellular controls and cues additional to the stem cells' own intrinsic program, helping to maintain progenitor function and/or quiescence and to direct tissue homeostasis. Similarly, tumour cells interact with surrounding stroma in a reciprocal manner, and can influence the stroma such that it is more conducive to tumour growth, and it is proposed that CSC reside within a "Cancer Stem Cell Niche", which maintains their function and identity. Disruption of tumour cell niches may represent a valuable means of therapeutic intervention.

CD44 is involved the homing of normal haematopoietic progenitors to the bone marrow niche through interactions with HA and also other ligands expressed on BM endothelium such as E-selectin (Dimitroff et al. 2001). It appears to play an analogous role in the arrest of tumour cells on bone marrow endothelium (Draffin et al. 2004), and may also promote preparation of a "premetastatic niche" in other metastatic predilection sites such as lymph node and lung (Jung et al. 2009). As a component of the peritumour stroma, the CD44 ligand HA may itself play a role in tumour progression, and represents another potential therapeutic target. Like direct blockade of CD44, disruption of HA may inhibit retention of disseminated tumour cells at metastatic sites such as bone marrow (Draffin et al. 2004).

Yu et al. (1997) disrupted CD44 function in a highly metastatic murine carcinoma cell line, and showed that whilst cells could adhere to and penetrate the lung endothelium and enter the interstitium, they then underwent apoptosis. This would be consistent with the idea of a distinction between cancer cells which disseminate but fail to develop further, and a CSC population which may give rise to active metastases through their ability to prepare an appropriate niche. Similarly, in a murine model of AML, ligation of CD44 with monoclonal antibodies prevented the engraftment of transplanted leukaemic stem cells. Cells were unable to home effectively to their microenvironmental niches, and their self-renewal and repopulation abilities were impaired, leading to their eradication (Jin et al. 2006).

The involvement of CD44 in establishing putative CSC niches thus represents an important avenue for further investigation.

Clonal Evolution and *De Novo* Generation of CD44+ Cancer Stem Cells

One key implication of the CSC hypothesis for cancer therapy is the concept that the behaviour of cancer cells will be predictable, based on their status as CSC (tumourigenic) or non-CSC (non-tumourigenic). This implies that therapy could effectively "ignore" certain cells within a tumour – as long as treatment eliminates the tumour-propagating CSC population, the rest of the cells should be unable to sustain the malignancy and the tumour will regress.

However, increasing evidence indicates that the reality is more complex than was at first promised by the CSC model. Although it is the basis for much of CSC theory, the observation that some cancer cells are more tumourigenic than others is also consistent with clonal evolution (Nowell 1976). In this model, prevailing pressures (which may include the host immune response, interactions with the surrounding microenvironment, or anticancer therapy) may give rise to phenotypically and functionally distinct cancer cell subpopulations, but this is not a predictable developmental hierarchy like that proposed by the CSC hypothesis. This complicates any notion of targetted therapy based on a predictable CSC phenotype, as all of these populations may be capable of sustaining and propagating the tumour.

Data from a detailed genetic profiling study by Shipitsin et al. (2007) demonstrated that, whilst $CD44^{+}CD24^{low/-}$ breast cancer cells did show more "stemness"-associated characteristics and correlated with indicators of prognosis, $CD24^{+}$ cells were not only prevalent in distant metastases in drug-refractory patients but also showed a distinct genetic signature. This suggested that they represented a divergent subpopulation, rather than simply the terminally differentiated progeny of the $CD44^{+}$ cells (Shipitsin et al. 2007). Indeed,

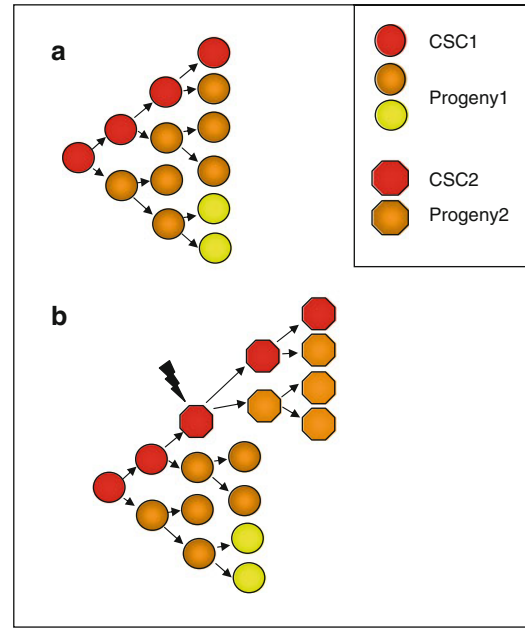

Fig. 23.2 Clonal evolution of $CD44^{+}$ CSC will complicate their identification and targetting. (**a**) Cancer stem cell hierarchy – CSC maintain the tumour, through self-renewal and differentiation into all relevant cellular lineages. They have a predictable phenotype (e.g. $CD44^{+}$). Progeny have more restricted potential, are incapable of extensive proliferation and are non-tumourigenic. (**b**) Clonal evolution of CSC – If CSC are subject to clonal evolution, additional diverse CSC populations may develop during tumour progression. If this is associated with variation in surface phenotype, targetting CSC on this basis will not be a reliable approach

evidence from both haematological and solid tumours has indicated that CSC themselves may undergo clonal evolution (Barabe et al. 2007; Hermann et al. 2007). If both CSC and clonal evolution processes may occur within a single tumour, it will be difficult to prospectively identify or target a putative CSC fraction on the basis of surface phenotype and be confident that only the cells with a given expression profile are capable of tumour maintenance (Fig. 23.2a, b).

There is also evidence that expression of a $CD44^{+}$ CSC phenotype within cell populations may be unstable – that is, that $CD44^{-}$ cells may acquire the $CD44^{+}$ phenotype. A central tenet of the model is a hierarchy in which CSC may give rise to non-CSC, and not vice versa, with no transition of tumour cells from non-CSC to CSC

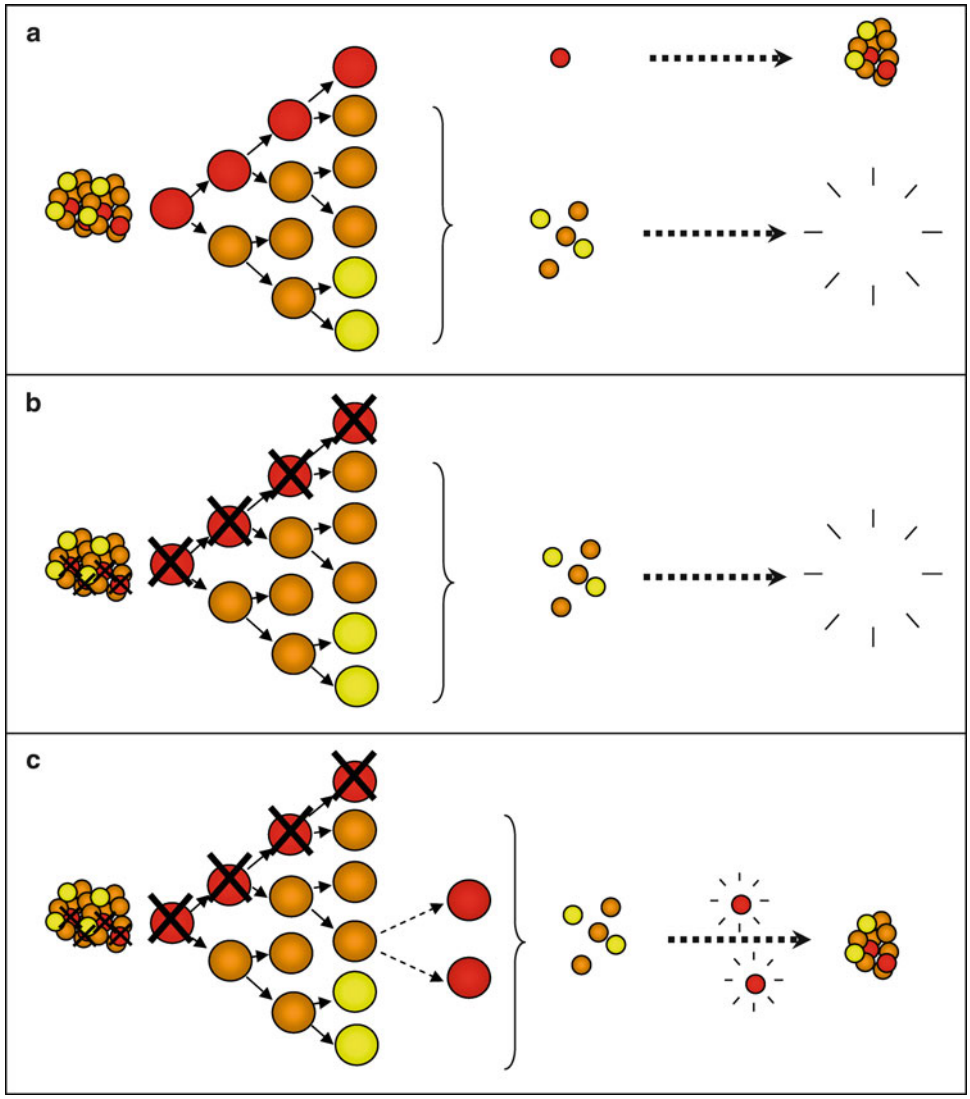

Fig. 23.3 Therapeutic targetting of CSC may not be valid in the face of an unstable differentiation hierarchy. (**a**) CSC model with strict differentiation hierarchy. Only CSC have the potential to sustain extensive proliferation and maintain the tumour, to form metastases, or to form new tumours in xenotransplantation experiments. (**b**) CSC targetting. If CSC are selectively eliminated, residual non-CSC are incapable of repopulating the tumour, which will regress. (**c**) Absence of a strict developmental hierarchy – "CSC" may arise within "non-CSC" populations, such that these are not biologically distinct with regards tumourigenic potential. Where "non-CSC" have the *potential* to maintain or form new tumours, applicability of the CSC hypothesis (and associated nomenclature) is questionable

"compartments". It now appears that, for at least some $CD44^+$-defined putative CSC subsets, this is not the case, and that cells expressing the putative stem cell phenotype may arise from the non-stem fraction, both *in vitro* and *in vivo* (Chaffer et al. 2011; Gupta et al. 2011). This has important implications for the clinical relevance of the CSC hypothesis, for if $CD44^+$ CSC may arise *de novo* from $CD44^-$ non-CSC, therapy will still have to target all cells in the tumour (Fig. 23.3a–c).

These reports investigate a particular putative CSC phenotype ($CD44^+CD24^{low/-}ESA^+$) in cultured cells, whose behaviour may be different from that of cancer cells *in situ*. For example, levels of CD44

surface expression in some culture systems fluctuate according to transit of cells through the cell cycle, and this is associated with altered biological behaviour. $CD44^{high}$ cells in several canine cancer cell lines comprise a greater proportion of dividing (G2/M-phase) cells than do $CD44^{low}$ cells, leading to the appearance of CSC-associated traits (including greater proliferative capacity and formation of tumourspheres) in assays. However, as both populations can recapitulate the expression pattern of the parental cell line, in this context the marker does not distinguish "CSC" from "non-CSC" (Blacking et al. 2011).

In the human gastric adenocarcinoma cell line MNK45, upregulation of v4, v5, and v7 splice variants in G2/M phase is associated with increased adhesion to endothelial cells (Oertl et al. 2005). Harper et al. (2010) examined the cell cycle profiles of both primary and cultured human head and neck carcinoma cells as well as normal oral mucosa, and found that $CD44^{high}$ cells showed increased clonogenicity and a lower rate of apoptosis, associated with a greater proportion of cells in G2. By contrast, in the seminal breast CSC study by Al-Hajj et al. (2003) (which examined cells directly *ex vivo*) cell cycle profiles of tumourigenic and nontumourigenic cells were similar, and so would not account for the differences between the biological behaviour of $CD44^{high}$ and $CD44^{-/low}$ cells. Thus, given that the implications of CD44 expression appear to vary considerably between systems, these should be interrogated carefully in every instance.

Future Directions for Evaluating CD44 as a Cancer Stem Cell Marker

There is now considerable evidence to support the existence of cellular hierarchies within multiple tumour types, particularly haematopoietic malignancies. However, one of the most enticing prospects raised by the CSC hypothesis was the possibility that there might be a consistent, universal marker enabling identification of CSC in diverse tissues. No such candidate marker has emerged, and it is this author's opinion that, particularly in light of the possibility of *de novo* generation or clonal evolution of CSC subsets, it may never be possible to definitively predict which cells represent a CSC fraction (such that all other cells may be considered non-tumourigenic) without examining tumours on a case-by-case basis. Where the conditions of the CSC model are not demonstrated to apply, it is inappropriate to use associated nomenclature on the basis of surface phenotype alone. The CSC hypothesis has, however, focussed attention on the existence of and interplay between heterogeneous cell types within tumours, and the possible significance of specific phenotypes. Thus, careful further investigation of the role of CD44 expression by putative CSC fractions is certainly warranted.

It is surprising, given the breadth of data about CD44's potential role in cancer progression and the numerous studies implicating it as a putative CSC marker, that there exists much less experimental data examining what actual functional role it may have in the context of the CSC. For example, where unfractionated breast tumour cells are predominantly $CD44^{+}$, but only $CD44^{+}CD24^{low/-}$ (and not $CD44^{+}CD24^{+}$) cells show enhanced tumourigenicity (Al-Hajj et al. 2003), this might suggest that (in this context at least) expression of CD44 itself does not convey a specific functional advantage. It is functional capacity (self renewal, ability to give rise to all relevant lineages), not phenotype, which defines the putative CSC. Thus, further investigation of whether CD44 is contributing to these stem cell characteristics, or merely identifying their likely presence, will be valuable. This may require investigation on a tumour type-specific (if not case-specific) basis, as the phenotypic profile of CSC is variable and frequently unpredictable. For instance, in some colon cancers expression of CD24 (which is associated in its own right with tumour progression, invasive and metastatic properties) is reported to correlate more closely with clonogenicity than CD44, and the tumour-initiating fraction of pancreatic adenocarcinoma has been reported as $CD44^{+}CD24^{+}$ (Li et al. 2007; Vermeulen et al. 2008).

The frequencies of *de facto* tumour-initiating cells within $CD44^{+}$ putative CSC fractions are often low (requiring inoculums of hundreds or thousands of cells), even in highly manipulated

tumourigenic cell lines (Al-Hajj et al. 2003; Chaffer et al. 2011). By contrast, in some models, transplantation of as few as one cancer cell can recapitulate the parental tumour (Quintana et al. 2008). There are numerous caveats to interpreting and comparing the results of tumourigenicity assays in the literature, as they may be influenced by so many factors. Significantly, the inability of a cell to survive or proliferate when inoculated as a xenograft (particularly where this is not orthotopic, i.e., at the natural tumour site) may not accurately reflect its potential in the original tumour, where it is surrounded by a network of supportive cells, and subject to local cell-cell interactions. However, given that the clinical relevance of CSC research requires that one can identify as precisely as possible the tumourigenic cell fraction it will be of great value to seek additional discriminatory features, be they phenotypic or functional.

For example, the use of CD44/CD24 profile to define "stem-like", "luminal" or "basal" cellular compartments in human breast originated and is employed mostly within the CSC field. Many studies of normal breast use different means to isolate stem and progenitor cells, including alternative surface markers or functional assays such as expression of Aldehyde Dehydrogenase (ALDH). A greater understanding of cellular hierarchies within normal tissues and further fractionation of neoplastic $CD44^+$ populations using these additional markers could facilitate more precise identification of tumourigenic subsets.

A significant factor when considering the use of CD44 as a CSC marker, and thus for potential therapeutic targetting, is its ubiquitous expression in normal tissues. Studies of murine models suggest that widespread inactivation of CD44 produces less disruption to normal tissues than might be expected, given its role in critical processes such as cell adhesion and leukocyte trafficking (Protin et al. 1999; Jin et al. 2006; Zeilstra et al. 2008). Nonetheless, it is unlikely that this approach would be feasible in a clinical setting. Bispecific antibodies, which bind to a cell on the basis that it expresses two different antigens, might facilitate more precise targetting of candidate cells (Zoller 2011). Such approaches will still require identification of a second, more tumour-specific antigen than CD44 to define the target cells.

The role of specific CD44 variants remains largely unexplored in the context of the CSC hypothesis – for example, antibodies used in flow cytometric studies most commonly recognise the standard form of the molecule, or identify both standard and variant forms without discrimination. As discussed, there is abundant evidence that CD44 variants play a role in the pathogenesis of different tumour types, and that blockade of these may impede or even prevent tumour progression.

Defining more precisely the spectrum (i.e., CD44s and/or the multitude of CD44v isoforms) of expression in normal and neoplastic tissue may provide valuable information to help identify and characterise putative CSC. For example, a change in the proportions of CD44 isoforms, and particularly increased expression of variant exons v4 and v6, is an early event in colorectal neoplasia (Wielenga et al. 1999). Recently, Olsson et al. (2011) published analysis of CD44 variant expression in 187 human breast tumours and 13 breast cancer cell lines. They demonstrated that standard and variant forms were expressed heterogeneously within tumours, with particular subtypes dominated by one isoform. Moreover, expression patterns were altered when cells were propagated as tumourspheres, although whether this represented enrichment of a specific subpopulation or altered expression patterns was not clear. Although CD44s was the predominant isoform in Basal B type tumours, it was not detected in tumours with the $CD44^+CD24^-$ phenotype, with which the CD44v3-v10 isoform showed the strongest correlation. Interestingly, there was also no correlation between the $CD44^+CD24^-$ phenotype and expression of the functional CSC marker ALDH1, although this latter was associated with strong CD44s expression (Olsson et al. 2011).

These complex altered splice variant patterns will need to be further dissected if the role of CD44 in any putative CSC population is to be adequately determined. Moreover, whilst particular variants may be more frequently associated with individual tumour types or indices of progression such as metastasis, alternative splicing

patterns of CD44 may be further modulated by DNA damage (Filippov et al. 2007). This is likely further to complicate attempts to fully understand the role of CD44 as a marker of CSC, particularly as it implies that expression patterns may change during the course of cancer therapy. If specific CSC are shown to express specific variant isotypes of CD44, blockade of these could provide a vital means of targetting CSC whilst limiting adverse effects on normal $CD44^+$ populations.

In conclusion, although CD44 remains the focus of considerable interest within the CSC field, there remain (as for the hypothesis as a whole) many unanswered questions regarding its role in the identification of these elusive cells. Its expression appears to identify putative CSC in numerous tumours and cancer cell lines, but the potential for clonal evolution of $CD44^-$ CSC populations and for $CD44^+$ cells to arise in $CD44^-$ populations may make it an unpredictable marker. Moreover, its ubiquitous nature and function in normal tissues is likely to complicate notions of therapeutic blockade. A fuller understanding of the molecule's function in the context of CSC, how it interacts with the putative CSC niche, and the roles of CD44v isoforms, may prove vital to our understanding of its relevance and potential role as a therapeutic target.

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NK-92 Cytotoxicity Against Cancer Stem Cells in Hematologic Malignancies

24

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Contents

Abstract

The discovery that many cancers are driven by a rare population of cancer stem cells has raised new questions as to how the immune system recognizes these cells. There is evidence to support an immunophenotypically defined stem cell in acute myeloid leukemia and multiple myeloma, two common hematologic malignancies. While chemotherapy can cure a minority of AML patients, multiple myeloma is generally incurable with this approach. However, allogeneic stem cell transplantation is the most effective therapy for AML implying an important role for the graft-versus-leukemia effect while multiple myeloma is typically treated with autologous bone marrow transplantation. One novel emerging therapeutic approach is the use of immune effector cell lines that have a broad tumour killing capacity such as NK-92. Here we review the role of NK-92 in the recognition and killing of cancer stem cells in leukemia and multiple myeloma and outline the application of clonogenic cytotoxicity assays to study these immunologic interactions.

Introduction

The field of cancer immunology has focused on the interaction of the immune system with bulk tumour cells. The discovery of cancer stem cells has raised new questions as to how the immune system recognizes these cells, and has significant

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therapeutic implications. In the case of acute myeloid leukemia and multiple myeloma, two common hematologic malignancies, the stem cell compartment comprises a rare subpopulation (Lapidot et al. 1994; Bonnet and Dick 1997; Matsui et al. 2008). While chemotherapy can cure a minority of AML patients, multiple myeloma is generally incurable with this approach.

AML accounts for approximately 90% of all acute leukemias in adults and has an incidence of 2.4/100 000/year (Lowenberg et al. 1999). More than 13,000 new cases of AML are diagnosed annually in the US and approximately 9,000 deaths will be due to this disease (Ries et al. 2008). The mainstay of therapy for AML is based on a core regimen of daunorubicin and cytarabine and although 70–85% of AML patients treated with current chemotherapy protocols are able to achieve a morphologic remission, (Hurwitz et al. 1995; Ribeiro et al. 2005) many relapse because of recurrence from minimal residual disease (MRD) leading to a 5 year survival of approximately 40% (Lowenberg et al. 1999). Allogeneic bone marrow transplantation has improved outcomes for high risk patients in part from a graft-versus-leukemia effect (GVL). However, only 60% of patients will have a matched sibling or unrelated donor, leaving a large proportion of patients without a transplant option (Ruggeri et al. 2002). Although GVL was initially thought to be solely mediated by T cells, there is evidence supporting a role of NK cells in mediating GVL (Ruggeri et al. 2002). However, allogeneic stem cell transplantation is the most effective therapy for AML, implying an important role for GVL in curing patients and by inference in eliminating leukemic stem cells.

Multiple myeloma accounts for 1% of malignancies and 13% of hematologic cancers, with a median age at diagnosis of 70 years (Palumb and Anderson 2011). Combination therapies including immunomodulatory agents (e.g. thalidomide and lenalidomide), or proteosome inhibitors (e.g. bortezomib) with steroids (e.g. dexamethasone) are replacing traditional MM chemotherapy drugs in induction regimens prior to autologous stem cell transplantation (Palumbo and Rajkumar 2009). Thalidomide is currently used for maintenance therapy, with lenalidomide under investigation. In patients ineligible for transplant, combinations of alkylating agents (e.g. melphalan) and steroids (e.g. prednisone), with either thalidomide or bortezomib are the standard of care (Palumbo and Rajkumar 2009). Despite this treatment strategy, MM remains largely incurable with a median survival around 3–6 years.

Allogeneic transplantation in MM may lead to longer remission, lower relapse rates and long-term survival in subsets of patients (Bruno et al. 2009). This suggests a possible graft-versus-myeloma (GVM) effect with the potential to eradicate MM stem cells in some patients in whom conventional chemotherapy treatment alone would lead to eventual disease relapse. Reduced-intensity conditioning with allogeneic transplantation reduces treatment-related mortality and an increased overall survival in patients receiving HLA matched sibling allogeneic transplantation after an initial autologous transplant compared to patients receiving single or tandem autologous transplants (Bruno et al. 2009). Nevertheless, the reason for the increased toxicity of allogeneic bone marrow transplantation in MM patients, due in part to graft versus host disease (GVHD), needs to be better understood. Additional studies should aim to enhance the GVM effect important for increasing survival after allogeneic transplant, while minimizing GVHD (Bruno et al. 2009).

The GVL and GVM are mediated by lymphocytes (T and NK cells) that recognize host and tumour-specific antigens. A particularly novel emerging therapeutic approach is the use of immune effector cell lines with broad tumour killing capacity, such as the NK cell line, NK-92. Here, we review the role of NK-92 in the recognition and killing of cancer stem cells in leukemia and multiple myeloma and focus on the use of clonogenic cytotoxicity assays to study immune effector interactions with cancer stem cells.

Role of Natural Killer Cells in the Immune System

The cellular immune system comprises lymphocytes and myeloid cells which work in coordination primarily to fight microbial infection.

The lymphocyte subset includes T and NK cells which play important roles in tumour immune surveillance (Swann and Smyth 2007). T and NK cells both contain cytotoxic granules containing perforin and granzymes that can be exocytosed onto target cells leading to apoptosis. In addition, they can kill via death ligands on their surface such as Fas Ligand or TRAIL. Cytotoxic T-cells recognize foreign peptides in the context of class I HLA allowing for the immune system to probe internal proteins produced by cells while NK cells play a complementary role.

NK cells are able to recognize and destroy cells that have downregulated HLA class I molecules on their surface. This enables NK cells to destroy virally-infected cells or tumor cells unrecognized by T cells which rely on antigen presentation in the context of HLA class I. NK cells express killer immunoglobulin like receptors (KIRs) that, when engaged by self HLA class I molecules, inhibit NK cells from killing. Therefore, when cells downregulate HLA class I, they are susceptible to NK cell recognition and cytolysis as described by the "missing self" hypothesis (Karre et al. 1986). Further, work by Ruggeri et al. (2002) in AML patients treated with haplotype bone marrow transplants demonstrates that mismatch of the recipient's HLA-type with the donor's NK cell KIR receptors leads to improved survival. NK cells also have a number of activating receptors that recognize ligands on target cells, such as NKG2D, implicated in recognition of leukemic blasts (Diermayr et al. 2008). A balance of inhibitory and activating signals determines whether an NK cell kills its target (Sutlu and Alici 2009).

Cell Line Therapy for Cancer

The ability to create cell lines from primary tumour samples has been critical to facilitate experimental work leading to our current understanding of tumour biology and also in the development of effective cancer therapies. Malignant transformation can occur in the cells of the immune system such as T or NK cells, with occasional retention of the ability to recognize and lyse other tumour cells. Application of these cells to treat cancer is like fighting 'fire with fire' and can be accomplished by utilizing Good Manufacturing Practice (GMP) facilities to scale up large therapeutic doses of cells in a standardized manner.

The first attempt to utilize cell lines as therapeutic agents was with cell line, TALL-104 derived from a patient with T-cell acute lymphoblastic leukemia which had the ability to kill a wide range of tumour targets in an MHC unrestricted manner (Cesano and Santoli 1992). It was tested in a phase I study to treat breast cancer and most toxicities were limited to grade I/II with one patient experiencing grade IV liver toxicity (Visonneau et al. 2000). NK-92, a cell line derived from a patient with a NK-cell non-Hodgkin lymphoma (Gong et al. 1994), has undergone extensive preclinical development (Tonn et al. 2001). NK-92 recently underwent a Phase I trial of patients with metastatic renal cancers and melanoma with no major toxicities reported (Arai et al. 2008). The use of such cell lines for cancer treatment is a novel approach, and has several advantages over other forms of adoptive immunotherapy that use endogenously-derived autologous or allogeneic NK cells, including standardization of therapy and cell manufacture as well as unlimited potential for *ex vivo* cell expansion. We are currently evaluating NK-92 in a Phase I clinical trial for advanced hematological malignancies at the Princess Margaret Hospital and have not observed any significant adverse reactions to date.

Immune Therapy for Cancer Stem Cells

The best evidence that cellular immune therapies can eliminate cancer stem cells is cure of leukemia after allogeneic transplantation. While the benefit of allogeneic hematopoietic stem cell transplantation is greatest for chronic myeloid leukemia (Arora et al. 2009), there is still benefit in acute myeloid leukemia (Litzow et al. 2010) and acute lymphoblastic leukemia (Burke et al. 2009). However, a traditional understanding of the graft-versus-leukemia (GVL) effect has centered

around the role of T-cells recognizing tumour cells via major and minor histocompatibility elements (Pierce et al. 2001). In addition to GVL, is the risk of GVHD that increases with the number of HLA loci mismatches. However, the observation that some patients benefit from fully matched donors without any GVHD, suggests a tumour specific element to the GVL or a greater susceptibility to killing of malignant tissue compared with normal tissue. Cure of patients with allogeneic transplantation relies on engraftment of donor hematopoietic stem cells, the establishment of chimerism and reconstitution of a functional immune system.

NK cells were implicated in GVL when AML patients treated haplotype transplants (half-matched) with KIR ligand mismatches had better survival outcomes than those without mismatches (Ruggeri et al. 2002). There is, however little direct evidence to support the notion that the cure of hematologic malignancy by bone marrow transplantation is the result of elimination of cancer stem cells. Many clinical study outcomes measure morphologic parameters of bone marrow, and even when minimal residual disease is assessed, it is rarely done with specificity for cancer stem cells. Furthermore, there is a paucity of *in vitro* and *in vivo* studies that directly study the interaction of immune effectors with cancer stem cells (Costello et al. 2000; Langenkamp et al. 2009; Williams et al. 2010; Swift et al. 2012), leaving much to be understood about the cellular and molecular immunology behind the recognition of cancer stem cells. This is, in part, related to difficulties in defining and studying cancer stem cells and in studying the interactions of immune cells with rare cell subpopulations.

Clonogenic Assays – Historical Development

The clonogenic assay was developed by Puck et al. (1956) initially as a system to grow HeLa cells and then to grow epithelial cells from normal human tissue (liver, conjunctiva, kidney and appendix) (Cieciura et al. 1956). This involved the use of semi-solid medium supplemented with growth factors and nutrients to grow colonies derived from single cells. This predated the discovery of hematopoietic stem cells by McCulloch and Till (1960) using the *in vivo* spleen colony forming assay. An effective means to generate murine bone marrow derived colonies was done in 1966 using agar plates with mouse kidney or embryonic feeder layers (Bradley and Metcalf 1966). The first attempt to grow primary tumours in semi-solid medium was with murine myeloma colonies were grown by McCulloch's research group (Park et al. 1971). Subsequently, they applied this approach to study sensitivity of murine and human hematopoietic stem cells to chemotherapy drugs (Ogawa et al. 1973). Further applications of an agar based colony forming assay for primary acute myeloid leukemia samples was demonstrated to predict clinical response and resistance (Park et al. 1980).

Today, the clonogenic assay involves growing the cells either in methylcellulose or agar with various growth factors and cytokines. Proliferation of a subset of cells results in the appearance of colonies that can be enumerated typically at 10–14 days.

The value of clonogenic assays is that it allows assessment of single cells with high proliferative capacity. Tumour stem and progenitor cells have the ability to form colonies in a semisolid state matrix consisted of either agar or methylcellulose providing a readout for testing cytotoxic agents. However, limitations of clonogenic assays include low frequency of clonogenic cells, two log range for cytotoxicity evaluation and clump artifacts for some types of cells (Hoffman 1991). Clonogenic chemotherapy assays are good at predicting patients likely to be resistant to a particular chemotherapy agent, but not as accurate at determining those who will be sensitive. Clonogenic assays have the potential of individualizing patient treatment as they can predict clinical responses, but have not been incorporated into standard of care for any given cancer, possibly because of the inability to grow a high proportion of primary tumours using this approach.

Application of the Clonogenic Assay to Study Cytotoxicity of Cellular Agents

Few studies have examined the *in vitro* sensitivity of cancer stem cells to immune effector killing. In one study, lymphokine activated killer (LAK) cells and allogeneic lymphocytes were shown to exert a modest cytotoxic effect on AML cancer stem cells (CD34+ CD38−) that were intrinsically resistant to the chemotherapeutic agent, daunorubicin (Costello et al. 2000). This was done by sorting the cells and performing a chromium release assay. A limitation of that study was that the LAK cells were a mixed population of IL-2 activated T and NK cells, making it difficult to discern the contribution of either cell type.

Another study using the clonogenic assay to examine the role of KIR mismatched NK cells against primary AML showed a reduction in colonies (Langenkamp et al. 2009). This study also utilized the chromium release assay and reported secondary replating of clonogenic cells. Similarly, autologous activated marrow-infiltrating T-lymphocytes (MILs) have demonstrated superior *in vitro* cytotoxicity against clonogenic MM cells compared to activated peripheral blood lymphocytes (PBLs) isolated from the same patient. Normal hematopoietic progenitors were not affected by MILs or PBLs, providing pre-clinical evidence for the safety of this therapy (Noonan et al. 2005). However, a direct comparison between bulk tumor and clonogenic cell killing was not considered in this work. Further, neither study utilized appropriate controls to differentiate killing during the 4 h co-incubation versus over the 2 week low density incubation in methylcellulose, making a comparison of methods difficult to interpret.

We therefore developed an assay to control for the effect of NK-92 on targets cells during low density interactions over the 2 week methylcellulose incubation following the initial high density interaction for 4 h in a 96 well plate. This allowed for a better comparison to the standard 4 h chromium release assay in an attempt to compare the killing of bulk versus cancer stem cells by immune effectors (Fig. 24.1) and also to quantify the killing that occurred during both conditions of co-incubation (Fig. 24.2).

The clonogenic assay provides an additional useful functional readout independent of the variability of the immunophenotype of cancer stem cells. It is noteworthy that patients whose primary MM samples form colonies *in vitro,* have poorer survival outcomes than those who do not (Takahashi et al. 1985). Moreover, the sensitivity of clonogenic leukemic cells to chemotherapy predicts the clinical response in AML (Park et al. 1980). These data suggest that clonogenicity is a clinically relevant parameter. While clonogenic cells are not cancer stem cell *per se*, they include a subset of malignant progenitors and stem cells with significant proliferative capacity and the ability to contribute to disease progression. Inhibition of colony formation therefore, is an appropriate surrogate measure of cancer stem cell toxicity or cytostasis.

NK-92 as a Cancer Stem Cell Targeting Agent – Leukemia and Multiple Myeloma

We tested NK-92 against cell line KG1, an acute myeloid leukemia cell line, utilizing chromium release (CRA), flow cytometric (FCCA) and methycellulose (MCA) and liquid culture (LCA) cytotoxicity assays (Williams et al. 2010), all standardized to the same 4 h co-incubation in a 96 well plate. While additional killing was detected with an Annexin V based FCCA, it still did not predict the results of killing in the clonogenic assay at a 10:1 effector:target ratio. In fact, at this ratio, percent lysis in the CRA was 78% while all colonies were ablated in the MCA, leading to a cytotoxicity readout of 100%. We interpreted this to indicate preferential killing of leukemic stem cells in the cell line KG1 relative to bulk cells. Given that KG1 has stem cell capacity outside the classically defined CD34+CD38− population, this was a useful technique to measure cytotoxicity against leukemic stem cells. Further, our results

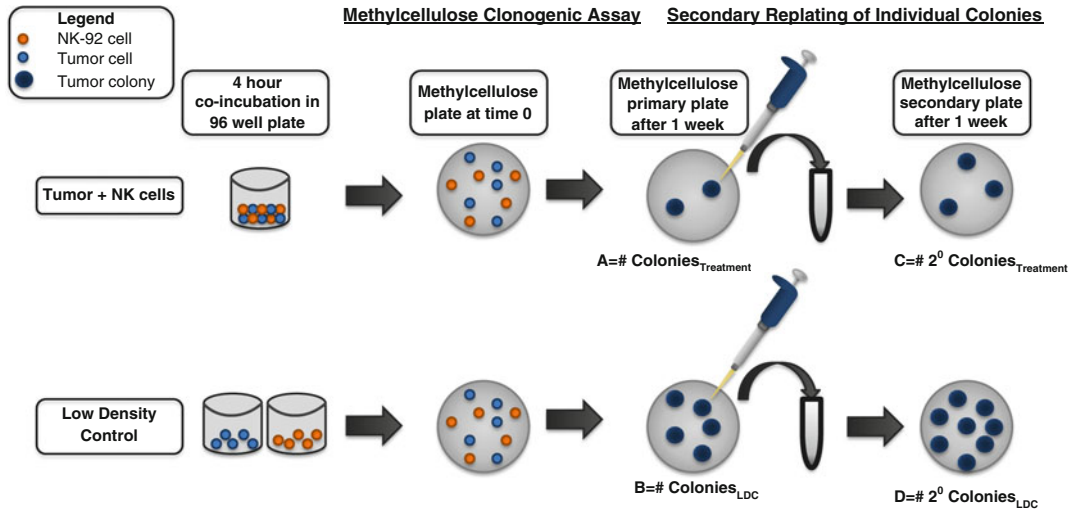

Fig. 24.1 Methylcellulose cytotoxicity assay (primary and secondary). Target cells are added to individual wells of a 96 well-U bottom plate. NK-92 are added to treatment wells at varying effector:target ratios. Low density control samples are prepared by separate incubation of target and effector cells in the 96 well plate followed by co-injection into methylcellulose to evaluate the impact of NK-92 cells on target cell capacity to form colonies after 1–2 weeks. The plates are centrifuged at 500 rpm for 5 min and then incubated for 4 h at 37°C and 5% CO_2. Subsequently, the cell suspension is injected into 3.8 ml of methylcellulose, vortexed and plated in triplicate in 35 mm Petri dishes and incubated at 37°C, 5% CO_2 and 100% humidity. After 1–2 weeks colonies are enumerated on each plate. Target and effector cells are injected separately into methylcellulose to identify the baseline number of colonies and to demonstrate that NK-92 cells cannot form colonies. Primary colonies from the low density control and treatment groups are replated in a secondary methylcellulose assay with enumeration of secondary colonies after 1–2 weeks. The concentration of cells was adjusted to achieve the same plating density as the primary culture

with the clonogenic assay were confirmed by a liquid culture proliferation cytotoxicity assay in which no residual proliferative capacity remained relative to controls at a 10:1 effector:target ratio.

Similarly, we demonstrated that clonogenic MM cells in the RPMI 8226 and NCI-H929 cell lines were also preferentially killed by NK-92 relative to bulk cells by comparing CRA, FCCA and MCA data. Bulk RPMI 8226 and NCI-H929 cell death was 25 and 30%, respectively in the chromium release assay at a 10:1 effector to target ratio and increased to 50% in the more sensitive FCCA. Notably, the clonogenic growth of RPMI 8226 and NCI-H929 was inhibited by 70 and 85%, respectively at the same effector to target ratio (Swift et al. 2012).

Further, secondary replating of residual colonies enables a more precise calculation of cytotoxicity against clonogenic MM cells to be obtained, as self-renewal of residual colonies are accounted for after NK cell treatment. Secondary replating can be performed by two methods: individual colonies can be plucked and replated (Fig. 24.1) or colonies (and intervening cells) from the entire plate can be pooled and replated at the initial seeding density. The first method addresses the residual colony-forming capacity of individual colonies, while the second pools all cells on the plate (including non-colony forming cells) which has the potential to detect quiescent stem cells that may remain between the colonies. Both methods compare the clonogenicity of residual cells after NK cell treatment to the clonogenicity of colonies in the low density control which involves incubating NK cells and targets in separate wells for 4 h and then co-introducing them into methylcellulose.

Replating pooled residual RPMI 8226 and NCI-H929 colonies after NK-92 treatment formed significantly fewer colonies than did the low density control. Similarly, replating ten individual residual colonies after NK-92 treatment

$$\%1^0\ Clonogenic\ Inhibition = \left[\frac{B-A}{B}\right] \times 100\%$$

$$\%\ Residual\ Primary\ Colony\ Growth = 100 - \%1^0\ Clonogenic\ Inhibition$$

$$\%\ Additional\ 2^0\ Clonogenic\ Inhibition = [\%\ Residual\ Primary\ Colony\ Growth] \times \left[\frac{D-C}{D}\right]$$

$$\%\ Cumulative\ Clonogenic\ Inhibition = \left[\%1^0\ Clonogenic\ Inhibition\right] + \left[\%\ Additional\ 2^0\ Clonogenic\ Inhibition\right]$$

Fig. 24.2 Methylcellulose cytotoxicity assay calculations. Cytotoxicity calculations are determined using terms A, B, C, and D described in Fig. 24.1

produced fewer secondary colonies than replating ten colonies from the low density control. This suggests that NK-92 treatment reduced the clonogenic potential of residual MM cells. Furthermore, pooled and individual replating methods produced similar results for residual RPMI 8226 and NCI-H929 colonies after NK cell treatment compared to the low density control, suggesting that cells interspersed between colonies do not have clonogenic potential (Swift et al. 2012).

Secondary replating by either method facilitates the calculation of cumulative clonogenic inhibition and for NK-92 against RPMI 8226 and NCI-H929 detected an additional 3–6% increase in the cytotoxicity when factoring in self-renewal of secondary colonies (Swift et al. 2012). Therefore, the approach of secondary replating provides a more accurate enumeration of toxicity against cancer stem cell if colonies remain in the primary assay.

In conclusion, NK-92 is one of two clinically tested cell lines with potential to treat hematological malignancies. The ability to effectively kill clonogenic cells, a surrogate measure of cancer stem cell killing, indicates their therapeutic potential. Our work suggests that NK-92 can preferentially target malignant stem cells versus bulk tumour by as yet to be determined mechanisms. We expect that identifying mechanisms that mediate the interaction of cellular immune effectors and the cancer stem cell will lead to more effective cancer immunotherapies.

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Cancer Stem Cells in Head and Neck Squamous Cell Carcinoma

25

Mark E. Prince and Steven B. Chinn

Contents

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide. Despite advances in diagnostic and treatment modalities, overall survival has not significantly changed over the last 30-years. Better understanding of the molecular pathophysiology of HNSCC may allow the introduction of novel diagnostic and therapeutic modalities that could impact survival. At the cellular level, cancer stem cells represent the mechanism for cancer tumorigenesis, metastasis and treatment failure. Head and neck cancer stem cells are a subpopulation of cancer cells with the unique ability for tumorigenesis, self renewal and the ability to recapitulate the heterogeneity of head and neck squamous cell carcinoma. Head and neck cancer stem cells have been shown to have a higher capacity for tumorigenesis and have greater metastatic potential in-vitro and in-vivo. In addition their slow growth and ability to survive adverse conditions makes them a great candidate as mediators of treatment resistance and cancer recurrence. Better understanding of the molecular mechanisms of head and neck cancer stem cells may allow more targeted therapies and improved survival.

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Introduction

Cancer remains the second leading cause of death in America. Worldwide, head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer, affecting over 400,000 patients, and leading to over 200,000 deaths. In the United States, 50,000 Americans are affected leading to over 11,000 deaths annually (Jemal et al. 2011). Head and neck squamous cell carcinoma arises from the upper aerodigestive epithelium of the oral cavity, oropharynx, nasopharynx, larynx and upper esophagus. Metastases typically present regionally in the lymph nodes of the neck and distantly in the lungs. Treatment typically involves a combination of surgical resection and/or radiation at the primary site and regional cervical lymph drainage sites. Chemotherapy is reserved as a synergistic adjunct for radiation therapy, for systemic therapy, and for advanced disease. Treatment failure, local recurrence and regional metastasis predicts poor outcome, with a 50% decrease in their overall survival. Distant metastases are nearly uniformly fatal. Despite the increasing depth of knowledge and development of advanced diagnostic and therapeutic interventions, overall survival of HNSCC remains unchanged over the last 30 years. Better understanding of the mechanisms associated with treatment failures and metastasis promises improved outcomes for HNSCC patients.

As research furthers our understanding of the mechanisms of cancer, current evidence supports the analogy of tumor growth to organogenesis. Just as an organ contains stem cells that are capable of producing heterogeneous progeny that make up a complex organ, a subpopulation of cells in tumors are also designed in a hierarchal fashion with the ability to create a complex heterogeneous conglomeration of cells. This subpopulation of tumorigenic cells in human malignancy has been deemed cancer stem cells (CSC). Cancer stem cells were originally identified in hematogenous malignancies (Lapidot et al. 1994), however over the past several years research into their role in tumorigenesis, metastasis and treatment failure in solid organ malignancies has intensified. Cancer stem cells are defined as a subset of cancer cells with the ability to form new tumors, recapitulate the heterogeneity of the original tumor, and have the capacity for self-renewal. Cancer stem cells have recently been identified as a novel and potential target for head and neck squamous cell carcinoma.

Background

Tumorigenicity has been classically described based on the stochastic model of tumorigenesis. In this model, cancer arises from multiple different mutations within cells. These mutations eventually lead to immortalized cells, all of which are capable of tumor growth. This concept if valid effectively means there is no selectivity between cancers cells that lead to a heterogeneous tumor; every cell is capable of tumorigenesis and metastasis (Fig. 25.1a). The cancer stem cell theory of tumorigenesis has long been established, but more recently has gained increasing popularity due to identification of CSC in solid malignancies. In the cancer stem cell theory, there is a hierarchy of cells capable of tumorigenesis and metastasis. Cancer stem cells are capable of giving rise to other CSC as well as to differentiated progenitor cells that make up the vast majority of a heterogeneous tumor population. Only the CSC are capable of tumorigenesis while the progenitor cells have limited to no ability to form tumors. Malignant transformation within normal squamous epithelial stem cells or in differentiated cells creates a subgroup of cells with a cancer stem cell phenotype. The cancer stem cell phenotype is a rare subset of cells with the ability for self-renewal, regeneration of the heterogeneous original tumor and the ability to form cancer. In the CSC theory, this subgroup of cells is responsible for initiation of tumor growth and spread, whereas non-CSC are incapable of regeneration of progeny or the ability to recapitulate a tumor (Fig. 25.1b) (Reya et al. 2001). Despite these two contrasting views on tumorigenesis, the stochastic and cancer stem cells models are not mutually exclusive. Whether CSC are derived from mutated stem cells in a stochastic mechanism or differentiated cells are altered forming dedifferentiated cells with stem cell properties remains unclear. However, what is known is that there

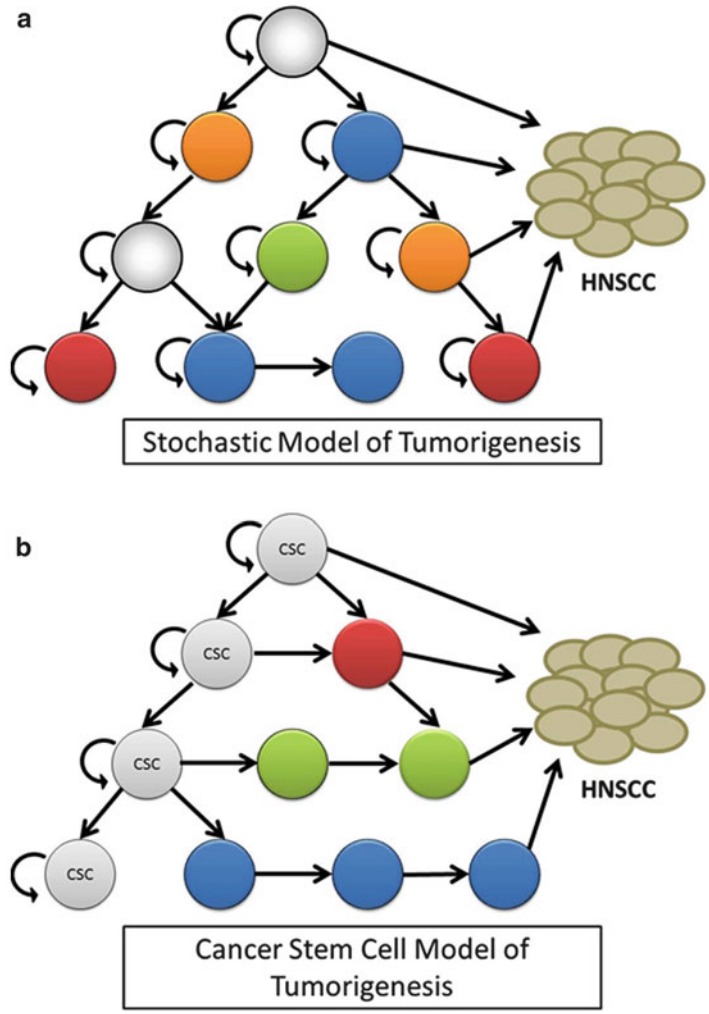

Fig. 25.1 (**a**) Stochastic model of tumorigenesis where every cell is capable of tumor growth. (**b**) Cancer stem cell model of hierarchal tumorigenesis where only a small subset of cells (*CSC*) are capable of tumor growth and renewal

appears to exist a subpopulation with greater capacity for cancer formation relative to other cells within a heterogeneous tumor cell population.

Subpopulations of highly tumorigenic cells, or CSC, were first described in hematologic malignancies (Lapidot et al. 1994). Since then they have been identified in multiple tumor types, both hematologic and solid, using a variety of cellular markers. Studies performed in leukemia were the first to demonstrate the ability of leukemia CSC to form tumors, regenerate the primary leukemia based on the cellular markers CD34 and CD38 (Bonnet and Dick 1997). Breast cancer was the first solid organ malignancy to be identified containing CSC. Work by Al-Hajj et al. (2003) revealed that breast cancer cells with the cellular markers $CD44^{+}/CD24^{-/low}/Lineage^{-}$ cells were isolated from human breast carcinoma and orthotopically injected into immunocompromised mice. These cells were capable of serial regeneration, recapitulation of the primary tumor and self-renewal of the tumor initiating cells. Since then, CSC have been identified in CNS, pancreatic, lung, colon and recently head and neck squamous cell carcinoma.

Work by Prince et al. (2007) identified a subpopulation of HNSCC tumor cells with stem cell-like phenotypes initially using the cellular marker CD44 and more recently with aldehyde dehydrogenase (ALDH) (Chen et al. 2009; Clay

et al. 2010). Like other cancer stem cells, these cells have stem cell-like qualities: self-renewal, tumorigenesis and the ability to recapitulate a heterogeneous tumor. This marked the first identification of CSC in HNSCC.

Identification of Cancer Stem Cells

Cancer stem cells are often identified by specific molecular markers or enzymatic reactions. Using these CSC specific markers allows the use of flow cytometry to identify and sort a select subpopulation of cancer cells. Fluorescence activated flow cytometry enables rapid identification and distribution of cells by suspending them in a stream of fluid and each cell placed into a single droplet. The droplets are then passed through a laser whose light is then deflected based on the fluorescent nature of the cell. The deflected light activates an electrical charge to the cell based on its fluorescence. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. This rapid distribution of cells allows high throughput analysis based on physical and/or chemical properties of the cells. The cellular makers CD44, ALDH and CD133 are used to identify head and neck cancer stem cells by fluorescent activated flow cytometry.

CD44

CD44 is a type-1 transmembrane glycoprotein, also known as Phagocytic Glycoprotein-1 (PGP-1). This transmembrane protein primarily serves as a receptor for hyaluronate and is involved in adhesion of leukocytes to endothelial cells, extracellular matrix and stromal cells. The function of CD44 can be altered by a combination of alternative splicing of its variable region and alternative glycosylation of both the constant region and the variable region. Downstream effects of CD44 binding include invasion and metastasis (Wang et al. 2009). In HNSCC, the CSC populations were initially identified based on elevated expression of CD44. Supporting evidence for CD44 as a marker for CSC in HNSCC has shown cells expressing high levels of CD44 have a more primitive morphologic appearance, express higher levels of the stem cell marker BMI-1, and co-stain with the basal cell marker cytokeratin 5/14. In vivo, cells with high CD44 ($CD44^{high}$) expression are capable of tumorigenesis, whereas cells with low CD44 ($CD44^{low}$) expression do not (Prince et al. 2007). The identification of CD44 by flow cytometry has traditionally used fluorochrome-conjugated anti-CD44 antibodies against epitopes within the conserved region of the CD44 extracellular domain. The top 1% of cells expressing are defined as $CD44^{high}$ and those with less than 1% expression are defined as $CD44^{low}$ (Fig. 25.2a).

ALDH

The aldehyde dehydrogenase (ALDH) superfamily is made up of 19 separately encoded genes. The expression and activity of the ALDH product is found in nearly all intracellular components. ALDH is a $NAD(P)^+$-dependent enzyme involved in oxidation of aldehydes for both physiologic and pathologic cellular mechanisms. The isoforms ALDH1A1 and ALDH3A1 have been shown to play a critical role in embryonal tissues as well as adult stem cells. ALDH was initially identified as a normal physiologic stem cell marker and then as a cancer stem cell marker in myeloma, breast cancer and leukemia (Ginestier et al. 2007). ALDH1 has been shown to be a putative marker for CSC in HNSCC and colon cancer (Clay et al. 2010; Chen et al. 2009).

Identification of ALDH1 activity by flow cytometry uses the fluorescent substrate BIODIPY aminoacetaldehyde (BAAA) assay (Aldefluor Assay®). The BAAA substrate is added to cell culture and passively diffuses into cells. The ALDH enzyme then oxidized BAAA to BIOPDIPY aminoacetate (BAA−). BAA^- is unable to escape the cell and emits a highly fluorescent light (Storms et al. 1999). Cells containing high levels of ALDH (ALDH+) and low levels (ALDH−) can be identified and sorted based on the level of BAA^- fluorescence emitted using fluorescent activated cell sort (Fig. 25.2b).

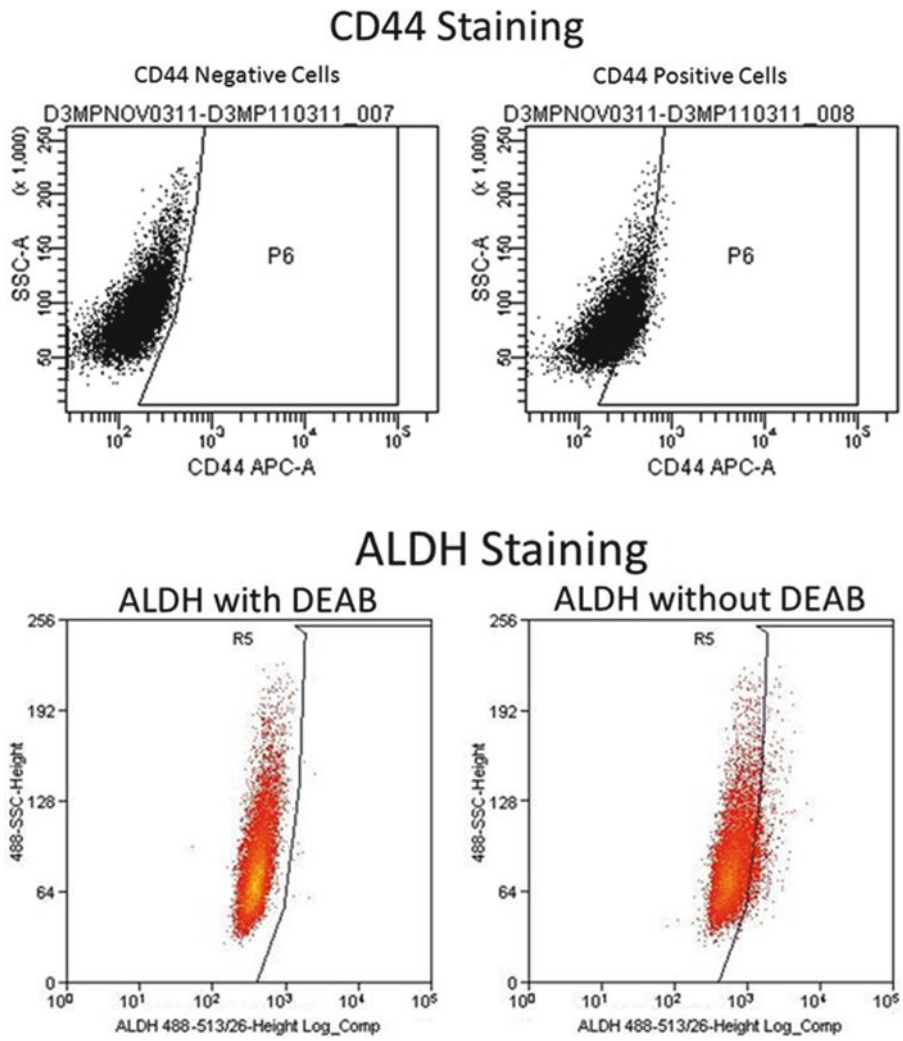

Fig. 25.2 Flow cytometery. (**a**) CD44 and (**b**) ALDH flow cytometry for identification of HNSCC CSC

CD133

CD133 is a five-transmembrane glycoprotein localizing to cell membrane protrusions and microvilli. It has also been found expressed on endothelial precursor cells, glial stem cells and prostate epithelial stem cells. CD133 was originally described as a CSC marker in leukemia and glioblastoma. Zhou et al. (2007) identified CD133+ cells to have a CSC phenotype and as a putative marker of laryngeal squamous cell carcinoma. Similar to CD44, fluorochrome-conjugated anti-CD133 antibodies are used to identify cells with high levels of CD133 and sort them by fluorescent activated flow cytometry for analysis.

Tumorigenesis

The ability to form a tumor mass and reproduce indefinitely defines cancer. Cancer stem cells represent a mechanism for tumorigenesis. Understanding their role is critical to developing therapies. As described previously, HNSCC CSC have been shown to have greater rates of tumor initiation. Prince et al. (2007) were able to demonstrate

as few as 5,000 cells with CD44^{high} expression were capable of tumor growth in the flanks of NOD/SCID mice. Correspondingly, cells with CD44^{low} expression had limited ability to grow, even when >1×10^6 cells were injected. Tumors that were grown from CD44^{high} cells were then extracted and analyzed for histology and cellular composition. The histology of the tumors demonstrated recapitulation of a heterogeneous tumor that mimicked the original primary tumor. When these tumors where reanalyzed by flow cytometry, the tumor cell make-up was heterogeneous and there was renewal of the CD44^{high} cells. Additionally, analysis of the CSC transcriptome demonstrated greater expression of BMI-1, an established tumorigenic stem-cell related gene, in the CD44^{high} tumor and cell populations. This was the first description of a subgroup of HNSCC cells with the CSC phenotype.

In two independent studies evaluating the role of ALDH in tumorigenesis, it was shown that cells isolated from primary tumors with ALDH+had greater rates of tumorigenesis in mouse flank and neck injections (Clay et al. 2010; Chen et al. 2009). This work has been confirmed and repeated by several groups. Advancing their work, Chinn et al., were able to demonstrate an enhanced ability for HNSCC cells expressing CD44^{high} and ALDH+to have greater tumorigenesis and significantly elevated rates of tumor growth using an oral cavity orthotopic mouse model (Chinn et al., unpublished work). Tumor histology and flow cytometry confirmed the ability for recapitulation of a heterogeneous tumor, and self-renewal (Fig. 25.3a and c).

Based on these studies, CD44^{high} and ALDH+ cells have been shown to be highly tumorigenic and maintain the CSC phenotype. In contrast to the stochastic theory on tumorigenesis, only a highly select subset of cells is capable of tumorigenesis. This evidence strongly supports the cancer stem cell theory; that a select group of cells have the capacity to form a new cancer with repopulation of the heterogeneous cellular population of the original tumor and form new cancer stem cells with the same capacity for tumor regeneration. The cells lacking cancer stem cell markers are incapable of tumorigenesis and thus recapitulation and self-renewal.

Metastasis

Regional and distant metastasis in most solid organ malignancies predicts extremely poor prognosis. Current treatment modalities for distant metastases are often only palliative in nature. Head and neck cancer metastasis has been associated with clinical and pathologic parameters such as tumor size, perineural invasion, tumor differentiation, extracapsular spread from regional metastasis and tumor location. However, the exact cellular and molecular mechanisms of invasion and metastasis remain unknown, thus presenting a critical area of study to develop potentially novel therapies.

Although CSCs are known to exhibit increased tumorigenicity compared with the rest of the tumor population, their invasive and metastatic potential has only recently been elucidated. Cancer stem cells represent a novel area of study for metastasis as they have the ability to form spheroids and can grow independent of attachment. Given the ability for "independent" growth, in theory, a single cancer cell can invade either a blood vessel or the lymphatic system (Brabletz et al. 2005). This cell can then travel to regional or distant site, bind to and invade through regional or distant organs. Cancer stem cells make an ideal mediator of metastatic spread given their propensity for survival in adverse conditions and more importantly their independent tumorigenetic capability. Understanding and targeting these cells is vital to advancing treatment strategies for HNSCC.

Work in breast cancer has been shown that approximately 30% of patients present with occult disseminated tumor cells in their bone marrow. Analysis of bone marrow metastasis demonstrated a higher proportion of the cells expressing the cancer stem cell marker phenotype (CD44+/CD24−) (Balic et al. 2006). Additionally, when reimplanted into a mouse model, these metastatic cells were capable of regenerating cells with the cancer stem cell phenotype (tumorigenesis, recapitulation of a heterogeneous tumor and self-renewal). Analysis of the breast CSC marker ALDH demonstrated greater metastatic potential and predicted poor outcomes in inflammatory

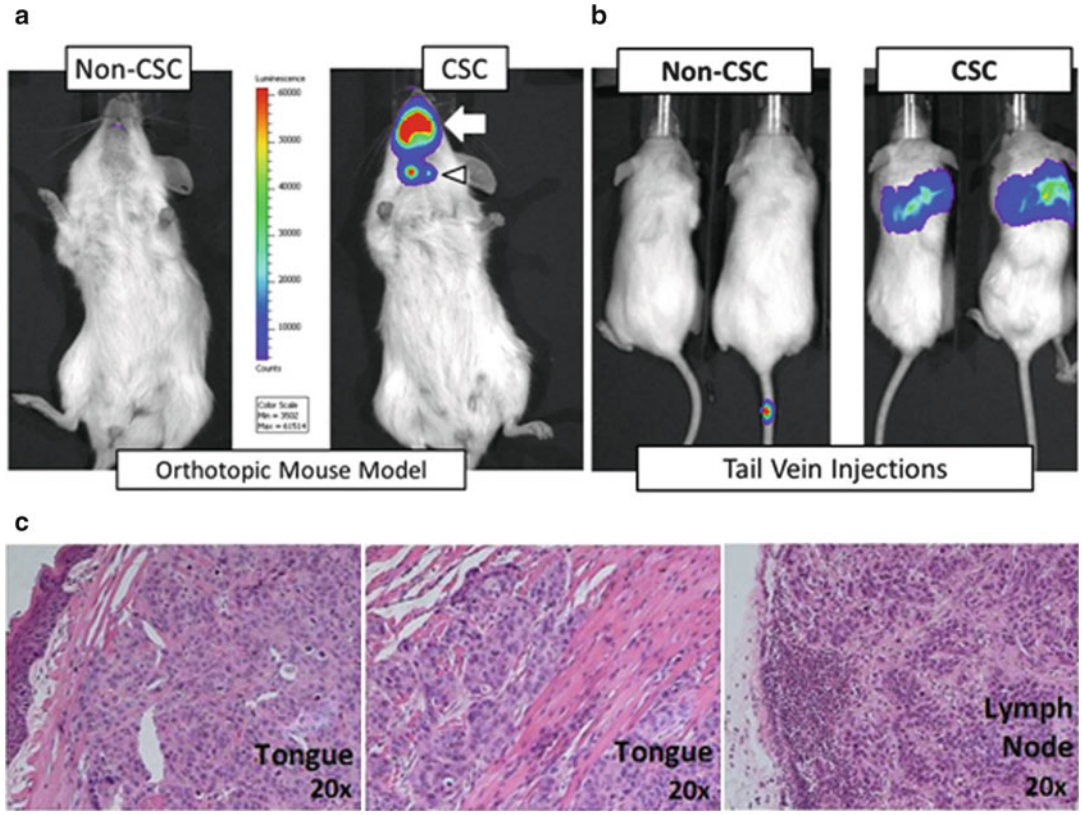

Fig. 25.3 Animal models of CSC mediated tumorigenesis and metastasis. (**a**) CSC orthotopic tip of tongue injections had greater rates of tumorigenesis in the tongue (*arrow*) and regional metastasis to the lymph nodes (*arrow head*). (**b**) CSC tail vein injections had greater rates of spread to the lungs compared to non-CSC. (**c**) Tongue and lymph node histopathology demonstrating recapitulation of a heterogeneous SCC

breast cancer (Charafe-Jauffret et al. 2010). Pancreatic adenocarcinoma has also demonstrated CSC as potential mediators of metastasis. Hermann et al. (2007) were able to identify a subgroup of pancreatic CSC ($CD133^{+}$ $CXCR4^{+}$) with an enhanced metastatic phenotype.

In HNSCC, expression analysis of biomarkers (CMET, HIF, TWIST, SNAIL) associated with increased metastatic potential have been shown to have enhanced expression in cells with CSC-like phenotypes. In addition, CSC have been shown to be highly enriched within the epithelial-mesenchymal transition (EMT) (Sun and Wang 2011; Chen et al. 2011). The EMT represents a physiologic step in embryology and for wound healing allowing transition of cells to switch morphology and function to form specific functions. In cancer, this phenomenon allows cancer cell to transition from an epithelial morphology to a more mesenchymal morphology. This transition allows increased invasion, loss of cellular adhesions and ultimately the capacity for regional and distant migration. Taken together, the elevated expression of invasive metastatic cancer genes and the findings of CSC enrichment at the EMT further supports the concept that CSC mediate the invasive and more metastatic borders of tumor growth and likely control metastatic potential.

In vitro work by Chinn et al. (unpublished) and Davis et al. (2010) has shown that CSC expressing $CD44^{high}$ have an increased rate of wound closure and have an increased ability to

migrate through a porous barrier compared to non-CSC. In-vivo work using a tail vein mouse model by Davis et al. (2010) were the first to show that $CD44^{high}$ cells efficiently formed lung lesions, whereas non-CSCs did not give rise to any distant disease (Fig. 25.3b). The metastatic potential of these cells was confirmed in an orthotopic mouse model. Chinn et al. (unpublished) were the first to demonstrate significantly higher spontaneous regional and distant metastases in mice injected with cells expressing $CD44^{high}$ and $CD44^{high}$/ALDH + compared to those injected with $CD44^{low}$ and $CD44^{low}$/ALDH- cell (Fig. 25.3a). Histologic and flow analysis of the primary tumor, regional lymph node metastasis and distant lung metastasis confirmed recapitulation and self-renewal (Fig. 25.3c). Additionally, flow analysis of primary patient specimens has shown CSC enrichment in the metastatic lesions. Collectively, these findings support CSC as an important mediator and potential target in HNSCC metastasis.

Treatment Failures

Similar to metastasis, treatment failure portends a poor prognosis in HNSCC. One of the mechanisms of treatment failure is treatment resistance. Increasing amount of work in other solid organ malignancies has shown a propensity for CSC to be a primary mediator of chemotherapy and radiation resistance (Fig. 25.4a). Given CSC ability for tumorigenesis, incomplete CSC eradication can lead to treatment failure and recurrence. Work in glioblastoma and breast cancers have shown that CSCs have a greater ability to have sustained growth and survival in harsh conditions and may use innate resistance mechanisms against treatment induced cell death (Bao et al. 2006). Cancer stem cells are thought to possess the mechanisms necessary for treatment resistance based on two vital characteristics: CSC pump activity and slow cycling cells (Clarke et al. 2006).

Okamoto et al. (2009) studied CD44+ cells in a head and neck cancer cell line and found higher expression of CD133 and ABCG2 levels compared to CD44- cells. In addition, CD44+ cells were found to have greater chemotherapeutic resistance. Likewise, Yang et al. (2011) evaluated laryngeal SCC cells expressing CD133+ and demonstrated significantly lower cell death when these cells were co-cultured with 3 common HNSCC chemotherapy agents. Higher levels of ABGC2 expression was found in CD133+ cells and likely corresponds with chemoresistance. CSC expressing CD44 and CD133 appear to have greater chemoresistance likely mediated through ABCG2 mechanisms.

Koukourakis et al. (2012) recently demonstrated that cells expressing the CSC markers CD44, CD24, Oct4 and integrin-b1 were associated with poor radiation therapy outcomes. Hypoxia has been shown to be associated with radiation resistance, higher rates of local-regional recurrences and may lead to poor differentiation (Kim et al. 2009). Cancer stem cells have been shown to have a high capacity for sustained growth and survival under hypoxic conditions, suggesting another mechanism for radiation resistant tumors. Radiation induced tumor cytotoxicity relies upon oxygen derived oxygen free radicals; tumor hypoxia coupled with CSC ability for hypoxic survival provides the mechanism and role of CSC in radiation resistance and treatment failure. As mentioned previously, HIF genes are critical in the hypoxia pathways and have been shown to be overexpressed in CSC and may be responsible for some aspect of radiation-resistance and post-radiation recurrence in HNSCC. The enhanced mechanisms of CSC to evade treatment survival may help explain treatment failure and poor outcomes. Further study of these CSC-specific mechanisms may potentially lead to novel CSC-specific targets.

Tumor Microenvironement/Niche and Supporting Cells

Stem cells rely on surrounding signals within their native microenvironment to signal, regulate and support stem cell proliferation, differentiation, and apoptosis. In adverse conditions, there is an increase in stem cell activity in an effort to preserve normal organ function. In cancers, a similar phenomenon takes place. Recently the stem cell environment and the need for supporting cells to

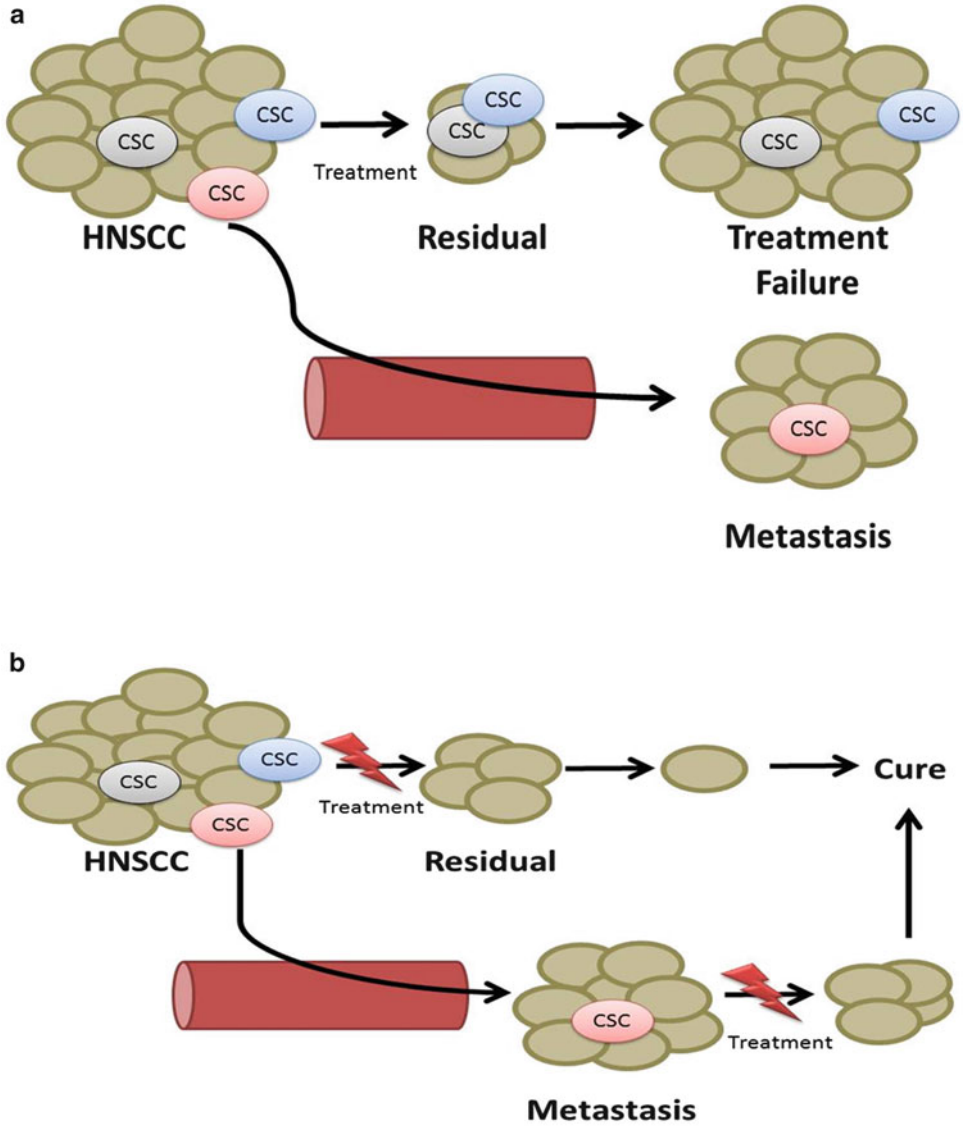

Fig. 25.4 (**a**) Standard treatment with cancer stem cell survival serves as potential mechanisms of treatment failure. A single cancer stem cell may also be capable of forming metastasis. (**b**) Cancer stem cell targeted treatment (*red arrow*) may lead to more effective and specific tumor treatment

facilitate cancer stem cell function and maintenance of stemness has been described. This environment has been described as the cancer stem cell "tumor niche" with supporting cells (endothelial, extracellular matrix, inflammatory cells, etc.) providing inter-cellular signals to maintain the CSC hierarchy in tumors, enhance tumor growth and stimulate invasiveness (Fig. 25.5). The cancer stem cell niche has been previously described in glioblastoma and colon cancer. In glioblastoma, there is a close relationship between glioblastoma cancer stem cells and endothelial cells. Additionally, the relationship with angiogenesis was symbiotic; CSC were found to secrete vascular endothelial growth factor (VEGF), thus enhancing local angiogenesis leading to a cancer promoting feedback loop.

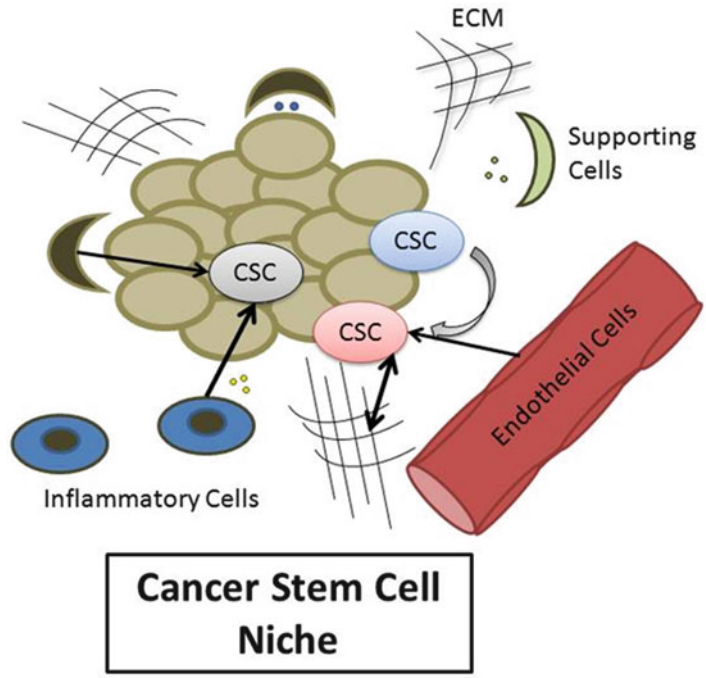

Fig. 25.5 Cancer stem cell tumor niche. Surrounding microenvironment stimulates CSC proliferation, inhibition of apoptosis and stimulate invasion and metastasis

In colon cancer, CSC maintenance has been shown to be associated with colonic stroma, particularly tumor associated myofibroblasts. Myofibroblasts produce Wnt-ligands. Wnt has been shown to control stemness and dedifferentiation. This association with supporting cells supports the impact and importance of the cancer stem cell niche (Korkaya et al. 2011). Furthermore, the cancer stem cell niche has also been shown to "dedifferentiate" cells. This is of particular importance in the epithelial-mesenchymal transition (EMT). The EMT region of tumor growth has been shown to be an early and critical step in invasion, metastasis and ultimately poor prognosis.

The cancer stem cell niche and the EMT has been recently described in head and neck cancer. Krishnamurthy et al. (2010) were the first to describe the "tumor niche" in HNSCC CSC. They were able to demonstrate a propensity for CSC to aggregate around tumor vessels and depend on endothelial cells for maintenance of their stemness and may enhance survival and treatment resistance. These "tumor niche" may act via specific cellular interactions and mediators of cancer stem cell survival, growth and differentiation. Likewise, cancer stem cells were shown to have higher levels of EMT-associated genes (SNAIL, TWIST, etc.). These cells had a higher propensity for metastasis compared to those lacking the EMT associated expression patterns.

Similar to the supporting cells, tumor environment has also been shown to increase CSC activity. Hypoxia has long been associated with tumor aggressiveness, metastasis, treatment failure, and poor survival (Lu and Kang 2010). Hypoxic conditions, such as in rapidly growing tumors often switch intracellular metabolism to a more oxygen independent pathways. In addition, activation of pro-stem cell pathways has been described. OCT3/4 has recently been identified in HNSCC CSC and associated with poor outcomes.

Clinical Implications and Future Aims

The ultimate goal for research into CSC is to apply the findings to patient care. This can be done twofold: diagnostics and targeted therapy. Joshua et al. (2012) evaluated primary HNSCC patient tumors. They found that tumors with elevated

CD44+ cells (>36%) had a significantly increased risk of recurrence of their primary tumor compared to tumors with lower CD44+ levels (<15%). When they implanted these cancers into mice there was a significant correlation with in-vivo tumor growth and primary tumor recurrence. Cancer stem cells represent a subset of tumor cells that drive tumorigenesis, metastasis and treatment failure. This correlate in humans further supports the role of CSC in HNSCC. By designing CSC-specific therapies, there is potentially a greater likelihood that we will be able to treat patients with distant metastasis and traditional chemotherapy and radiation-treatment failures (Fig. 25.4a). Numerous studies in other solid organ malignancies are studying the effects of CSC-targeted therapies. This can be done either based on inherent factors associated with cancer stem cells, such as CSC-specific genes, immunity, behavior or in modulating the surrounding niche that supports CSC proliferation.

At the genomic level, by identifying CSC-markers it may be possible to detect and diagnose HNSCC at presentation or during surveillance after treatment. Faber et al. (2011) were able to demonstrate higher concentrations of CD44 in peripheral blood samples of HNSCC patients compared to healthy controls. In addition, recent evidence has shown that despite CSC being a highly specific subgroup of cancer cells, cellular heterogeneity within CSC subgroups lead to variations in tumor behavior. Further research to delineate the differences between CSC that are capable of providing tumor resistance to treatment and invasive and metastatic spread is critical. Further defining CSC subgroups may allow for better characterization of tumor behavior and better predict prognosis.

The findings that head and neck cancer stem cells are vital for tumorigenesis, metastasis and treatment failure make them attractive area of study. Targeted therapies directed at CSC may reduce remnant cells that lead to recurrence and metastasis and allow for more targeted therapy (Fig. 25.4a and b). Future work to better understand the CSC specific molecular pathways will be critical to understand the mechanisms of tumorigenesis, metastasis and treatment failures with the ultimate goal to development of novel CSC diagnostics and therapeutic targets.

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Cancer Stem Cell Antigens from Autologous Tumor Cell Lines in Patient-Specific Active Immunotherapy for Metastatic Cancer

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Abstract

Cancer is lethal because of metastatic spread, and is seldom curable, even in patients who can be rendered free of disease. Autologous, proliferating, self-renewing tumor cells (cancer stem cells and/or early progenitor cells), which are responsible for metastatic tumors, could be excellent sources of antigen for vaccines that could be used for the active specific immunotherapy of patients with advanced cancer. We have established proliferating pure tumor cell cultures from cancer samples, followed by further expansion for patient-specific therapeutic purposes. In clinical trials, patients were treated with a series of s.c. injections of irradiated autologous tumor cells (TCV), or autologous dendritic cells (DCV), loaded with antigens from their tumor cell line. Cell lines were successfully established for nearly half of patients, with the highest success rates in melanoma, renal cell, sarcoma, and glioblastoma. For patients with melanoma and renal cell, who were treated with TCV, observed 5-year survival rates were nearly three times longer than national figures. For patients with melanoma who were treated with DCV, the 5-year survival rate was five times greater than national estimates. In a randomized trial comparing DCV and TCV in patients with metastatic melanoma, at a median follow up of 2 years, survival is better in patients treated with DCV. Patient-specific active immunotherapy with antigens from autologous proliferating, self-renewing tumor cells is a feasible approach. Such

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treatment is associated with encouraging long-term survival rates, and results are superior in patients treated with DCV loaded with antigen from such cells. A higher success rate of establishing cancer stem cell cultures is needed, and additional clinical trials will be required to establish the clinical benefit.

Introduction

Cancer continues to be the number one health concern in Western countries. Targeting cancer stem cells by the endogenous immune system should provide benefit to cancer patients, perhaps not so much as a rapid acting cytotoxic therapy, but rather by providing long-term benefit by prevention of new tumor sites that result from cancer stem cells. If there are common antigens on cancer stem cells, then perhaps a vaccine approach could actually prevent cancers in populations. In this chapter we describe the rationale for pursuing cancer stem cells as a source of antigen for inducing or enhancing an anti-cancer immune response. We describe many of the issues which confront investigators in trying to develop an appropriate vaccination, or immune enhancing strategy, and the reasons we have chosen autologous proliferating cell cultures as the antigen source, and why we are focusing on autologous dendritic cells as the means of presenting such antigens. Most importantly, we summarize the results of technically challenging, laborious, bench-to-bedside clinical trials, which are complicated to execute, and the encouraging long-term survival data that has emerged from this effort, especially in metastatic melanoma.

Background and Rationale

Cancer: An Unmet Need

In the United States, cancer is the number one cause of death in younger populations (<age 80 years). Cancer deaths have increased dramatically as longevity has increased, thanks to advances that have decreased the risk of dying earlier from other maladies, especially cardiovascular disease. Cancer survival rates have steadily improved thanks to technologies that allow earlier diagnosis and more sensitive detection of cancer in the body, and improvements in surgical procedures, radiation therapy technology, and especially systemic therapies. In the modern age, cancer usually is lethal because of the spread of cancer throughout the body—metastatic disease—rather than effects of localized cancer. For this reason systemic therapies that can target cancer anywhere in the body are crucial to improve long-term cancer survival. Although progress has been made in the treatment of metastatic disease, older systemic treatments such as chemotherapy are very toxic, and contribute little to long-term survival. Newer targeted therapies, such as monoclonal antibodies and tyrosine kinase inhibitors often must be given indefinitely because of their mechanisms of action on cell surface molecules and intracellular transduction pathways. Hence their total contribution to the cost of cancer therapy is increasingly an issue. Furthermore, as we gain more experience with these products, it is evident that most targeted therapies do have undesirable toxicities and side-effects, and have improved survival rates only marginally. Newer treatment strategies are needed that are potentially curative, are associated with minimal side effects, and can be delivered over a relatively short time period, but induce long-lasting beneficial effects.

Cancer Immunotherapy

Immunotherapy is one of several systemic modalities that, to date, have produced only limited improvement in cancer survival (Dillman 2011). Approved immunotherapeutics include the cytokines interferon-alpha (IFN-α), interleukin-2 (IL-2), and granulocyte-macrophage colony stimulating factor (GM-CSF), which have been available for more than two decades, and more recently the monoclonal antibody ipilimumab (Hodi et al. 2010) and the cell therapy sipileucil-T (Kantoff et al. 2010) These agents induce changes in the immune system, which result in anti-cancer effects. The three cytokines unleash a variety of immune

effects, and are relatively non-specific in their actions. Ipilimumab targets cytotoxic T-lymphocyte antigen-4 (CTLA-4) to unleash autoimmune effects, which again are not cancer-specific. Sipileucil-T product consists of autologous dendritic cells (DC) that have been co-incubated with a fusion protein of GM-CSF and prostatic acid phosphatase (PAP). Although the sipileucil-T was intended to induce specific anti-PAP activity on prostate cancer cells, this product consists of heterogeneous white blood cells, including DC, and T-lymphocytes. Sipileucil-T is the first DC-product approved for cancer therapy. Although most monoclonal antibodies were originally designed to be cytotoxic to tumor cells (TC) via interaction with complement and/or effector immune cells, it is increasingly evident that their primary mechanism of anti-tumor effects is altered cell biology secondary to targeting membrane receptors (e.g. rituximab, trastuzumab, cetuximab, etc.) or blocking cancer-stimulating ligands (e.g. bevacizumab) (Dillman 2009).

Cancer Vaccines and Active Specific Immunotherapy

The ideal cancer immunotherapy would induce and/or enhance the cancer patient's endogenous immune system to recognize his/her cancer-associated antigens resulting in long-lasting anti-cancer effects with minimal toxicity. Such a products is typically classified as a cancer vaccine. However, the term "vaccine" classically was used to describe products that are injected to immunize (induce an immune response) against antigens to prevent a malady, such as infection. When such vaccinations are used to induce and/or enhance immune effects against an existing malady, the approach is more accurately referred to as "active specific immunotherapy." Using these definitions, all cancer vaccine approaches to date would be classified as active specific immunotherapy, since their testing has been in patients who already have been diagnosed with cancer. However, because most products have been administered with the intent of preventing new sites of metastases, rather than elimination of detectable cancer, there are aspects of this approach that overlap. Although it may actually work as an adoptive cell therapy, sipileucil-T has been characterized as both a vaccine and active specific immunotherapy. Similarly, in this paper the term "vaccine" is used to characterize products injected with intent to prolong survival in patients who are already diagnosed with metastatic disease, regardless of whether metastatic cancer is detectable at the time of treatment.

Immune Targeting of Cancer Stem Cells

We continue to believe that the best source of antigens for cancer vaccines are the patient's own proliferating cancer cells (Dillman et al. 1993). Furthermore, we believe it is better to use relatively pure proliferating, self-renewing autologous cells as the source of antigen rather than whole tumor, in order to target cancer stem cells. In terms of antigen-presentation, we now have clinical evidence that it is better to present such antigens via autologous DC that have been loaded with antigen exogenously rather than endogenously (Dillman et al. 2006, 2009, 2010, 2012). We feel that injection into subcutaneous tissue is an adequate site for antigen exposure. We believe it is advantageous to administer these products with an immune-enhancing adjuvant.

Why Proliferating Cancer Cells?

Cancer patients die because of the spread of cancer cells and proliferation of these cells. Cancer cells that self-renew and proliferate in cell culture are derived from such cells and should express antigens that are uniquely associated with this process (Dillman et al. 1993). Such cell cultures contain tumor stem cells and/or early progenitor cells. In contrast, the malignant cells in a depot of tumor are mostly more differentiated TC which have lost the ability to proliferate indefinitely, and therefore stop proliferating in cell culture after a few passages. The malignant cell composition of a tumor sample ranges from 5 to 95% of the cells in the specimen. Depots of tumor contain varying amounts of immune cells, stromal elements, and mesenchymal cells, in addition to varying numbers

of TC. These non-cancer tumor elements also die out during cell culture. Cell preparations from tumor deposits, whether whole cells, or lysates, contain large quantities of antigens from non-malignant cells. This may be the explanation for the failure of autologous approaches that utilized heat shock proteins (Testori et al. 2008).

Why Cells Rather Than Specific Antigens?

Vaccination with specific antigens, including monovalent or polyvalent peptides and other components of cell membranes, have been useful for proof-of-principal for cancer vaccines, because of the known antigen, but have failed to produce meaningful survival benefit in clinical trials (Kirkwood et al. 2001; Lawson et al. 2010; Schwartzentruber et al. 2011). These approaches are limited by the complexity and heterogeneity of humans and their malignancies, as opposed to microbes in which such approaches have been effective for preventive vaccines. Inducing the immune response to focus on such a specific antigen, or a small number of common antigens, may actually be detrimental because of distracting the immune system away from more important antigens, some of which may be unique to different patients. This hypothetical concern is supported by results from clinical trials. For instance, in a melanoma vaccine trial, patients receiving a ganglioside vaccine had a much worse survival than patients who received IFN-α (Kirkwood et al. 2001). In the pivotal ipilimumab trials, patients receiving gp100 peptide vaccine alone had a worse survival compared to ipilimumab or ipilimumab plus gp-100 (Hodi et al. 2010). Was ipilimumab really more effective, or did it overcome the negative effects of immunizing with gp100 alone?

Why Autologous Rather Than Allogeneic Cells?

Autologous TC express any and all antigens that are relevant to that patient's cancer, especially any unique neoantigens that are specific to mutations or epigenetics in that individual's cancer. Compared to whole tumors, proliferating allogeneic cells offer the same benefits as proliferating autologous TC, in terms of purity and self-renewing proliferation. They also express antigens that are common to that cancer cell type. However, allogeneic TC also express many irrelevant antigens and alloantigens that may dilute the immune response to tumor-associated antigens, or actually distract the immune response away from some important antigens. This may explain why in the CancerVax® melanoma trials in patients with resected stage III or stage IV disease, survival was better in the Bacille-Calmette-Guerre (BCG)-placebo arms than the active therapy arms that included BCG with allogeneic TC (Morton et al. 2007). Numerous melanoma trials utilizing lysates or cells from allogeneic lines have also failed to demonstrate benefit, perhaps for similar reasons (Hersey et al. 2002; Mitchell et al. 2007; Sondak et al. 2002; Wallack et al. 1998).

What Is the Best Vaccine Dose?

For each vaccination, we decided to inject the antigens associated with about 10 million TC. We had no way of knowing which antigens were most important; therefore, cell number was our surrogate measure for the quantity of any and all relevant antigens. We selected 10 million TC based on the encouraging clinical results reported in colorectal cancer patients who exhibited cellular and humoral immune responses after injections of about 10 million TC from whole tumors with BCG (Hoover et al. 1993). However, our TC product was clearly different because it consisted of pure, proliferating TC, while the products in the literature utilized cell suspensions from whole tumor with variable numbers of TC. While we could have examined much lower numbers of TC, we believed that in terms of potency, more antigen exposure was probably better. It was hard to give more TC because of practical limitations related to incubator space and cost. We also quickly recognized that TC from individual patients varied in viability, sensitivity to radiation, and tolerance of freezing and thawing, but were consistent across frozen aliquots. Therefore we elected to examine the potency issue retrospectively over the range of cells that were actually given to different patients during the first three vaccinations (Dillman et al. 2005). Using correlation with survival, over the range of cells that were injected,

2–10 million per injection, we could not determine a preferred or undesired dose. Therefore, we have continued to administer whatever number of cells are available for each given patient. Although there was substantial inter-patient variability, there was minimal intra-patient variability for each thawed TC-vial at the time of injection.

In our most recent work we utilized DC as the antigen presenting cell rather than the tumor cells themselves (Dillman et al. 2006, 2009, 2010, 2012). We incubated DC and TC at a ratio of about 2:1, but because this was arbitrary, and because of biological variability and practical considerations, we did not try to match the numbers of viable DC and TC for co-incubation. For this reason the number of cells injected ranged from 5 to 33 million (median 15) per injection, and the number of viable cells injected ranged from 4 to 24 million (median 10). Because samples were aliquoted, and because of their biologic similarity, at the time of each treatment, approximately the same number of cells were injected for that specific patient.

What Is the Best Route of Administration?

In all of our trials we have administered the autologous cell-based vaccine products by subcutaneous (s.c.) injection. Exposure to antigens in various sites of the body can induce or enhance immune effects. Various animal models have supported certain routes over others, but there is no consensus regarding an optimal route for vaccinations. It would be costly and impractical to conduct large comparative human trials to definitively establish a preferred route of administration. In addition to the s.c. route, successful immunization has been documented by exposure to antigen via oral (p.o.), intravenous (i.v.), intradermal (i.d.), intramuscular (i.m.), and intranasal routes. We felt that i.v. might be an inferior route of administration because of the opsonization of tumor cells and elimination in the lung, liver, and spleen via that route. The i.v. route may not be as effective as cutaneous-lymph node lymphatic pathways. The i.v. route would also increase the risk of possible anaphylactic reactions. In our studies we chose skin injections because of the common use of this approach, and chose s.c. over i.d for practical reasons. It is technically easier to inject s.c. than i.d.; larger volumes can be injected s.c. than i.d.; and it is easier to include the entire product in one s.c. injection rather than multiple painful i.d. injections. Once there was suggestion of benefit, we saw no reason to rigorously investigate other routes of administration.

What Is the Best Adjuvant?

We have been giving our patient-specific vaccines with GM-CSF. When we started these studies the literature strongly supported the benefits of giving a non-specific immune stimulating agent along with a vaccine. Our bias was toward the immune-stimulating cytokines rather than more traditional adjuvants such as BCG or alum. When we started our first trials, IFN-α, IFN-γ, and GM-CSF were commercially available, but not IL-2. Animal models had suggested that GM-CSF was a particularly good adjuvant (Warren and Weiner 2000). Our first exploratory trials included the injections of irradiated autologous tumor which were given with a variety of different adjuvants by different physicians. Subsequently we conducted a randomized phase II trial that enabled some comparison of IFN-γ, to GM-CSF (Dillman et al. 2003). Although we saw no difference between these two, there was a suggestion that either or both might be better than no adjuvant, BCG, or dinitrophenol, based on limited retrospective comparisons (Dillman et al. 2003). When we began using the DC product, we thought that co-administration with GM-CSF might be advantageous for those cells. Given the encouraging results we have observed, we have seen no reason to explore other adjuvants. By the time our DC trials were initiated, the FDA allowed us to suspend the cells in 500 micrograms of GM-CSF under the same investigational new drug exemption (IND), rather than having to inject the GM-CSF separately.

What Is the Best Schedule for Administration?

We have injected our patients weekly for 3 weeks and monthly for 5 months. We have no idea if this is the optimal schedule. A variety of schedules have been proposed based on animal models, and

various investigators have utilized a variety of different schedules in clinical trials. Most have used weekly, biweekly, or monthly, schedules for induction, and monthly, semi-annual, or annual for maintenance. When we started our work, we were influenced by publications of results in hamster animal models, and encouraging clinical results associated with injections of BCG and autologous tumor in patients with colorectal cancer (Hoover et al. 1993). Based on those reports, we chose the weekly times three induction schedule, and monthly booster schedule. We were limited to a total of eight injections because of the number of tumor cells available, and the intent to utilize antigen from about 10 million cells at each injection.

Methods

We have used two patient-specific products in clinical trials: a tumor cell vaccine (TCV) and a dendritic cell vaccine (DCV). The sources of antigen for both products are autologous proliferating cancer cells, putative cancer stem cells and/or progenitor cells. In both instances the first requirement is the ability to establish an autologous TC line in cell culture. The TCV relies on endogenous DC for antigen presentation. The DCV relies on DC loaded exogenously with antigens from TC.

Tumor Cell Vaccine (TCV)

Detailed descriptions of the methods used to establish autologous tumor cell lines have been previously published (Nayak and Dillman 1991; Dillman et al. 1993, 2011; Selvan et al. 2010) Fresh tumor samples were transported to a cell biology laboratory in a transport kit that included media and antibiotics. In a laminar flow hood, using sterile technique, cell suspensions were created using mechanical mincing and enzyme-digestion. Cell suspensions, either were placed directly into flasks containing enriched RPMI-1640 culture media, or cryopreserved for subsequent efforts to establish a cell line. An important step in this process was the serial reduction of protein concentration to inhibit fibroblast overgrowth (Nayak and Dillman 1991; Selvan et al. 2010). Successful establishment of a cell line was defined by serial passages associated with exponential growth and expansion to 50 million tumor cells that had the immunohistochemical phenotype and morphological appearance for that given cancer. This process typically took from a few weeks to months depending on the contamination with fibroblasts, and the rate of tumor cell growth. Patients and their referring oncologists were notified when a cell culture had been successfully established. Depending on desires of the patient, and the direction from the managing oncologist, the cells then were either cryopreserved, or expanded to 150–200 million cells for possible treatment. The time for additional expansion, which was dependent on the exponential growth rate of the culture, typically ranged from 1 to 2 months. After completion of expansion, cells were irradiated with 100 Gy in a cesium irradiator, then apportioned into aliquots and cryopreserved for therapeutic use, and for skin tests and future laboratory tests. Whether patients were ever treated depended on how well their disease had been controlled during the time needed to grow the cells, and the direction of their managing physician. At the time of treatment, an aliquot of cells was thawed, washed, and suspended in a syringe for injection.

Dendritic Cell Vaccine (DCV)

The first step for this product was the establishment of a tumor cell line as described for the TCV. After a TC line had been established, patients underwent a single 4 l leukapheresis from which peripheral blood mononuclear cells (PBMC) were isolated. DC were generated from PBMC by incubating in the presence of GM-CSF and Interleukin-4 (IL-4) (Cornforth et al. 2011c). In the initial phase II melanoma and renal cell trials, PBMC were isolated by Ficoll-Hypaque density centrifugation. For the subsequent randomized melanoma trial, PBMC were isolated using a closed system (Elutra® Cell Separation

System CaridianBCT, Lakewood, CO.) About 400 million PBMC were placed in T225 flasks containing GM-CSF and IL-4 and incubated for 6 days to generate DC. Adherent DC were incubated overnight with TC, during which time tumor cells were phagocytosed by the DC. For melanoma and renal cell patients treated during 2001–2006, the TC were incubated in the presence of IFN-γ prior to irradiation in an effort to increase expression of histocompatibility antigens (Cornforth et al. 2011b). Approximately 100 million tumor cells were incubated with 100–200 million DC, then that product was aliquoted and cryopreserved in vials that were thawed just prior to injection. For the randomized phase II trial conducted during 2007–2011, DC were co-incubated with the contents of the single-dose TC vials that would have been thawed for injection if the patient had been randomized to receive TCV. Thus, producing the DCV took about four additional weeks, one for the DC production process and incubation with TC, and 3 weeks for quality control tests.

Results

We have been conducting clinical trials with these patient-specific products since 1990. For all of these trials the therapeutic products were produced in the Cell Biology Laboratory of the Hoag Cancer Center in Newport Beach, California. The treatment schedules for all protocols called for injections weekly for 3 weeks, and then monthly for 5 months. All trials were reviewed and approved by Institutional Review Boards for the protection of human subjects.

Establishment of Cell Lines

The first requirement for treatment with such a patient-specific TC-based immunotherapy is the successful establishment of a cell line. During 1990–2001 efforts were made to grow a variety of tumor types. Experience with samples from the first 695 patients was previously published (Dillman et al. 2002), and specifically for ovarian cancer (Dillman et al. 1999), sarcoma (Dillman et al. 2004a), renal cell (Dillman et al. 2004b), and melanoma (Dillman et al. 2007, 2009). During 2001–2006 the focus was limited to melanoma and renal cell cancer per request of the U.S. Food and Drug Administration. During 2007–2011 the focus was on melanoma. All together, between 1990 and 2011 there were 1,018 tumor samples submitted from 943 individuals, including 222 primary tumors and 752 metastatic lesions. This total includes tissues that were contaminated, or of poor quality, and efforts that were aborted because of knowledge of clinical deterioration and demise of patients. There were 406 cell lines established for an overall success rate of 40, or 43% based on individual patients. The success rate has varied by tissue. The highest success rates were for the mesenchymal tumors (melanoma, renal cell, sarcomas, and glioblastomas) for which the success rate was 414/811 (51%). The ratios of successful cell lines established to number of individual patients and numbers of patients treated were 300/647 for melanoma (170 treated), 59/97 for renal cell cancer (36 treated), 41/71 for sarcoma (23 treated), 14/26 for glioblastoma (7 treated), 19/82 for lung (5 treated), 21/68 for ovary (3 treated), 8/50 for colorectal (3 treated), 8/44 for breast (1 treated), 3/10 for pancreas (none treated), and 3/10 for anaplastic astrocytoma (none treated). The success rate for melanoma cell lines has ranged between 50 and 60% in all three eras.

Results of Clinical Trials

TCV 1990–2001

TCV were given per Cancer Biotherapy Research Group (CBRG, formerly National Biotherapy Study Group) protocols 91–08 (November 1990 through September 1993) and 92–12 (June 1992 and March 2001). TCV were shipped to various sites that had laboratory facilities capable of storing and thawing samples. The CBRG 91–08 protocol allowed treating physicians to administer an adjuvant of their choice or none. The CBRG 92–12 protocol prescribed randomization to either GM-CSF or IFN-γ which were injected

separately from the TC. There were 177 evaluable patients enrolled for TCV therapy between 1990 and 2001, 42 per CBRG 92–08 and 125 per CBRG 92–12 (Dillman et al. 2002). Some tumor-specific data was previously published for patients with ovarian cancer (Dillman et al. 1999), sarcoma (Dillman et al. 2004a), renal cell cancer (Dillman et al. 2004b), and melanoma (Dillman et al. 2007). The only tissue type for which objective tumor responses were documented was melanoma.

Melanoma. The 74 evaluable melanoma patients had a median age of 50 years, 45 were male, 53 were given GM-CSF and/or IFN-γ as an adjuvant, and 36 had no evidence of disease at the time treatment started. At baseline 8/70 (11.4%) had a positive delayed type hypersensitivity (DTH) reaction to autologous tumor cells at baseline, but after three injections, 14/50 (28%) who were retested had a positive DTH. Three of the 36 patients with detectable disease at baseline had an objective tumor response (8.3%), one complete and two partial. No surviving patients were lost to follow up. With longer follow up, 22 patients are known to have survived more than 5 years. The updated median survival is 20 months and the 5-year survival rate is 28%.

Renal Cell Carcinoma. The 27 renal cell cancer patients had a median age of 62 years, 21 were male, 20 were given GM-CSF and/or IFN-γ as an adjuvant, and 11 had no evidence of disease at the time treatment started. Only 1/25 had a positive DTH reaction to an i.d. injection of autologous tumor cells at baseline while 6/19 retested after three injections had converted to a positive reaction. Using more recent versions of the American Joint Committee on Cancer staging systems (versions 6 and 7), six of these patients had disease localized to the kidney (three stage I and three stage II) as their most advanced stage of disease. None of the 16 patients who had detectable disease at baseline had an objective tumor response. No patients were lost to follow up and all surviving patients had been followed more than 5 years at the time of this analysis. The median survival is 26 months and the 5-year survival rate is 41%. The stage distribution of the 11 patients who were still alive after 5 years were 3-stage I (small localized), 2-stage II (larger but localized), 2-stage III (regionally advanced), and 4-stage IV (distant metastatic). Four of the 19 patients whose most advanced stage of disease had been stage IV survived more than 5 years. Only one of the eight patients who had stage I-III disease did not survive 5 years and he had not recurred at the time of death.

Sarcoma. The 23 sarcoma patients had a median age of 50 years, 11 were male, 19 were given GM-CSF and/or IFN-γ as an adjuvant, and only three had no evidence of disease at the time treatment started. Five had leiomyoscarcoma, four liposarcoma, two osteosarcoma, two synovial sarcoma, one Ewing's sarcoma, and nine unspecified. At baseline 0/21 had a positive DTH to autologous tumor cells at baseline, but after three injections, 7/16 that were retested had a positive DTH. None of the 18 patients with detectable disease at baseline had an objective tumor response. Two patients were lost to follow up, one at 14 months and one at 20 months. Two surviving patients had been followed more than 5 years at the time of this analysis. The median survival was 11 months and the 5-year survival rate was 11%. Two patients survived more than 5 years after treatment: one was a 16 year old male with recurrent and metastatic osteosarcoma, and the other was a 43 year old male with synovial sarcoma. Each had lung metastases as their only site of distant metastases.

DCV 2001–2006

Patients were enrolled in the open-label, phase I-II DCV trial between December 2000 and October 2006. For these trials TC were exposed to IFN-γ *in vitro* before being loaded on DC. The final DCV was suspended in 500 micrograms of GM-CSF for injection. Only patients with renal cell cancer or melanoma were treated per protocols specific for each tumor type. In these trials all therapeutic products were administered in Newport Beach per request of the US Food and Drug Administration.

Renal Cell Cancer. There were nine patients with metastatic renal cell cancer treated with DCV plus GM-CSF during 2001–2004 (NCI-V01-1647, NCT00014131). Most advanced stage of disease was stage IV for eight, and stage III for one. Their median survival is 26 months and 5-year survival is 33%. We have compared these 9 DCV patients to a comparable subset of 21 renal cell cancer patients (19 stage IV, 2 stage III) treated with TCV during 1990–2001 This latter group had a median survival of only 12 months, but 5-year survival was 29%. The survival curves did not differ for the DCV and TCV patients ($p = 0.42$).

Melanoma. We previously reported the results for 54 melanoma patients treated with DCV during this era (NCI-V01-1646, NCT009480) (Dillman et al. 2009). None of the 15 patients with measurable disease at the time of treatment had an objective tumor response. The updated survival curve for these patients reveals a median survival of 62 months and a 5-year survival rate of 50%.

DCV vs TCV 2007–2011

In this era only patients with metastatic melanoma were treated per an open-label, randomized phase II trial (NCT00436930). Patients were enrolled between October 2007 and February 2011. In contrast to the previous trial, TC were not exposed to IFN-γ in the production of the TCV. Once a cell line had been established, and the patient referred to begin treatment, he/she was stratified by extent and measurability of metastatic melanoma, then randomized to receive either TCV or DCV, both of which were suspended in GM-CSF. This trial was stopped prematurely when Hoag Hospital, which had supported these trials financially, abruptly closed the laboratory for fiscal reasons. Although the randomized trial was stopped early, which greatly reduced the power to detect a difference in outcome, an unplanned analysis performed at a time when 21 patients had died, showed a better survival for patients in the DCV arm with a 73% reduction in death (Dillman et al. 2012). The median survival had not been reached in the DCV arm vs 15.9 months in the TCV arm, and respective 2-year survival rates were 72 and 31% ($p = .007$). A comparison of patient characteristics and prior treatments revealed no differences between the study arms. Variables compared included most advanced stage of disease and whether measurable disease was present at the time of treatment; these two variables were used for stratification at the time of randomization. There was also no difference between the two arms in age, gender, Karnofsky performance status, lactate dehydrogenase level, or prior therapies.

At the time of the analysis, the survival curve for the 18 patients in the DCV arm is quite similar to that generated for the 54 patients treated with DCV during 2001–2006, and the survival curve for the 24 TCV patients is quite similar to that generated for the 74 melanoma patients treated with TCV during 1990–2001 (21 per CBRG 91–08 and 53 per CBRG 9212). An analysis of data pooled data from these three trials included 98 TCV and 72 DCV patients. Characteristics were similar in terms of age (median 51 vs 52 years), gender (38% female in both), no evidence of disease at the time of treatment (46 vs 47%), and presence of M1c visceral disease at the time of treatment (13 vs 14%). Overall survival is about three times longer in patients treated with DCV (median 63 vs 21 months), and 5-year survival rates are twice as high (51 vs 26%) with a significant difference in the Kaplan-Meier survival curves ($p < 0.001$).

Prognostic and Predictive Markers

In the course of these trials we have examined a variety of possible biological markers and clinical features that are associated with therapeutic benefit. Because of issues of sample size, most of these analyses have been in the context of clinical trials in patients with metastatic melanoma. In subsets of patients levels and changes of serum gangliosides and thymus activation-regulated chemokine (TARC) have shown some correlation with progression free survival (time to progression) (Selvan et al. 2008; Cornforth et al. 2009). Elevated serum levels of S100b and lactate dehy-

drogenase (LDH) have been prognostic of worse survival (Schiltz et al. 2008) but do not appear to be independent variables (Dillman et al. 2011). Because of the difficulties in accurately defining dates of disease progression, overall survival is still the best endpoint for such correlations.

Delayed Type Hypersensitivity Reaction to Autologous Tumor Cells

DTH reaction to i.d. injections of autologous tumor cells appeared encouraging in our initial trials combining all tumor types (Dillman et al. 2002), but longer follow up and injection of tumor cells grown in serum free media, have failed to support this observation. A major issue is whether such tests are a predictive marker of successful immune enhancement, or merely a prognostic marker for inherent immune competence as an epiphenomenon of disease burden. As we developed better tumor samples for skin testing using serum free media, the rates of DTH response decreased to a level for which meaningful correlation was no longer possible. In the most recent analysis, of 139 patients treated with TCV who had the tumor DTH test measured at baseline, only 13 had a positive DTH test at baseline. Their survival was the same as for the 126 patients who had a negative DTH test (median 20 months for both, 5-year survival 23 vs 27%).

Only 109 of the 139 patients had a DTH test at week-4 after three weekly injections of DCV. The most common reason for not being retested was rapid progression of disease. Of these 109 patients, six were positive at both baseline and week-4, three changed from positive to negative, and 27 converted from negative to positive. Survival for the 33 patients who had a positive DTH at week-4 was not better than for the 76 patients who had a negative DTH at week-4 (median 22 vs 21 months, 5-year survival 29 vs 32%). Theoretically patients whose immune systems are already recognizing their tumor, but their disease is progressing anyway, are unlikely to benefit from further boosting of the immune system. A comparison of the 27 patients whose DTH converted from negative to positive, to the 76 who were negative at both baseline and week-4, also showed no difference in survival (median 21 months for both, 5-year survival 28 vs 32%).

We examined this issue again in the 54-patient DCV trial in metastatic melanoma (Dillman et al. 2009). The skin test used in this trial differed from that used in the TCV trials in that cells used in the DTH test had all been grown in serum free medium. Such an analysis also introduced a bias because only patients who were doing well had an opportunity to get repeated DTH tests. No patients had a clearly positive test at baseline, and only 5 were DTH-positive to tumor cells at week-4. One of these patients died 6 weeks after enrollment because of a cerebrovascular event that was unrelated to melanoma, one died at 46 months of progressive disease, and three are still alive with more than 6 years of follow up. An updated analysis of those 54 patients, using the variable of whether patients ever had an equivocal or positive DTH test to autologous tumor at baseline, week-4 or week-12 ($n=13$) or not ($n=41$) does not suggest a difference in survival, with median of about 5 years in both groups ($p=0.74$).

Clinical Variables

Baseline clinical variables can be prognostic of survival. Cancer trials repeatedly identify older age, poor performance status, advanced stage of disease, and tumor burden as poor prognostic factors. Lactate dehydrogenase (LDH) as a marker of tumor burden is also prognostic of poor outcome in many studies. For the 54-patient DCV trial, univariate analyses identified eight features associated with improved survival: ECOG performance status (PS) of 0, no measurable disease at study entry, receiving eight vaccinations, age <50 years, normal baseline LDH, no history of visceral metastases, and anergy to standard skin tests (Dillman et al. 2011). Multivariate logistic regression for survival at 3.5 years identified six independent features: prior radiation therapy, younger age, male gender, Eastern Cooperative Oncology Group performance status of 0 (asymptomatic and no limitations in activity), higher numbers of cells administered during the first three injections, and lower numbers of viable cells administered during the first three injections (Dillman et al. 2011). This model correctly classified survival for 28/32 patients (87%) and death for 20/22 (91%). Most studies have suggested that female gender, as opposed to male gender, is better prognostic variable, but this has not been true in

all trials, and gender was not an important variable in the univariate analysis. Performance status is highly correlated with tumor burden and visceral metastases. Older age is an independent variable. Radiation has been suggested to induce a form of apoptosis that is more favorable for antigen loading onto DC, and may also decrease circulating regulatory T cells.

Laboratory Variables

It is interesting that the multivariate analysis included two variables related to the vaccine product (Dillman et al. 2011). The total number of cells injected should be a surrogate for quantity of antigen injected. The inverse relation to viable cells is consistent with a concept that apoptosing, but not yet phagocytosed tumor cells present in the final product may be contributing to immune stimulation. In univariate analyses the only laboratory variable associated with prolonged survival was *in vitro* resistance to IFN-γ (Cornforth et al. 2011a). In the 54-patient DCV melanoma trial, tumor cells were exposed to IFN- γ *in vitro* to enhance expression of histocompatibility antigens (HLA-1, HLA-2) (Cornforth et al. 2011b). However, this may have been an indirect assay for the presence of stem cells in the culture, since they typically express none or very low levels of HLA-1and HLA-2. When this variable was included in the multivariate analysis, the predictive accuracy increased to 27/29 (93%) for survival and 19/20 (95%) for death (Dillman et al. 2011). Laboratory variables that were not associated with survival included the expression of S100 on the original pathology specimen, baseline and week-4 levels of disialogangliosides, baseline and week-4 levels of thymus activation-regulated chemokine (TARC), the time and passages needed to reach 50 million TC, the level of expression of various melanoma-associated antigens including Mage, Mart-1, Mel-5, HMB45, S100, and tyrosinase level, increase of HLA-1 and HLA-2 expression, ELISPOT detection of autologous lymphocyte reaction to autologous tumor cells, and any of various DC variables including the numbers of lymphocytes and monocytes collected during leukapheresis, and the levels and changes in expression of CD11c, CD80, CD83, CD86 (Dillman et al. 2011).

Summation, Discussion, and Conclusions

In this chapter we summarize two decades of bench-to-bedside translational research in which cancer patients were treated with cell-based vaccine preparations derived from autologous self-renewing tumor cells.

Feasibility and Efficiency

Our experience over the past two decades shows that patient-specific active specific immunotherapy with antigens from autologous proliferating, self-renewing tumor cells is a feasible approach, but the production process needs to be more efficient and expeditious. The success rate for establishing cell cultures to produce the antigenic product needs to be higher, and ideally 100%. This will greatly increase the numbers of patients who might be treated with such products. In addition, the time required to grow a sufficient number of cells also needs to be decreased. During the time it takes to produce the product, many patients experience progressive disease and go on to other therapies from which they usually derive minimal or no benefit, and therefore the patient is never in a favorable clinical condition for potential benefit from such a therapy, which by design, will not have anti-cancer effects on more differentiated tumor cells, but rather is designed to prevent new cancer sites from emerging, or recurrence of existing sites of metastases that can be treated with other modalities. Expedience and efficiency should be achievable with a focused effort.

Efficacy

This patient-specific anti-tumor stem cell immunotherapy is associated with encouraging long-term survival rates, and results are superior in patients treated with DC loaded with antigen from such cells. It is generally held that metastatic melanoma and renal cell cancer are associated with 5-year survival rates of only about 10%.

However, such patients treated in our TC trials had 5-year survival rates between 25 and 30%, and the phase II trial with DCV suggested a 5-year survival of about 50% for melanoma. Because of the unintentional patient selection bias secondary patient referral, and to the time needed to develop the vaccine product, only randomized trials can establish the true degree of benefit. In metastatic melanoma, the results of our 42-patient randomized phase II trial are consistent with the results obtained in the earlier in the 74-patient TCV trial and 54-patient DCV trial. Randomized trials would be needed in other tumor types as well.

Toxicity

The toxicities associated with these treatments have been minimal, and generally limited to local injection-site reactions. This active specific immunotherapy is not associated with dermatologic, gastrointestinal, hematologic, hepatic, renal, or neurologic toxicities, or fatigue or hair loss. Although autoimmune reactions were a theoretical concern for this approach, such toxicities have not been observed.

Cost

The cost of cancer treatment, and the value of such treatments (benefit to cost), are garnering increasing scrutiny. Unfortunately many targeted therapies, including monoclonal antibodies and tyrosine kinase inhibitors have to be given indefinitely because of their mechanism of action; and, therefore are quite costly over time. These patient-specific therapies have been given in the outpatient setting intermittently over a few months. Although the initial unit cost for production is relatively high, the total cost associated with such therapy is much less than the $100,000 a year charges applied to recently approved therapies such as sipileucil-T and ipilimumab.

Cancer Patient Populations

This approach can be applied to tumor types other than melanoma and renal cell cancer. Even with the current limitations that are a consequence of the amount of time required to grow a sufficient number of cells for treatment, this approach could easily be applied to ovarian cancer, glioblastoma, and regionally advanced lung cancer, because these malignancies are all associated with a high mortality rate, for which there is initial standardized multidisciplinary therapy that takes several months to deliver. Many of these patients have a limited tumor burden after completing the standard therapy, and would be ideal candidates for such an adjunctive immunotherapy approach.

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Safe Resection of Hemangioblastoma Using Indocyanine Green Videoangiograghy

27

Yasuo Murai and Akira Teramoto

Contents

Abstract

The aim of the present study was to assess the usefulness of a new technique for surgical microscope-based indocyanine green videoangiography (ICGVAG) in the removal of cerebellar haemangioblastomas (HBs). Cerebellar HB was detected in five patients presenting with symptoms of vertigo and/or headaches and diagnosed on the basis of preoperative magnetic resonance imaging (MRI) and cerebral angiographic findings. None of the patients underwent any procedure prior to ICGVAG that would affect the ICG findings, such as perilesional haemostatic coagulation or ablation. In each patient, it was possible to judge the approximate location of the tumour in relation to the brain surface and to differentiate between the feeding and draining vessels. Following resection of the tumour, ICGVAG images confirmed that the mural nodule had been eliminated. None of the patients required blood transfusion, either during or after the surgery. For each patient, the lesion was pathologically confirmed as HB, postoperative contrast-enhanced MRI confirmed the absence of a residual tumour and diffusion-weighted MRI revealed no ischaemic changes. Differentiation between the feeding and draining vessels in the region of the lesion is particularly important for successful surgical removal of HBs. ICGVAG findings enabled easy vascular differentiation and were also useful for confirming that there was no residual tumour. Thus, ICGVAG is useful for the safe resection of HBs.

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Introduction

Currently, a critical issue (Wang et al. 2006) in the successful removal of cerebellar haemangioblastomas (HBs) is intraoperative assessment of feeding arteries, draining veins and the location of the mural nodule (Jagannathan et al. 2008). Additional techniques such as preoperative embolization have been suggested (Murai et al. 2012) to prevent haemorrhagic complications. Intraoperative digital subtraction angiography (DSA) is the gold standard (Murai et al. 2011b; Wang et al. 2006) technique for evaluating feeding arteries and draining veins. However, there are some drawbacks (Ferroli et al. 2011) to the DSA technique, including its technical complications, the time and cost and the use of an ionized agent in the operating room (Murai et al. 2009). A noninvasive technique for direct intraoperative evaluation of vessel patency is needed (Kim et al. 2011). The usefulness of indocyanine green videoangiography (ICGVAG) as a tool for intraoperative monitoring has been reported since approximately 1998 (Sakatani et al. 2000), and its use is particularly indicated during the neurosurgical treatment of vascular disorders (Raabe et al. 2003). Subsequently, ICGVAG has been reported to be an easy, rapid and inexpensive modality (Raabe et al. 2005). With regard to intracranial lesions, ICGVAG has been reported to be useful in cases (Woitzik et al. 2006; Takagi et al. 2007) of cerebral arterio venous malformation (Killory et al. 2009), cerebral aneurysm (Murai et al. 2011a), vascular reconstructive surgery (Woitzik et al. 2005), and cerebral infarction (Kuebler et al. 1998). ICGVAG is considered to be useful for confirming aneurysm occlusion, residual blood flow through perforators of approximately 0.3 mm in diameter and patency of reconstructed blood vessels (Raabe et al. 2005). However, there have been only a few reports (Britz et al. 2002; Hwang et al. 2010) indicating the usefulness of ICGVAG with regard to tumourous lesions (Murai et al. 2011c). Here we report the intraoperative ICGVAG findings in cases of cerebellar haemangioblastoma (HB) and discuss the usefulness of this imaging modality.

Technical Note

Tumour removal for cerebellar HB was performed in five cases using the Carl Zeiss Surgical Microscope OPMI Pentero INFRARED 800 (Carl Zeiss Co.; Tokyo, Japan). After craniotomy and dural incision, 10 mg/kg (25 mg/10 mL of distilled water) of ICG was intravenously infused through a peripheral blood vessel, followed by flushing with 10 mL of physiological saline. The feeding artery, draining vein and tumour lesion were then observed using ICGVAG (Murai et al. 2009).

Representative Case Presentation

A 43-year-old man presented with vertigo and headaches. Computed tomography, magnetic resonance imaging (MRI) and cerebral angiography revealed a cerebellar HB with the posterior inferior cerebellar artery (PICA) as its feeding artery. Craniotomy was performed through a midline suboccipital approach.

Following incision of the dura, the cisterna magna was opened and the cerebrospinal fluid was aspirated. The fluid content of the cyst was also aspirated and the operative field was secured. The site of the abnormal tortuous vessel around the lesion was confirmed from above the arachnoid (Fig. 27.1). ICG, at 10 mg/kg (25 mg/10 ml of distilled water), was intravenously infused through a peripheral blood vessel, flushing was performed with 10 ml of physiological saline, and visualization was achieved 9 s after ICG infusion.

The feeding artery (peripheral segment of PICA) (Fig. 27.2) was visualized before the arterial phase, while the draining vein (Fig. 27.3) was visualized before the venous phase. Thus, the feeding and draining vessels could be clearly differentiated. ICGVAG performed following tumour resection showed no abnormal staining that would correspond to a tumour shadow. Postoperative contrast-

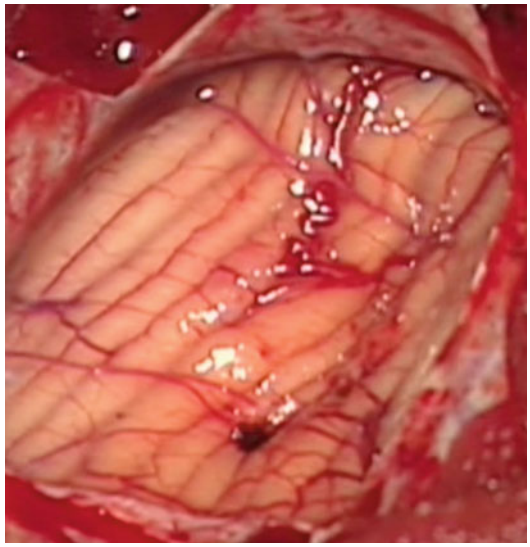

Fig. 27.1 The lesion site is unclear in the visual field of an ordinary microscope. It is difficult to distinguish the feeders and the drainers

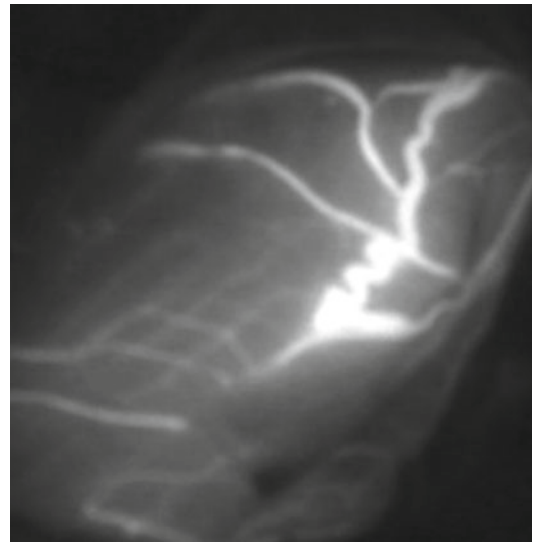

Fig. 27.3 An ICGVAG image taken 25 s after ICG infusion. The tortuous draining vessel has been enhanced

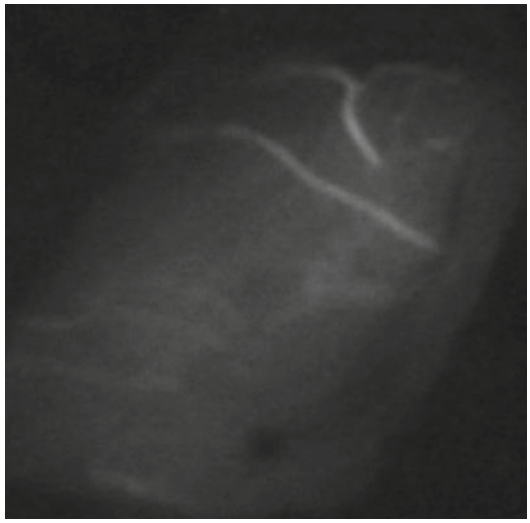

Fig. 27.2 An ICGVAG image taken 9 s after ICG infusion. The feeding artery is visualized; however, the draining vessel has not yet been enhanced

enhanced MRI on day 2 after resection also confirmed that the tumour lesion had been totally removed. The postoperative course was uneventful and the tumour was pathologically diagnosed as HB. On day 43 after resection, ICGVAG revealed no abnormal staining that would correspond to a tumour shadow, and postoperative contrast-enhanced MRI also confirmed complete removal of the tumour.

Indocyanine Green Videoangiography for Neurosurgery

ICGVAG is a new intraoperative vascular imaging modality in the field of neurosurgery. Raabe et al. (2003) reported their clinical experience with ICGVAG as an intraoperative vascular imaging technique for evaluating cerebral vascular surgery. With regard to its usefulness in comparison with invasive intraoperative cerebral angiography, ICGVAG is an easy (Murakami et al. 2011), rapid and inexpensive modality (Takagi et al. 2007). With regard to intracranial vascular disease, ICGVAG is useful in cases of cerebral aneurysm (Raabe et al. 2005), vascular bypass surgery (Woitzik et al. 2006), cerebral vascular malformations (Takagi et al. 2007) and ischaemic lesions (Kuebler et al. 1998). There have also been reports of the usefulness of this imaging modality in a small number of patients with tumorous lesions (Murai et al. 2011c; Hansen

et al. 1993). Hwang et al. (2010) and the authors (Murai et al. 2011a) reported that ICG may provide visual enhancement of such lesions. There have been only one report (Murai et al. 2011a) of its application in patients with cerebral cavernous angiomas located in the optic chiasma. In comparison with digital subtruction angiography, which is a radiographic test, ICGVAG is a fluorescence test and is unable to depict the lesion site if the suffusing light is blocked by the brain tissue, cranial nerves or vessels (Utsuki et al. 2010). Therefore, a lesion in the brain parenchyma can only be observed if the perilesional tissue is removed. However, procedures such as coagulation, compression or ablation around the lesion may affect the ability to depict the lesion itself and/or the image findings, thereby making it difficult to achieve objective evaluation of the lesion. As cerebral cavernous angiomas are usually located in the deep brain parenchyma, it is comparatively rare to obtain ICGVAG cavernoma images that are unaffected by surgical procedures. We are unaware of any such reports to date. In this study (Murai et al. 2011a), the cavernous angioma was depicted as an avascular area, a finding similar to the usual findings with cerebral angiography. Collection of data from more such cases is required. In comparison to cerebral cavernous angioma, HB lesions are often found as mural nodules located close to the brain surface (Wang et al. 2001). Therefore, ICGVAG retain the potential to be useful tools as intraoperative imaging to remove the HB. However, there are only a small number (Murai et al. 2011b; Kim et al. 2011) of clinical reports regarding the application of ICGVAG in patients with cerebellar haemangioblastomas.

Indocyanine Green Videoangiography for Haemangioblastoma

Manipulation of tumour or vascular anatomy and normal brain tissues or vessels is always important in brain tumour surgeries (Wang et al. 2006). ICGVAG has been indicated during the surgical resection of cerebral arteriovenous malformations (AVM) (Takagi et al. 2007). Because HB is angiographically similar to such malformations, it is probable that ICGVAG can be conveniently applied to the surgical removal of HBs. In our study (Murai et al. 2011a), the HB, feeding artery and draining veins could be clearly visualized by ICGVAG, thus aiding in the clarification of tumour borders and decreasing the extent of necessary resection. There have recently been additional reports concerning ICGVAG findings of haemangioblastomas in the cerebellum and spinal cord. (Hwang et al. 2010; Murakami et al. 2011) both reported cases with spinal haemangioblastomas.

HB lesions more often present as mural nodules located close to the brain surface (Wang et al. 2001) rather than in the deep areas. The lesion is partially exposed on the cerebellar surface, and the blood vessels from the tumour form an abnormal vascular network on the brain surface directly above. These anatomical characteristics make ICGVAG convenient for intraoperative examination of HBs. For cases of AVM, which are often located deep in the brain (Takagi et al. 2007), it is necessary to employ cerebral angiography, which is a radiographic test capable of seeing through the brain. In comparison with cerebral angiography, ICGVAG, which is a fluorescence test, is unable to depict the lesion site if the light is blocked by the brain parenchyma (Raabe et al. 2003). Therefore, a lesion in the brain parenchyma can only be observed with ICGVAG if the perilesional tissue is first removed. ICGVAG is currently under evaluation for future application in the differential diagnosis of imaging findings (Ferroli et al. 2011), and the present cases provide important examples of intraoperative ICGVAG imaging of HBs.

In addition to confirming the location of the tumour body, successful resection of HBs requires differentiation between the feeding and draining vessels and evaluation of the presence/absence of residual tumour tissue (Britz et al. 2002). Wang et al. (2006) reported that their surgical principles for HB are similar to that for AVM: selective division of feeding arteries, preservation of the main draining veins and protection of vessels across the tumour surface during dissection, and they are crucial for total tumour resection. Cleavage of the main draining veins, which are

extremely dilated, should be carried out at the last moment. Jagannathan et al. (2008) also reported that care should be taken to coagulate and sharply interrupt vessels individually as they enter or leave the tumour capsule and that vessels supplying the tumour should be cauterized and sharply transected. With regard to differentiating between the feeding and draining vessels, the blood in the draining vessels is predominantly arterial blood due to shunting, but both types of blood vessels are abnormal compared to ordinary vessels. Therefore, it is not easy to differentiate between the feeding and draining vessels on the basis of their appearance. However, ICGVAG allows ready differentiation because it permits observation of the direction and time flow of blood (Takagi et al. 2007). This may also contribute to the prevention of intraoperative blood loss. Wang et al. 2006 reported the usefulness of intraoperative sonographic guidance and (Utsuki et al. 2010) suggested that fluorescence diagnosis performed using 5-aminolevulinic acid can provide useful information when making the choice to remove HBs. In addition, the existence of a residual tumour can be confirmed on the basis of marked contrast enhancement of the lesion with ICGVAG.

In conclusion, ICGVAG has many advantages compared to intraoperative digital subtraction angiography. ICGVAG was found to be very useful for detecting the feeding artery, draining vein and tumour mass before tumour removal. After resection of the tumour, ICGVAG findings immediately indicated the patency of the peritumoural normal vessels and the presence of any residual tumour.

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28 Overcoming Chemotherapy Resistance by Targeting Hyaluronan/CD44-Mediated Stem Cell Marker (Nanog) Signaling and MicroRNA-21 in Breast, Ovarian, and Head and Neck Cancer

Lilly Y.W. Bourguignon

Contents

Abstract

Multidrug resistance frequently contributes to the failure of chemotherapeutic drug treatments in patients diagnosed with solid tumors such as breast, ovarian and head and neck cancers. It is now certain that oncogenic signaling is directly involved in chemotherapeutic drug resistance and tumor progression. A number of studies have aimed at identifying those molecules which are expressed specifically by epithelial tumor cells and correlate with metastatic behavior and chemotherapy resistance. Among such candidate molecules is hyaluronan (HA), the major glycosaminoglycan component of extracellular matrix (ECM). HA serves not only as a primary constituent of connective tissue extracellular matrices but also functions as a bio-regulatory molecule. Pertinently, HA is enriched in many types of tumors. HA is capable of binding to CD44 which is a ubiquitous, abundant and functionally important receptor expressed on the surface of many normal cells and tumor cells.

Recent evidence indicates that HA-CD44 interaction with the stem cell marker, Nanog promotes downstream, intracellular signaling pathways that influence multiple cellular functions. In particular, certain microRNAs such as miR-21 (small RNA molecules with ~20–25 nucleotides) have been shown to play roles in regulating tumor cell survival and chemotherapy resistance. In this article, a special focus is placed on the role of Nanog overexpression in activating oncogenic signaling

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molecule(s) and miRNA-21 function leading to the concomitant onset of HA/CD44-mediated tumor cell activities (e.g., survival and chemoresistance) and tumor progression. Conversely, donwregulation of Nanog not only inhibits miR-21 expression/function and HA/CD44-mediated tumor cell behaviors but also enhances chemosensitivity. This new knowledge could serve as groundwork for the future development of new drug targets to inhibit the stem cell marker (Nanog) signaling and miR-21 function in order to overcome chemotherapy resistance in the progression of HA/CD44-induced solid tumor cancers such as breast, ovarian and head and neck cancer.

Introduction

Myriad studies have attempted to identify specific adhesion molecule(s) expressed in solid tumor cancer [e.g., breast cancer, ovarian cancer and head and neck squamous cell carcinoma (HNSCC)] that correlate with tumor cell invasive behavior(s). Among such candidate molecules is hyaluronan (HA) which is a nonsulfated, unbranched glycosaminoglycan consisting of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine (Lee and Spicer 2000). HA, which is detected in the extracellular matrix (ECM) of most mammalian tissues, is synthesized by specific HA synthases and digested into various smaller-sized molecules by hyaluronidases. Most importantly, HA has been found to accumulate at tumor cell attachment sites and appears to play an important role in promoting cancer-specific behaviors (Toole et al. 2002).

CD44 denotes a family of cell-surface glycoprotein receptors which are expressed in a variety of human solid neoplasms, particularly those classified as breast cancer, ovarian cancer and HNSCC (Bourguignon et al. 2008; Wang and Bourguignon 2011). Nucleotide sequence analyses reveal that many CD44 isoforms (derived by alternative splicing mechanisms) are variants of the standard form, CD44s (Screaton et al. 1992). The presence of high levels of CD44 variant (CD44v) isoforms is emerging as an important metastatic tumor marker in a number of cancers including human breast cancer, ovarian cancer and head and neck cancers (Bourguignon 2008; Wang and Bourguignon 2011). Recent studies have shown that CD44 is also expressed in tumor stem cells which have the unique ability to initiate tumor cell-specific properties (Al-Hajj et al. 2003). In fact, CD44 appears to be an important surface marker for cancer stem cells (Al-Hajj et al. 2003). All CD44 isoforms contain an HA-binding site in their extracellular domain and thereby serve as a major cell surface receptor for HA. Importantly, both CD44 isoform and HA are overexpressed/elevated at sites of tumor attachment (Toole et al. 2002). HA binding to CD44 isoform not only affects cell adhesion to extracellular matrix (ECM) components, but also is involved in the stimulation of a variety of tumor cell-specific functions leading to cytoskeleton function and tumor progression (Bourguignon 2008). Multidrug resistance frequently contributes to the failure of chemotherapeutic drug treatments in patients diagnosed with solid tumors, such as breast cancer, ovarian cancer and head and neck cancer (Bourguignon et al. 2008, 2012; Wang and Bourguignon 2011). P-glycoprotein (P-gp), the product of the *MDR1 (ABCB1)* gene, is a transmembrane ATP-dependent transporter molecule known to play a critical role in drug fluxes and chemotherapeutic resistance in a variety of cancers (Higgins 1992). Interestingly, recent studies indicate that both HA and CD44 are also involved in chemotherapeutic drug resistance in many cancer types (Bourguignon et al. 2008, 2012; Wang and Bourguignon 2011). Specifically, HA binding is capable of stimulating *MDR1* expression and drug resistance in tumor cells (Bourguignon et al. 2008). In addition, CD44 interacts with P-gp to promote cell migration and the invasion of tumor cells (Bourguignon et al. 2008). Furthermore, HA/CD44-mediated ErbB2 signaling and PI3 kinase/AKT-related survival pathways are also involved in chemotherapeutic drug resistance in tumor cells. Previously, we reported that activation of several other HA-CD44-mediated oncogenic signaling pathways [e.g., intracellular Ca^{2+} mobilization, epidermal growth factor receptor (EGFR)-mediated ERK signaling and topoisomerase activation] leads to multidrug resistance in

HNSCC cells (Wang and Bourguignon 2011). However, the cellular and molecular mechanisms controlling the ability of CD44 isoform-positive solid tumor cells to migrate, invade and resist chemotherapy treatment remain to be poorly understood.

The stem cell marker, Nanog is an important transcription factor involved in the self-renewal and maintenance of pluripotency in the inner cell mass (ICM) of mammalian embryo and of embryonic stem (ES) cells (Mitsui et al. 2003). Nanog signaling is regulated by interactions among various pluripotent stem cell regulators (e.g., Rex1, Sox2 and Oct3/4) which together control the expression of a set of target genes required for ES cell pluripotency (Mitsui et al. 2003). These findings confirm the essential role of Nanog in regulating a variety of cellular functions. Recently, several tumor cell types have been shown to express common embryonic stem cell markers including Nanog (Bourguignon et al. 2008, 2009a, 2012). The Nanog family of proteins has also been found to act as growth-promoting regulators in different tumor cells (Bourguignon et al. 2008, 2009a, 2012). Our recent work indicates that HA binding to epithelial tumor cells promotes Nanog protein association with CD44, followed by Nanog activation and the expression of pluripotent stem cell regulators (e.g., Rex1 and Sox2) (Bourguignon et al. 2008). These findings strongly suggest that HA/CD44 signaling and Nanog function are tightly linked. Several studies have focused on enhancing chemosensitivity using various Nanog signaling perturbation approaches. This review will focus on stem cell marker, Nanog and its role in regulating tumor cell survival and multi-drug resistance during HA/CD44-activated signaling pathways in breast cancer, ovarian cancer and head and neck cancer.

Stem Cell Marker (Nanog) Expression and HA/CD44-Mediated Chemotherapy Resistance in Breast and Ovarian Cancer

Multidrug resistance is a challenging clinical problem in the treatment of both breast and ovarian cancers (Harnett et al. 1986). The most commonly used anti-tumor agents in the treatment of disseminated breast or ovarian cancers include doxorubicin and paclitaxel (taxol) (Gewirtz 1999). The ability of doxorubicin to bind DNA and/or produce free radicals is thought to be possible mechanisms to induce cytotoxic effects on tumor cells (Gewirtz 1999). Paclitaxel is known to bind a subunit of the tubulin heterodimers that form cellular microtubules (Bhalla 2003). The paclitaxel-cytoskeletion interaction accelerates tubulin polymerization and inhibits the depolymerization of microtubules resulting in an inactivation of the mitotic checkpoint and tumor cell killing (Bhalla 2003). Resistance to chemotherapeutic drugs used in breast and ovarian cancer treatments (e.g., doxorubicin and paclitaxel) is often associated with overexpression of the multidrug resistance gene 1 [MDR1 or P-glycoprotein (P-gp)] (Higgins 1992; Baker and El-Osta 2004). The MDR1 gene product functions as a drug efflux pump actively reducing intracellular drug concentrations in resistant tumor cells (Higgins 1992; Baker and El-Osta 2004). However, the biological factors determining the ability of certain breast and ovarian epithelial cells to overexpress the *MDR1* gene and display the multidrug resistant phenotype remain to be poorly understood. It is currently very difficult to predict the efficacy of any particular chemotherapeutic drug treatment. Thus, there is a great need to clarify the tumor biology mechanisms underlying the clinical behavior of multidrug resistance in both breast and ovarian tumor cells.

A number of studies indicate that HA, CD44 and Nanog are overexpressed in solid tumors including breast and ovarian cancers, and that these three molecules are all implicated in the initiation and development of malignancy (Bourguignon et al. 2008, 2009a, b, 2012). Thus, identifying the specific extracellular matrix-surface receptor (i.e., HA-CD44)-mediated signaling events plus the Nanog function(s) required for activation of epithelial tumor cells could be very important for understanding the disease mechanisms occurring during breast and ovarian tumor progression. Our recent results reveal that HA binding to breast or ovarian tumor cells promotes Nanog protein association with CD44 followed by Nanog activation and the expression

of pluripotent stem cell regulators (e.g., Rex1 and Sox2) leading to doxorubicin and paclitaxel (taxol) resistance (Bourguignon et al. 2008).

Furthermore, we found that overexpression of Nanog by transfecting tumor cells (e.g., breast or ovarian tumor cells) with Nanog cDNA stimulates Rex1/Sox2 gene expression and breast/ovarian tumor cell behaviors (e.g., MDR1 overexpression, and multidrug resistance) (Bourguignon et al. 2008). Downregulation of Nanog signaling by transfecting tumor cells with Nanog siRNA not only blocks HA/CD44-mediated breast/ovarian tumor cell behaviors but also enhances chemosensitivity (Bourguignon et al. 2008). Taken together, these findings suggest that targeting HA/CD44-activated Nanog signaling pathways may represent a novel approach to overcome chemotherapy resistance in some breast and ovarian tumor cells displaying stem cell marker properties during tumor progression.

Stem Cell Marker (Nanog) Signaling and MicroRNA-21 (miR-21) Function in HA/CD44-Mediated Chemotherapy Resistance in Breast Cancer

The microRNAs (miRNAs) are evolutionarily conserved and function as negative regulators of gene expression by inhibiting the expression of mRNAs that contain complementary target sites referred to as the "seed region" (Cowland et al. 2007). Previous data has revealed that human miRNAs are processed from capped and polyadenylated transcripts, that are precursors to the mature miRNAs (pri-miRNAs) (Cai et al. 2004). In mammalian miRNA biogenesis, primary transcripts of miRNA genes (pri-mRNAs) are subsequently cleaved to produce an intermediate molecule containing a stem loop of ~70 nucleotides (pre-mRNAs) by the nuclear RNase III enzyme DROSHA and exported from the nucleus by Exportin 5 (Cowland et al. 2007). A second RNase III enzyme Dicer then generates the mature miRNA which is loaded into the RNA-induced silencing complex (RISC) in association with the argonaute protein (Ago) that induces silencing via the RNA interference pathway (Valencia-Sanchez et al. 2006). Although Dicer has an important role in the silencing action of miRNAs, recent studies have shown that silencing can still occur in cells that lack Dicer (Giraldez et al. 2005). It has recently been shown that the nuclear p68-RNA helicase is required in the uptake of certain miRNAs into the silencing complex (Salzman et al. 2007). p68 belongs to a family of proteins that are involved in RNA metabolism processes such as translation and RNA degradation (de la Cruz et al. 1999). A previous study showed that miR-21 processing or biogenesis (via the precursor pri-miR-21) required p68 and DROSHA in breast tumor cells (Davis et al. 2008). Several transcription factors, including Nanog, also appear to be involved in the regulation of pri-miRNA expression during development (Lee et al. 2004).

Accumulating evidence indicates the involvement of non-coding microRNAs (miRNAs, approximately 22-nucleotides) in both cancer development and multidrug resistance (Bourguignon et al. 2009a, 2012). Analysis of the array profile of miRNA expression in normal breast and breast carcinoma tissues reveals that miRNA-21 (miR-21) is abundantly produced in tumors compared to normal tissues. The functional significance of miR-21 has been elucidated in several recent studies following the discovery of its specific targets (Asangani et al. 2008). miR-21 is now one of the most studied miRNAs due to its involvement in cancer progression. It has recently been shown that miR-21 plays a role in the inhibition of a tumor suppressor protein such as Program Cell Death 4 (PDCD4) via a conserved site within the 3'-UTR (3'-untranslated region) of the mRNA (Asangani et al. 2008). Downregulation of PDCD4 expression by miR-21 leads to tissue invasion and metastasis (Asangani et al. 2008). Thus, miR-21 is currently considered to be an oncogene.

In a recent study we investigated a novel HA/CD44-mediated Nanog signaling mechanism that regulates the biogenesis of miR-21 production leading to the tumor suppressor protein (PDCD4) reduction, inhibitors of the apoptosis family of proteins (IAPs)/MDR1 (P-gp) overexpression, anti-apoptosis and chemoresistance in breast tumor cells (Bourguignon et al. 2009a). Inhibition of either Nanog signaling or silencing miR-21 expression/function by transfecting breast tumor

cells with Nanog siRNA or anti-miR-21 inhibitor not only results in PDCD4 upregulation and PDCD4-eIF4A complex formation, but also causes a reduction of IAP/MDR1 (P-gp) and an enhancement of apoptosis and chemosensitivity (Bourguignon et al. 2009a). These findings provide important new insights into understanding the roles that HA/CD44-mediated Nanog-miR-21 signaling play in regulating anti-apoptosis and chemotherapy resistance in breast tumor cells.

Stem Cell Marker (Nanog)-Regulated Stat-3 Signaling and MicroRNA-21 (miR-21) in HA/CD44-Mediated Chemotherapy Resistance in Head and Neck Cancer

Head and neck squamous cell carcinoma (HNSCC) is a malignancy that involves cancers of the lip, oral cavity, pharynx, hypopharynx, larynx, nose, nasal, sinuses, neck, ears and salivary glands (Parkin et al. 2005). Advanced HNSCC is an aggressive disease associated with major morbidity and mortality. The 3-year survival rate for patients with advanced-stage HNSCC treated with standard therapy is only 30–50 %. Resistance to standard therapy continues to be a limiting factor in the treatment of HNSCC. Nearly 40–60%of HNSCC patients subsequently develop chemoresistance, locoregional recurrences or distant metastases (Parkin et al. 2005). Thus, clarification of key aspects of tumor cell functions underlying the clinical behavior of HNSCC is greatly needed.

Cisplatin is the most common anti-head and neck chemotherapy used today. The ability of this drug to induce tumor cell death is often counteracted by the presence of anti-apoptotic regulators leading to chemoresistance (Wang and Bourguignon 2011). Several lines of evidence point toward the IAP family (e.g., c-IAP-1, c-IAP-2 and XIAP) playing a role in oncogenesis via their effective suppression of apoptosis (Hunter 2007). The mode of action of IAPs in suppressing apoptosis appears to be through direct inhibition of caspases and pro-caspases (primarily caspase 3 and 7) (Hunter 2007). IAPs also support chemoresistance by preventing tumor cell death induced by anticancer agents. Although certain anti-apoptotic proteins (e.g., Bcl-xL) have been shown to participate in anti-apoptosis and chemoresistance in HA/CD44-activated breast tumor cells (Bourguignon et al. 2009b), the involvement of IAPs in HA/CD44-mediated HNSCC cell survival and chemoresistance has just begun to be elucidated.

The "signal transducer and activator of transcription protein 3" (Stat-3) was initially identified as APRF (Acute Phase Response Factor), an inducible DNA binding protein that binds to the IL-6-responsive element within the promoters of hepatic acute phase genes (Darnell 1997). Accumulating evidence indicates that Stat-3 also plays an important role in regulating cell growth, differentiation, and survival. Nanog and Stat-3 also appear to be both structurally linked and functionally coupled in HA/CD44 signaling during epithelial tumor cell activation (Bourguignon et al. 2008).

A number of studies indicate that more than 50%of microRNA [(miRNA), small RNA molecules with ~20–25 nucleotides] are located in cancer-related genomic regions or fragile sites, suggesting that miRNA may be closely associated with the pathogenesis of a variety of cancers including HNSCC (Chang et al. 2008). Analysis of an array profile of miRNA expression in HNSCC tissues reveals that miR-21 is abundantly produced in these tumors compared to normal tissues. The functional significance of miR-21 has been elucidated in several recent studies following the discovery of its specific targets (Asangani et al. 2008), making miR-21 one of the most-studied miRNAs due to its involvement in cancer progression. Importantly, miR-21 plays a role in the inhibition of tumor suppressor proteins such as Program Cell Death 4 (PDCD4), via a conserved site within the 3'-UTR (3'-untranslated region) of the mRNA (Asangani et al. 2008; Bourguignon et al. 2009a). Downregulation of PDCD4 expression by miR-21 leads to tumor cell growth, survival, chemoresistance, invasion and metastasis (Bourguignon et al. 2009a). Thus, miR-21 is currently considered to be an oncogenic miRNA.

In our recent study we discovered a new matrix HA/CD44-mediated Nanog/Stat-3 signaling mechanism that regulates miR-21 production

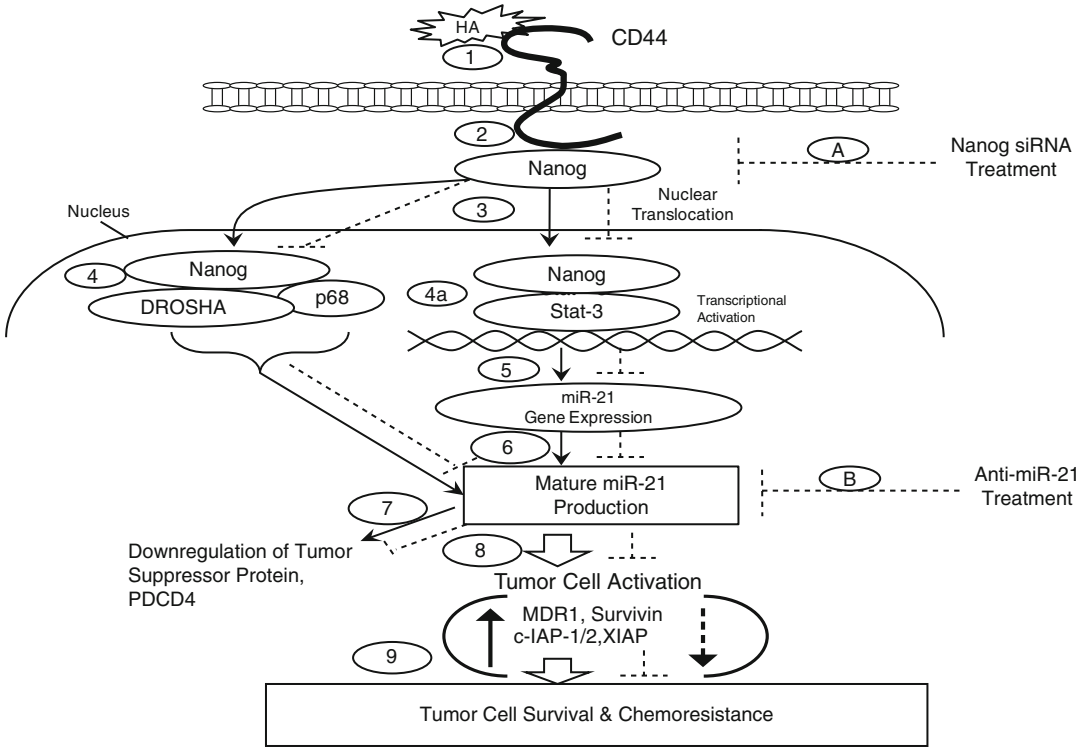

Fig. 28.1 A proposed model for HA/CD44-mediated Nanog signaling in the regulation of oncogenesis and chemotherapy resistance in solid tumor progression

and cisplatin chemoresistance in HNSCC cells (Bourguignon et al. 2012). Specifically, our results indicate that HA/CD44 activates Nanog-Stat-3 signaling which, in turn, stimulates miR-21 expression and function. These events lead to the reduction of the tumor suppressor protein, PDCD4, upregulation of survival proteins, IAP family (e.g., c-IAP-1, c-IAP-2 and XIAP) and cisplatin chemoresistance in HNSCC cells (Bourguignon et al. 2012). Inhibition of either Nanog/Stat-3 signaling or silencing miR-21 expression/function not only results in PDCD4 upregulation, but also causes a reduction of survival protein expression and an enhancement of cisplatin chemosensitivity to cisplatin (Bourguignon et al. 2012). Thus, our findings strongly support the contention that Nanog, Stat-3, and miR-21 form a functional signaling axis that regulates tumor cell survival and cisplatin chemoresistance in HNSCC cells.

Proposed Models for Stem Cell Marker (Nanog) Signaling and MicroRNA-21 Function in Oncogenesis and Chemotherapy Resistance in Solid Tumor Progression

The stem cell marker (Nanog)-mediated tumor cell survival and chemoresistance are closely linked to oncogenic signaling pathways and miRNA-21 production in HA/CD44-associated solid tumor cancers including breast, ovarian and head and neck cancers. Our current models for illustrating HA-dependent and CD44-specific Nanog activation and miR-21 signaling pathways are described as follows:

Specifically, HA binding to tumor cell surface(s) (Fig. 28.1-step 1) promotes CD44 association with the stem cell marker, Nanog (Fig. 28.1-step 2) which then becomes translocated

from the cytosol to the nucleus (Fig. 28.1-step 3, indicated by the solid line) and interacts with the microprocessor complex containing the RNAase III (DROSHA) and the RNA helicase (p68) (Fig. 28.1-step 4, indicated by the solid line) and/or Stat-3 (Fig. 28.1-step 4a, indicated by the solid line) which then binds to an upstream/enhancer region (containing Stat-3 binding sites) of the miR-21 promoter (Fig. 28.1-step 5, indicated by the solid line), resulting in miR-21 gene expression and mature miR-21 production (Fig. 28.1-step 6, indicated by the solid line). The resultant miR-21 then functions to downregulate the tumor suppressor protein (PDCD4) (Fig. 28.1-step 7, indicated by the solid line). Subsequently, these changes result in the expression of MDR1 and survival proteins, IAPs (e.g., survivin, c-IAP1/2 and XIAP) (Fig. 28.1-step 8, indicated by the solid line) and stimulation of anti-apoptosis, survival and chemoresistance (Fig. 28.1-step 9, indicated by the solid line) in tumor cells. In direct contrast, downregulation of Nanog or miR-21 [by treating tumor cells with Nanog siRNA (Fig. 28.1-step A, indicated by the dash line) or anti-miR-21 inhibitor (Fig. 28.1-step B, indicated by the dash line), respectively] blocks Nanog nuclear translocation (Fig. 28.1-step 3, indicated by the dash line) and Nanog-p68/DROSHA complex formation (Fig. 28.1-step 4, indicated by the dash line), abolishes Nanog-Stat-3 binding/miR-21 gene expression (Fig. 28.1-step 5, indicated by the dash line) and inhibits miR-21 production (Fig. 28.1-step 6, indicated by the dash line) leading to the expression of tumor suppressor protein (PDCD4) (Fig. 28.1-step 7, indicated by the dash line). Subsequently, these changes result in the inhibition of MDR1 and IAPs (survivin, c-IAP1/2 and XIAP) expression (Fig. 28.1-step 8, indicated by the dash line), stimulation of apoptosis/cell death as well as enhancement of chemosensitivity (step 1–step 9, indicated by the dash line) in tumor cells. This newly-discovered Nanog signaling pathway leading to miR-21 functioning should provide important drug targets for sensitizing tumor cell apoptosis and overcome chemoresistance in HA/CD44-activated cancer cells such as breast, ovarian and head and neck cancers.

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Part VI

Stem Cells for Regenerative Medicine

Urinary Bladder Regenerative Medicine

29

Manoj V. Rao and Arun K. Sharma

Contents

Abstract

One of the greatest challenges in urology is the surgical replacement of urinary bladder tissue. In a variety of pathological conditions, the bladder must be replaced or its capacity expanded in order to store urine at a low pressure, avoiding potential damage to the kidneys. The gold standard in current practice involves the incorporation of a vascularized patch of detubularized bowel into the urinary bladder, leading to a wide variety of complications with the potential loss of the ability to empty urine in a volitional manner. In an effort to circumvent these issues, multiple investigators have utilized different scaffolding materials in hopes of avoiding the continued reliance on intestinal tissue in bladder replacement surgery. The optimal biomaterial would be able to reproduce the function, elasticity, vascularity, and innervation of the native bladder tissue so as to recreate normal voiding, ideally combined with multipotent stem/progenitor cells. Although the histological and physiological function of the urinary system is complex, decades of research into bladder tissue engineering has lead to significant progress in the quest for the ideal biomaterial.

Introduction

The normal function of the urinary bladder is to store and expel urine at a low pressure with the appropriate neurological cues. This ability is due

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to the compliant viscoelastic bladder wall receiving the appropriate neural signal to accommodate increasing volumes of urine. While multiple disease states exist in which the bladder is unable to perform this task, the management of spinal cord injury patients circa World War I highlights the importance of normal bladder function (Silver 2011). At the time of World War I, a major cause of death amongst spinal cord injury patients was pyelonephritis attributable to the mismanagement of the dennervated bladder. Many of these patients, due to the inability to expel urine against a closed or a dyssynergic urinary sphincter, stored urine at a high bladder pressure. A landmark discovery in the care of patients with spinal cord anomalies was the importance of maintaining a low bladder leak point pressure (under 40 cm H_2O) in order to prevent urine refluxing into the kidneys leading to kidney injury and pyelonephritis (McGuire et al. 1983).

With long standing outlet obstruction, the urinary bladder wall (likely due to ischemia) undergoes a variety of microscopic architectural changes ultimately resulting in the loss of its compliance due to decreased elasticity. Using the formula compliance = volume/pressure, as the volume of urine increases in the urinary bladder, a poorly compliant bladder will have increasing internal pressures, leading to pressure transmission to the kidneys. Although there is no universally agreed upon value for a compliant urinary bladder wall, the trend of the bladder pressure as it distends with fluid may be used to determine compliance in the setting of urodynamic studies. With the loss of compliance, hypertrophy and scarring of the bladder wall may also lead to a decreased bladder capacity. The ability to maintain a safe bladder pressure with an adequate capacity is an invaluable part of treatment in any surgery aiming to replace or expand the bladder capacity.

Medical treatments are utilized to maintain a low bladder pressure in patients with spinal cord anomalies, such as myelodysplasia or traumatic spinal cord injuries, and those who have end organ damage from bladder outlet obstruction, such as in posterior urethral valves or long-standing benign prostatic hyperplasia. Anticholinergic medications, to relax the bladder wall, and clean intermittent catheterization, to empty the bladder before high pressures are reached, are typically utilized as a first-line treatment for patients with high pressure, poorly compliant or small capacity bladders in order to prevent hydroureteronephrosis and potential kidney damage. However, when medical interventions fail, the gold standard surgical treatment remains augmentation cystoplasty using bowel interposition to increase the volume of urine the bladder can accommodate at a low pressure as well as to increase the bladder capacity.

The underlying principle inherent to any bladder augmentation or replacement surgery is LaPlace's Law, in which bladder pressure = wall tension/radius. Assuming the wall tension remains constant, the goal of any bladder surgery is to provide a large radius in order to maintain a low bladder pressure, preventing reflux of urine to the kidneys. Using this law, the optimal reservoir for urinary storage should be spherical in shape similar to a healthy urinary bladder. Other than patients with spinal cord abnormalities and bladder outlet obstruction, bladder replacement surgery may also be utilized for multiple urological conditions.

In 2012, over 70,000 patients will be diagnosed with bladder cancer and about 15,000 will die from this disease (Siegel et al. 2012). The cost per patient for bladder cancer is higher than all other cancers due to rigorous tumor surveillance protocols – the overall annual cost of bladder cancer is nearly $4 billion in the United States (Botteman et al. 2003). Although over 70% of bladder cancer patients will be diagnosed with non-muscle invasive bladder cancer, select cases of bladder cancer, such as cancer within a bladder diverticulum or an urachal remnant, a partial cystectomy with intestinal augmentation may be used. Patients with more advanced or aggressive forms of disease may require complete surgical removal of the urinary bladder and pelvic lymph nodes with incorporation of the ureters into a detubularized segment of bowel as a means to drain urine at a low pressure. Other than cancer and myelomeningocele requiring bladder surgery, a small capacity bladder due to radiation therapy, tuberculosis, or bladder exstrophy also may require augmentation of the bladder with intestinal tissue.

Any portion of the gastrointestinal tract from stomach to the colon may be utilized to increase capacity and decrease pressure in augmentation cystoplasty or replace the urinary bladder in the setting of malignancy. The addition of bowel to the urinary tract may lead to a multitude of problems including electrolyte imbalances, vitamin deficiencies, diarrhea, mucus production, stone formation, pyelonephritis, lack of bladder sensation, rupture of the bowel diversion, altered medication metabolism, altered linear growth in children and even malignancy in the bowel segment contacting urine (Rubenwolf et al. 2009). Relative contraindications to using a large segment of intestine exist in those with a shortened or a diseased gastrointestinal tract, as seen in cloacal exstrophy or Crohn's disease, respectively. In hopes to prevent this wide array of potential complications and avoid removing a portion of the gastrointestinal tract, multiple investigators have studied various scaffolds in different animal models in hopes to engineer an appropriate replacement for the human bladder with its abilities to expand and accommodate urine at a low pressure as well as contract and empty urine in a volitional manner. These scaffolds have been combined with various cell types including terminally differentiated bladder tissue constituents as well as multipotent stem cells. Over the past decades, tremendous growth has occurred in bladder regenerative medicine. The focus of this chapter aims to examine the various scaffolding materials being studied in the design of the ideal bladder replacement while briefly touching upon the use of stem cells within this context.

The Goals of Scaffolds

In order to improve upon the surgical treatment for high-pressure low capacity bladder, multiple biomaterials have been studied in lieu of the standard detubularized intestinal segment. There are several categories of biomaterials that deserve mention- synthetic non-biodegradable materials, naturally-derived materials, acellular extracellular matrices and synthetic biodegradable polymers. When discussing each biomaterial, a key issue is the usage of seeded or unseeded technology. While modern techniques involve seeded scaffolds with cultured cells grown on the biomaterial prior to implantation, traditional scaffolds utilize an unseeded technology in which the material alone is incorporated into the bladder wall allowing for native tissues to engulf and proliferate over the material.

When studying the outcomes of various biomaterials, an understanding of the intricate microscopic architecture of the bladder is important. The bladder has a multilayer urothelial mucosa lining its inner aspect with tight junctions between the umbrella cells to prevent the absorption of excreted urinary toxins from the kidney back into the bloodstream. The multilayered smooth muscle is a support to the urothelium and its underlying submucosa, providing the ability to contract and also stretch and expand with the appropriate signal. The signals to the organ originate from the central nervous system and reach the bladder via nerve fibers that can be readily identified within the muscular and submucosal layers. While sympathetic nerve fibers originate from cell bodies in its paraspinal plexus, the parasympathetic fibers originate from ganglion within the bladder wall itself. Various molecules, such as collagen and elastin, within the extracellular matrix provide the mechanical framework for the bladder. An increased amount of Type III to Type I collagen, seen with chronic obstruction, reduces bladder wall compliance and elasticity. Another important feature of the urinary bladder is its robust vascular channels providing nutrients and removing metabolic byproducts from the organ. The aim of tissue engineering is to recreate the physical and functional properties of the healthy human bladder with the appropriate histological and biochemical makeup as well as the ability to store and empty urine at a low intravesical pressure.

Synthetic Non-biodegradable Materials

The history of bladder tissue regenerative medicine can be traced back to the mid twentieth century. At that time a host of unseeded, non-biodegradable,

non-biologic materials were studied in the context of bladder augmentation cystoplasty. After Bohne et al. (1955) first described using acrylic molds in bladder substitution, investigators utilized various synthetic scaffolds such as polytetrafluoroethylene (Teflon®), polyvinyl sponges, rubber, gelatin sponges, and vicryl matrices, to name a few. These materials proved to be inadequate due to a multitude of shortcomings including encrustation with stones, bacterial colonization and inflammation with contractures. Many of the animals in these experiments died due to multitude postoperative issues. The animals that survived were able to provide guidance for future studies.

Many of these materials were created as spherical molds to allow tissue growth, but then required a second operation to remove the material. Any re-operative surgery is challenging in that scarring and obliteration of tissue planes is commonly encountered. Ideally, biomaterials used for augmentation cystoplasty would not require removal and instead may biodegrade within the host. Few synthetic materials were able to recreate the histological makeup of the bladder, specifically the muscular backing, whereas a urothelium lined luminal surface was noted by most investigators, affirming the robust regenerative capacity of the urothelium noted by many investigators. The ability to recreate the intravesical vasculature and neural network were not a consideration at that time. Also important, synthetic materials proved difficult to manipulate in the operating room; in some cases, the material would not be useful if it became saturated with bodily fluids. Some synthetic materials required further chemical modification so as to withstand this shortcoming. Although initial attempts were unsuccessful, the knowledge gleaned lead to further advances in the bladder regenerative medicine.

Naturally Derived Materials

Initial forays into engineering a bladder substitute lead to the realization that the biomaterial of choice should be non-immunogenic, biodegradable and able to reproduce the histological and functional ability of the normal healthy bladder. Naturally derived materials were first utilized as a bladder tissue substitute in the early to mid 1900s in the form of fascial grafts (Neuhof 1917). Fascial grafts proved inadequate due to bone formation with poor histological regeneration of the bladder. Since that time a variety of other naturally derived substances have been studied as well including dura, placental membrane, peritoneum, pericardium and even canine bladder.

Most animal studies using naturally derived materials were able to demonstrate varying degrees of urothelial cell uptake, muscular growth, and occasionally, bladder capacity increase. In a very interesting series of experiments, early success with the usage of a canine bladder transplant to other dog bladders prompted Tsuji et al. (1963) to attempt to use dog bladder xenografts in humans. They sought to perform two augments in patients with tuberculosis-induced contracted bladders, one partial cystectomy, and one radical cystectomy. Unfortunately all surgeries had poor results. The first two patients failed with no improvement in functional capacity and the other two needed further surgical procedures, including cutaneous ureterostomy due to severe contraction in the radical cystectomy patient.

In another study, readily available dura mater, the tough outermost layer of the brain's connective tissue, was used for bladder replacement in 34 humans requiring surgery for a variety of conditions. Although 13 patients had successful outcomes with increased capacity and continued ability to empty their bladder, there is was no demonstrable muscular generation over the graft (Kelami 1975). Hradec (1970) described using a peritoneal flap in two cases of subtotal cystectomy. The lack of muscular growth on histological exam raised questions about the regenerative capacity of the human bladder wall. Others were able to demonstrate muscle growth however results were not consistent across studies. Although human studies were unable to consistently reproduce the initial promise seen in animals, continued interest in the area of naturally derived materials lead to the discovery of acellular

extracellular matrices in the field of bladder augmentation.

Acellular Extracellular Matrices and Cell Seeding

Acellular extracellular matrices consist of mechanically and chemically treated organs harvested from cadaveric humans or animals. The two main types of acellular extracellular matrices utilized in bladder augmentation are small intestine submucosa (SIS) and bladder acellular matrix (BAM). SIS, derived from porcine small intestine, is mechanically processed to remove the mucosal luminal surface as well as the outer muscular and serosal layers. The resultant translucent submucosal tissue is about 1/10 mm in thickness and contains a variety of growth factors. Its utility in bladder augmentation followed successful unseeded SIS patch grafting of canine arteries (Lantz et al. 1990). Numerous studies have been performed on bladder augmentation with SIS.

In a series of studies, canines underwent partial cystectomy with an unseeded SIS graft to replace 35–40% of the anterior bladder wall. Kropp et al. (1996a) was able to demonstrate regeneration of urothelium, muscularis and serosal layers of the bladder. On closer inspection the smooth muscle bundle size, orientation and the ratio of connective tissue to muscle was abnormal in the SIS-bladder augmented canines. One canine out of 13 had a single bladder stone within the augmented bladder, but none of the augmented areas showed scarring and contracture. SIS-augmented bladders demonstrated good compliance, appropriate capacity, and even signs of nerve tissue within the augmented areas. The response to various chemical and electrical stimuli was diminished in comparison to normal bladders perhaps due to the altered muscle composition and concentration (Kropp et al. 1996b).

Overall the initial animal studies showed promise but the consistency of results has been variable between studies likely due the origin of the intestinal segment used to create the SIS graft as well as size of the bladder augment. A recent analysis compared proximal versus distal intestinal SIS segments in a partial cystectomy rat model. The defect created was 40–50% of the bladder capacity. Inflammatory reactions in the proximal SIS-augmented bladders had higher amounts of mast cells and eosinophils with decreased attrition rates of macrophage and neutrophil in comparison to distal intestinal SIS augments. This suggests more of an atopic reaction with the proximal SIS grafts, whereas the distal SIS grafts had a more appropriate healing reaction. When analyzing the final histological changes, the authors noted a higher proportion of scarring and collagenous tissue in the proximal SIS augmented bladders (Ashley et al. 2010). When creating a larger bladder defect with removal of 90% of the bladder wall, SIS augments fail to maintain bladder capacity due to graft scarring with calcifications noted (Zhang et al. 2006). The lack of uniformity in SIS grafts and the inability to regenerate more than half the bladder wall are concerning.

The introduction of cell-seeding may overcome some of the shortcomings that are encountered in unseeded SIS grafts. When describing cell-seeding, it is important to have a cursory knowledge of stem cells currently used in bladder regenerative medicine. Stem cells can be obtained from a variety of sources. Embryonic stem cells demonstrate a pluripotent differentiation capability with the ability to recreate any of the three germ cell layers. However they are plagued by political and ethical debate which limits the access to newer lines. They also may form a cancerous teratoma when implanted in vivo. These issues limit their widespread usage in urological regenerative medicine.

Alternatively, mesenchymal stem cells, which can be obtained from bone marrow, peripheral blood, connective tissue or muscle, have been shown to be multipotent with the ability to differentiate into tissue other than their original source (e.g., marrow stem cells can form muscle and adipose stem cells can form nerve). Mesenchymal stem cells, especially those which are marrow-derived, have been studied extensively. This is due to the lack of political and ethical debate, the ability to regenerate multiple tissues, and also the ease with

which they may be obtained. A simple iliac crest marrow harvesting procedure can be performed on an outpatient basis. Concerns exist about the lack of proliferative capacity in vitro with mesenchymal stem cells. Other sources of stem cells include adipose tissue, umbilical cord, placenta and amniotic fluid. More recently investigators have even demonstrated the ability to isolate stem cells from urine (Wu et al. 2011). However, further study must be conducted on urine-derived stem cells.

In a recent study, SIS seeded on both sides with autologous marrow-derived mesenchymal stem cells was used in a partial cystectomy non-human primate model (Sharma et al. 2011). A complete layer of urothelial mucosa was seen in both unseeded and seeded SIS grafts. While the unseeded SIS control demonstrated scarring within the graft along with inflammatory infiltrates, seeded SIS animals demonstrated muscle content nearly identical to native bladder tissue without scarring or inflammation. Markers demonstrated the origin of the regenerated muscle was from the stem cells. The ability of seeded stem cells to assist in the regenerative process may add to the future applicability of SIS grafts.

Bladder acellular matrix (BAM) is a collagenous xenograft created after mechanical and chemical processing of bladder tissue from sacrificed animals. The resultant scaffold is composed of collagen and elastin along with growth factors and adhesive molecules, facilitating cell adhesion and ingrowth of regenerated tissue. Its consistency mirrors that of normal bladder tissue reportedly making it easy to handle during surgery. Furthermore, the inflammatory potential may also be reduced as most antigens are removed by the processing of the graft. In a rat partial cystectomy model, BAM grafts utilized lead to stone formation, infection and contracture in four animals despite antibiotic treatment (Obara et al. 2006). The regenerated bladder tissue within the scaffold displayed urothelium, smooth muscle and nerve fibers; however the ratio of muscle to collagen was significantly inferior from normal bladder tissue. Although rat models have a well-recognized predisposition to stone formation, the inability of antibiotic treatment to rid the grafted animals of pyuria is concerning.

Extrapolating outcomes from rats to humans is difficult due in part to the smaller size, urinary lithogenicity, and the relatively quick regenerative capacity of the urinary bladder in healing wounds. As opposed to the vast majority of research done in this area, the benefits of the aforementioned non-human primate model although the beyond the scope of this chapter deserve mention. Most investigators utilized rat models due to the cost, minimal upkeep, and accessibility. However, their urinary bladder histological makeup differs from humans in that none of their neuronal cell bodies are found within the bladder wall. The entire innervation to the bladder comes from the pelvic ganglion (McMurray et al. 2006). Non-human primates are structurally and histologically more similar to human beings. Few utilize these animals in bladder regenerative medicine though they are more representative of humans likely due to cost and accessibility.

Addressing the limitations of using a rat model, Probst studied a canine partial cystectomy model in which the control animals' bladder specimens were processed into unseeded BAM grafts for usage in seven experimental canines. The unseeded BAM grafted animals demonstrated a significantly larger bladder capacity than those without augmentation. Five experimental dogs had urinary tract infections 1 month post operatively; and at the time of death, three animals showed significant bacterial contamination. No mention was made of any further antibiotic treatment after 48 h from the perioperative period. The BAM graft was about 1.5 times the size of the cystectomy defect and some shrinkage in the absence of scarring was noted at the time of harvest. Besides the complete urothelial lining of the BAM graft, muscular and serosal layers along with nerve fibers throughout the wall and submucosal portions of the graft were identified 7 months after surgery. Detailed analysis of the ratio of collagen to smooth muscle was not performed and a portion of the original BAM graft was still visible at the end of the study. The rate of infection and

bacterial colonization in BAM graft studies using different animals is concerning.

Others have compared outcomes of BAM grafts seeded with adipose-derived stem cell cells versus unseeded grafts in a rabbit model (Zhu et al. 2010). The seeded BAM grafts demonstrated improved bladder capacity and regenerated smooth muscle and neural tissue. However no muscle or nerve fibers were identified in the unseeded BAM grafts. Furthermore the smooth muscle and nerve stains utilized for identification, namely alpha smooth muscle actin and S-100, respectively, are non-specific markers and the investigators were also unable to demonstrate if the regenerated layers were derived from the stem cells or from the native bladder tissue. Recently, investigators demonstrated the ability to seed rat-derived hair follicle stem cells onto BAM and demonstrated better growth and shape of regenerated bladder tissue than acellular BAM (Drewa et al. 2009). The most important aspect of this study was the novel description of hair follicles as a source for stem cells in bladder tissue engineering.

The concerns regarding the origin of the regenerated tissue in the BAM graft model has also been studied in vitro. Marrow-derived stem cells seeded onto BAM grafts and subjected to exogenous growth factors have been shown to differentiate into smooth muscle using multiple histological stains (Antoon et al. 2012). To our knowledge, this scaffold with growth factors and marrow-derived stem cells has yet to be applied in an animal model. Although there are positive studies with acellular extracellular matrices, there has been robust growth in the study of synthetic polymers in bladder augmentation surgery.

Synthetic Biodegradable Polymers

The appeal of a synthetic, biodegradable polymer lies in a multitude of factors which are highlighted by a comparison to the aforementioned biomaterials. Permanent synthetic materials routinely demonstrated mechanical failure along with stones, bacterial colonization and scarring. In some cases re-operative surgery was required to remove the material and urothelial lining was the only layer consistently found. On a microscopic level, naturally derived materials at best were able to demonstrate a scant amount of detrusor muscle at the periphery of the graft. When applied in clinical trials, most patients had poor outcomes and required further interventions. Acellular matrices, such as SIS and BAM, have been studied in detail more recently and have shown promise. However, biodegradable synthetic polymers may be more valuable in their reproducibility and known degradation characteristics. This is highlighted by the inconsistent results noted with SIS grafts and the variable reactions noted based on its origin. When using naturally derived materials or acellular matrices, the host from which the matrix is derived may not be identical to future sources of material and this is a confounding variable of any investigation using acellular matrices. Furthermore, the health of the host must be extensively evaluated in order to prevent the transmission of disease to the recipient.

Biodegradable polymers are created to seamlessly mesh with the existent architecture of the host tissue and can be tailored to degrade gradually as the regenerative process evolves of the target tissue. The safety profile of biomaterials is evidenced by PLGA or poly(lactic-co-glycolic acid). PLGA biodegradable polymers have already been FDA approved after rigorous testing to be used in medical devices, controlled release systems and even surgical sutures. Its ability to undergo hydrolysis in vivo and reform its original monomers, lactic and glycolic acid which are naturally produced in the human body with metabolism, makes it an appealing biomaterial. By altering the ratio of lactic to glycolic acid, molecular weight, and crystallinity, the degradation rate can be predictably be altered from weeks to even years. Also, due to its thermoplasticity, alterations can also be made to create three-dimensional structures with a rough surface allowing for easier and more robust ingrowth of regenerated tissue and proteins. Polymer-based biodegradable constructs can be seeded with growth factors to help generate more robust vascular and neural regeneration.

The work of Vacanti and Langer generated a great deal of interest in the utilization of polymer-based scaffolds for bladder tissue engineering. These investigators demonstrated that autologous cells could be seeded and expanded on these scaffolds in vitro prior to their re-introduction back into the host. Many authors have studied the application of this technology to bladder augmentation.

In a landmark study, Oberpenning et al. (1999) first described seeding of autologous cells onto a bladder-shaped biodegradable polymer composed of polyglycolic acid (PGA) and coated with poly-DL-lactide-co-glycolide (PLGA) in a canine trigone-sparing partial cystectomy model. After performing a transmural bladder biopsy, the urothelial cells were seeded on one side with the detrusor muscle cells on the other side of the construct and then expanded in culture. Cell-free polymers were unable to duplicate the results of the seeded polymers in a variety of measures including bladder capacity, bladder wall compliance, distensibility, shape, and histologically. The unseeded scaffolds displayed fibroblast deposition, scarring and graft contracture. The seeded polymers demonstrated an intact urothelial lining, complete muscular layer, along with neural tissue at 6 months after implantation. This trial set the groundwork for the first and only clinical trial utilizing a polymer-based seeded scaffold in bladder augmentation.

Seven pediatric myelomeningocele patients underwent augmentation cystoplasty due to high pressure or poorly compliant bladders (Atala et al. 2006). All patients underwent suprapubic bladder biopsy from the dome of the bladder to obtain urothelial and smooth muscle cells for seeding onto scaffolds. Four patients received a seeded collagen matrix from acellular bladder submucosa, one of whom also had an omental wrap. Three patients received a collagen-PGA hybrid seeded scaffold with omental wrap. After 7 weeks of expanding the seeded cells ex vivo on the construct, patient underwent augmentation cystoplasty. All patients underwent a rigorous bladder cycling protocol beginning 3 weeks after surgery. Patients underwent cystoscopic biopsy of the engineered and native bladder tissue for histological analysis six to eight separate times.

Post-operative analysis in the patients who received a collagen matrix scaffold demonstrated minor improvements in leak point pressures and bladder wall compliance. Patients without omental wraps actually had a decreased bladder capacity and one required reoperative intervention with augmentation cystoplasty. The collagen-PGA seeded scaffold patients had minor improvements in leak point pressures as well as bladder capacity and wall compliance. Bladder biopsies demonstrated the collagen-PGA scaffolds regenerated all layers of the bladder wall. There was no mention of the vascular or neural supply to this tissue. Without question, this study is groundbreaking, but there are shortcomings that deserve mention.

First, all patients post-operatively required intermittent catheterization to drain their bladders and were continued on anticholinergic medications. One of the goals of tissue engineered augmentation cystoplasty is the neural regeneration may allow these patients to be free of catherization and avoid the well-known side effects of anticholinergic medications. Histological analysis using stains specific to neural tissue was not performed. Another concern is the cells used to seed the scaffolds were obtained from neurologically impaired patients and had been subjected to years of stress. It is unknown whether these cells will develop into normal healthy tissue or if the patients will simply redevelop their prior bladder pathology. Studies have since demonstrated the regenerative capacity of urothelial cells from diseased bladders is diminished when compared to healthy patients (Subramaniam et al. 2011). Finally although the usage of the omental wrap to provide enhanced blood supply in reconstructive surgery or to avoid overlapping suture lines with potential fistula formation is common, the inflammatory mediators released may alter the characteristics of the scaffold, leading to unknown consequences. Since this trial, multiple studies on different scaffolds have been performed due in part to some to of the inherent shortcomings of PGA and PLGA scaffolds.

PGA and PLGA scaffolds lack a few fundamental properties required for the ideal bladder augmentation scaffold. First and foremost, bladder tissue must be capable of expanding and contracting in order to accommodate and expel

urine. Both of these scaffolds may be too rigid to provide this function. Although they have been shown to maintain their shape on cystogram, neither trial, specifically the study by Oberpenning et al. (1999) in which dogs were able to spontaneously void, analyzed the ability of the scaffold to contract similar to the native bladder tissue in response to the appropriate signal. Studies have shown the ability to contract and expand leads to better muscle growth and contractile ability, so without a flexible scaffolding material, the muscle layer may not develop appropriately (Heise et al. 2009). Also, these materials may lose their original configuration and mechanical properties with repeated stress of the contracting and expanding surrounding bladder tissue.

Although glycolic and lactic acid are normal byproducts of metabolism, there is a risk for systemic acidosis should they be absorbed during polymer degradation. One of the key complications of intestinal augmentation cystoplasty is the systemic acid–base imbalance created by the absorption of urinary waste products across gastrointestinal mucosa. The gastrointestinal tissue lacks the tight junctions seen between urothelial cells and is built to absorb nutrients and chemicals from digestion. When using ileum or colon, ammonium chloride is absorbed which can lead to systemic acidosis that in children may cause linear growth retardation due to the leaching of calcium from the axial skeleton. In the clinical trial by Atala et al. (2006), systemic acidosis was not seen in their population however, the size of the graft and the usage of rigorous intermittent catheterization protocol may be the explanation as the acid byproducts may have been drained before systemic absorption occurred. Although the tight junctions seen in the urothelium impede absorption of most metabolites, the urothelial lining is capable of absorbing chemicals with the risk for systemic toxicity as is seen with intravesical instillation of the anti-cholinergic medication oxybutynin (Lehoranta et al. 2002). Furthermore even if the acid byproducts are not absorbed they will alter the local tissue environment for the regenerating and native bladder cells and this may be detrimental to their appropriate development.

The poly(1,8-octanediol-co-citrate) elastomer (POC) is formed from the reaction of citric acid with the water-soluble 1,8-octanediol. It has been recently utilized as a scaffold for in bladder augmentation cystoplasty as well as in other tissue engineering areas such as vascular grafting and cartilage replacement. The differences between POC and the aforementioned synthetic, biodegradable scaffolds such as PGA, make it an exciting addition to bladder tissue engineering arsenal. Its mechanical property is an important aspect of any augmentation cystoplasty scaffold as it may reproduce the viscoelastic properties seen in healthy bladder walls. As previously mentioned, the ability of the scaffold to expand and contract helps regenerate healthy smooth muscle. POC has been to shown to withstand burst pressures and contractile forces of small arteries. By altering various parameters of POC, including crosslinking temperature, time for crosslinking, or the concentrations of the two monomers, the degradation rate can be altered but the mechanical strength remains the same. Similar to other FDA-approved polyesters, it has been shown to be non-toxic and non-immunogenic in immunocompetent animals and likely will demonstrate a similar profile in humans. Unlike the acidic load released by PGA, which may influence the surrounding cellular milieu, the byproducts of POC are negligible and mainly consist of citric acid, a byproduct of the Krebs cycle.

In a recent study, POC scaffolds were tested in three different arms of a rat partial cystectomy model: POC seeded on opposite sides with human marrow-derived protein-labeled mesenchymal stem cells and a commonly used urothelial cell line; POC seeded on opposite sides with the same urothelial cell line and protein-labeled smooth muscle; and unseeded POC (Sharma et al. 2010). After seeding the stem cells at a low density, the POC scaffold allowed for massive stem cell expansion, demonstrating its non-toxic properties and its ability to allow stem cell regeneration ex vivo similar to that seen with PGA and PLGA constructs. The elastomeric properties of the scaffold allowed for stretching with forceps up to 137% of its length without deformity and suture placement with no damage to the biomaterial, an

attribute lacking in PGA scaffolds. The animals were harvested at 4 and 10 week time points and at that time stem cell seeded POC continued to demonstrate the elasticity and pliability sought in regenerative bladder engineering.

Upon extensive histological studies, the stem cell/urothelium seeded scaffolds demonstrated smooth muscle of human-origin based on human-specific protein staining in a greater muscle to collagen ratio than the other arms with intact urothelial lining, beginning at 4 weeks. The replicative capability of stem cells may explain the greater amount of muscle tissue seen in comparison to the muscle/urothelial seeded POC group. Whereas the smooth muscle/urothelial seeded constructs showed decreasing smooth muscle expansion from the 4 to the 10-week period. There was no evidence of inflammation in the upper tracts in nearly all of the rats. However, stone material was seen on bladder tissue, a well-recognized pitfall of this animal model. Encrustation was not on the POC scaffold but instead on the host bladder tissue. Further study is needed to determine why this occurred; however it may be due to citric acid chelating with urinary byproducts overproduced in rats. This initial study prompted further manipulation of the POC scaffold in future studies.

With the addition of the vasculogenic CD34+ hematopoietic stem cells (HSCs), POC scaffolds augmented into partial cystectomy rats displayed more robust neovascularity and were the only scaffolds to have early nerve fiber regeneration (unpublished data). The degree of neovascularization in the setting of CD34+ HSC POC constructs was five times higher than that of the native bladder tissue. Also perhaps more important, the source of mesenchymal stem cells whether adults, pediatric myelomeningocele patients, or age-matched healthy pediatric patients all functioned the same both in vivo and in vitro. The implications and possibilities raised by this research are quite profound.

If nerve regeneration is possible, with CD34+ HSC-augmented POC grafts in a spinal injury model would animals be able to void spontaneously? Furthermore, can these cells be used in bladder cancer patients, in whom there may be a pan-urinary tract cancer predilection, to be augmented without risk for cancer recurrence? The questions raised by this study are numerous and ongoing research work from multiple labs across the world continues to show promise in the search for the optimal scaffold for bladder tissue engineering.

In conclusion, bladder tissue regenerative medicine in the context of biomaterials has evolved tremendously over the years. At the onset of this journey, investigators have and continue to seek a tissue replacement capable of recapitulating the histological makeup and functional ability of a normal healthy bladder. Besides the scaffolding material, other pertinent variables exist in this search such as the source of and materials to seed onto this biomaterial. Human studies have been conducted over the years with some success but current investigators need to exploit animal models that are more representative of humans who may benefit from this research in order to obtain translatable data. Should the target population be myelomeningocele patients, then large animal models with a similar voiding behavior, anatomical makeup, and neurological deficit must be used to evaluate scaffolds. Nearly a century after the initial studies, the field of regenerative biomaterials has made significant progress but much remains to be discovered.

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Tissue Engineering and Regenerative Medicine: Role of Extracellular Matrix Microenvironment

30

Ngan F. Huang

Contents

Abstract

Tissue engineering and regenerative medicine hold tremendous promise for repairing diseased or dysfunctional tissues with functional biological replacements. To date, a number of engineered tissues are FDA-approved or are undergoing clinical trials. An important component of engineered tissues is the extracellular matrix (ECM) which provides mechanical and physical stability to the tissue, while also providing instructive signaling cues. In the advent of technological advancements, ECMs can be engineered with greater spatial and mechanical organization to better mimic physiological properties of native ECMs. The ECMs used in the generation of artificial skin and vascular grafts are highlighted as examples of engineered tissues under development and commercialization.

Introduction

Tissue engineering is broadly defined as the application of engineering and life science principles to create biological tissue replacements. Since the time that tissue engineering was popularized by Langer and Vacanti in the 1990s, the field continues to hold promise for replacing diseased or injured tissues with functional substitutes. In the past several decades, tissue engineering made a number of technological advancements, including the fabrication of three-dimensional scaffolds that promote cell

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survival, incorporation of stem cell-derived cells, and the identification of inducing factors that modulate cell behavior. Through these technological breakthroughs, tissue engineering has achieved some clinical successes, including engineered skin and vascular conduits.

The classical tissue engineering triad (cells, extracellular matrix (ECM), and inducing agent) remains as important components in the successful fabrication of engineered tissues. Among the triad, the ECM provides both structural support as well as dynamic signaling cues that influence cellular organization and function. Here, we first overview the types of ECMs and methods to modulate the organization of ECMs. Then, using artificial skin and vascular grafts as prime examples, we will then highlight the role of ECMs in the development and commercialization of engineered tissues.

Types of Extracellular Matrices

The desirable qualities of biomaterials consist of the ability to match the mechanical and structural properties of the native tissue ECM, as well as being biocompatible and biodegradable. A three-dimensional porous scaffold is ideal for enabling cellular ingrowth, proliferation, and the formation of interconnected three-dimensional tissue structures. ECMs can be broadly classified as naturally-derived or synthetic. Naturally-derived ECMs, which can be harvested from donor tissues, are favorable materials due to their biocompatibility. However, the disadvantage of naturally-derived ECMs is the limited control of the ECM's mechanical and chemical properties, as well as the potential exposure to xenoproteins when originating from non-human sources. As an alternative to naturally-derived ECMs, synthetic materials overcome the limitation of naturally-derived ECMs in the ability to tune the mechanical and physical properties using chemistry. To date, many naturally-derived or synthetic ECMs can be commercially obtained for medical or research use (Table 30.1). Below are descriptions of some commonly used ECMs.

Naturally-Derived Extracellular Matrices

About one third of the proteins in the body consist of collagen, a family of helical fibrillar proteins that provides structural integrity and sustains mechanical loads (Myllyharju and Kivirikko 2001). Among the various types of collagen, collagen type I is most abundant and is found in the heart, blood vessels, bone and skin. Some types of collagen have more specialized functions. For example, collagen type II is prevalent in hyaline cartilage and is associated with the absorption of shock in the joints and vertebra. Collagen is first synthesized within the cell in a procollagen form before being secreted to the extracellular space, where it is cleaved by proteolytic enzymes to enable collagen fibril assembly (Diegelmann 2001). Enzymes that break down collagen include acid phosphatase, leucine amino peptidase, collagenase, and matrix metalloproteinases (Lauer-Fields and Fields 2002).

Fibrin is formed by the process of thrombin-mediated fibrinopeptide release that converts fibrinogen to fibrin, followed by a crosslinking step to form a stabilized fibrin matrix (Ho et al. 2006). Fibrin becomes degraded by fibrinolytic enzymes such as plasmin (Sierra 1993). Fibrin can be easily extracted, purified from blood plasma, or embedded with exogenous growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to provide further instructive cues. Fibrin is already FDA-approved as a surgical sealant matrix.

Matrigel is a basement membrane extract derived from mouse Engelbreth-Holm-Swarm (EHS) sarcoma. Matrigel is composed of a mixture of matrix proteins such as laminin, fibronectin, and collagen IV, as well as a number of growth factors including bFGF and transforming growth factor-β (TGF-β) (Kleinman et al. 1982). As a temperature-sensitive material, Matrigel remains a liquid when maintained at 4 °C and forms a gel at physiological temperature. One limitation of Matrigel is that it is not well chemically defined, so it can vary between batches.

Hyaluronic Acid (HA) is a carbohydrate that is prevalent in articular cartilage, and it plays a

Table 30.1 Partial list of commercial ECMs

Product	Company	Material	Form	Use
Medical Grade				
AlloDerm	Lifecell	Human skin	Sheet	Reconstruction of the abdominal wall, breast, head, neck
TissueMend	TEI Biosciences	Fetal bovine skin	Sheet	Surgical repair and reinforcement of soft tissue in rotator cuff
PriMatrix	TEI Biosciences	Fetal bovine skin	Sheet	Wound management
Permacol	Tissue Science Laboratories	Porcine skin	Sheet	Soft connective tissue repair
Axis dermis	Mentor	Human dermis	Sheet	Pelvic organ prolapsed
Graft Jacket	Wright Medical Tech	Human skin	Sheet	Foot ulcers
Oasis	Healthpoint	Porcine small intestinal submucosa (SIS)	Sheet	Wound and burn repair
CuffPatch	Arthrotek	Porcine SIS	Sheet	Reinforcement of soft tissues
Surgisis	Cook SIS	Porcine SIS	Sheet	Soft tissue repair and reinforcement
Restore	DePuy	Porcine SIS	Sheet	Reinforcement of soft tissues
Tisseel	Baxter Biosciences	Human fibrin from plasma	Gel	Surgical hemostasis
Research Grade				
Matrigel	BD Biosciences	Basement membrane extract from EHS mouse sarcoma	Gel	Cell culture
BD PuraMatrix	BD Biosciences	Synthetic self-assembling peptides	Gel	Cell culture
Extracellular Matrix	BD Biosciences	Human ECM derived from placenta	Gel	Cell culture
3D Collagen Composite Scaffolds	BD Biosciences	Bovine skin	Sheet	Cell culture

role in resisting compressive forces between joints in the body. HA is comprised of disaccharide repeats of glucuronic acid and N-acetyl glucosamine. HA is synthesized by HA synthases and becomes degraded by hyaluronidases (Watanabe and Yamaguchi 1996).

Decellularized tissue scaffolds represent another class of natural-derived ECMs.

Decellularized tissues are obtained by chemically treating the tissues to remove cellular components, leaving behind only the intact ECM (Cho et al. 2005). The advantages of decellularized tissues are the preservation of complex three-dimensional structure, mechanical integrity, and the presence of multiple types of ECMs. However, the limitations of decellularized tissues are the inability to control its mechanical or chemical properties and the potential presence of xenoantigens if the tissues are harvested from non-human sources.

Synthetic Polymers

Synthetic polymers, which can be produced by chemical synthesis or recombinant DNA technology, have well-controlled chemical structure and mechanical properties. A number of synthetic biomaterials are already FDA-approved, such as polyglycolic acid (PGA) sutures. Medical-grade polyester and expanded polytetrafluoroethylene (ePTFE) are other examples of synthetic ECMs

that have been used in medical devices. Among the types of synthetic polymers, PGA and poly-L-lactic acid (PLLA) are aliphatic polyesters that are widely studied as biocompatible and biodegradable materials for tissue engineering applications. These polymers can be synthesized by ring-opening polymerization reaction (Wu and Wang 2001). Since aliphatic polyesters can have poor cell attachment and can undergo failure after prolonged cyclic strain, they can be modified to promote cell attachment and improved elastic properties. To enhance cell attachment on synthetic materials, polymers can be modified with cell binding domains such as RGD (Liu et al. 2004), YIGSR, and IKVAV peptide sequences derived from laminin (Santiago et al. 2006). The elasticity and tensile strength of these materials can be tuned by the cross-linking reactive residues interspersed within the elastin-like domains (Liu et al. 2004).

Control of Extracellular Matrix Organization

To mimic the spatial geometry of physiological tissues, numerous approaches have been taken to control ECM spatial patterning in two-dimensional and three-dimensional scales, as is reviewed extensively elsewhere (Huang and Li 2011). Briefly, among the approaches to pattern at the two-dimensional scale include soft lithography, dip pen nanolithography (DPN), and electron-beam lithography. Soft lithography is a conventional method for patterning cells at the microscale by creating pre-designed patterns on a silicon wafer and then transferring the patterns to deformable materials such as polydimethylsiloxane (PDMS) (Huang and Li 2011). The PDMS can then be used directly for cell culture or as a template for microcontact printing onto another substrate. Microchannels of patterned PDMS can also be used to create microfluidic patterns onto substrates. Besides soft lithography is DPN, which is a technique that creates nanoscale patterns using a contact-based printing technique by transferring molecules from an atomic force microscopy (AFM) tip to a substrate. Other forms of contact-based cantilevers can be used to create microscale patterns (Huang et al. 2010). In addition, electron-beam lithography utilizes electrosensitive resist to create nanopatterns with up to 3-nm resolution (Huang and Li 2011). The advantage of DPN and electron-beam lithography is the ability to create defined patterns in the absence of a silicon wafer.

To mimic the three-dimensional structure and geometry of native ECMs, some commonly used methods include salt-leaching, stacking of microfabricated films, three-dimensional printing (3DP), and electrospinning. Conventional methods to create porous three-dimensional scaffolds utilize salt-leaching or gas-foaming approaches to create cavities within the scaffold that promote cellular ingrowth. However, these approaches provide very limited control over the architecture of the scaffold. In contrast, controllable three-dimensional structures can be created by stacking of microfabricated films. Another approach involves computer-assisted three-dimensional design and printing of binder materials into sequential layers, enabling precisely-controlled spatial patterning. 3DP can be used to create complex geometric structures, including interconnected pores. Electrospinning is an approach to create nanofibrous polymers by the deposition of charged polymeric solution onto a grounded collector plate. Electrospinning can be used to create networks that mimic native nanofibrous ECM. Depending on the configuration of the collector plate, the nanofibers can be spun in geometric patterns such as parallel-oriented nanofibers (Xu et al. 2004). These methods to modulate ECM structure and geometry enable the formation of engineered tissues that better resemble the architecture of native tissues.

Extracellular Matrix Chemical Signaling

Cell-ECM interactions are involved in a wide range of biological processes, from the formation of embryonic organs to pathological remodeling in disease states. In chick embryos, for example, fibronectin is critical for embryonic development

and is expressed in the dorsal aorta of embryonic day 6 (E6) , and laminin is prevalent in the aortic vascular wall at E10 (Risau and Lemmon 1988). Heparan sulfate proteoglycans like perlecan appear during embryonic development on E10 in mice during development of the heart and blood vessels (Handler et al. 1997). In the adult vasculature, for example, collagen IV is highly abundant in the basement membrane of blood vessels (Francis et al. 2008).

ECM-mediated regulation of cell behavior is largely due to heterodimeric transmembrane adhesion receptors known as integrins. The extracellular domain of integrins binds to ECMs, whereas the intracellular domain connects to the actin cytoskeleton through the focal adhesion complex. ECMs modulate cell behavior and function in part by recruiting specific integrins into focal adhesions. Among the proteins that cluster at focal adhesions is focal adhesion kinase (FAK), a 125-kDa cytoplasmic protein-tyrosine kinase that interacts with both integrins and intracellular signaling pathways. FAK mediates signal transduction processes that affect a variety of cellular functions, including cell proliferation, migration, and adhesion (Gronthos et al. 2000). For example, endothelial cells cultured on fibronectin display FAK-dependent Rac activation that stimulates transition to S phase of cell cycle by cdk4 phosphorylation, whereas this effect is not achieved on laminin (Planat-Benard et al. 2004a). This observation is consistent with previous studies suggesting enhanced proliferation on $\alpha_5\beta_1$ and $\alpha_v\beta_3$ ligands (such as fibronectin), as compared to $\alpha_2\beta_1$ ligands (like laminin) (Planat-Benard et al. 2004b). ECM-integrin interactions have been shown to activate downstream signaling pathways related to cell survival and function, including mitogen activated protein kinase (MAPK) and Akt pathways. These findings implicate the fundamental importance of ECM-integrins in the control of cellular behavior and function, which is important for engineering biological tissues.

In the advent of high-throughput combinatorial screening approaches, it is now feasible to examine the effects of numerous ECM compositions on cell behavior simultaneously. ECM microarrays can be fabricated using a robotic printing technology that deposits nanoliter volumes of ECMs onto substrates with printed areas spanning hundreds of microns. Cells can then be cultured on the arrays and assayed for biological responses. When used in conjunction with automated image acquisition and analysis software, this can be a powerful approach to examine the effects of biomaterials on cell function, as well as in determination of stem cell fate commitment. Such ECM microarrays have been utilized to show differential effects of ECM compositions on hepatic function, as well as differentiation of ESCs into hepatic or cardiac lineages (Flaim et al. 2005, 2008). This approach can be used to examine the effects of not only naturally-derived ECMs but also synthetic polymers such as acrylates (Anderson et al. 2004).

Cell Sources

Besides the ECM, another important component of engineered tissues is cells. To date, there are numerous sources of cells for engineered tissues, ranging from somatic cells to stem cells. Somatic cells such as fibroblasts and endothelial cells are already being utilized for clinical-grade engineering of vascular grafts by Cytograft. Although somatic cells such as dermal fibroblasts can be easily harvested and cultured, others such as cardiomyocytes are generally non-proliferative and are therefore impractical to isolate for therapeutic applications. Furthermore, since cells derived from patients may be diseased or dysfunctional, somatic cells may have limited therapeutic potential.

Therefore, stem cells and progenitor cells serve as an alternative source of therapeutic cells for tissue regeneration. Stem cells can be generally categorized as pluripotent, multipotent, or unipotent, based on the degree of plasticity to differentiate. Pluripotent stem cells, which consist of embryonic stem cells or induced pluripotent stem cells (iPSCs), have the greatest flexibility to differentiate into cells derived from all three germ layers (Bilic and Izpisua Belmonte 2012). ESCs are harvested from the inner cell mass of fertilized embryos. In contrast to ESCs, iPSCs can be

derived autologously from somatic cells, overcoming the ethical concerns associated with the generation of embryos. Multipotent stem cells, such as bone marrow mesenchymal stem cells (MSCs), can differentiate into many lineages include bone, cartilage, fat, and skeletal muscle (Prockop 1997). In contrast, unipotent stem cells, including those found in mammary epithelium (Visvader and Lindeman 2011), are generally committed to one lineage. Due the ability to differentiate stem cells into therapeutic cells, stem cells remain a promising cell source for tissue engineering applications.

Extracellular Matrices in Tissue Engineering Models

Among the earliest success stories of engineered tissues are that of biological skin equivalents. Owing to the simplicity in the structure and thickness of skin, a number of acellular as well as cellularized skin substitutes are currently in the market for treatment of burns and ulcers. However, complex organs such as the heart or liver will require further research in order to engineer biological equivalents. Among the more complex three-dimensional tissues, engineered vascular conduits have made considerable advancements towards clinical use. To illustrate the role of ECMs in tissue engineering applications, we briefly describe the progress in the development and commercialization of artificial skin grafts and vascular conduits (Table 30.2).

Artificial Skin Equivalents

As the largest organ in the human body, skin is comprised of the epidermis and a dermis layers. The stratified and keratinized epidermis serves as a physical barrier to the body, whereas the dermis provides strength and support. The dermis contains a biochemical framework of collagen, elastin, HA, and proteoglycans that enables the skin to regenerate and repair. For treatment of wound injuries or disease, skin autografting is effective but is invasive and can lead to morbidity or infection. As a result, alternative treatments including bioengineered skin grafts are in demand.

Artificial skin grafts are one of the first successful tissue engineered products to be FDA-approved. The emergence of skin grafts was motivated by the need to cover burn injuries for patients with insufficient autologous skin for grafting. However, skin grafts were later shown to have broader applications for treatment of chronic wounds and skin diseases and could be an off-the-shelf therapy.

The ideal properties of an engineered skin graft would include good contact to the wound site upon application, accelerate tissue regeneration and wound repair, mimic the structure and function of native skin, and not be rejected immunologically by the host tissue. Today there are a number of companies that have commercialized acellular or cell-seeded grafts for treatment of burns, skin wounds, and skin diseases. A hallmark feature of these grafts is the presence of an ECM scaffolding structure that provides structural support for the ingrowth of cells.

Acellular porous scaffolds such as those composed of collagen provide a physical structure for cell migration, which is necessary for the wound healing process. Moreover, scaffolds derived from decellularized skin tissue retain some aspects of the native biochemical composition and fibrous structure. For example, AlloDerm (LifeCell) consists of decellularized cadaveric human skin that retains the dermal matrix components and basement membrane. As the scaffolds become bioresorbed with time, it is replaced by the ECM secreted by the host cells.

In addition to acellular scaffolds, cellularized scaffolds using keratinocytes and/or fibroblasts can be used in conjunction with ECM scaffolds to generate biological skin grafts. These cells are harvested from donor full-thickness skin biopsies and can be used autologously or allogenically. One commercial dermal graft is Dermagraft (Advanced Biohealing), which is indicated for the treatment of full-thickness diabetic foot ulcers. Dermagraft consists of a three-dimensional bioresorbable polyglactin mesh scaffold containing allogenic fibroblasts. The cells proliferate within the scaffold as well as secrete matrix

Table 30.2 Skin and vascular grafts

Type	Product Name	Composition
Skin Graft	AlloDerm (LifeCell)	Decellularized human cadaveric skin with dermal matrix and basement membrane
	Apligraf (Organogenesis)	Bovine collagen gel containing a layer of allogenic fibroblasts and a layer of allogenic keratinocytes
	Biobrane (Smith and Nephew)	Porcine collagen bonded to nylon mesh and silicone film
	Bioseed-S (BioTissue Technologies)	Suspension of autologous keratinocytes in fibrin glue
	Dermagraft (Advanced Biohealing)	Polyglactin mesh scaffold containing allogenic fibroblasts
	Epicel (Genzyme BioSurgery)	Cultured epidermal sheet of autologous keratinocytes
	Integra (Integra Life Sciences)	Bilayered skin substitute containing an inner layer of bovine collagen and glycosaminoglycans and an outer layer of silicone
	Laserskin (Fidia Advanced Biopolymers)	Hyaluronic acid membrane with laser-drilled micropores and autologous keratinocytes
	OrCel (Forticell Bioscience)	Bovine collagen scaffold containing a layer of allogenic fibroblasts and a layer of allogenic keratinocytes
Vascular Graft	Artegraft (Artegraft)	Bovine collagen conduit
	AlboGraft (LeMaitre Vascular)	Polyester vascular graft
	Gore Propaten (Gore Medical)	Expanded polytetrafluoroethylene (ePTFE) conduit with bonded heparin
	Humacyte (Humacyte)	Human ECM-derived vascular graft
	Lifeline (Cytograft)	Autologous fibroblast sheets in tubular form with a lining of autologous endothelial cells
	Lifespan (Edwards Life Sciences)	Expanded ePTFE conduit

proteins and cytokines. This dermal graft works by restoring the dermal bed to facilitate wound healing. Another class of biological skin substitutes is epidermal grafts. Epicel (Genzyme Biosurgery) is an example of an autologous epidermal graft consisting of multiple layers of keratinocytes. It is indicated for patients who have deep dermal or full thickness burns to prevent infection and fluid loss at the wound bed. Although Epicel does not utilize any exogenous matrix, another epidermal graft called Bioseed-S (BioTissue Technologies) utilizes fibrin matrix to adhere the keratinocytes to the wound bed. In addition to epidermal and dermal replacements, composite skin grafts contain both epidermal and dermal components. Apligraf (Organogenesis) is a bilayered tissue construct that contains allogenic fibroblasts cultured in purified acid-soluble collagen I gel, upon which a layer of stratified keratinocytes are grown. Apligraf is used for the treatment of diabetic foot and venous leg ulcers. Another composite skin graft, OrCel (Forticell Bioscience), contains a cross-linked bovine collagen I scaffold with asymmetric pore distribution such that one side contains macroscopic pores and the other side is a non-porous. Within this asymmetric scaffold, the porous side is cultured with fibroblasts, whereas the non-porous side is coated with gelatin and cultured with keratinocytes from the same donor. Taken together, these commercial skin grafts illustrate the prevalence of ECMs for therapeutic skin repair.

Recent research suggests that engineered tissue constructs cultured with stem cells may accelerate wound healing. Not only can stem cells provide beneficial paracrine factors but also differentiate into vascular lineages. For example, in a porcine model, bone marrow-derived MSCs

cultured on collagen glycosaminoglycan polymer scaffolds showed promise for treatment of partial thickness burn wounds, as measured by contraction area, degree of keratinization, and angiogenesis. The stem cell-seeded scaffolds contracted significantly less than the control no treatment group, while displaying improved keratinization and angiogenesis, and histologically the stem cells appeared to persist in the tissue (Liu et al. 2008). Bone marrow-derived MSCs have also been shown to differentiate into keratinocytes when cultured on electrospun collagen/poly(l-lactic acid)-co-poly(3-caprolactone) nanofibrous scaffolds. The differentiated keratinocytes were morphologically round in appearance and expressed known markers including keratin 10 and filaggrin (Jin et al. 2011). In conjunction with nanofibrous polymers that mimic the nanotextured environment of native skin, these engineered constructs may be useful for engineering of biological skin equivalents. In summary, artificial skin equivalents are among the most successful engineered tissues in clinical use, and further research in the incorporation of stem cells or ECM organization will likely lead to improved products for medical use.

Vascular Conduit

Blood vessels have a trilayered structure consisting of endothelial cells in innermost layer, smooth muscle cells in the medial layer, and an adventitial layer composed of fibroblasts. Blood vessels are responsible for transporting blood and nutrients throughout the body. The endothelium plays a dominant role in preventing obstructions of the vessel by modulating vessel tone and by reacting with circulating blood components. However, endothelial cells that become dysfunctional are a major cause of prevalent cardiovascular diseases. Any obstruction to blood vessels may result in myocardial infarction, stroke, or atherosclerotic disease. When donor vessels are not available for bypass grafting, vascular conduits are an attractive alternative.

Synthetic polymer-based acellular grafts are commercially available off-the-shelf products for vascular intervention. Grafts composed of ePTFE and polyester are flexible and can withstand suturing. Gore Propaten (Gore Medical), for example, is an ePTFE conduit that is bonded with heparin to further reduce thrombogenesis. Gore Propaten has been shown to reduce neointimal hyperplasia in a baboon model of aortoiliac bypass (Lin et al. 2004).

Naturally-derived polymeric acellular grafts have also shown therapeutic promise. Artegraft, for example, is a woven and crosslinked collagen conduit derived from bovine carotid artery. This conduit is indicated for usage as an arterio-venous (A-V) shunt, arterial replacement, arterial bypass, arterial patch, and femoropopliteal bypass. A recent prospective, randomized controlled clinical study demonstrated improved primary patency in the patients treated with Artegraft, in contrast to those receiving grafts composed of expanded ePTFE (Kennealey et al. 2011). Although derived from bovine tissue, Artegraft appears to be non-antigenic and negative for bovine spongiform encephalopathy. Another approach for the production of naturally-derived ECMs for vascular grafting is based on the technology from Humacyte using human cell-derived ECM. The advantages of this approach include the absence of xenoproteins and the ability to be delivered together with cells or drugs. Humacyte's vascular grafts appear to be non-immunogenic, leakproof, easily sutured, and have a long-term shelf life. A recent publication demonstrated that the human vascular grafts had similar mechanical properties as native vessels, and could be implanted in a baboon model for A-V access for hemodialysis (Dahl et al. 2011).

Although acellular grafts are clinically effective and patent as large-diameter vascular grafts, the patency of acellular grafts becomes reduced when less than 6 mm diameter due to thrombus formation and intimal hyperplasia. Therefore, there is a tremendous need to develop small-diameter biological vascular grafts as a therapeutic option for replacement of small diameter vessels. Cell-based vascular grafts that promote the formation of a luminal layer of endothelial cells are a promising therapeutic option, since endothelial cells play an important role in resisting adhesion of

lipogenic proteins and monocytes. However, with respect to commercialization, cell-based vascular grafts are further behind in clinical testing. Cytograft is currently undergoing clinical trials for grafts used for hemodialysis access (McAllister et al. 2009). These grafts also have therapeutic potential for bypass grafting. Cytograft is an autologous vascular graft formed by completely endogeneous ECMs. From a patient skin biopsy, the fibroblasts and endothelial cells are harvested and expanded *in vitro*. The patient's fibroblasts are then cultured in the form of cell sheets before rolling together to form a tube-shaped graft with sufficient mechanical integrity. The vascular graft is maintained within a bioreactor before lining the lumen with endothelial cells. In the first clinical trial for hemodialysis access in a multicenter cohort study, ten patients were recruited who had end-stage renal disease with high risk of access graft failure (McAllister et al. 2009). Among the ten patients, five had grafts for hemodialysis that were functional for 6–20 months after implantation. The primary patency rates were 60% at 6 months post-implantation. This study shows promise of these grafts for clinical use.

To simplify the manufacturing process and to reduce manufacturing costs, Cytograft propose a number of potential modifications to future generation products (Peck et al. 2012). For example, the presence of the endothelium may not be necessary due to the high flow rates in A-V shunts, and eliminating the endothelial aspect may reduce the complexity of the graft. In addition, an allogenic source of cells could also reduce manufacturing costs associated with autologous generation of vascular grafts. Furthermore, a non-living graft could be produced in which the cells are devitalized, leaving behind the matrix which is responsible for the mechanical integrity of the product. Ultimately, the generation of a non-living graft, similar to the strategy of Humacyte, would simplify the production process and increase the storage life, while reducing the manufacturing cost and eliminating the need for patient-specific biopsies.

Recent research strategies aim to develop stem cell-derived vascular grafts. For example, autologous ovine bone marrow-derived MSCs were cultured on decellularized ovine carotid arteries before implanting as an interpositional graft in the carotid artery (Zhao et al. 2010). The cell-seeded grafts remained patent for over 5 months, whereas the acellular graft became occluded within 2 weeks. Histologically, endothelial and smooth muscle cells infiltrated the graft, and ECM components elastin and collagen could be detected. Some stem cells persisted up to 2 months after transplantation. When bone marrow-derived MSCs were cultured on circumferentially oriented poly-L-lactide nanofibrillar scaffolds, not only did the cells align along the direction of the polymer nanofibrils, but they also conferred some anti-thrombogenic properties that promoted graft patency and deposition of native collagen and elastin ECM proteins (Hashi et al. 2007). When implanted as interpositional grafts, the cell-seeded conduits formed an intimal endothelial layer and medial smooth muscle layer. Although the majority of MSCs were no longer detectable by 7 days after engraftment, the short-term persistence of MSCs appeared to have a functional impact on graft patency. In summary, acellular conduits are effective for large-diameter vascular grafts, and cell-based grafts are a promising approach for small-diameter grafts.

Future Directions

In summary, ECMs are a critical component of engineered tissues by modulating cell behavior while providing structural support. Although naturally-derived ECMs are biocompatible and easily purified, synthetic materials enable greater control over the chemical and mechanical properties. Increasingly important will be the successful generation of three-dimensional scaffolds that mimic the complex spatial geometry of endogenous tissues. To date, artificial blood vessels and skin substitutes are examples of engineered tissues that have achieved some degrees of clinical success. However, a number of refinements to the ECM are necessary to further their functionality. Current approaches to generate three-dimensional porous scaffolds with complex physiological architecture are limited in long manufacturing

time, requirement of specialized equipment, or lack scalability. Therefore, technological advancements are needed to improve the scalability of three-dimensional scaffolds with preformed spatial organization. Additionally, the mechanical and physical properties of the ECM should match that of the native tissue, and the degradation rates of the scaffolds should match that of native ECM production. Due to the diffusion limits of three-dimensional scaffolds, a bioreactor system may help perfuse nutrients to the cells to maintain viability. By furthering the advancement of novel ECM fabrication technologies, it is anticipated that engineered tissues will become a more widely used therapy.

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