Stem Cells and Cancer Stem Cells 12
Therapeutic Applications in Disease and Injury

M.A. Hayat
Editor

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Volume 12
Therapeutic Applications in Disease and Injury

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Edited by

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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

Preface

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

In human history, immortality has been one of the most cherished, but unrealistic, wishes of human beings. Indeed we are still hoping to cure serious diseases to achieve immortality, but medical treatments have been proven to result in less than impressive success. An excessive emphasis on medical therapies has diverted attention from non-therapeutical efforts to prolong life, i.e., to slow down the inevitable aging process. In fact, unfortunately some treatments may shorten life instead of prolonging it.

This is volume 12 of the multi-volume series, *Stem Cells and Cancer Stem Cells: Therapeutic Applications in Disease and Tissue Injury.* The discovery that stem cells possess unique capability of self-renewal and indefinite growth and differentiation into almost every cell type in the human body has allowed us to explore the possibility of cell therapy applications. Various types of stem cells, including cancer stems cells, are available for specific applications. By expressing four transcription factors in somatic cells, these cells can give rise to almost any other type of cell in the human body. The ethical limitations of embryonic stem cells have been overcome by producing induced pluripotent stem cells which like the former cells can give rise to almost every cell type. In other words, induced pluripotent stem cells have similar properties to those possessed by embryonic stem cells. The current understanding of molecular mechanisms underlying human somatic cell reprogramming to generate induced pluripotent stem cells is explained. Experts have discussed the advantages and limitations of the applications (e.g., transplantation) of some of the stem cell types (pluripotent stem cells, neural stem cells) in this volume.

It is well-established that stem cells have the unique capabilities of self-renewal, grow indefinitely, and differentiate into multiple types of cells. Many different types of stem cells exist, but they are found in very small populations in the human body; for example, in circulating blood there is 1 stem cell in 100,000 cells. Stem cell markers can be used for distinguishing stem cells from other types of cells. Specific stem cell markers are also available for identifying and isolating embryonic mesenchymal, hematopoietic, neural, skin, muscle, fat, endothelial, pancreatic, and tumor stem cells.

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 to therapy. These types of stem cells are discussed in this series. Different sources of cancer stem cells are discussed. Potential clinical importance of cancer stem cells in normal lung and lung cancer is also explained.

Adipose tissue functions as a critical organ for energy regulation, inflammation, and immune response through intricate signals. Mature adipocytes can be reprogrammed through their gene expression profile into different cytotypes. Because adipose-derived stem cells are of autologous tissue origin, they are non-immunogenic. Although these cells are of mesodermal origin, their regenerative capacity extends to both ectodermal and endodermal tissues and organs. Human adipose derived stem cells can be isolated in a greater number than those from blood or bone marrow. A method for isolating multipotent endothelial-like cells from human adipose tissue is presented. These cells are suitable for clinical applications in cell therapy and regenerative medicine. It is known that endothelial progenitor cells are capable of self-renewal and participate in vasculogenesis, angiogenesis, and arteriogenesis. Adipose derived stem cells are ideal for practical regenerative medicine because they can be produced in large quantities. The authors describe their proliferation and differentiation capacities in a variety of regenerative medicine, including cartilage defects due to injuries, brain (stroke), lung injury, or diseases, including cancer.

Neural stem cells in the brain possess proliferative and self-renewal capabilities, and thus are able to generate neurons and glial cells. Under normal physiological conditions, these properties are tightly controlled via signaling pathways. However, when these pathways are deregulated, they may promote neoplastic transformation of neural stem cells into cancer stem cells, resulting in the formation of gliomas. It is pointed out that an understanding of these pathways will explain brain cancer development and progression. Histamine is one such factor that modulates both neural stem cells and tumor cells.

Dendritic cells occupy a pivotal role in the human immune system. Antigen presentation elicits an aggressive immune response or imposes a state of immunological tolerance. These cells serve as an intervention for therapeutic purposes. Therefore a reliable source of patient-derived dendritic cells is needed. Alternatively, autologous induced pluripotent stem cells can be used. Because the use of patient's own peripheral blood may be inadvisable for some reason, an alternative protocol for the derivation of dendritic cells from human induced pluripotent stem cells is detailed here for clinical applications of the former cells; for example, in immunotherapies.

It is well-established that somatic cells can be reprogrammed to induced pluripotent stem cells having similar properties to those possessed by embryonic stem cells. The former cells can also give rise to almost every other cell type in the human body, and they also lift ethical limitations in the use of human embryonic stem cells. The current understanding of molecular mechanisms underlying human somatic cell reprogramming to generate induced pluripotent stem cells is explained.

Hematopoietic stem cells give rise to multiple lineages of mature cells. This process is tightly controlled by signaling network regulated by cytokines. Notch signaling represents one of the major pathways activated during the interaction between hematopoietic progenitor cells and bone marrow stroma. The critical role of notch in the differentiation and function of dendrite cells and its effects on the immune response is explained.

Mesemchymal stem cells are multipotent progenitor cells that modulate and suppress immune responses. One of their immunoregulatory properties is the suppression of dendritic cell differentiation, maturation, and effector functions. It is emphasized that careful consideration is needed when selecting the source of mesenchymal stem cells for cellular therapy because different sources of these cells exert varying immunoregulatory effects.

The most serious late complication of allogenic stem cell transplantation is the graft versus host disease (GVHD). Up to a minimum of 100 days following stem cell transplantation, ~ 50% of patients will experience some degree of GVHD. The most efficient preventive strategy for GVHD consists of an immunosuppressive regimen although this treatment is immunologically nonspecific, and thus is only partially effective.

The use of endothelial progenitor cells and mesenchymal stem cells in renal repair in the experimental artherosclerotic renal artery stenosis is explained. The incidence of this problem, accompanied by increased cardiovascular morbidity and mortality, rises as the age of the population increases.

Adipose tissue functions as a critical organ for energy regulation, inflammation, and immune response through intricate signals. Mature adipocytes can be reprogrammed through their gene expression profile into different cytotypes. Human adipose-derived stem cells can be isolated in a greater number than those from blood or bone marrow. A method for isolating multipotent endothelial-like cells from human adipose tissue is presented. These cells are suitable for clinical application in cell therapy and regenerative medicine. It is known that endothelial progenitor cells are capable of self-renewal and participate in vasculogenesis, angiogenesis, and arteriogenesis.

It is known that hemophilia A is caused by mutations within the factor VIII (FV III) gene, which leads to the depleted protein production and inefficient blood clotting. Several attempts at gene therapy have failed for various reasons, including immune rejection. Liver is the primary source of FV III synthesis. The capacity of bone marrow stem cells to transdifferentiate into hepatocytes and liver sinusoidal cells has created profound interest in the use of these cells in the treatment of liver injury and acute or chronic liver failure. The author proposes here that the severity of the bleeding disorder can

be ameliorated by partial replacement of mutated liver cells with bone marrow-derived hepatocytes and endothelial cells, which can synthesize FV III in liver and correct bleeding. It seems that bone marrow stem cell therapy is a potential alternative approach to managing hemophilia A.

As the field of stem cell research advances, there will be an ongoing and increasing need for mathematical and other quantitative tools to facilitate research and discovery. The author has discussed several mathematical models related to the cancer stem cell hypothesis and their use in studying stem cell differentiation.

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 51 contributors representing 12 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in these important areas of disease and injury. I respect and appreciate the hard work and exceptional insight into the nature of cancer and other diseases provided by these contributors. The contents of the volume are divided into five subheadings: Cancer Stem Cells, Pluripotent Stem Cells, Dendritic Stem Cells, Regenerative Medicine, and General Applications 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 Dr. Dawood Farahi and Philip Connelly for their encouragement to continue the endeavor to publish these volumes. I am also thankful to my students for their help in many ways in completing this project.

Union, NJ, USA M.A. Hayat
September, 2013

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Part I

Cancer Stem Cells

Histamine in the Neural and Cancer Stem Cell Niches

1

Maria Francisca Eiriz, João Oliveira Malva, Fabienne Agasse*, and Liliana Bernardino*

Contents

Abstract

Neural stem cells (NSCs) present in the subventricular zone and in the subgranular zone of the adult brain possess proliferative and self-renewal capacities and are able to generate neurons and glial cells. Under physiologic conditions, these properties are tightly regulated at the neurogenic niche consisting of soluble factors and cell-to-cell interactions that control signaling pathways and genetic expression related to the stemness state. The deregulation of these pathways has been suggested to promote the neoplasic transformation of NSCs into cancer stem cells (CSCs) and the formation of gliomas. In fact, NSCs and CSCs share several characteristics including cell surface receptors and intracellular signalling pathways, several cell markers of immaturity, and affinity for blood vessels. Therefore, understanding the cellular and molecular pathways controlling NSCs properties will shed light on brain cancer development and progression. Among soluble factors able to modulate both NSCs and tumoral cells, histamine is raising attention due to its ability to modulate proliferation, differentiation, and survival of both cell types. This may suggest that the modulation of the histaminergic system could emerge as a novel approach to promote brain repair by neurogenesis stimulation and to hamper the development of brain tumors. In this chapter we discuss recent findings regarding the role of histamine in both neurogenesis and tumorigenesis.

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Introduction

The discovery of persistent populations of neural stem cells (NSCs) in the adult mammalian brain has challenged the dogma of the immutable adult brain. NSCs reside in a specific microenvironment, the stem cell niche, which contributes to the maintenance of their intrinsic capacities. Identification of the cellular and molecular components of this niche is fundamental to the understanding of how stemness is maintained and may provide crucial targets for the expansion of stem cells for therapy purposes.

The biology of NSCs took a surprising turn when Reya and collaborators (2001) suggested that glioma may arise from the deregulation of the control mechanisms that restrain stem cells to their niches. Gliomas are the most common form of primary brain tumors of glial cell origin. Gliomas are classified into ependymomas, astrocytomas, oligodendrogliomas and oligoastrocytomas according to tumor composition. Diffuse gliomas remain clinically intractable, due to their highly infiltrative nature and resistance to radiation and chemotherapy. Glioblastoma multiform (grade IV malignant astrocytoma), the most malignant form of gliomas, shows a very poor prognosis as only 3% of the patients survived until the fifth year following the diagnosis of the disease. The standard of care for glioblastoma includes surgical resection of the tumor, radiotherapy and single-agent chemotherapy such as temozolomide. However, classical chemo- and radiotherapies lead to tumor mass reduction but are inefficient on tumor regrowth from spared tumor cells. It has been showed that the spared cells are cancer stem cells, resistant to anti-cancer therapy and responsible for tumor relapse (Chen et al. 2012a). For long persisted the idea that tumor initiating cells, the so-called stem cells, arise from differentiated glial cells (astrocytes or oligodendrocytes) following rare events of mutagenesis that led to dedifferentiation. Recently, the neural stem/progenitor cells present in the SVZ have been proposed as better candidates. Many tumors are indeed either periventricular or contiguous with the SVZ and experimental exposure to oncogenic viruses or administration of carcinogens results in the preferential formation of tumors in germinal regions giving rise to more highly migratory and invasive tumors as opposed to the non-proliferative brain parenchyma. In fact, NSCs and CSCs also share several properties, including their capacity for proliferation and self-renewal, expression of cell markers for immaturity (such as nestin, Musashi-1, and Sox2 among others) and the close association with the vasculature. However, while proliferation and self-renewal are tightly controlled in normal stem cells, these processes are exacerbated in CSCs. A striking demonstration of these deregulations is the capacity of CSCs to develop new tumors upon orthotopic implantation (Reya et al. 2001; Galli et al. 2004).

Mechanisms responsible for the neoplastic transformation of NSCs to CSCs become to be unveiled and involve epigenetic regulation of gene expression as well as DNA mutations. Also, there is an increasing body of evidences that perturbation in the microenvironment homeostasis may play a role in this transformation. Histamine is an amine that acts as a neurotransmitter and neuromodulator in the central and peripheral nervous system. It is present in the brain in three main cellular stores: neuronal, mast and microglial cells. Its actions can be mediated through several receptors: two postsynaptic (H1R, H2R), one presynaptic (H3R), and a forth receptor mainly present in the immune system (H4R). All receptors belong to the family of rhodopsin-like class A receptors coupled to guanine nucleotide-binding proteins (G proteins). Histaminergic neurons, located exclusively in the tuberomammillary nucleus of posterior hypothalamus, have widely distributed projections throughout the adult brain. Therefore, histamine is involved in a broad range of brain functions including the sleep-wake cycle, emotion, learning and memory (for review see Brown et al. 2001). Recently, our group and others have shown that histamine is an important mediator of cell proliferation and neuronal differentiation of neural stem cells (Bernardino et al. 2012; Rodriguez-Martinez et al. 2012). Interestingly, histamine, released at

high amounts in tumors, can also modulate brain tumor cells proliferation, survival and differentiation. Unraveling the molecular and cellular effects of histamine on both neurogenesis and tumorigenesis is highly relevant for the use of anti-histaminics and histamine related molecules in brain repair therapies, either by boosting neurogenesis in a context of acute brain injury, or by inhibiting brain cancer growth and progression.

Neurogenesis in the Adult Mammalian Brain

Adult neurogenesis is an ongoing process which occurs in two restricted areas of the adult mammalian brain: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus. Both the SVZ and the SGZ harbors stem cells, i.e., a population of self-renewing and multipotent cells able to give rise to neurons and glial cells. New neurons produced in the SVZ migrate towards the olfactory bulb where they differentiate into mature interneurons and achieve the complex task of integrating into existing circuitries. In the hippocampus, newly-born neurons migrate locally from the SGZ to the granular cell layer (GCL) and differentiate into granule neurons by elongation of axons towards the CA3 region and extension of dendritic trees to the molecular layer (for reviews see Zhao et al. 2008; Ming and Song 2011). Addition of new neurons in the olfactory bulb and in the dentate gyrus (DG) is correlated with improvements in olfactory functions, and memory and learning, respectively.

Neurogenesis is very well documented in rodents, and has also been demonstrated in primates and humans. In fact, SVZ actively produce neurons in the infant human brain under 18 months of age destined for the olfactory bulb and the prefrontal cortex (Sanai et al. 2011). Importantly, SVZ stem cells extracted from the adult human brain retain the capacities to produce neurons *in vitro*, suggesting that neurogenesis in the SVZ may be boosted under proper stimulation. The presence of NSCs in the hippocampus of the adult human brain was firstly described by the seminal study of (Eriksson et al. 1998). Interestingly, it was also shown that a significant number of cells obtained from human biopsies from the temporal lobe of epileptic patients are also capable of expansion and multipotency *in vitro* (Vaysse et al. 2012).

However, we should keep in mind that most of studies done in humans do not directly measure neurogenesis but instead evaluate stem cell markers expression in tissue biopsies and cell proliferation, without knowing whether immature neurons derived from stem cells become integrated and functional in the host tissue. Yet, cell replacement therapy using NSCs (either by cell transplantation or by the mobilization of endogenous progenitors) may comprise a stronger potential avenue for the repair of neurodegenerative diseases.

SVZ and SGZ, in Brief

The adult SVZ is situated in the lateral ventricle walls. It is delineated on one side by the monolayer of ependymal cells that separate the brain from the cerebrospinal fluid (CSF) contained in the ventricles, and on the other side by the striatum. At this neurogenic niche, B1 cells expressing nestin, sex-determining region Y-box 2 (Sox2), and astrocyte markers such as glial fibrillary acidic protein (GFAP) and the glutamate-aspartate transporter (GLAST), have been identified as the resident stem cells of the SVZ. In fact, B1 cells (distinguished from B2 astrocytes which are located close to the striatum) present characteristics of radial glial cells, the neural precursor cells of the development, such as specialized endfeet on blood vessels at their basal domain and an apical cilium contacting the ventricle lumen (for reviews see Ihrie and Alvarez-Buylla 2011; Fuentealba et al. 2012) (Fig. 1.1a). B1 cells divide asymmetrically to originate C cells which in turn actively proliferate to give rise to immature neuroblasts or A cells. Newly born neurons tangentially migrate along the rostral migratory stream (RMS), a specialized astrocytic tubular structure with blood vessels serving as a

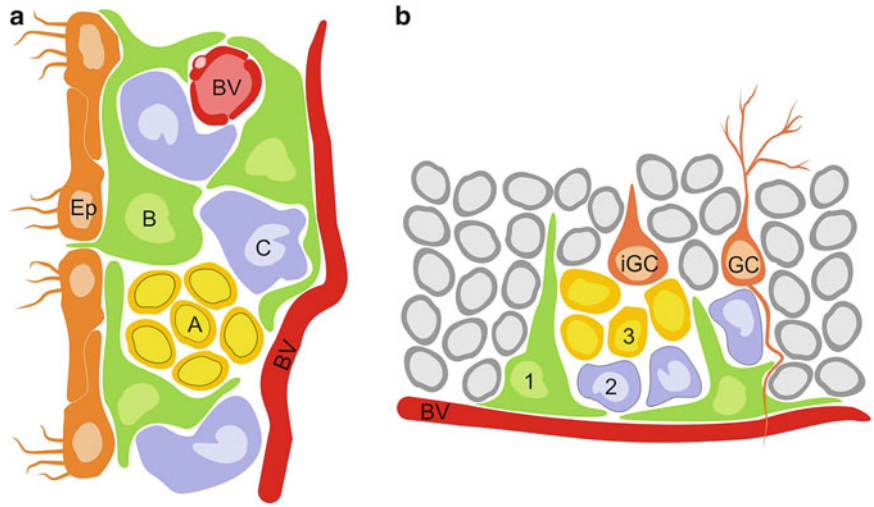

Fig. 1.1 The subventricular zone (*SVZ*) and the subgranular zone (*SGZ*) in the adult mammalian brain. (**a**) The SVZ is separated from the ventricle lumen by the monolayer of ependymal cells (*Ep*). B cells, known as the neural stem cells, extend a cilium through the ependymal layer and contact with blood vessels (*BV*). C cells or transient amplifying progenitors generate neuroblasts (A cells) that migrate in chains out of the SVZ towards the OB. (**b**) In the SGZ, radial astrocytes or type 1 cells (1) are stem cells that divide to give rise to fast proliferating type 2 cells (2). As maturation proceeds, type 3 cells (3) express markers for immature neurons and continue migration towards the granule cell layer. Immature granule cells (*iGC*) extend axons and elaborate dendritic trees so as to complete maturation into granule neurons (*GC*)

scaffold for migration, until the OB. Neuroblasts migration in the RMS is directed by guidance cues either attractive such as prokineticin 2 released by OB cells, or repulsive like Slit that is secreted by the choroid plexus and distributed as a caudal to rostral concentration gradient in the CSF that parallels the RMS flow. When reaching the OB, A cells change their mode of migration; they detach from chains and start migrating with a radial pattern. This detachment has been demonstrated to be promoted by mitral cell's reelin and also tenascin-R. Neuroblasts then differentiate into granule cells and periglomerular cells in the granule (GCL) and glomerular (GL) layers respectively and replace dead ones. Only half of the newly incorporated cells survive beyond 30 days and survival mostly depends on cellular activity related to sensory experience. These newly born neurons participate in processes such as olfactory memory, and thus are extremely important for olfactory behaviors in rodents (for review see Zhao et al. 2008).

In the human brain, the cytoarchitecture of the SVZ is different from the one found in the rodents. In adult humans, the SVZ is also located on the lateral walls of ventricles but comprises four distinct layers: Layer I, a monolayer of ependymal cells surrounding the lateral wall of the ventricles is apposed to Layer II, a layer devoid of cell bodies, also known as the hypocellular gap; Layer III consists of astrocytes surrounding the hypocellular gap; and Layer IV is enriched in myelin and separates Layer III from the brain parenchyma. No clear evidence of a RMS in the human brain was demonstrated to date consistent with an absence of neurogenesis beyond 18 months of age (Sanai et al. 2011).

In the adult DG of the hippocampus, the SGZ situated between the hilus and the GCL harbors stem cells that are responsible for the production of about 10,000 new neurons per day. The cellular organization of the neurogenic DG is similar to what has been described for the SVZ. Type-1 cells (or radial astrocytes) are considered the NSCs of the DG since they self-renew and are multipotent according to *in vivo* fate mapping studies. Type-1 cells share similarities with B1 astrocytes: they extend a primary cilium and

contact blood vessels at their "hilar side" and express GFAP, nestin, and Sox2 (Fig. 1.1b). Controversy persists regarding the stem cell properties of these cells essentially because the sphere forming capacities of these cells *in vitro* are limited: few primary spheres are obtained from adult SGZ cells, they do not passage indicating no self-renewal capacity, and are mainly unipotent generating astrocytes (Clarke and van der Kooy 2011). However, the sphere forming assay as a tool to identify stem cells in a tissue has its limitations. It may not detect quiescent stem cells (such as Type 1 and B1 cells) but rather favor sphere formation from cycling progenitors and "activated" stem cells. In the DG, Type-1 cells asymmetrically divide to generate fast-proliferating nestin-positive cells first negative (Type 2a) and then positive (Type 2b) for double-cortin. These cells give rise to immature neurons or Type-3 cells which are much less proliferative and nestin-negative. Finally, Type-3 cells migrate locally to the GCL, become post-mitotic and differentiate into calbindin positive mature granule neurons (for review see Zhao et al. 2008). New granule neurons extend an elaborated dendritic arborization in the molecular layer and an axon on the CA3 region at around 20 days after their birth. However maturation, including spine growth, continues beyond 8 weeks. As in the OB, the survival of these young neurons is dependent on neuronal activity: a majority of them dies within 3 weeks even after establishing synaptic connections.

Regulation of Neurogenesis

Neurogenesis comprises five cardinal steps: proliferation, migration, differentiation, survival and integration, all regulated by several intrinsic and extrinsic factors. Intrinsic mechanisms regulating neurogenesis and stemness include epigenetic regulators of genes such as transcription factors, microRNAs and enzymes modifying the DNA structure and chemistry without altering its sequence. We will just give here some examples of these mechanisms, and refer to Mu and collaborators for extensive review (Mu et al. 2010). Briefly, transcription factors such as Sox2 and NANOG are classic examples of relevant modulators of differentiation. They have been used to reprogram adult somatic cells such as fibroblasts into NSCs, through an intermediate induced pluripotent stem cell (iPSC) state. Forced expression of these transcription factors artificially tune somatic cells for neurogenesis. This is an extreme example of how transcription factors responsible for the maintenance of stemness and downregulation of differentiation can modulate neuronal differentiation. Sox2 expression is a hallmark of NSCs and is related to stem cell maintenance and proliferation. On the other hand, certain genes are classified as "proneurogenic". This is the case of the basic helix-loop-helix transcription factors Mash1 and Neurogenin-1 and the homeobox transcription factor Dlx2. These factors, expressed in C and A cells promote proliferation and neuronal commitment of the progenitors. MicroRNAs or miRNAs are non-coding RNA that regulates gene expression. Among them, miRNA124 downregulates Sox9 expression, a necessary step for neuronal differentiation in C cells. Finally, changes in DNA structure and chemistry regulate gene expression. These inheritable changes may be through DNA methylation and histone modification, among other molecular chromatin alterations. Histone modifications include a variety of posttranslational changes, from which acetylation is the most studied. Acetyltransferases (HATs) are enzymes that acetylate conserved lysine residues on the N-terminus of the histone protein core of the nucleosomes. Upon this acetyl group addition, the DNA packing into heterochromatin is loosed into euchromatin, which makes it more prone to be actively transcribed.

Regarding extrinsic regulation, panoply of diffusible and contact factors has been identified to modulate different steps of neurogenesis. These factors, that include growth factors, cytokines, hormones, morphogens, neurotransmitters and neuropeptides, are secreted by the cellular components of the niche. Ependymal cells, for instance, secrete Noggin to antagonize the progliogenic effect of bone morphogenetic protein on SVZ cells and thereby favoring neuronal

production. The same antagonism exists in the DG with astrocytes secreting Neurogenesin-1. Astrocytes of both neurogenic areas are a source of proinflammatory cytokines and morphogens like sonic hedgehog (SHH) that induces proliferation and neuronal differentiation. In the SVZ, dopamine and serotonin innervations promote proliferation. Neuropeptides co-released with neurotransmitters such as the neuropeptide Y induces proliferation and neuronal differentiation in the DG and the SVZ. Most interestingly, regulatory loops have been identified to control the expansion of populations of proliferating cells in the niche. Indeed, GABA is released by SVZ neuroblasts and acts in an autocrine manner to decrease their proliferation and speed of migration. Moreover, NSCs GABA decreases proliferation of NSCs *via* $GABA_A$ receptor activation. GABA signaling therefore regulates neuroblasts production. On the other hand, NSCs release glutamate that increases proliferation in SVZ cells. Also, progenitors regulate proliferation and self-renewal of SVZ stem cells through a mechanism involving the interaction of the epidermal growth factor receptor and Notch pathway. The vasculature is another key cellular component of the stem cell niche. The blood brain barrier present in the SVZ is modified and stem cells contact directly the endothelial cells without the presence of astrocytes end feet or pericytes. Nestin expressing astrocytes in the DG extend a long apical process through the molecular layer and contact blood vessels. These interactions with blood vessels contribute to controlling stemness and neurogenesis. Endothelial cells secrete factors supporting self-renewal and maintenance in an undifferentiated state of NSCs. Other endothelial-derived factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), stroma cell-derived factor-1 (SDF-1) promote neurogenesis. Moreover, extracellular matrix molecules secreted by brain endothelial cells, namely laminin, regulate adherence, stemness and proliferation of SVZ stem cells and progenitors. Finally, environmental factors that induce neuronal activity such as physical exercise, social interactions and enrichment of the environment, stimulate neurogenesis in the DG while aging and stress are usually deleterious for neuronal production in both niches. We provided here some few examples of factors controlling stem cells niches and neurogenesis, more exhaustive lists and references are reviewed in (Mu et al. 2010; Ihrie and Alvarez-Buylla 2011; Ming and Song 2011).

Histamine as a Modulator of Neuronal Differentiation

Histaminergic neurons project broadly throughout the CNS, including the cortex, hippocampus, basal ganglia and also into the epithelial ventricular zone, thus reaching both neurogenic niches. Moreover, histamine present in the CSF can affect stem cells either *via* ependymal cells (paracrine effect) or directly *via* the cilium of SVZ GFAP-positive stem cells (type B cells) that contact the ventricle lumen. In the healthy brain, the "bulk" concentration of histamine is very low. In fact, several studies reported that histamine is present at nanomolar concentrations in the CSF of humans and rodents. However, circulating levels of histamine are robustly increased following brain injury, degeneration or infection (Soya et al. 2008; Kanbayashi et al. 2009). Under these pathological conditions, the inflammatory response may trigger degranulation of mast cells in the choroid plexus, leading to a massive release of histamine in the CSF and blood brain barrier permeability. Therefore, present in the milieu, histamine may play a role in neurogenesis. In fact, it was shown that histamine receptors are expressed in undifferentiated neural progenitors and that histamine transiently increases intracellular calcium free levels ($[Ca^{2+}]_i$) *via* H1R activation in SVZ immature/stem cells, embryonic stem and carcinoma cells (Agasse et al. 2008).

It was firstly shown by (Molina-Hernandez and Velasco 2008) that histamine induces proliferation and neuronal differentiation in NSCs cultures from rat embryonic cerebral cortices. More recently, we proved that histamine, *via* H1R activation, induces neuronal differentiation in early postnatal SVZ precursor cells from mouse. Histamine triggered histone H3 trimethylation

on lysine K4 on the promoter regions of the proneurogenic genes *Mash1*, *Dlx2* and *Ngn1*, leading to their increased expression, ultimately favoring neuron commitment (Bernardino et al. 2012). In agreement with our results, (Rodriguez-Martinez et al. 2012) showed that histamine is required during cell proliferation in order to increase neuron differentiation of cortex neuroepithelium progenitors. This effect is mediated by an increase in Ngn1 expression, complementary to our data, and also in Prox1. Altogether these reports suggest that histamine can amplify the neurogenic capabilities of NSCs and thus be used for stem cell-based therapies aimed to promote brain repair of the diseased brain.

Ontogeny of Gliomas: Genesis in the SVZ?

The idea that NSCs could be the cell of origin for glioma is based on several evidences including: periventricular localization of most of brain tumors and detection of positive immunoreactivity for immature markers such as nestin in human tumor cells. Moreover, CSCs from glioma have been isolated using laboratorial methods classically used in stem cells biology. Harvested from the tumor, they are able to divide and form spheres in serum free medium in the presence of growth factors and absence of adherence. When plated in adherence and induced to differentiate with serum, CSCs can differentiate into neurons, astrocytes and oligodendrocytes although there is a huge variability among tumors as well as aberrant co-expression of markers in the cells generated, which discriminates CSC's progeny from the one differentiated from NSCs. Moreover, CSCs are resistant to conventional chemo- and radio-therapies, that result in tumor shrinkage, and are responsible for tumor recurrence and spreading. It seems that anti-cancer therapies mainly target the differentiated population of cells in the tumor representing the bulk of the tumor cells, while sparing the CSCs (Fig. 1.2). Thorough understanding of CSCs biology represents therefore a major challenge in order to develop efficient anti-cancer therapies. A frantic effort in unveiling the molecular basis of chemo- and radio- resistances is currently being made by the scientific community. Interestingly, exacerbation of the activation of signalling pathways involved in NSCs self-renewal and stem cell maintenance, referred as to "stemness", have been shown to mediate CSCs resistance to therapies such as the Notch and the sonic hedgehog pathways and targeting these pathways has already been proved to be efficient to chemosensitize the CSCs to classical anti-glioma drugs such as temozolomide.

CSCs differ from NSCs in their capacity to survive chemo- and radio-therapies. In fact, NSCs are sensitive to anti-cancer therapies and decrease in neurogenesis following treatment might partly account for the cognitive decline observed in patients. Some of the mechanisms conferring resistance to CSCs have been unveiled. CSCs survive ionizing radiation treatment by activating the DNA checkpoint kinases Chk1 and Chk2 which allows the DNA damages caused by the radiation to be repaired (Bao et al. 2006). Also, it has been suggested that CSCs express higher levels of the O6-methyl-guanine-DNA-methyltransferase, the enzyme that repairs cytotoxic DNA damages caused by temozolomide. CSCs display higher levels of pump of drug efflux such as the ABC transporter ABCG2 as compared to NSCs, which may play a crucial role in resistance. However, temozolomide has been found not to be a substrate of ABCG2 (Bleau et al. 2009).

Since CSCs are resistant to both chemo- and radiation therapies, the identification of the cell of origin of glioma and the mechanisms regulating proliferation and self-renewal are under intense investigation with the aim to find new treatments for the cure of gliomas. Several efforts have also been made in order to find a unique marker for both NSCs and CSCs. Among them, CD133 (prominin-1), has been detected in a number of normal and cancer stem cells including brain tumors suggesting a possible lineage relationship between neural and brain tumor stem cells. In the mouse brain, CD133 expression was found on neural stem cells in the embryonic brain, on an intermediate radial glial/ependymal cell type in the early postnatal stage, and on ependymal cells in the adult brain (references in

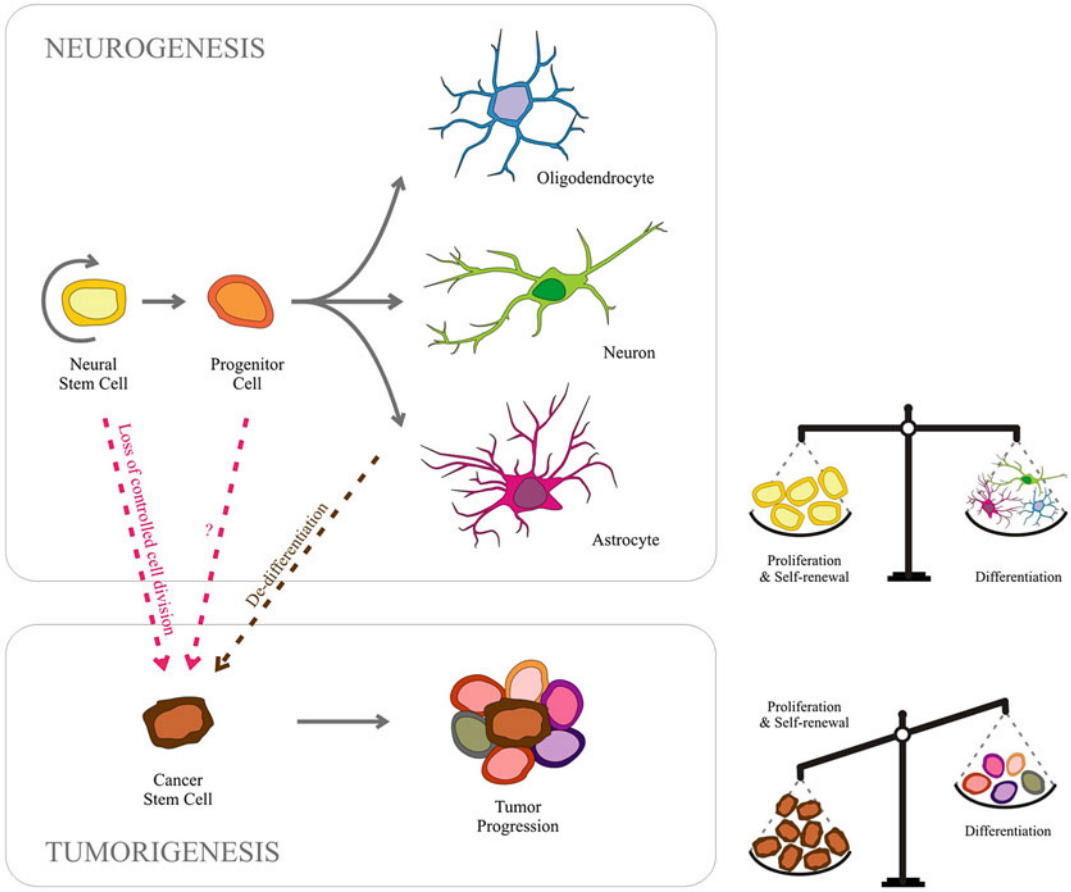

Fig. 1.2 Relationship between neurogenesis and tumorigenesis processes. Neural stem cells in the SVZ give rise to progenitors that differentiate either in neurons, astrocytes or oligodendrocytes (*upper left panel*). Neurogenesis is a balanced mechanism involving proliferation of stem/progenitor cells followed by cell differentiation (*scale on the upper right panel*). Cancer stem cells may arise from the deregulation of the mechanisms that control stem/progenitor cell proliferation and self-renewal and/or the dedifferentiation of more mature cell types. Cancer stem cells initiate and sustain tumor growth. Glioma consists of astrocytes and/or oligodendrocytes often not completely differentiated (*lower left panel*). Cancer stem cells display exacerbated proliferation and self-renewal capacity with aberrant and incomplete differentiation of their progeny (*scale in the lower right panel*)

(Ihrie and Alvarez-Buylla 2011). CD133+ glioma cells are more radioresistant than CD133- tumor cells, implying that they may be the cancer stem cell population responsible for disease recurrence after therapy (Bao et al. 2006). However, evidences suggest that CD133 is not a specific marker for CSCs, instead it may be an indicator of an intermediate state related to adaptability to the hypoxic conditions observed in regions of tumor outgrowth. Moreover, tumor development was observed from CD133 negative cells. There is a crucial need to identify a unique marker for CSCs or a specific pathways that could be targeted in order to deplete CSCs without damaging other cells types including NSCs.

Genetic and Environmental Factors Involved in the Neoplastic Transformation

Mechanisms responsible for the neoplastic transformation of NSCs to CSCs become to be unveiled and involve genetic alterations and

perturbations in the homeostatic mechanisms that maintain the integrity of the niche. Causes of their occurrences *in vivo* remain elusive. Acquisition of genetic mutations that confers CSCs properties to stem cells have been demonstrated experimentally. Genetic mutations found in glioma mainly affect three core signaling pathways namely the p53, retinoblastoma (Rb) and receptor tyrosine kinase (RTK) pathways (reviewed in (Chen et al. 2012b). In the normal stem cell niches, these pathways regulate proliferation, cell-cycle, apoptosis and senescence. Mutations in the p53 tumor suppressor gene are frequent in human glioma. P53 expression in the adult brain is confined to germinal areas where it restrains proliferation. The loss of p53 associated with fetal exposure to the mutagen ENU, results in the formation in adult mice, of periventricular areas of cellular hyperplasia, characterized by clusters of GFAP+cells, mature glia, and neuroblasts. The neoplastic transformation is characterized by dramatic changes in the properties of the quiescent adult SVZ cells, including enhanced self-renewal, recruitment to the fast-proliferating compartment and impaired differentiation (Gil-Perotin et al. 2006). A recent study (Jacques et al. 2010) provides further evidence for the SVZ stem cells being the cells-of-origin for glioma. Using Cre-Lox targeted ablation of key tumor suppressors specifically in GFAP expressing cells, it has been shown that combined ablation of p53 and PTEN in the SVZ stem cell compartment leads to the growth of gliomas containing GFAP expressing cells. Instead, p53 and Rb deletions generate primitive neuroectodermal tumors, i.e. expressing neuronal markers. The combined ablation of the 3 genes reduces the latency to tumor development resembling the p53/Rb generated tumors. These tumors are similar to the ones observed in humans. This study supports the idea that combination of disrupted genes in SVZ stem cells may account for the histological diversity observed in gliomas. Also, transgenic mice overexpressing activated K-Ras and AKT in nestin positive cells, developed glioblastomas. Homozygous deletion of the INK4a-ARF locus, often observed in gliomas, exacerbates tumor incidence from K-Ras and AKT overexpressing neural precursors cells. INK4a and ARF are tumor suppressor genes encoding the Rb negative regulator proteins $p16^{INK4a}$ and $p14/p19^{ARF}$. K-Ras and AKT are both elicited by RTK signaling. Ras proteins are a family of small GTPases which role consists in transmitting the signals from upstream growth factor receptors such as epidermal growth factor (EGF) receptor and platelet-derived growth factor (PDGF) receptor, to down-stream cytoplasmic effectors, such as mitogen activated protein kinases (MAPKs) and AKT promoting cell growth and division. In accordance with this hypothesis, constitutive activation of K-Ras in GFAP-expressing SVZ cells of mice leads to deregulated proliferation and glioma growth (Abel et al. 2009). Increase in the number of copies of the ras gene is indeed seen in glioma samples from patients however, these mutations are not prevalent in glioma. Nonetheless, there is an increase in the Ras activity observed in gliomas. This increase is mainly due to the hyperactivation of the up-stream growth factor receptors as mutations of the EGFR receptor to the EGFRvIII constitutively activated form is a common feature in gliomas, or to the mutation of Ras inhibiting regulators (Abel et al. 2009). Other mutations are reported across the literature and reviewed in (Chen et al. 2012b).

There is an increasing body of evidences that perturbation in the microenvironment homeostasis may act synergistically with genetic mutations in the process of transformation of NSCs into CSCs. Overexpression of growth factors in the niche exacerbate SVZ cell proliferation and lead to neoplasm formation. Indeed, infusion of EGF in the lateral ventricles leads to the proliferation of C cells acquiring a glial phenotype with increase capability of invasion. Similarly, injection of PDGF stimulates proliferation of B cells and the growth of astrocytoma-like structures in the ventricles (reviewed in Ihrie and Alvarez-Buylla 2011).

Also, sensitivity to hypoxia is a common feature in both the neural and cancer stem cells niches (Mannello et al. 2011). In culture, the conditions are considered hypoxic when cells are exposed to 10% O_2 as compared to normoxia

(20% O_2). *In vivo*, secretion of factors known to stimulate proliferation, survival and migration of neural/progenitor cells [among them VEGF, erythropoietin (EPO), stem cell factor (SCF), SDF-1α and monocyte chemoattractant protein-1 (MCP-1)] is upregulated under hypoxia. Moreover, hypoxia promotes stemness in NSCs cultures while normoxia increase astrocytic differentiation and apoptosis in a p53 dependent manner. These effects rely on the stabilization of the transcription factor subunit hypoxia-inducible factor-1α (HIF-1α) upon hypoxia. HIF-1α heterodimerizes with β subunit (HIFβ/ARNT) to form a transcription factor. The heterodimer binds hypoxia response elements and activate multiple target genes. Interestingly, physical interaction of HIF1α to Notch intracellular domain may increase the half-life of Notch and participate to inhibition of differentiation in NSCs. In tumors, the peri-necrotic area is highly hypoxic and low levels of oxygen correlate with aggressiveness of the disease and poor clinical outcome. Hypoxia expands CD133+ CSCs in glioma and stimulates, *via* HIF-1α, the expression of genes related with multipotency such as Sox2, NANOG and OCT4 in cancer cell lines.

In addition and similarly to what happens in the NSCs, the interactions between the vasculature and the CSCs play a crucial role in controlling CSCs dynamics. In particular, the laminin and α6β1 integrin system has been described in this modulation. Firstly, CSCs express high levels of α6 integrin and selecting for α6 integrin is more efficient in enriching for stem cells from glioma cells over the canonical CD133 marker (Lathia et al. 2010). Moreover, α6 integrin signaling is involved in survival and expansion of CSCs *via* the activation of the AKT pathway. Specific downregulation of α6 integrin expression decreases self-renewal and tumor formation capacities of CSCs (Lathia et al. 2010). These effects may depend on the interaction of α6β1 integrin with endothelial cells-derived laminin as protocols describe successful propagation of CSCs in monolayers by using laminin coating. Moreover, endothelial cells also sustain self-renewal and proliferation capacities of CD133+ CSCs and specific elimination of CD105+ endothelial cells decreases Notch effectors mRNA levels in tumor cells, demonstrating that endothelial cells-derived paracrine factors, including contact factors, promote stemness *via* activation of the Notch pathway (Hovinga et al. 2010). Other endothelial-derived factors yet to be identified have been shown to stimulate stemness and survival of CSCs *via* the mTOR pathway (Galan-Moya et al. 2011). It is therefore plausible that deregulation of the vasculature secretome may favor CSC emergence from NSCs or at least participate in CSCs expansion, tumor growth and tumor relapse. In the light of this, a thorough understanding of the NSC niche will give the keys to decipher its deregulation in tumorigenesis and help to design new therapeutics in order to eradicate CSCs and to avoid tumor relapse from residual CSCs.

The Role of Histamine in Cancer: Focus on Glioma

NSCs in the SVZ are regulated by soluble and contact factors referred as the stem cell niche (for review see Ihrie and Alvarez-Buylla 2011). In addition to genetic alterations, perturbation of the stem cell niche may account for the emergence of CSCs from NSCs. Among other factors, histamine is found in the host milieu of several cancer types, thus suggesting that it may be a crucial factor involved in tumorigenesis. In fact, levels and activity of histidine decarboxylase (HDC), the only enzyme responsible for the generation of histamine from L-histidine, are significantly increased in both experimental and human tumors. Moreover, the coexistence of endogenously produced histamine and its specific membrane receptors strongly suggests an autocrine/paracrine loop for histamine in cancer (for review see Falus et al. 2010).

Many evidences have been shown that histamine may be involved in several cancer cell types' development and progression. Experiments performed in malignant cell lines and experimental tumors *in vivo* suggest that histamine modulates diverse biological responses related to tumor growth, including proliferation, survival,

differentiation, migration, modulation of inflammation and angiogenesis (Table 1.1). Interestingly, one report (not present in the Table 1.1), showed that immature myeloid cells, known as myeloid-derived suppressor cells, regulate cancer development *via* their synthesis of histamine. *Hdc*-deficient mice showed increased susceptibility to chemically induced colon and skin cancers, indicating that histamine restricts tumor growth. These transgenic mice showed higher IL-6 production and concomitantly IL-6 deficient immature myeloid cells were unable to trigger tumor growth, suggesting that IL-6 may regulate tumor growth and proliferation and, in normal conditions, this pathway is inhibited by histamine (Yang et al. 2011). This study highlighted the benefits of using histamine-based therapies in cancer. On the other hand, some clinical trials showed increased survival of patients with gastric and colon cancer after treatment with H2 receptor antagonists, such as cimetidine or ranitidine (Matsumoto et al. 2002). These two reports exemplify the often contradictory effects mediated by histamine in cancer cell biology.

There are fewer studies showing the role of histamine in gliomas. It was already shown that HDC expression is higher in gliomas, and several brain tumor cells display changes in Ca^{2+} concentrations upon stimulation with histamine, suggesting the presence of functional histamine receptors (for review see Panula et al. 2000). It has been reported that histamine may enhance cell proliferation and consequently tumor growth of several tumors, including glioma, whereas cimetidine can reverse these effects. Moreover, co-administration of cimetidine and TMZ to nude mice with U373-derived human brain tumors enhances survival as compared to treatment with TMZ alone (Lefranc et al. 2006). (Hernandez-Angeles et al. 2001) showed that histamine stimulated proliferation of a human astrocytoma cell line by H1R activation and the downstream stimulation of PKC. The combined immunotherapy of two immune factors has been also suggested as a mechanism to burst the inflammatory reactions against tumor cells, leading to a better prognosis of the disease. In agreement, (Johansson et al. 2000) showed that the combination of IL-2 and histamine reduced intracerebral glioma growth in an orthotopic rat glioma model, in opposite with the lack of effect when IL-2 or histamine were administered alone. In this study, reduction in microvessel density was observed in the group of animals treated with both the drugs. Besides these reports, there is yet a long road to clarify the role of histamine in cancer stem cell transformation and in the progression of gliomas.

Discussion

NSCs divide and generate new neurons constitutively throughout life in the adult mammalian SVZ and SGZ neurogenic niches. Adult neurogenesis is of physiological relevance as newly-born neurons participate to olfaction and in learning and memory in mammals although no clear evidence of SVZ neurogenesis was shown in humans. It has been suggested that the deregulation of the mechanisms controlling stem cell homeostasis could trigger the neoplastic transformation of NSCs to CSCs. Indeed, even if the precise identity of the cell of origin for gliomas remains elusive, there are strong evidences supporting NSCs as candidates. In fact, both populations share cellular and molecular characteristics including self-renewal, proliferation, multipotency and antigen expression. Main differences between the two stem cells types reside in the fact that only CSCs are able to generate and grow brain tumors and that CSCs are highly resistant to both radiation and chemotherapy. More in-depth knowledge regarding NSCs' biology will help to unravel the cellular and molecular pathways controlling CSCs maintenance and survival, which is warranted in order to promote the development of novel and more effective anticancer therapies.

Histamine is an endogenous biogenic amine that has been demonstrated to modulate cell proliferation, survival and differentiation of NSCs and several types of tumor cells. Presence of histamine in both normal and cancer stem cells niches along with expression of functional histamine receptors suggest that histamine could modulate both NSCs and tumoral cells. So far, the effects of histamine in neurogenesis and

Table 1.1 Effects of histamine and its receptors in several cancer types

	Experimental models	Effects of histamine	Histamine receptors involved	References
Breast cancer	Mammary biopsies; MDA-MB-231 cell lines	Increased cell proliferation and migration via H3R and inhibited proliferation and induced cell cycle arrest and apoptosis via H4R activation	H3R and H4R	Medina et al. (2008)
	MDA-MB-231 and MCF-7 cell lines	H4R agonists inhibited cell proliferation and induced cell senescence and apoptosis	H4R	Medina et al. (2011)
Colon cancer	C170, LIM2412 cell lines and xenographs in vivo	Stimulated cell proliferation and tumour growth	H2R	Adams et al. (1994)
	Colorectal cancer biopsies and HT29, Caco-2, and HCT116 cell lines	Stimulated cell proliferation and angiogenesis	H2R and H4R	Cianchi et al. (2005)
Melanoma	HT168 cell lines and xenographs in vivo	H2R antagonists blocked tumour cell proliferation	H2R	Szincsak et al. (2002a, b)
	Malignant melanoma implants	Stimulated growth of malignant melanoma implants	H2R	Tomita et al. (2005)
	WM35 and M1/15 cells lines and melanoma biopsies	Inhibited proliferation and induced cell senescence	H4R	Massari et al. (2011)
Pancreatic cancer	PANC-1 cell lines	Inhibited cell proliferation and induced cell cycle arrest	H2R	Cricco et al. (2006)
	PANC-1 cell lines	Cell proliferation increased through H3R activation and diminished via H4R activation	H3R and H4R	Cricco et al. (2008)
Biliary cancer	CCA cell lines	H4R agonist decreased cell proliferation and inhibited xenograft tumor growth	H4R	Meng et al. (2011)
	Human cholangiocarcinoma cell lines	Promoted cell proliferation, tumor growth and VEGF expression	H1R	Francis et al. (2012)
Gastric cancer	SGC-7901 and MGC-803 cell lines	Cimetidine induced apoptosis of gastric cells and inhibited tumor growth	H2R	Jiang et al. (2010)
Leukemia	Leukemic precursors from ALS patients and ALL cell lines	Induced cell proliferation	H1R	Malaviya and Uckun (2000)
Lung Cancer	A549 and LLC cell lines	Enhanced cell proliferation	H1R, H2R and H4R	Stoyanov et al. (2012)
Salivary gland cancer	Human salivary gland tumor cells	Cimetidine induced cell apotosis	H2R	Fukuda et al. (2008)

NSCs are consistent: histamine promotes neuronal differentiation in NSCs cultures (Molina-Hernandez and Velasco 2008; Bernardino et al. 2012; Rodriguez-Martinez et al. 2012). Next steps of investigation must determine the effects of histamine in the neurogenic niches *in vivo*. Nevertheless, these results pave the way for the use of histamine as a pro-neurogenic factor to boost neurogenesis from endogenous stem cells in the diseased brain. Also, histamine may be a crucial mediator in cancer development and progression as it modulates diverse biological responses related to tumor growth such as proliferation, angiogenesis, differentiation, survival and modulation of the immune responses. In fact, the majority of the reports are consistent with the view of histamine as an autocrine/paracrine growth factor stimulating tumor growth. However, in several experimental cancer models and clinical trials, the effects of histamine are contradictory either stimulating or inhibiting tumor growth. This can be due to the type of receptor activated and consequently the different downstream regulatory pathways induced, affinity and abundance of each receptor subtype, the endogenous/exogenous histamine concentration and the cancer cell types under study. Even if the comprehension regarding the regulatory pathways activated by each histamine receptor in both neurogenesis and tumorigenesis is warranted, there are already several evidences that strengths the idea that histamine and its receptor agonists/antagonists are promising new proneurogenic and anticancer agents.

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Emerging Concepts of Stem Cell Organization in the Normal Lung and in Lung Cancer

2

Joanne L. Van der Velden, Ivan Bertoncello, and Jonathan L. McQualter

Contents

Abstract

Gaps in our knowledge about the organization and regulation of regenerative cells in the normal and diseased adult lung have been a major impediment to the identification of targets around which improved therapies for intractable lung diseases and lung cancer could be developed. During the last decade, specific lung injury models, the availability of genetically engineered reporter mice, and the development of robust cell separative methods and clonogenic assays for the identification and characterization of adult lung epithelial stem cells have provided the field with powerful tools for the identification and validation of predictive stem cell biomarkers and critical molecular pathways and microenvironmental signals which regulate their behavior. These approaches are key to the development of new therapies to restore healthy lung epithelium following injury or disease.

Introduction

The respiratory epithelium forms a physical barrier to inhaled foreign particles and microbes, protecting the lung from infections. Epithelial maintenance in the steady-state, and epithelial regeneration and repair after damage, is thought to be mediated by the proliferation of continuously renewing endogenous stem cells and their differentiation into the specialized epithelial cells of the conducting airway and alveolar beds.

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However, progress in characterizing epithelial stem and progenitor cells in the adult lung and in identifying the critical microenvironmental signals which regulate their activity has been hampered by the lack of specific markers for their isolation and of robust functional assays to measure their potential. These limitations are being overcome with the increasing availability of instructive, genetically engineered reporter mouse models and the development and refinement of flow cytometric cell sorting strategies and *in vitro* clonogenic assays for their identification and quantitation. These approaches provide powerful tools for the analysis of the organization and regulation of regenerative cells in the healthy lung, and in advancing our knowledge of the dysregulated behavior of lung stem cells following injury, and in understanding the nature and properties of lung cancer initiating cells. In this chapter we discuss what is known about the identity and regulation of epithelial stem and progenitor cells in the developing and adult lung, and how this knowledge informs the characterization of lung cancer initiating cells and the development of novel therapies for lung diseases.

Lung Development

The analysis of fetal lung development has contributed significantly to our understanding of adult lung regeneration and repair after injury. The embryonic lung originates from epithelial progenitors located in the anterior foregut endoderm which form the bronchial airway tree by the process of branching morphogenesis. Briefly, following the outpouching of the foregut, epithelial lung buds grow into the surrounding mesenchyme where fibroblast growth factor-10 (Fgf-10) secreted by smooth muscle progenitors acts to promote epithelial progenitor cell proliferation and inhibit differentiation. As the airway branches, the descendants of these progenitors remain in the airway stalks where they begin to differentiate, while the progenitors continue to proliferate at the growing distal tips of the branching lung epithelium (Rawlins 2008). Cell lineage-tracing has confirmed that self-renewing epithelial progenitor cells give rise to all lung epithelial cell lineages during the pseudoglandular stage of lung development and are characterized by the expression of inhibitor-of-differentiation-2 (Id2) (Rawlins et al. 2009a). Tracheobronchial and bronchiolar airway lineages are subsequently established during the transition from the pseudoglandular to canalicular stage of lung development when proximal airway cells lose the ability to respond to mesenchymally-derived signals capable of inducing distal airway differentiation (Hong et al. 2004a).

Although the process of adult lung regeneration and repair is largely thought to recapitulate ontogeny (Warburton et al. 2001) current evidence suggests that separate epithelial progenitor cell populations are responsible for building the lung during fetal development, and for maintaining and replenishing epithelial cell lineages in the adult lung. This includes the observation that the majority of genes expressed in distal tip progenitor cells in the embryonic lung are not expressed in the adult lung (Rawlins 2008) and that Id2-positive progenitor cells which play a significant role in lung development are not retained in the adult lung (Rawlins et al. 2009a).

Heterogeneity of Adult Mouse Lung Epithelial Stem and Progenitor Cells

The composition of cells that comprise healthy adult lung epithelium varies along the proximal-distal axis of the lung. The tracheobronchial airways are lined by pseudostratified basal, non-ciliated club (previously named Clara cells) and ciliated cells, interspersed with neuroendocrine cells and collecting duct cells which extend into submucosal glands (SMG). The bronchiolar airways are lined by columnar epithelium largely comprising ciliated and club cells, as well as foci of neuroendocrine cells which are referred to as neuroepithelial bodies (NEB). Proximal airways and bronchiolar epithelia also contain interspersed mucus-producing goblet cells which increase markedly in abundance in chronic lung

diseases such as asthma and chronic obstructive pulmonary disease (COPD). On the other hand, alveolar epithelium comprises alveolar type I (squamous) and type II (cuboidal) cells which are the primary sources of surfactant protein-C (SPC, Sftpc).

While it has long been accepted that damaged and senescent epithelial cells in the healthy lung are continually replenished by proliferating and differentiating stem and progenitor cells throughout life, it is only recently that significant inroads have been made into understanding the precise identity and organization of epithelial stem and progenitor cell pools responsible for epithelial homeostasis, regeneration and repair. This has been made possible by exploiting the selective toxicity of drugs to analyze the temporal pattern of epithelial cell regeneration following ablation of specific lung epithelial cell lineages; the development of genetically-engineered reporter mouse models for cell lineage tracing; and the development of robust flow cytometric cell separative strategies for the identification and prospective isolation of candidate lung stem and progenitor cells, and functional *in vitro* clonogenic assays to measure their incidence and proliferative and differentiative potential. The naphthalene-injury model has proven especially useful in describing the organization and properties of airway epithelial stem and progenitor cells. Mature club cells, which comprise the majority of the proximal airway epithelium, are selectively ablated by systemic administration of naphthalene because they express the CYP2F1 and CYP2F2 isoforms of cytochrome P450 enabling them to metabolize naphthalene to generate a cytotoxic derivative, while their precursors are spared (Buckpitt et al. 1992; Reynolds et al. 2000).

Temporal analysis of airway epithelial regeneration in naphthalene and hypoxic injury models has identified distinct progenitor cell populations in the submucosal glands (SMG) and proximal airways. In particular, experiments have identified that murine SMG duct cells that express Krt14 are resistant to severe hypoxic-ischemic injury (Hegab et al. 2011). This population was shown to be self-renewing and was capable of differentiation along ciliated and secretory lineages to SMGs tubules and ducts, as well as the epithelium overlying SMGs, suggesting that there is a multipotent progenitor cell responsible for maintaining the SMGs. In contrast, basal cells in the proximal airways act only as progenitors responsible for repopulation of airway epithelium (Hong et al. 2004b; Rawlins et al. 2009b). Lineage tracing using a cytokeratin-14 (Krt14)-CreER transgene to follow the fate of basal cells in the tracheobronchial airways of mice following naphthalene injury, showed that Krt14-positive basal cells were able to self-renew and labeled basal, ciliated and club cells (Hong et al. 2004a, b). Other studies have demonstrated that Krt5-labeled basal cells are also able to give rise to both ciliated and club cells in both the steady state and following injury (Rock et al. 2009). The relationship between Krt5 and Krt14-positive cells is unclear.

In the bronchiolar airways, epithelial stem/progenitor cells have been identified as naphthalene-resistant cells that also express club cell secretory protein (CCSP, Scgb1a1). These 'variant club cells' are located at the bronchioalveolar duct junction (BADJ) and are associated with calcitonin gene-related peptide (CGRP) expressing neuroepithelial bodies in the branching airways (Giangreco et al. 2002; Reynolds et al. 2000). Variant club cells are transit amplifying cells that give rise to mature club cells and ciliated cells following naphthalene-induced epithelial injury (Hong et al. 2004a, b). Another study has shown that progenitor cells at the BADJ express both airway (CCSP) and alveolar (SPC) epithelial lineage markers. Termed bronchioalveolar stem cells (BASCs), these cells are resistance to naphthalene-treatment and have been shown to proliferate in response to both bronchiolar and alveolar injury (Kim et al. 2005).

In the alveoli, it has long been established that alveolar type II (AT2) cells function as progenitor cells capable of giving rise to alveolar type I (AT1) cells. A recent study by Chapman and colleagues has shown that a subset of murine alveolar epithelial cells that express the integrin-receptor α6β4, but are deficient in pro-SPC, serve as alveolar progenitors during lung regeneration following bleomycin induced lung injury (Chapman

et al. 2011). More recently, studies have also shown that despite being absent from the distal lung in the steady state, p63-expressing cells proliferate and contribute to alveolar regeneration in response to epithelial damage following H1N1 influenza infection (Kumar et al. 2011). The cell surface marker CD74, has also been identified as a positive-selection marker that enriches murine AT2 cells capable of generating alveolar-like colonies when cultured in Matrigel (Lee et al. 2012).

The diversity of epithelial progenitor cells identified in the adult lung in the various injury models suggests that the fate of epithelial stem and progenitor cells is context dependent. One study using an aggregation chimera mouse model showed that lung epithelium was maintained by randomly distributed progenitor cells during homeostasis and repair after moderate injury. In contrast stem cells associated with putative stem cell niches (BADJ and NEB) were necessary to regenerate the denuded lung epithelium after severe injury (Giangreco et al. 2009). Other studies using flow cytometry-based sorting strategies in combination with *in vitro* colony-forming assays (Bertoncello and McQualter 2011) have identified multipotent EpCAM^{pos} Sca-1^{low} α6^{pos} β4^{pos} CD24^{low} epithelial stem/progenitor cell in the adult mouse lung (McQualter et al. 2010). When co-cultured with EpCAM^{neg} Sca-1^{pos} lung mesenchymal feeder cells in a Matrigel-based clonogenic assay, these cells form distinct airway, alveolar or mixed lineage epithelial colonies. Furthermore, when mixed-lineage colonies are dissociated and reseeded they are able to form lineage-restricted airway and alveolar colonies as well as reform mixed lineage colonies, while reseeding of airway and alveolar colonies gives rise to their respective lineages (McQualter et al. 2010). Separate studies have demonstrated that α6^{pos} β4^{pos} progenitor cells give rise to airway and alveolar epithelial cells (Chapman et al. 2011). These findings support the existence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung, in which multipotent epithelial stem/progenitor cells are able to self renew and contribute descendants to airway and alveolar lineages.

Another recent study also reports that three different progenitor populations can be segregated from the heterogeneous EpCAM^{pos} CD24^{low} epithelial stem/progenitor cell population in mice on the basis of the differential expression of an Sftpc-GFP transgene (Chen et al. 2012). The different subsets were distributed along the proximal-distal lung axis, with GFP expression ranging from undetectable in the proximal conducting airways, to low in the terminal bronchioles and high in the alveoli. Furthermore, when placed into culture, GFP^{neg} cells largely produced colonies that resembled pseudostratified epithelium, while GFP^{low} and GFP^{hi} cells formed epithelial colonies that resembled distal airway epithelium. These findings support the concept that local hierarchies of epithelial progenitors are responsible for maintaining different epithelial regions along the proximal-distal lung axis.

Despite the recent advances in the identification of multipotent epithelial stem cells and lineage restricted progenitor cell populations in the adult lung, it remains an open question whether the adult lung epithelium is maintained by an epithelial stem/progenitor cell hierarchy with a single multipotent stem cell subpopulation that gives rise to regional lineage-specific progenitors or by discrete functionally distinct progenitor cells that maintain the anatomically diverse regions of the airways and alveoli.

The Role of the Niche Microenvironment in Regulating Stem and Progenitor Cell Potential

It is also important to remember that the regenerative potential of stem and progenitor cells is not only determined by their intrinsic potential, but also by external regulatory factors provided by their microenvironment. These include cytokines, adhesion molecules, stromal cells, immunomodulatory cells and extracellular matrix proteins. For example, *in vivo* studies have shown that mesenchymal stromal cells (MSC) are involved in re-epithelialization following naphthalene-induced lung injury (Volckaert et al. 2011), and we have also demonstrated that the colony-forming potential of mouse lung epithelial stem/progenitor cells in culture is conditional on the

presence of co-cultured CD45^{neg} CD31^{neg} Sca-1^{pos} lung MSC (McQualter et al. 2010).

In vivo interaction between stem cells and the microenvironment is likely to be even more complex considering that the adult lung comprises at least 40–60 different cell types. Importantly, stem and progenitor cell behavior will also be influenced by the significant changes in the composition of the microenvironment in chronic respiratory diseases, such as smooth muscle hyperplasia, matrix deposition, angiogenesis, and inflammation and cytokine production. Consequently, the physiological and pathophysiological behavior of lung epithelial stem/progenitor cells will be dictated by temporospatial changes in their physical microenvironment and elaboration of regulatory factors in the steady state or following perturbation or injury. Further work is needed to understand if these changes in the airway microenvironment are coincidental or contributory to dysregulation of epithelial stem/progenitor cells and the associated epithelial remodeling.

Human Lung Epithelial Stem and Progenitor Cells

To date, most studies aimed at identifying, isolating and characterizing stem and progenitor cells in the adult lung have concentrated on the analysis of mouse models, and comparatively few have reported progress in isolating and characterizing stem and progenitor cells in the human lung. This may be attributed to the limited availability of adequate amounts of normal human tissue, which is generally restricted to samples from the trachea and proximal airways, and also to the lack of specific cell surface markers to enable their isolation. While some progress has been made despite these impediments, the nature and properties of candidate human epithelial stem and progenitor cells in the normal lung remains unclear at this time.

One of the first reports describing the properties of human lung stem/progenitor cells, showed that unfractionated normal lung cell suspensions were able to form bronchospheric colonies of mixed alveolar, airway and mesenchymal cells (Tesei et al. 2009). More recently Kajstura et al. (2011) described a population of putative human lung stem cells isolated on the basis of their differential expression of c-kit (Kajstura et al. 2011). *In vitro* culture of c-kit^{pos} cells in this study showed that they were clonogenic and multipotent. When injected into an injured mouse lung they appeared to regenerate bronchiolar and alveolar epithelium as well as vasculature, challenging the concept that cells of embryonic endodermal and mesodermal origin remain as distinct lineages in the adult lung. However, significant reservations have been raised about the experimental designs employed in this study (Lung stem cells: Looking beyond the hype. 2011. Nat. Med. 17: 788–789) and the results need to be interpreted with care. More recently, Oeztuerk-Winder et al. (2012), have described an E-cadherin ^{pos}Lrg6^{pos} lung progenitor cell cohort able to regenerate damaged bronchioalveolar epithelium following bleomycin-induced lung injury in mice, as well as regenerate bronchioalveolar tissue when transplanted under the kidney capsule. Significantly, c-kit^{pos} cells isolated in this study were not able to do so.

Perhaps, a recent study of epithelial cells isolated from human trachea provides the most compelling analysis of candidate human lung stem/progenitor cells (Hegab et al. 2012). In this study, basal and SMG duct stem/progenitor cells were isolated from the surface epithelium of human trachea on the basis of their differential expression of nerve growth factor receptor (NGRF), CD166 and CD44. When cultured in a three-dimensional Matrigel-based assay, both basal and SMG duct progenitors demonstrated the capacity to self-renew and differentiate under various conditions in culture.

Lung Stem/Progenitor Cells as Cancer-Initiating Cells

While the precise cellular origin of lung cancer is unknown, comparisons between normal tissue stem cells and cancer cells have revealed many similarities in their behavior, including the capacity for self-renewal and ability to differentiate

into a variety of cells. The concept that cancer is a caricature of normal tissue development was originally proposed by Pierce 40 years ago (Pierce 1974; Pierce and Speers 1988) and has been influential in framing the cancer stem cell paradigm which proposes: (1) that cancers arise from the genetic mutation or epigenetic transformation of rare normal stem or progenitor cells; (2) that the hierarchical organization of cancer stem cells and their progeny within a tumor mimics that of regenerative cells within normal tissue; (3) that cancer stem cells retain the potential to renew, proliferate and generate descendent cell lineages characteristic of their normal counterparts; and (4) that like their normal counterparts, their proliferation and differentiation is dictated by their intrinsic potential and by microenvironmental cues (Castano et al. 2012; Sneddon and Werb 2007).

The regional distribution of specific lung tumor sub-types broadly reflects specific genetic and epigenetic changes in distinct regional stem/progenitor cells distributed along the proximal-distal axis of the airway tree (Asselin-Labat and Filby 2012; Sullivan et al. 2010; Sutherland and Berns 2010), as does the analysis of mice harboring oncogenic mutations. For example, squamous cell carcinomas are generally located in the main bronchus and upper airways, while small cell lung cancers are found in bronchiolar airways, and adenocarcinomas and bronchioalveolar carcinoma are restricted to the alveoli and bronchiolar airways. Evidence that SP-C^{pos} AT2 cells and/or BASC are the cells of origin for lung adenocarcinoma is also consistent with studies reporting an increased incidence of BASC in mice harboring the oncogenic K-ras mutation (Kim et al. 2005) expressed in 15–22% of non-small cell lung cancers (Riely et al. 2009), and the development of lung adenocarcinoma in p53-deficient mice that conditionally express K-ras in SP-C^{pos} cells, but not CCSP^{pos} cells (Xu et al. 2012). On the other hand, another study utilizing cell type-restricted adeno-Cre viral vectors targeting neuroendocrine, club and AT2 cells has shown that neuroendocrine cells are the predominant cell of origin of small cell lung cancer following inactivation of the Trp53 and Rb1 tumor suppressor genes in these lineages (Sutherland et al. 2011).

However, efforts to establish the precise identity of lung cancer initiating cells is confounded by the development of heterogeneity within the tumor during disease progression due to the clonal evolution of tumors resulting from genomic and epigenetic instability (Marusyk and Polyak 2013; Nowell 1976). These temporal genomic and epigenetic changes during tumor progression result in plasticity in the immunophenotypic signature profiles of cancer cells during disease progression. This blurs differences between cells of differing proliferative and differentiative potential and makes it difficult to identify, prospectively isolate and hierarchically order cancer stem cells and their progeny to establish the precise relationship of cancer initiating and propagating cells to their normal counterparts (Sneddon and Werb 2007; Sullivan and Minna 2010).

The analysis of human lung cancer stem cells has been significantly impeded by the intrinsic biological variability and heterogeneity of primary lung cancer biopsied tissue, as well as the lack of defining biomarkers and robust assays for their identification, prospective isolation and functional characterization. Cell-separative strategies devised for the prospective isolation of candidate lung cancer stem cells in human cancer cell lines and primary tumor biopsies have mostly relied on the differential expression of cancer associated biomarkers including aldehyde dehydrogenase (ALDH), CD133, or Hoechst-33342 dye efflux as a measure of multidrug resistance gene activity (Eramo et al. 2010). Candidate lung cancer stem cells isolated on this basis (ALDH^{pos}, or CD133^{pos}, or Hoechst^{dull} side population cells) exhibit proliferative characteristics *in vitro* and in *in vivo* tumor xenografted mouse models that are consistent with the characteristics of stem cells. This includes the ability to recapitulate the histology, heterogeneity and growth characteristics of the parent tumor when xenografted in immune-compromised mice, and the ability to renew and exhibit clonal growth and a high proliferative potential *in vitro*. The ability of these xenografts to mimic the original tumor and to clone *in vitro*

will enable the correlation of biomarker expression profiles with functional assay readouts and patient outcomes, as well as identify key signaling pathways and mutations important in driving the dysregulated behavior of lung cancer propagating cells.

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Part II

Pluripotent Stem Cells

Differentiation of Dendritic Cells from Human Induced Pluripotent Stem Cells

3

Alison Leishman and Paul J. Fairchild

Contents

Abstract

Dendritic cells (DCs) occupy a pivotal role in the immune system by determining the outcome of antigen presentation, either eliciting an aggressive immune response or imposing a state of immunological tolerance. As such, DCs serve as an obvious point of intervention for therapeutic purposes: while targeting DCs to enhance the response to tumour associated antigens (TAAs) remains the goal of cancer immunotherapy, reducing deleterious immune responses is critical for the treatment of autoimmunity and allograft rejection, or in situations in which therapeutic proteins may prove immunogenic upon administration. Functional heterogeneity among DCs suggests that distinct subsets may prove suitable for different applications. In terms of cancer vaccination, for example, much interest has focused on the recently-described subpopulation of $CD141^{+}XCR1^{+}$ DCs, capable of cross-presenting exogenous antigens in an MHC class I-restricted manner, since these may recruit cytotoxic T cells to the eradication of transformed cells. Furthermore, conventional DCs may be rendered pro-tolerogenic upon exposure to various pharmacological agents such as rapamycin and interleukin 10 (IL-10). For all such applications, a well-defined source of patient-derived DCs is highly desirable which is tractable for *in vitro* culture, expansion and manipulation. Given that for many disease states amenable to such strategies for therapeutic intervention, use of the patient's

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own peripheral blood is contraindicated, we have investigated the use of autologous induced pluripotent stem cells (iPSCs) as an alternative source of DCs. Here we describe our recently-published protocols for the derivation of DCs from human iPSCs that yield around 90–95% CD11c$^+$ DCs, of which up to 40% express the CD141$^+$XCR1$^+$ phenotype of the subset capable of antigen cross-presentation. These protocols therefore form a promising foundation for the future clinical application of DCs to immunotherapy.

Introduction

The outcome of the immune response determines the prognosis of many disease states, not merely infectious diseases but cancer, autoimmunity and end-stage organ failure, for which solid organ transplantation remains the treatment of choice. Whereas the eradication of transformed cells or infectious microorganisms requires strategies for vaccination, the induction of immunological tolerance is essential to restrain deleterious immune responses targeted against self components or tissue allografts.

Evidence accrued over several decades has implicated dendritic cells (DCs) as the fulcrum on which the balance between tolerance and immunity pivots. As professional antigen presenting cells, DCs are exquisitely adapted to the capture of exogenous foreign antigens and their processing for presentation to MHC class II-restricted helper T cells (Th cells). Furthermore, recent findings have identified in humans a minor subset of DCs in the peripheral blood and lymphoid tissues, defined by their co-expression of CD141 and XCR1 (Bachem et al. 2010; Crozat et al. 2010). This subset, equivalent to CD8α$^+$ DCs in the mouse, differs from conventional DCs in its ability to acquire exogenous antigens and process them for presentation on MHC class I (Sachamitr and Fairchild 2012). This process of cross-presentation is responsible for eliciting cell mediated immunity by recruiting cytotoxic T lymphocytes (CTL), essential for the eradication of viral infection and transformed cells expressing tumour associated antigens (TAAs). In order to exploit the unique properties of DCs for adoptive immunotherapy, many groups have made use of DCs differentiated from a patient's own peripheral blood monocytes. Indeed, such a source of autologous cells has been used extensively in Phase I clinical trials for cancer immunotherapy (Engell-Noerregaard et al. 2009) and as a therapeutic vaccine for chronic HIV-1 infection (Lu et al. 2003; Garcia and Routy 2011). Although such a source of DCs has yet to be used *in vivo* in regimens for the induction of antigen-specific tolerance, a variety of different pharmacological agents has been used to reduce the immunogenicity of moDCs, including rapamycin, 1α,25-dihydroxyvitamin D_3, dexamethasone, aspirin, IL-10 and TGF-β (Hackstein et al. 2001; Horibe et al. 2008; Unger et al. 2009; Boks et al. 2012). These modulators have been shown to generate DCs whose loss of co-stimulatory molecules, reduced T cell stimulatory capacity, increased expression of immunoinhibitory receptors and increased capacity for induction of regulatory T cells (Tregs), augurs well for applications requiring the establishment of systemic tolerance (Leishman et al. 2011).

Although clinical trials using monocyte-derived DCs (moDCs) for vaccination purposes have been shown to be inherently safe, the efficacy of the approach for securing tumour regression and eradication of HIV-1 has enjoyed only moderate success. Various factors have contributed to this disappointing outcome, including significant donor-to-donor variation in the yield and quality of moDCs which is frequently exacerbated by the very disease states such an approach seeks to address. Exposure to long-term chemotherapy for the treatment of malignancy, for example, has a detrimental impact on the capacity of the bone marrow to replenish populations of blood-borne monocytes. Furthermore, chronic HIV-1 infection has been associated with changes in the functional potential of monocytes and the DCs differentiated from them, while the long term culture of peripheral blood from HIV-1 infected individuals poses significant hazards. Most importantly, however, the limited capacity of moDCs to cross-present exogenously-acquired

antigen significantly restricts the nature of the responses they can elicit, and, in particular, the cell mediated immunity required for viral and tumour clearance.

Given the seminal findings of Takahashi and Yamanaka (2006) and Takahashi et al. (2007), and recent advances in cellular reprogramming that permit the generation of induced pluripotent stem cells (iPSCs) in a patient-specific manner, we reasoned that iPSCs might serve as an alternative source of DCs for immunotherapy. The generation of a pluripotent cell line capable of indefinite self renewal, would provide a potentially unlimited source of DCs on demand that could be subject to extensive quality control prior to administration to patients. Accordingly, we recently described protocols for the directed differentiation of DCs from human iPSCs which were able to generate not only conventional DCs indistinguishable from moDCs, but cells co-expressing CD141 and XCR1 with the functional capacity for antigen cross-presentation (Silk et al. 2012). Unlike their moDC counterparts, this subset was able to elicit primary T cell responses to the TAA, Melan A, among naïve $CD8^+$ CTL from the peripheral blood of a healthy donor. Here we describe how human iPSCs are cultured in the laboratory, expanded as required and guided by growth factors to differentiate down the hematopoietic lineage to ultimately give rise to DCs (ipDCs), a strategy first applied to the differentiation of human embryonic stem cells (hESCs) (Tseng et al. 2009). This protocol may be performed in an animal product-free manner for downstream clinical use and yields around 90–95% $CD11c^+$ ipDCs of which up to 40% constitute the $XCR1^+CD141^+$ population, capable of antigen cross-presentation (Bachem et al. 2010; Crozat et al. 2010).

Methods

Coating Tissue Culture Plates with Matrigel™

To provide cells with a means of attachment, phenol red-free, growth factor-reduced Matrigel™ (BD Biosciences, cat# 356231) is used as a basement matrix for iPSC culture. Matrigel™ may be stored as aliquots at −80 °C. We store Matrigel™ as a 1:2 dilution in ice-cold knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) (Invitrogen, cat# 10829-018). When working with Matrigel™, care should be taken to keep it cold at all times, which involves pre-cooling of tubes and pipettes, in order to prevent the formation of aggregates. Matrigel™ aliquots are thawed on ice and further diluted 1:15 in ice-cold KO-DMEM to make up a final Matrigel™ dilution of 1:30. Care should be taken not to introduce bubbles while mixing the dilutions. The surface of individual wells of a 6-well tissue culture plate (Corning, cat# 3335) are covered with 1 ml/well of diluted Matrigel™ and incubated overnight at 4 °C or for 1 h at room temperature before use. It is essential that Matigel™ covers the entire surface of each well and that no bubbles have been introduced. When Matrigel™ covered plates are stored at 4 °C overnight or longer, they should be sealed with cling film or a similar product to prevent evaporation.

Before use, Matrigel™ is removed and the wells of tissue culture plate optionally washed with Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, cat# 14190). This Matrigel™ can be reused at least once by pooling and replating 1 ml per well. Since Matrigel is an animal product, we have also shown that we are able to culture the iPSCs in a Matrigel™-free culture system using xeno-free Corning Synthemax tissue culture plates (Corning, cat# 3979XX1) and thereby proving the clinical applicability of our protocols.

Culture of Human iPSCs

Human iPSCs are cultured in complete mTeSR1 medium (Stemcell Technologies, cat# 05850) on 6-well plates coated with Matrigel™ as described above. mTeSR1 medium is left to warm up to room temperature before use. iPSCs are fed daily, except the day following passaging in order to allow more time for the cells to attach to the substrate.

Passage of Human iPSCs

Cells are passaged as clusters of about 0.5 mm diameter every 6–7 days. For passage, wells are washed with DPBS and treated with 1 ml of 1 mg/ml dispase (Stemcell Technologies, cat# 07913) per well for 4–5 min at 37 °C, according to the manufacturers' instructions. Once colonies start to round up at the edges, dispase is removed and cells are washed with DPBS. Cells are then scraped off into mTeSR1 medium containing 10 μM ROCK inhibitor (Reagents Direct, cat# 53-B85) using a cell scraper (Corning, cat# 3010) and gently broken into clusters by pipetting up and down using a 10 ml pipette. Cell clusters are diluted in complete mTeSR1 medium to passage at 1:12 and spread evenly on the Matrigel™-covered 6-well plates by gentle rocking. All cell cultures are incubated in a humidified incubator at 37 °C and 5% CO_2.

Differentiation of hiPSCs

Cell Counting

iPSC cultures are observed in order to identify a typical well which is representative of the confluence and number of cells in other wells: this well is then sacrificed in order to estimate, as accurately as possible, the average number of cells per well. The medium is removed and the wells washed with DPBS. TrypLE™ Express (Gibco, cat# 12604) equilibrated to room temperature is added at 1 ml per well and incubated for 5 min at 37 °C. To deactivate trypsin, medium containing serum is added. Colonies are dislodged and dispersed into a single cell suspension using a 5 ml pipette or 1 ml Gilson pipette. Cells are counted in a haemocytometer and the total number of cells available for differentiation determined by considering the total number of wells. The number of 6-well ultra-low attachment (ULA) plates (Corning, cat# 3471) required to set up an individual differentiation is calculated on the basis of these results, planning to plate 3×10^6 cells per well.

Differentiation Set Up

Medium should be prepared in advance. For the initial harvest, all growth factors are added to complete mTeSR1 medium, while for all following feeds the base medium is supplemented X-VIVO-15 (Lonza, cat# 04-744Q), thereby permitting the gradual transition of cells from mTeSR1 into X-VIVO-15 (Fig. 3.1) which improves cell survival.

To make up the initial differentiation medium, mTeSR1 is supplemented with 50 ng/ml of recombinant human bone morphogenetic protein-4 (BMP-4) (R&D Systems, cat# 314-BP/CF), 50 ng/ml of recombinant human vascular endothelial growth factor (VEGF) (PeproTech, cat# 100-20), 20 ng/ml of recombinant human stem cell factor (SCF) (R&D Systems, cat# 255-SC/CF) and 50 ng/ml of recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) (PeproTech, cat# 300-03).

In order to set up a differentiation culture, iPSCs are harvested in the same manner used for passaging, but in the absence of ROCK inhibitor. First, colonies are washed with DPBS, followed by dispase treatment. If handling a large number of wells simultaneously, dispase can be incubated at room temperature while working sequentially through the wells. Once colonies start to round up at the edges, dispase is removed and wells are washed with DPBS. Colonies are scraped into mTeSR1 medium, supplemented as described above. The harvested cells are then plated at 3×10^6 cells per well onto ULA plates in a total volume of 4 ml of supplemented mTeSR1.

Feeding Differentiations

After 2 days, the medium is topped up with 2 ml of X-VIVO-15 medium, supplemented with 1 mM sodium pyruvate (PAA Laboratories GmbH, cat# S11-003), 0.1 mM MEM non-essential amino acids (PAA Laboratories GmbH, cat# M11-003), 2 mM L-glutamine (PAA Laboratories GmbH, cat# M11-004), 5 μM 2-mercaptoethanol (Sigma, cat# M7522) and the growth factors BMP-4, VEGF, SCF and GM-CSF, as described above, to produce a total volume of 6 ml. All made up media are filter-sterilised before use. Subsequent feeding

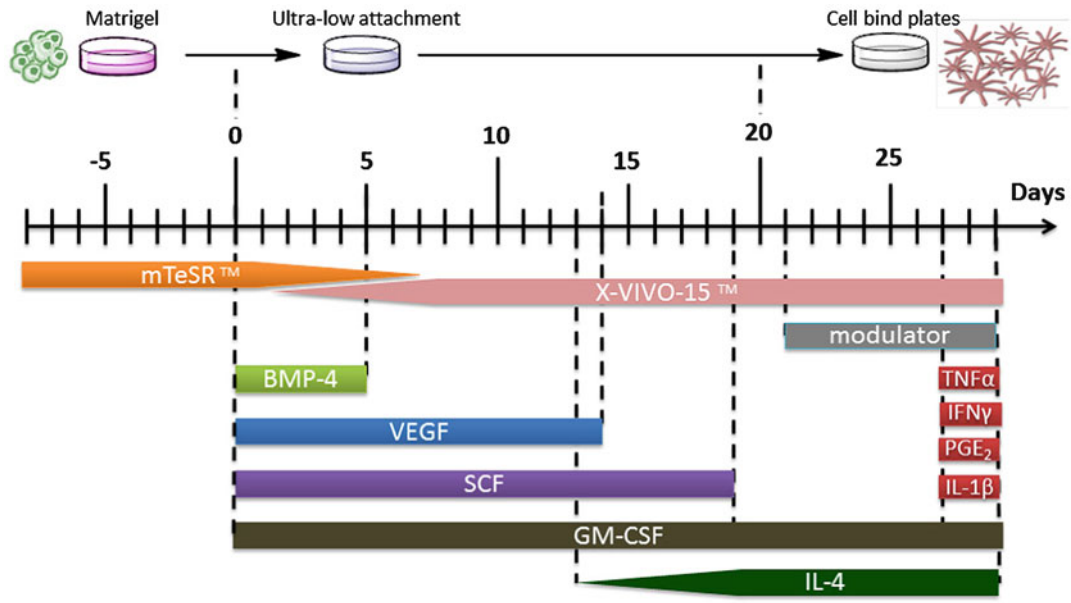

Fig. 3.1 Illustration of the protocol for differentiation of iPSCs into mature ipDCs. iPSCs are expanded in mTeSR1 medium according to the manufacturer's instructions (Stem Cell Technologies). In order to initiate differentiation, iPSCs are harvested as clusters and plated onto ultra-low attachment plates (ULA) in 4 ml per well of mTeSR1 medium supplemented with human recombinant bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor (VEGF), stem cell factor (SCF) and granulocyte macrophage-colony stimulating factor (GM-CSF). With successive feeds, mTeSR1 is gradually replaced with X-VIVO-15 as the base medium. Differentiation cultures are fed every 2–3 days with medium from which growth factors are progressively removed, as indicated above. On approximately day 13, upon the appearance of macrophage-like cells in cultures, IL-4 is added to medium starting at 25 ng/ml and is gradually increased to 100 ng/ml. Differentiations can be harvested from around day 21 onwards onto Cell Bind plates. From this point onwards, DCs may be treated with different pharmacological agents such as rapamycin or 1α,25-dihydroxy vitamin D_3. After 5 days of culture on Cell Bind plates, DCs can be matured by addition of a maturation cocktail containing GM-CSF, IL-4, IFNγ, TNFα, IL-1β and PGE_2. Cells are harvested and washed after 48 h of maturation and used for subsequent experiments

is performed every 2–3 days by replacing 2–3 ml of old medium with new supplemented X-VIVO-15 medium from which growth factors are successively removed, starting with BMP-4 at day 5, followed by VEGF at day 14 and SCF at day 19 of differentiation [19]. Care is taken not to remove any cells or cell clusters from differentiation cultures during the feeding process.

During the first few days of differentiation, cells cluster together to form embryoid body (EB)-like structures, as shown for days 3 and 7 in Fig. 3.2. Throughout the first few weeks, EBs increase in size and form cyst-like structures as shown at days 7, 10 and 11 (Fig. 3.2). Between day 10 and 20, EBs start to release a large number for cells, among which monocyte like cells can be observed (day 17). From about day 17 onwards, the first DCs, characterised by their cytoplasmic protrusions, appear in the differentiation cultures (Fig. 3.2, day 19). Once macrophage-like cells are observed (days 13–17), 25 ng/ml of IL-4 (Peprotech, cat# 200-04) is added, which is increased stepwise to 100 ng/ml.

On days 20–24, monocytes and DCs are harvested by gentle pipetting, leaving behind adherent macrophages in the culture dish. The cell suspension is passed through a 70 μm cell strainer (BD Falcon, cat# 352350) to remove cellular debris, washed with DPBS and plated at $1–1.5 \times 10^6$ monocytes per well of a 6-well Cell

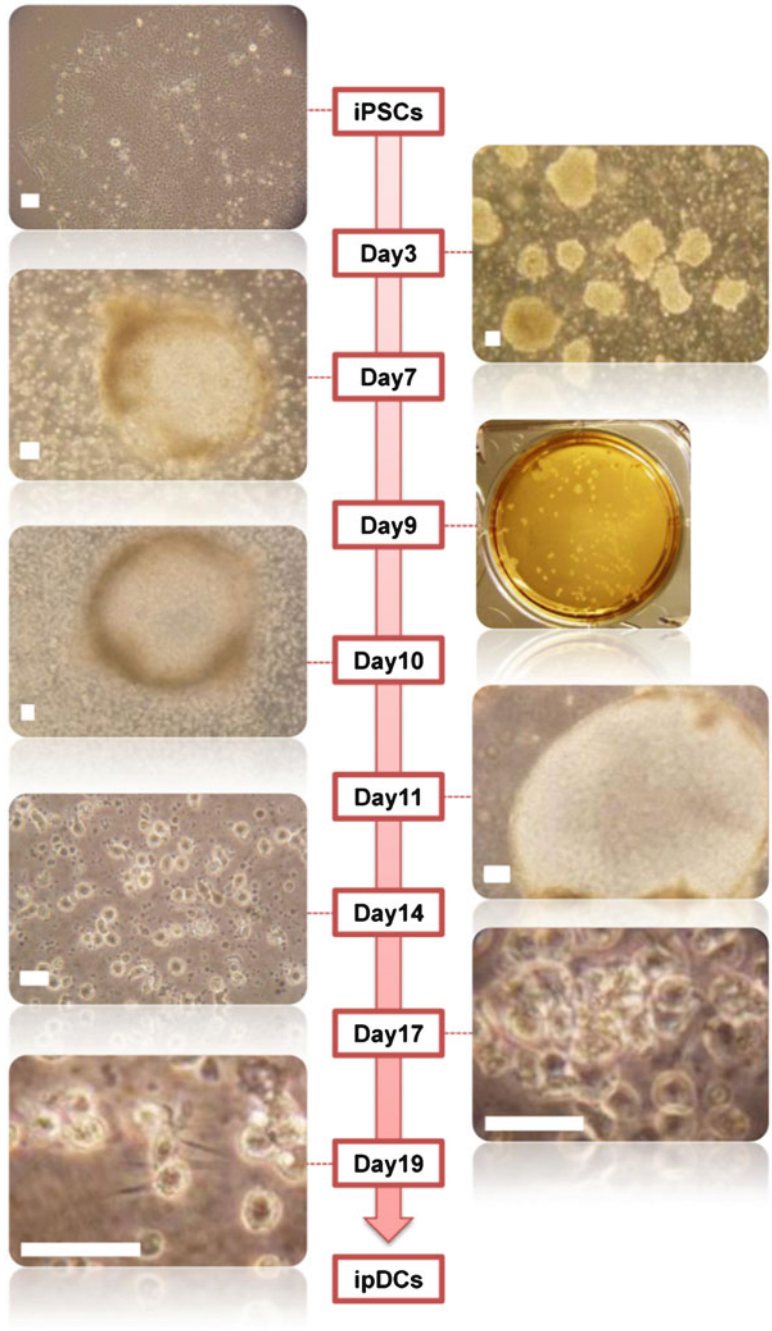

Fig. 3.2 Photomicrographs illustrating the differentiation process from iPSCs into DCs. During the first few days of differentiation, cells cluster together to form embryoid body (EB)-like structures, as shown for day 3. Throughout the first few weeks, EBs increase in size and form cyst-like structures as shown for days 7, 10 and 11. Between days 10 and 20, EBs start to release a large number for cells, among which monocyte-like cells can be observed (day 17 left). At around day 17, the first DCs, characterised by their cytoplasmic protrusions, can be observed in the differentiation cultures (day 17 and 19). *Scale bars* represent 100 μm

Bind plate (Corning, cat# 3335) in a total volume of 4 ml of X-VIVO-15 supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4.

DC Maturation and Pharmacological Treatment

At this stage, monocytes differentiating into DCs may be treated with pharmacological agents in order to generate functionally-modulated DCs. A variety of agents have been used to generate DCs with tolerogenic properties. In the past, our laboratory has focused on the use of rapamycin for the generation of modulated DCs derived from human embryonic stem cells (hESCs). The protocol involves the treatment of harvested ESC-derived monocytes with 5–10 ng/ml of rapamycin on day 3 prior to DC maturation on day 5.

For maturation, DCs are treated for 48 h using a maturation cocktail consisting of 50 ng/ml of GM-CSF, 100 ng/m IL-4, 20 ng/ml IFNγ (R&D Systems, cat# 285-IF/CF), 50 ng/ml TNFα (R&D Systems, cat# 210-TA/CF), 10 ng/ml of IL-1β (R&D Systems, cat# 201-LB/CF) and 1 μg/ml PGE_2 (Sigma, cat# P6532). This cocktail is made up as a 9× concentration in X-VIVO-15 and added as 500 μl per well. On day 7, DCs are harvested by gentle pipetting, and are washed prior to their use in experiments to prevent any carry-over of pharmacological agents or maturation cytokines. Maturation can be assessed by up-regulation of MHC class II and co-stimulatory molecules, such as CD86 (Fig. 3.3).

Discussion

While the use of DCs for immunotherapeutic intervention has been shown, in principle, to offer fine control over the outcome of pathogenic immune responses, limitations associated with the source of the DCs most commonly employed, have reduced the efficacy of such an approach. Here we have described protocols for the differentiation of DCs from patient-specific iPSCs, conventionally derived from a small punch biopsy taken from the skin. These so-called ipDCs contain a subpopulation defined by their co-expression of CD141 and XCR1, recently demonstrated to harbour the capacity for antigen cross-presentation, a property highly desirable for eliciting CTL responses to transformed or virally-infected cells. Such a novel source of DCs may, therefore, find application in situations in which the patient's own peripheral blood is inadequate as a source of monocytes, from which DCs are traditionally derived, such as the impact of long-term exposure to chemotherapy or chronic HIV-1 infection.

While the cross-presentation capacity of ipDC augurs well for their use in regimens for vaccination, our protocols are equally amenable to the pharmacological manipulation of ipDCs. Agents such as rapamycin, 1α,25 dihydroxyvitamin D_3 and IL-10 have been widely shown to enhance the tolerogenicity of moDC (Leishman et al. 2011), and have proven equally useful for modulating the function of hESC-derived DCs in our hands (Silk et al. 2011). The potential to reduce DC immunogenicity in favour of a tolerogenic phenotype may prove valuable in a variety of settings in which the immune response is itself pathogenic, rather than protective. Whereas the high precursor frequency of alloreactive T cells is likely to prove too great a barrier to the induction of transplantation tolerance using DCs alone, the effective treatment of various congenital conditions would be greatly facilitated by the induction of tolerance to single defined proteins whose therapeutic administration may overcome a patient's own endogenous deficiency. This scenario is most commonly encountered in the case of the lysosomal storage diseases (LSDs) in which the absence of a single enzyme from the lysosomal pathway results in the accumulation of waste products and consequent toxicity among critical cell types. Although LSDs, such as Pompe disease, may be treated by replacement of the missing enzyme, the induction of a robust immune response leading to clearance of the enzyme from the patient's circulation, prevents its efficient targeting to tissues (Banugaria et al. 2011, 2012). Whereas the use of DCs to establish immunological tolerance to individual enzymes in advance of their administration may prove

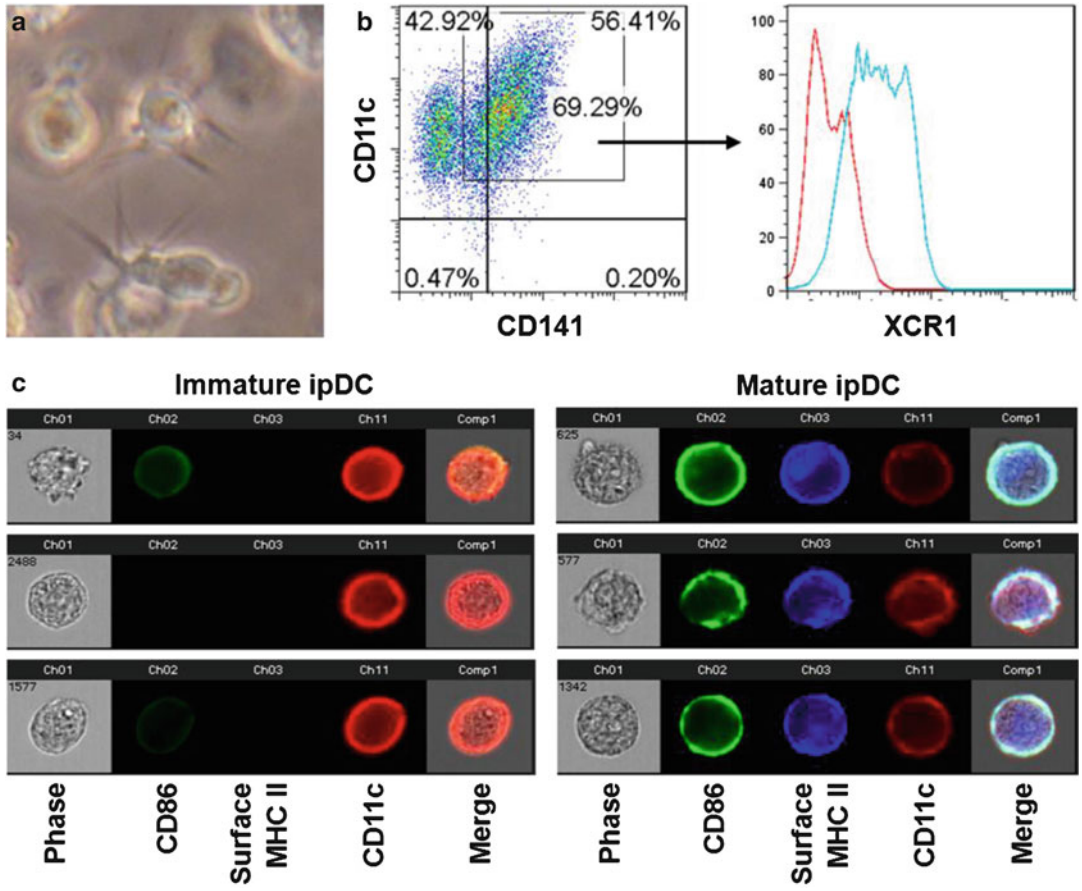

Fig. 3.3 (**a**) ipDCs featuring very long protrusions and dendrites. The generation of cells displaying this morphology coincides with the first appearance in cultures of cross-presenting DCs. (**b**) Differentiation cultures give rise to around 70% $CD11c^{+}CD141^{+}$ DCs amongst which cells express the cross-presentation marker XCR1 (blue histogram) which is a chemokine receptor for XCL1 (the red histogram denotes background staining with an isotype-matched control antibody) (Figure courtesy of Dr. Kate Silk). (**c**) iPSC were differentiated into ipDCs for a total of 30 days. On day 28, some cells were treated with GM-CSF, IL-4, IFNγ, TNFα, IL-1β and PGE_2 to induce their maturation while some were left untreated (immature). Image stream analysis of the expression of CD11c, MHC class II and CD86 demonstrates efficient maturation as immature cells express low or no CD86 and MHC class II, with the exception of a very low percentage of spontaneously matured cells, while matured ipDCs up-regulate both CD86 and MHC class II

feasible, the most severe cases of Pompe disease manifest in infanthood when the availability of peripheral blood monocytes is extremely restricted: under such circumstances, the establishment of an iPSC line from such individuals and its use as a potentially-unlimited source of DCs, offers an attractive alternative which is only minimally invasive.

Quite apart from being endowed with the capacity for antigen cross-presentation, the use of iPSCs as a source of autologous DCs has numerous advantages over conventional moDCs. As a cell line capable of indefinite self-renewal, iPSCs provide a potentially unlimited supply of DCs for repeated administration which are not prone to the same batch-to-batch variation observed among moDC and may be subject to rigorous quality control prior to use. Furthermore, their derivation using protocols that are free of animal products, makes them fully compatible

with downstream clinical use. Given that iPSCs are relatively tractable candidates for genetic modification, unlike fully differentiated DCs, the establishment of an iPSC line may enable the modulation of DC function through the forced expression of co-stimulatory or inhibitory molecules, thereby tailoring their activities to the individual needs of the patient. Once current regulatory hurdles surrounding the *in vivo* administration of cell types differentiated from iPSCs have been resolved, the use of autologous ipDCs may prove efficacious in the treatment of a broad spectrum of disease states.

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Molecular Mechanisms Underlying Human Somatic Cell Reprogramming to Generate Induced Pluripotent Stem Cells

4

Pauline Lieu

Contents

Abstract

The discovery by Yamanaka and Thomson has opened a "new era" for biology and regenerative medicine. They showed that by expressing four transcription factors in somatic cells, these cells can be reprogrammed to induced pluripotent stem cells (iPSCs) similar to embryonic stem cells and can give rise to almost every cell type in the human body. The creation of these special cells was major ground-breaking work in cell biology and opened the path for providing unprecedented access to patient-specific iPS cells for drug screening, disease modeling and cell therapy applications. Beside therapeutic issues, iPS cell technology opens the door for broader research on human pluripotent cells because ethical limitations are lifted with iPS cells as compared to hES cells. Therefore, it is not surprising that the methods for generating iPSCs have significantly evolved over the past few years. We are now able to convert essentially any somatic cell type into iPSCs with increased efficiency and at higher quality when compared to ESCs. Despite these advances, the molecular events occurring during various stages of reprogramming remain largely unknown. In this review we will discuss the current understanding of molecular mechanisms underlying human somatic cell reprogramming to generate induced pluripotent stem cells.

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Introduction

The first mouse embryonic stem cell line, established in the 1980s, demonstrated their ability to proliferate indefinitely and differentiate into three germ layers. Since then, embryonic stem cells have shown great promise for advancing the fields of drug discovery, disease modeling and regenerative medicine. In the past, it was widely believed that once stem cells differentiated, they could not revert back to an earlier development stage until the invention of somatic cell nuclear transfer (SCNT) (Briggs and King 1952). This technique replaces the nuclei of oocytes with those of somatic cells, resulting in the reversal of the fused cells from a differentiated to pluripotent status. Subsequently, many species of mammalian cells have been successfully cloned, including the famous cloned sheep "Dolly" (Campbell et al. 1996). However, this approach has low success rates and raises ethical issues regarding the use of embryos.

In 2006, Takahashi and Yamanaka successfully generated induced-pluripotent stem cells (iPSCs) with the novel approach of using viral integration of four transcription factors, Oct4, Sox2, Klf4 and cMyc. Subsequently, Yu et al. (2007) also successfully generated iPSCs using another set of four defined factors, Oct4, Sox2, Nanog and Lin28. Induced pluripotent stem cells share many features with embryonic stem cells in that they show similar gene and protein expression profiles and can generate viable, fertile live-born mice (Takahashi and Yamanaka 2006). This revolutionary technique has demonstrated the feasibility of using somatic cells to generate iPSCs. Patient specific iPSCs can now be derived from the cells of the same patient, thereby conceivably avoiding the immune rejection that occurs with SCNT or human embryonic stem cells (hESCs). In addition, the use of somatic cells as the starting material circumvents the ethical issues associated with the use of human embryos. Subsequently, many iPSCs have been generated from various types of somatic cells, including keratinocytes, neuronal stem cells and fibroblasts (Aasen et al. 2008) (Takahashi et al. 2007). These iPSCs display gene expression patterns, cell morphology and the capacity of forming teratoma in vivo similar to those of hESCS (Takahashi et al. 2007). The potential of iPS cell technology shows great promise to the research and medical communities. However, the actual molecular mechanism of induced reprogramming still remains undefined. In this chapter, we will summarize recent advances in iPS cells research and describe some of the molecular processes underlying pluripotency reprogramming.

Mechanisms of Transcription Factors Induce Pluripotency

The original discovery of Tahakashi and Yamanaka (2006) reported the use of four transcription factors Oct4, Sox2, Klf4 and cMyc (OSKM), to reprogram somatic cells to induced pluripotent stem cells (iPSCs). This combination of factors emerged from an initial screen of mouse fibroblasts based on co-transduction of 24 candidate genes (Takahashi and Yamanaka 2006). The generation of iPSCs with this method has now been reported from mouse and human somatic cells using various methods (Carey et al. 2011). Direct reprogramming is a slow and inefficient process, with estimated efficiencies in human cells ranging from 0.02 to 1% (Chin et al. 2009). Studies demonstrated that equal stoichiometry of all four reprogramming factor expressions is a critical contributing factor to successful hiPSC induction (Tiemann et al. 2011). In addition, the expression of four factors is required for a minimum of 12–16 days before activation of pluripotent markers emerge (Ruff et al. 2012).

It is thought that each reprogramming factor plays a distinct role in inducing reprogramming and maintaining pluripotency. The transcription factors Oct4, Sox2 and Nanog have essential roles in early development and are required for maintenance of embryonic stem cells (Boyer et al. 2005). Studies reported that Oct, Sox and Nanog collaborate to form regulatory circuitry consisting of autoregulatory and feedforward loops (Boyer et al. 2005). These factors occupy genes that encode

important developmentally homeodomain transcription factors as well as chromatin regulators in ES cells. In addition, it was shown that at the transcriptional level, Oct4 and Sox2 downregulate snail mRNA, the epithelial to mesenchymal transition regulator, during reprogramming (Li et al. 2010). Meanwhile, Klf4 induced E-cadherin mRNA and other epithelial markers (Li et al. 2010), while cMyc reduced TGFb signaling by repressing the TGFb1 and TGFb receptors (Kim et al. 2010). The target genes of these transcription factors are similar in iPSCs to those previously defined in ESCs (Plath and Lowry 2011).

cMyc has been shown to be dispensable for reprogramming, however, with dramatically reduced efficiency and kinetics (Nakagawa et al. 2008). Studies found that cMyc network is largely independent of the core ES cell pluripotency network and thus not involved in the upregulation of the pluripotency network during the final step of reprogramming (Plath and Lowry 2011). Rather, functions of cMyc include targeting genes that are involved in cell proliferation, metabolism, and biosynthetic pathways. A recent report also suggests that cMyc plays a major role in the release of promoter-proximal pausing of RNA polymerase II (Pol II) and thereby enhances the elongation of transcripts, rather than Pol II recruitment at its target genes (Rahl et al. 2010). These suggest that cMyc could enhance, but are not absolutely required for transcription of its target genes. Even though cMyc has been shown to be dispensable for reprogramming, cMyc over expression may lay the framework for the efficient induction of proliferation.

Although partially reprogrammed cells have successfully acquired a proliferative capacity, many pluripotency-related genes are not occupied by Oct4, Sox2 and Klf4, and the endogenous Nanog and Oct4 are not reactivated (Kim et al. 2010). One hypothesis is that in partially reprogrammed cells these transcription factors may need other proteins that are not yet available in pre-iPSCs to allow cooperative binding of the factors to promoter regions. One candidate is the transcription factor Nanog, which has extensive protein-protein interaction and co-localizes with Oct4 and Sox2 at a set of promoter regions in ES cells (Chen et al. 2008). In pre-iPSCs , many of the Nanog target genes completely lack binding of transcription factors as compared to ES cells. Nanog is absolutely essential for the generation of iPSCs, but required only during the final step of reprogramming (Theunissen et al. 2011).

Roles of miRNAs in Reprogramming

In 2004, Suh and colleagues identified a set of embryonic stem cell-specific miRNA which consequently have been found to contribute to embryo development. Moreover, deficiency in these miRNAs can cause detrimental defects in cell proliferation and differentiation. Among the highly expressed ESC-specific miRNAs, miR302/367 is highly expressed in early embryonic development and rapidly declines after differentiation (Ren et al. 2009). Several laboratories have reported the role of miR-302/367 in reprogramming (Subramanyam et al. 2011).

In 2008, Lin et al. and their peers demonstrated that a small noncoding RNA, called miR302, can replace all previously defined factors to reprogram human and mouse somatic cells to ESC-like iPSCs. Elevated miR302 expression triggers both global demethylation and coexpression of Oct4, Sox2 and Nanog in human iPSCs (Lin et al. 2011). MiR-302 is a 23 ribonucleotide microRNA expressed abundantly in human ESCs but is absent in all differentiated tissue cells (Kim et al. 2010). Specifically, it functions as a gene silencer and simultaneously downregulates multiple epigenetic regulators, including lysine specific histone demethylases 1 and 2 (namely AOF2/1, LSD1/2, or KDM1/1B), DNA cytosine 5-methyltransferase 1 (DNMT1) and methyl-CpG binding proteins 1 and 2 (MECP1/2) (Lin et al. 2011). DNA methyltransferase 1 (DNMT1), an essential regulator in DNA methylation, is then silenced in response to the down-regulation of AOF2, leading to genome-wide demethylation and consequently coactivation of pluripotency-promoting genes (Lin et al. 2011). Silencing of these epigenetic regulators induces global DNA demethylation, the first sign of somatic cell reprogramming.

In addition, miR302/367 also directly targets NR2F2, a member of the nuclear orphan receptor family of transcriptional factors and a negative regulator of Oct4 (Rosa and Brivanlou 2011). In hESCs, NR2F2 expression begins with differentiation and conversely correlates with the expression of Oct4 and miR302/367. Studies have also shown that Oct4, Nanog and Sox 2 bind to the promoter regions of miR302/367 and activate miR302 expression level (Marson et al. 2008). Through silencing of AOF1/2, MECP1/2 and DNMT1, we now understand that miR302 induces global demethylation and leads to Oct-4-Sox2-Nanog activation (Lee et al. 2002). The mutual stimulation between miR302 and Oct-Sox-Nanog forms a positive feedback regulation loop to maintain the pluripotent status of reprogrammed iPSCs. In addition, the expression of Lin28 and many other ESC marker genes was observed 1–3 days later, after the presence of Oct4-Sox2-Nanog elevation (Kuo and Ying 2012). The key to somatic cell reprogramming (SCR) is global demethylation, which unlocks and resets these differentiated gene expression patterns to a highly uniform ESC-like profile. This "unlocking" of a genome allows transcription machinery to access to the ESC specific genes and is required for iPSC formation.

To date, viral transfection has been the primary method of introducing four reprogramming factors into cells because of its high efficiency of delivery. However, this method is less ideal for two reasons. One is the possible viral integration of exogenous genes into the host genome. Second, in cases where non-integration viral delivery is used, cMyc and Klf4 are less ideal because both are potential oncogenes. Thus, these factors will affect the safety use of cells in clinical trials. If miRNAs can induce reprogramming events similar to those Yamanaka factors, they will provide a simple and safer way to generate iPSCs due to the fact that no oncogene is required. In addition, the dual function of miR302 in both reprogramming as well as tumor suppression will provide a convenient means to control the quality of iPSCs that are more ideal for human use.

Global Epigenetic Changes in Pluripotent Stem Cells

Previous studies with polycistronic cassettes that encode all four factors indicated that although most of the cells express all four reprogramming factors, only a small subset of cells gets converted to the pluripotent state, resulting in low efficiency between 0.02 and 1% (Jaenisch and Young 2008). Most cells expressing the reprogramming factors fail to successfully induce the first morphological change of proper reprogramming events, remain fibroblast-like and often undergo apoptosis, senescence or cell cycle arrest. Each of these processes is thought to be a barrier to reprogramming and suppressions of these responses result in higher reprogramming efficiency (Kawamura et al. 2009). For example, deletion of p53 and p21 or Ink4a/Arf was found to enhance the efficiency and kinetics of iPSC reprogramming (Kawamura et al. 2009). It is likely that promotion of cell proliferation through S-phase results in resetting chromatin landscapes and improves reprogramming (Ruiz et al. 2011). However, it is still unclear why the majority of cells failed to reprogram completely compared to the number of cells that are proliferative. Only a subset, approximately 3–5%, of the somatic cells that initially express the reprogramming factors eventually convert to the pluripotent state within this time frame. These findings indicate that induction of the pluripotent state requires additional mechanism to drive cells into fully pluripotent state.

Additional reports also suggest that the general reprogramming timeline may require secondary or stochastic events through which certain cells acquire unique advantages that permit transition to pluripotency (Hanna et al. 2009). Thus, the ectopic expression of the current set of embryonic factors appears insufficient to reset the somatic nucleus along, and the mechanism of action likely includes the activation of additional, yet unidentified downstream effectors.

It is currently believed that repressive chromatin comprises a major mechanistic barrier to transcription factor-induced reprogramming. Recent

large-scale analyses of DNA methylation and histone modifications revealed dynamic chromatin states and DNA methylation status at promoters and most CpG islands (Meissner et al. 2008), showing that the methylation state of H3K4 is a good indicator of promoter DNA methylation levels in mammalian cells. DNA methylation is a critical component of the epigenome that represses gene expression through promoter CG methylation, in addition to localization at heterochromatin and repetitive elements in the genome. This is consistent with prior studies indicating that H3K4 methylation disrupts DNA methylation by inhibiting contact of DNMTs with histones (Meissner et al. 2008).

Additional studies demonstrate that epigenomic landscape in hESC and lineage committed cells are drastically different and mainly differ in chromatin structure. Most changes arise from dramatic redistribution of repressive H3K9me3 and H3K27me3 marks (Harris et al. 2010). A large number of potential regulatory sequences also exhibit a high degree of dynamics in chromatin modifications and DNA methylation. This is mainly suggested by the ability of agents such as histone deacetylases, histone methyltransferases and demethylases, as well as DNA methyltransferase 1 inhibitors that liberate repressive chromatin states to enhance the process of reprogramming (Mikkelsen et al. 2007).

DNA methylation, and the loss of the repressive histone methylation marks, likely occurs at the end of the reprogramming process, concomitant with the binding of the reprogramming factors, Oct, Sox and Klf4, and transcriptional up-regulation of these genes (Mikkelsen et al. 2008). These findings are in agreement that the repressive chromatin state of promoters and enhancers of pluripotency-related genes may block engagement of the reprogramming factors. Thus reprogramming factors are not only inducing major transcriptional changes early on in the reprogramming process, but also affect the chromatin landscape in a global manner and may be by altering the activity or levels of chromatin remodelers or modifiers. Clearly, chromatin remodeling is critical to efficient reprogramming.

Phases of Reprogramming

Gene expression profiling in mouse fibroblast reveals that there are three phases during reprogramming: initiation, maturation and stabilization (Samavarchi-Tehrani et al. 2010). The first 5 days of reprogramming are characterized by an induction of a large number of epithelial associated genes. These genes include the epithelial junctional protein E-cadherin (Cdh1), as well as Cldns, -3, -4, -7, -11, Occuldin (Ocln), Epithelial cell adhesion molecule (Epcam) and Crumbs homolog 3 (Crb3), all of which are components of epithelial junctions. Knockdown of E-cadherin has been shown to interfere with reprogramming (Li et al. 2010). While the initiation phase has no embryonic stem cell factor expression, the maturation phase is marked by the expression of Nanog, Sall4, Esrrb, Rex1, Tcl1, Cripto and Nodal, which occurred at approximately day eight. Nanog drives broad changes in the transcription program that are associated with the acquisition of pluripotency and can push pre-iPSCs to the pluripotent state (Silva et al. 2009). Finally, the stabilization phase is marked by expression of Dnmt3I, Lin28, Utf1, pecam, Stella and Dppa4 started around day 21 (Samavarchi-Tehrani et al. 2010).

The molecular events in the early phases of reprogramming are still poorly understood. A high resolution time-lapse imaging approach demonstrates that the first noticeable changes in reprogramming include an increase in proliferation rate and a decrease in cell size, occurring as early as 24 h post induction of the reprogramming factors in mouse fibroblasts (Smith et al. 2010). These morphological and proliferative changes are accompanied by the induction of proliferation genes and down-regulation of the somatic expression program (Samavarchi-Tehrani et al. 2010). While most cells may be expressing reprogramming factors, only few cells undergo the rapid shift in proliferation that coincides with the reduction of cell size in this early phase of reprogramming. The initial phase is elastic because withdrawal of reprogramming factors after 5 days resulted in genes reverting

back to expression levels observed in the starting fibroblast population (Samavarchi-Tehrani et al. 2010).

A Mesenchymal-to-Epithelial Transition

One of the first noticeable changes during the reprogramming of fibroblasts, after suppression of the somatic transcriptional program, is their transformation into tightly packed clusters of rounded cells and coordinated changes in cell-cell and cell-matrix interactions, which corresponds with a loss of mesenchymal features and the acquisition of epithelial cell characteristics (Li et al. 2010). Modulations of signaling pathways, such as inhibiting the transforming growth factor TGFb, results in enhancing reprogramming because TGFb activity prevents mesenchymal-epithelial transition (MET) by inhibiting both the up-regulation of epithelial markers and the down-regulation of the mesenchymal transcriptional repressor Snail1 (Samavarchi-Tehrani et al. 2010). In addition, studies reveal the initiation phase consists of interaction of morphogenetic protein (BMP) with OKMS to induce miR-205 and miR-200 family members that in turn promote MET. Moreover, MET driven by the miRNA-200 family mimics synergized with OKMS to accelerate reprogramming and removed the requirement for BMP signaling during the early phase. This suggests that the major function for BMP during the early phase of MEF reprogramming is to induce MET (Samavarchi-Tehrani et al. 2010). These studies indicate that that MET is a critical step in fibroblast reprogramming.

Establishing Pluripotency

The sequential steps in establishing pluripotency are: the induction of proliferation, down-regulation of fibroblasts specific transcription, the acquisition of epithelial characteristics and activation of endogenous pluripotency genes, followed by silencing of transgenes used for reprogramming. Studies of sequential expression of pluripotency markers in mice demonstrate that alkaline phosphatase (AP) was activated first, followed by the stage specific embryonic antigen (SSEA1) at an intermediate state. While in human systems, alkaline phosphatase (AP) and the cell surface markers SSEA3, SSEA 4, TRA-1-81, TRA-1-60 and CD24 (Takahashi and Yamanaka 2006) (Chin et al. 2009) are early pluripotent markers that are widely used to detect iPS cells in culture. After cells have attained epithelial characteristics, activation of the core pluripotency network is established, i.e. up-regulation of pluripotent related genes such as Oct4, Sox2, Nanog and other pluripotent-related genes (Brambrink et al. 2008). The up-regulation of the core pluripotency network is considered the final step of reprogramming.

Additional basic tests that the iPS cell line has to meet in order to be considered true iPSC includes the ability to form teratomas; and when injected into blastocysts, iPS cells should contribute to the embryo tissues, including the germ line. Ultimately, the ability of iPSC to form a whole animal via tetraploid complementation is a clear indication of iPSC pluripotency and a nearly identical state to ESC (Stadtfeld et al. 2010).

Conclusion

Induced pluripotent stem cells (iPSCs) provide an invaluable resource for drug or toxicology screening, medical research and patient-specific cell therapy. The ground-breaking work of Yamanaka and Thomson showed that forced expression of just four transcription factors can reprogram mouse and human somatic cells to pluripotency, leading to the discovery of the induced pluripotent stem cells (iPSCs).

Pluripotent stem cells are able to self-renew indefinitely and differentiate into all types of cells in the body. These cells have opened up the opportunity to develop human disease models in vitro, drug and toxicity screening tools. In addition, they can be an inexhaustible source for future cell transplantation therapy to treat degenerative diseases that currently have no cure. Since non-autologous cells will cause immune rejection, induced pluripotent stem cell (iPSC)

technology can convert somatic cells to the pluripotent state and, therefore, offers a solution to this problem. Since the first generation of iPSCs, there has been an explosion of relevant research, from which we have learned much about the genetic networks and epigenetic landscape of pluripotency, as well as how to manipulate genes, epigenetics and microRNAs to obtain iPSCs. In the end, iPSCs continue to offer much promise for both clinical applications with personalized medicine and for basic research in developmental and cell biology. Before iPSC-based clinical applications can be initiated, detailed evaluations of the cells, including their differentiation potentials and tumorigenic activities in different contexts, should be investigated to establish their safety and effectiveness for cell transplantation therapies. Once their safety is confirmed, human-induced pluripotent stem cells (hiPSCs), which do not entail ethical concerns, may become a preferred cell source for regenerative medicine.

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Induced Pluripotent Stem Cells Differentiate into Functional Cardiomyocytes

5

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Contents

Abstract

The conversion of somatic cells into so called induced pluripotent stem cells (iPSCs) is generally considered as major breakthrough in stem cell research, and cardiomyocytes derived from human iPS cells are considered as promising cell source for disease modelling, drug screening and safety pharmacology, and future therapeutic applications.

This review gives an overview on the molecular basis of cardiac development, recent progress in directing differentiation of pluripotent stem cells into the cardiac lineage and characterisation of the resulting myocytes. Moreover, generation of cardiac cells from disease-specific iPSCs and application of iPSC-derived myocytes for engineering of functional heart tissue and heart repair is discussed.

Introduction

The intrinsic regenerative capacity of the human heart is very limited. Adult cardiomyocytes (CMs) have almost lost their potential for proliferation and cannot be expanded in culture. For many years, the lack of human myocytes represented a major obstacle in basic research in cardiology, in drug screening and safety pharmacology, and certainly also for development of cellular therapies and in vitro engineering of functional heart tissue.

Therefore, cardiomyocytes derived from human pluripotent stem cells (hPSCs) and especially

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from induced pluripotent stem cells (hiPSCs) hold great promise as a suitable cell source. Since the first report of cardiomyocyte generation from human embryonic stem cells (hESCs) (Kehat et al. 2001) much effort was put into establishing well defined conditions for more reliable and efficient differentiation protocols as well as selection procedures to provide highly enriched cell populations. The detailed molecular and functional characterization of the hPSC-derived cardiomyocytes demonstrated the parallel development of atrial, ventricular and pacemaker cells showing chronotrophic responses to cardioactive drugs, typically with a rather fetal than adult phenotype. In the following we provide an overview on developmental findings that provided (and still provide) important cues for targeted differentiation of pluripotent stem cells into cardiomyocytes and their specific sublineages, on established protocols for improved cardiac differentiation and maturation, and on different genetic and non-genetic strategies for enrichment of hPSC-derived cardiomyocytes. Furthermore, published data on the detailed molecular, structural and functional characteristics of hPSC-derived cardiomyocytes, and their suitability for drug screening and safety pharmacology, as well as first results of their application in disease modelling, tissue engineering and animal models are discussed.

Cardiomyocyte Formation During Heart Development

The development of the vertebrate heart has become more and more complex throughout the evolution. Nevertheless, basic mechanisms of heart development are conserved, and knowledge from different model organisms including zebrafish, xenopus leavis and mice critically contributes to the current understanding of human heart formation.

The major stages of mouse heart development are defined based on morphological and functional aspects and can be further characterized by the expression of specific transcription factors (Bruneau 2002). In a first step, heart development is initiated through the migration of cells from the primitive streak to the anterior region of the embryo where the cardiac crescent is formed. These initial cardiac progenitor cells are characterized by expression of the T-box transcription factors brachyury (T) and Eomes followed by the appearance of Flk1 (fetal liver kinase-1) (cardiac mesoderm) (development stage E7.0 in the mouse). These cells give the rise to the cardiac crescent (E7.5–7.75) with the expression of myocardin and Mesp 1 (mesoderm posterior homolog). Mesp 1 induces a complex network of cardiac transcription factors. Subsequently, the linear heart tube is formed (E8.0) by a later stage of cardiac progenitors specified by the expression of GATA4 (GATA binding protein 4) and Mesp 1. Then the heart tube loops (E8.5-E9.5 mouse, ~ d20-d25 human) (Sizarov et al. 2011) and heart cells start to express Nkx 2.5 (NK2 homeobox 5), dHand (heart and neural crest derivatives expressed), Tbx5 (T-box protein 5) and Mef2c (myocyte enhancer factor 2C). This early progenitor cell population which gives rise to the early heart tube represents the primary heart field. Cells from this first heart field contribute to the left ventricle and the atria. Anterior of the linear heart tube a second progenitor cell population forms. Cells from this so called anterior or secondary heart field were first identified by the expression of FGF10 and could be shown to contribute to the cardiac outflow tract. The expression of the LIM homeodomain transcription factor Isl1 (insulin gene enhancer protein) serves also as a marker for the secondary heart field. Isl1 positive cells contribute to the outflow tract, the right ventricle and the atria. A direct regulation of FGF10 transcription by Isl1 could be shown recently (Golzio et al. 2012). The subsequent chamber formation (E10-E12 mouse, ~ d26-38 human) is characterized by the expression of Nkx 2.5, Irx4 (iroquois homeobox 4), Tbx5 and Hand1/2. Whereas the expression of Irx4 is restricted to the ventricular myocardium, Hand1 is predominately expressed in the left and Hand2 in the right ventricle. HEY2 (hairy/enhancer-of-split related with YRPW motif 2) a downstream target of notch signalling reacts as a ventricle specific transcription factor. The atrial

cardiomyocytes are characterized by expression of HEY1, GATA4 and TBX5 (Brand 2003).

To enable the complex cardiac development the necessary signalling pathways are well-orchestrated. Positive as well as negative signals from the surrounding tissue are required. The neighbouring endodermal tissue provides positive signalling by members of the bone morphogenetic protein (BMP) family as well as fibroblast growth factors (FGFs). BMP2 and BMP4 contribute to induce the formation of cardiogenic cells from non-precardiac mesoderm, whereas FGF-signalling is responsible for the further proliferation and survival of these cells. For instance a combination of FGF4 and BMP2 could be shown to induce full cardiac differentiation in non-precardiac mesoderm (Brand 2003). In addition studies on Fgf10 and Fgfr2-IIIb mutant mice revealed that both the ligand as well as its receptor are required for normal heart development (Golzio et al. 2012).

WNT signalling provided by the mesoderm itself controls cardiogenesis via positive signals expressed in the precardiac mesoderm. Negative acting signalling molecules of the WNT/ßcatenin pathway, expressed in the cardiogenic mesoderm, have to be repressed by inhibitors like dickkopf (Dkk-1) to support early cardiogenesis (Burridge et al. 2012).

Initiated through the above extracellular signals and the appearance of various cardiac transcription factors, the increasing expression of different proteins of the contractile apparatus forms the basis for later heart function. Also, the different functional characteristics and distinct contractile properties of the heart chambers are defined by chamber specific structural proteins. The contractile protein myosin consists of two pairs of myosin light chains (MLC) and two heavy chains (MHC). The atrial subtype of myosin light chain (MLC2a) is initially expressed in the linear heart tube and later exclusively in the atrium whereas the ventricular subtype (MLC2v) is solely expressed in ventricular cells throughout development (Small and Krieg 2004). The two different isoforms αMHC and βMHC containing the ATP binding sites constitute the motor function of the myosin molecule, αMHC with high Ca^{2+} and actin activated ATPase activity results in faster shortening velocity of the cardiac fibers whereas βMHC with lower ATPase activity results in slower shortening velocity (Nadal-Ginard and Mahdavi 1989). In the ventricle of developing mammals ßMHC is the most abundant isoform, however directly before birth, this switches to the αMHC subtype. In small animals αMHC becomes the prevalent isoform in the adult ventricle (>90%), whereas in large mammals including humans the ventricular myocardium is predominantly composed of the slow βMHC isoform (>95%) (Miyata et al. 2000; Narolska et al. 2005). Finally the maturation/septation from E12 till birth (in humans between week 4 and 7) (Lamers and Moorman 2002) is specified by the expression of Nkx 2.5, TBX5, RXRα (retinoid X receptor alpha), FOG-2 (Zfpm2, zinc finger protein, multitype 2), GATA4, TEF-1 (TEA domain family member 1) and Sox4 (SRY-box containing gene 4). For establishing the left right identity of the atria the expression of pitx2 (paired-like homeodomain transcription factor 2) is required (Bruneau 2002). Septation of cardiac chambers and therefore the unidirectional bloodflow is ensured by functional cardiac valves. Signals between myocardium and endocardium mediated by BMP2, TBX3, TGFß, and the NOTCH pathway are necessary for correct positioning and function of the cardiac valves (Srivastava 2006). During mouse development these events take place during the first 12 days after fertilization whereas human development generally takes longer and cardiac valves are formed after 35 days (Sizarov et al. 2011). Even before this complex development is completed the primitive tubular heart begins to beat in mouse on day 8 and in human about 3 weeks after fertilization (Brand 2003).

During fetal development cardiomyocytes rapidly divide but exit the cell cycle postnatally. The majority of terminal differentiated cardiomyocytes do not reenter the cell cycle therefore an increase in cell size (hypertrophy) is responsible for increasing cardiac mass after birth (Burridge et al. 2012). Due to this limited proliferation potential adult cardiomyocytes cannot be expanded in culture. The opportunity to generate

virtually unlimited numbers of CMs differentiated from pluripotent stem cells might facilitate various applications of these cells.

Differentiation of Pluripotent Stem Cells Towards Cardiomyocytes

The first *in vitro* differentiation approach that resulted in spontaneously contracting cardiomyocytes was developed by A. Wobus and coworkers using murine embryonic stem cells (Wobus et al. 1991). After removal of feeder cells that support the maintenance of an undifferentiated state, cells were aggregated to form so called embryoid bodies (EBs). Within such EBs, the stem cells developed into various differentiated lineages mimicking at least in part the developmental processes that occur in a normal embryo. Since EB-based differentiation typically results in formation of a variety of different cell lineages, this approach is frequently considered as non-targeted or random differentiation. However, strictly speaking this is not correct. EB-based protocols are typically based on culture media contain fetal calf serum, which provides lot-dependent combination of cytokines and other differentiation-modulating compounds. Moreover, EBs consist of more or less organised tissue structures with distinct cell types that provide molecular signals for differentiation to neighbouring cells. For instance, EBs typically contain a surrounding cell layer of endodermal cells. Although not as well orchestrated as in a native embryo, molecular cues provided by a certain tissue component such as early endoderm result in specific differentiation pathways in individual cells of the EB.

More recently, EB-based differentiation protocols have been adapted to human ES cells (Kehat et al. 2001). Also human iPS cells have been differentiated into beating cells with phenotypic properties of functional cardiomyocytes (Haase et al. 2009; Zhang et al. 2009).

Typically, efficiencies of EB-based cardiac differentiation are relatively low, however, using pluripotent stem cell lines such as the hESC line (H9.2) or HES-3, efficiencies between 8 and 70% of beating EBs, which probably correspond to a few percent of myocytes, only, were obtained (Xu et al. 2002). In the meantime, these rather inefficient and barely reproducible approaches could be optimized by utilizing molecular insights from mouse development.

As already discussed above, developmental studies demonstrated that surrounding endodermal cells provide required inductive as well as inhibitory signals for the cardiac differentiation of pluripotent cells. Consequently, it was investigated whether endodermal cells can induce cardiogenesis in embryonic stem cells in vitro, also, and a mouse cell line (END-2) that resembles visceral endodermal cells was shown to induce cardiogenesis in mouse P19 and murine embryonic stem cells (Mummery et al. 1991). More recently, this finding could be transferred to human cells demonstrating induction of cardiogenesis in hESCs after co-culture with END-2 cells. Notably, different hESC lines could be differentiated to the cardiomyocyte lineage even if a standard EB based approach was not successful for generating mesodermal cell types from these specific hESC lines (Mummery et al. 2003). In addition, significant improvement of the protocol could be achieved by applying serum free conditions. Further work demonstrated that the inductive effect of END-2 cells does not depend on direct cell-cell-interaction but depends on soluble factors. Thus, serum free medium conditioned by END-2 cells was able to induce cardiomyogenesis, and depletion of insulin was identified as one underlying mechanism of the observed cardiogenic END-2 effect. On the other hand, insulin depletion alone was insufficient as other cell types with equivalent insulin-depleting activity did not show cardiomyogenic potential. Interestingly, microarray analyses revealed up-regulated expression of enzymes connected to the prostaglandin (PGI2) synthesis in END-2 cells. In fact supplementation of the serum free and insulin free differentiation medium with PGI2 could mimic the effect of END-2 cells or conditioned medium thereof on cardiomyogenesis (Xu et al. 2008a).

In the end, however, all extensive experimental efforts on barely controlled random differentiation,

in embryoid bodies and serum-containing media, or in coculture settings, did not lead to efficient and well reproducible protocols.

More recently, groundbreaking substantial work especially of the group of G. Keller (Kattman et al. 2011b) utilized the increasing knowledge on molecular cues and pathways in vertebrate heart development and led to differentiation protocols based on the sequential application of specific inducers for more efficient targeted differentiation.

Notably, development of such protocols and identification of crucial differentiation factors would have been barely possible without the recent advancements of technologies for generation of pluripotent stem cell lines that enable monitoring of cardiac differentiation through integration of transgenic reporters (Elliott et al. 2011). Long-term systematic research using suitable transgenic reporter cell lines step by step deciphered the molecular pathways underlying differentiation (Murry and Keller 2008).

In the meantime, sequential protocols for the efficient induction of cardiomyogenesis in hPSCs have been developed by several groups starting from feeder-based cultures as well as monolayer hPSC cultures. In stepwise approaches, the starting populations were manipulated by adding activators and/or inhibitors of signalling pathways, either as purified or as recombinant proteins, to sequentially form mesendodermal progenitors cells, cardiovascular and cardiac progenitors, and finally cardiomyocytes with typically rather immature phenotype (Burridge et al. 2012). Notably, establishment and optimization of these approaches would not have been possible through the commonly used endpoint analysis of beating cluster and expression of cardiomyocyte specific markers like cardiac troponin T (cTNT) or sarcomeric α actinin. In contrast, the stepwise design required a detailed characterization of the emerging intermediate progenitor populations with respect to lineage-specific cellular marker profiles and suitable molecular tools for their detection. Continued cardiac specification of the respective subpopulations was monitored especially by the appearance of transcription factors such as Mesp 1 and Nkx 2.5 (Kattman et al. 2011b), or by utilizing genetically engineered reporter cell lines which express fluorochromes under the control of a cell-lineage specific promoter, like Nkx 2.5 (Elliott et al. 2011). In combination with developmental data these tools and technologies allowed for targeted manipulation and tight monitoring of the necessary lineage decision during *in vitro* differentiation of pluripotent stem cells.

As a first step towards targeted generation of cardiomyocytes, induction of mesendoderm formation as visualized by expression of the T-box transcription factor brachyury was achieved by adding a combination of activin A and BMP4 in some cases supported by addition of FGF2 (Murry and Keller 2008; Burridge et al. 2012) Wnt and activin/nodal/TGF-ß signaling block ectoderm formation but induce mesendodermal differentiation. In addition, also BMP-4, has an inhibitory effect on ectoderm formation and supports mesendoderm formation, although apparently not being essential (Murry and Keller 2008). The subsequent formation of cardiac mesoderm with its continued expression of the T-box transcription factor brachyury could be visualized by temporal expression of the surface marker KDR (kinase insert domain receptor (human homolog to FLK-1)) in combination with PDGFR-α (Kattman et al. 2011b). During this 2nd stage of differentiation, activin dose-dependently supports endoderm and mesoderm formation, whereas now inhibition of Wnt-signalling is required for efficient formation of cardiac mesoderm (Murry and Keller 2008; Willems et al. 2011). TGFß/NODAL signalling as another important molecular pathway was modified by NOGGIN (Burridge et al. 2012). Notably, especially effects of activin/nodal/TGF-ß and BMP signaling critically depend on the dosage and timing (Kattman et al. 2011b). Further differentiation of the above specific subpopulations confirmed their cardiomyogenic potential through correlation of the early marker expression with increased cardiomyocyte content (Kattman et al. 2011b) which can be quantified by immunostaining for myocyte-specific structural proteins such as αMHC, cTNT or sarcomeric α actinin (Haase et al. 2009; Zhang et al.

2009). ßMHC can be utilized for detection of more mature human cardiomyocytes (Lundy et al. 2013).

In general, however, the protocols discussed above are typically highly cell line dependent, and time consuming adaption and fine-tuning of concentrations and application time points is necessary to obtain similar results for the cell lines of interest (Kattman et al. 2011a). Even more critical is the fact that the established protocols are based on recombinant/purified proteins. Such proteins are not only very expensive, but considerable lot-to-lot variations prevent the development of highly reproducible and robust differentiation protocols especially for clinically applicable large scale production of myocytes in a commercial scale.

In view of the above significant drawbacks of protein-based induction of differentiation, small chemically synthesized, inductive molecules continuously moved into the focus of stem cell research. Some compounds such as 5-aza-2′-deoxycytidine (5-aza-dC), retinoic acid (RA) or dimethyl sulfoxide (DMSO) have been reported already years ago to promote cardiomyogenesis in murine and human pluripotent stem cells (Xu et al. 2002; Yoon et al. 2006). By utilization of transgenic ES cells (αMHC-GFP) for screening a chemical library Takahashi et al. (2003) were able to show an inductive effect of ascorbic acid on the cardiac differentiation (Takahashi et al. 2003). Notably however, the above compounds presumably act rather unspecific and do obviously not support well orchestrated development of cardiac lineages.

Subsequently, a more selective administration of small molecules could also been shown for different pathways. Graichen et al. (2008) reported a regulating role of the p38 MAP (mitogen-activated protein) kinase pathway, which had also been described to be involved in the regulation of cardiomyocyte mitosis. This pathway could be manipulated by the compound SB203580. Addition of concentrations ≤10 μM during the first 24 h of differentiation lead to a remarkable increase in the number of beating areas and expression of cardiac specific genes in comparison to untreated cultures (Graichen et al. 2008).

Confirming the role of FGF signalling, the FGF receptor inhibitor SU5402 was shown to have a positive effect on the early stages of cardiomyogenesis in combination with BMP2 (Tomescot et al. 2007).

Again taking advantage of transgenic pluripotent reporter cell lines that enable monitoring of cardiac differentiation, more recently novel small compounds were identified that strongly support cardiac differentiation of hES and hiPS cells. By high content screening of two commercial available small molecule libraries on HESCs, which were predifferentiated under mesendoderm inductive conditions, an inhibitor of the canonical WNT pathway (IWR-1) was identified as a potent activator of cardiomyogenesis (Willems et al. 2011). Similar to the biological WNT/ßcatenin inhibitor Dkk-1 other WNT inhibitors (PORCN, 53AH, XAV939) which act on different levels of the same pathway were also able to induce cardiomyogenesis in this predifferentiated cell population (Willems et al. 2011). Based on mouse developmental studies positive BMP signalling and inhibition of WNT signalling promotes mesoderm and cardiac mesoderm formation. Therefore, the combination of BMP4 supplementation with inhibition of the ß-catenin signalling pathway by small molecules (IWR-1 as well as IWP-4) (Ren et al. 2011) could further increase expression of genes described for cardiac mesoderm or cardiac progenitor cells.

Also, replacement of previously identified inductive proteins through known activators and small compound inhibitors of the respective signaling pathways finally led to much more efficient and reproducible differentiation protocols. Subsequently a study by Lian et al. (2013) could demonstrate robust cardiac differentiation by manipulating the canonical WNT signalling by small molecules, only. Applying two small molecules, a Gsk3 inhibitor (CHIR 99021) for activation of the ß-catenin signalling for 24 h (mesoderm induction) and in a second step blocking of WNT signalling to initiate cardiac specification by addition of IWP-4 or IWP-2 in a well defined temporal manner resulted in a highly efficient and quite robust differentiation protocol with cardiomyocyte contents of up to 98% (Lian et al. 2013).

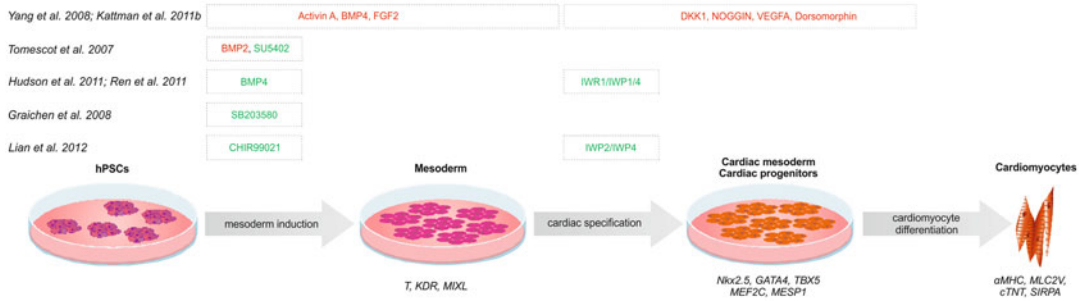

Fig. 5.1 Sequential differentiation protocols leading cells in the desired direction. By addition of growth factors and/or small molecules in a concentration and temporal dependent manner hPSCs are guided to develop into mesodermal cells, cardiac mesoderm, cardiac progenitors and finally cardiomyocytes. Induction and further specification can either be achieved by the use of specific growth factor combinations (*red*) additionally supported by small molecules or based on the addition of small molecules, only (*green*). Abbreviations: hPSCs, human pluripotent stem cells; BMP, bone morphogenic protein; FGF2, fibroblast growth factor 2; DKK1, dickkopf homolog 1; NOGGIN, BMP signalling inhibitor; VEGFA, vascular endothelial growth factor A; IWR1, inhibitor of WNT response 1; IWP4, inhibitor of WNT production 4; SU5402, FGF receptor inhibitor; CHIR 99021, inhibitor of Gsk3; SB203580, inhibitor of p38 MAP kinase pathway; T, T-box transcription factor brachyury; KDR, kinase insert domain receptor; MIXL, Mix paired-like homeobox; Nkx2.5, NK2 homeobox 5; GATA4, GATA binding protein 4; TBX5, T-box protein 5; MEF2C, myocyte enhancer factor 2C; MESP1, mesoderm posterior 1 homolog; αMHC, myosin heavy chain 6; MLC2v, myosin light chain 2V; cTNT, cardiac troponin T type 2; SIRPA, signal-regulatory protein alpha

Beside WNT signalling additional pathways (TGFß, BMP and FGF signalling) are involved in cardiomyogenesis. Controlling FGF as well as BMP signalling by small molecules (FGF receptor inhibitor SU5402 and dorsomorphin, respectively) was already been shown to improve cardiomyogenesis. Combining different small molecule based differentiation strategies might lead to even more robust and ideally cell line independent protocols.

The different signalling molecules and addition time points in the course of differentiation are summarized in Fig. 5.1. It is distinguished between growth factor dependent (red) and application of small molecules only (green) to induce mesoderm, for cardiac specification and finally for cardiomyocyte differentiation.

Selection of Generated Cardiomyocytes

Although substantial progress was made concerning the efficiency and reproducibility of differentiation protocols identification and further purification of the desired cell population still appears indispensible, especially as a safety measure to prevent teratoma formation after transplantation of PSC-derived cell preparations.

Different approaches and strategies which take advantage of specific properties of the desired cells are meanwhile available for the selection of cardiomyocytes from mixed populations. One method for the non-genetical selection uses the high mitochondria content of cardiomyocytes compared to other cell types. Staining of differentiation cultures with the non toxic mitochondrial dye tetramethylrhodamine methyl ester perchlorate (TMRM) results in populations with high, intermediate and low fluorescence activity. Cardiomyocytes with high mitochondria content could therefore be isolated from the highly positive population. Utilizing this reversibly labelling of mitochondria, cardiomyocyte populations with purities of up to 99% were reported (Hattori et al. 2010). However, in direct comparison with a common genetic selection strategy the TMRM based purification showed significantly lower purity and very much lower cell yields as the genetic selection approach (Kensah et al. 2012).

Interestingly, Tohyama et al. (2013) also established selection strategies, which take advantage of cell type specific metabolic differences. In contrast to most other differentiated hPSC derivatives, cardiomyocytes are able to metabolise lactate as energy source, and could therefore be enriched in lactate supplemented and glucose depleted medium (Kawamura et al. 2012; Tohyama et al. 2013) Likewise transferring differentiation cultures to hypertonic solutions with osmotic pressure ≥370 mOsm/kg resulted in enrichment of cardiomyocytes.

In the meantime, also surface marker based approaches for enrichment of cardiac progenitors and cardiomyocytes are available. As shown by Kattman et al. (2011a, b) identification of cardiac progenitors by expression of KDR^{pos}/C-KIT^{neg} or KDR^{pos}/PDGFR-α^{pos} and further propagation of the respective population under optimal conditions concerning application of growth factors results in an increase of cardiomyocyte content with up to 80% for the KDR^{pos}/PDGFR-α^{pos} fraction (Kattman et al. 2011b).

By extensive screens of known antibody libraries against human cell surface molecules two groups could recently identify markers which allow for sorting more mature cardiomyocytes from differentiation cultures. Uosaki et al. (2011) screened a library consisting of 242 antibodies and could identify vascular cell adhesion molecule 1 (VCAM1) as a cardiac specific surface marker which labelled 80% of the cardiac troponin T positive cells (Uosaki et al. 2011).

Dubois et al. (2011) utilized a Nkx 2.5-eGFP reporter cell line for screening a library of 370 anti CD antibodies and could identify signal regulatory protein alpha (SIRPA) as a marker for cardiomyocytes. Staining against SIRPA and subsequent sorting of positive labelled cells resulted in a 98% cardiac troponin t positive population (Dubois et al. 2011).

Another approach to identify a suitable surface marker used once more insights from the vertebrate heart development. The activated leukocyte cell-adhesion molecule (ALCAM, CD166) a marker with a specific temporal expression in the developing heart could be shown to be co-expressed with early cardiac markers like Nkx 2.5, GATA4, MEF2C and TBX5. 85% of the ALCAM positive population were also positive for αMHC (Rust et al. 2009).

Application of these strategies allows for generation of highly enriched cardiomyocyte populations either by fluorescence activated cell sorting (FACS) or magnet assisted cell separation (MACS).

Due to the initial lack of suitable surface markers for the selection of cardiomyocyte populations classical cardiomyocyte selection strategies were based on stable genetically modified cell lines that carry randomly inserted transgenes mediating an antibiotic resistance (e.g. aminoglycoside phosphotransferase meditating a neomycin resistance or the puromycin acetyl transferase) or the expression of a fluorescence marker (eGFP; mCherry) under the control of a cardiomyocyte type specific promoter. Constructs carrying a resistance gene for neomycin driven by the αMHC promoter were first established for the selection of cardiomyocytes from murine ESCs (Klug et al. 1996). Applying these strategies >99% pure cardiomyocyte populations could be obtained. These approaches were also adapted for the selection of human cardiomyocytes. The construct used for the murine ESCs was also applicable for the selection of cardiomyocytes from hESCs differentiation with purity of up to 99% (Xu et al. 2008b). Beside this first strategy the myosin light chain 2 v promoter (MLC2v) could be utilized for the specific expression of different reporter genes for the selection of cardiomyocytes with similar yields (Anderson et al. 2007; Huber et al. 2007) Notably, in case of random genomic integration of transgenes such genetically engineered cell lines will barely be clinical applicable due to potential insertional mutagenesis. Also, the time consuming generation of these lines, the dependency of expression levels on the genomic integration site and the frequently observed silencing of transgenes limits the usefulness of such lines. Notably, however, efficient site-specific gene editing techniques which allow for safe long term transgene expression at well defined genomic integration sites in hPSCs could recently be developed. Targeted induction of double strand breaks by employing

tailored designer nucleases, such as zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs) or RNA-mediated editing by CRISPR-Cas leads to enhanced homologous recombination based gene integration in hPSCs with very high efficiencies (Gaj et al. 2013). These techniques facilitate not only highly efficient generation of genetically engineered cell lines with more robust transgene expression and negligible transgene silencing but may also be in accordance to regulatory requirements for clinically applicable cell lines. Since the high purities of up to 100% (Kensah et al. 2012) combined with superior cell vitality that can be achieved through genetic selection procedures could so far not be obtained with any of the non-genetic selection techniques, the novel targeted genome engineering technologies discussed above may represent the most advanced safe, efficient and reliable cardiomyocyte enrichment approaches.

Characterization of Generated Cardiomyocytes

A thorough characterization of the generated CMs is an indispensible requirement for any conceivable application like disease modelling, drug screening and safety pharmacology, or cellular therapies.

Gene expression analysis is one crucial building block for the detailed characterization of generated cell populations. Functional cardiomyocytes should express common markers that are already present in cardiac progenitors like the transcription factors GATA4, MEF2C and Nkx 2.5, but additionally also cardiomyocyte specific marker proteins like cardiac troponin T (cTnT), sarcomeric α actinin and αMHC. In addition, markers with specificity for certain cardiomyocyte sublineages such as MLC2a and MLC2v or atrial natriuretic factor (ANF) are available (Kehat et al. 2001; Graichen et al. 2008). Moreover expression analysis of several cardiac specific ion channels e.g. Hyperpolarization-activated cyclic nucleotide-gated channels HCN1, -2 and-4 and Human Ether-a-go-go Related Gene (HERG) is utilized to demonstrate the cardiac phenotype of the generated cells (Guo et al. 2011; Honda et al. 2011). Maturation of human PSC-derived cardiomyocytes is commonly monitored on the immunocytological level through increased expression of ßMHC, and can also be assessed based on the arrangement of structural proteins and the striated pattern of myofibrillar bundles. Expression of structural proteins (e.g. cTNT, αMHC, MLC2a, MLC2v) could be shown for hPSC derived CMs which can be diffuse in early state and becomes more pronounced during maturation of the CMs (Kehat et al. 2001; Lundy et al. 2013).

Importantly, ultrastructural characterization of cardiomyocytes represents another component of a thorough characterization and can provide more detailed informations on the structure of myofibrillar bundles, Z-bands and intercalated discs in the generated cardiomyocytes (Kehat et al. 2001). With advancing maturation in culture, ultrastructural organization of the hPSC derived CMs develops from cells with unorganized myofibrils to elongated cells in which the myofibrils are more organized and well aligned. In these cells A, I and Z bands within the sarcomers as well as mitochondria between the sarcomeric structures could be shown by transmission electron microscopy (Kehat et al. 2001; Snir et al. 2003). Transverse tubules could not be found in any of the analyzed cells. In contrast to adult cardiomyocytes, which are typically multinucleated, cultured hPSC derived CMs are more immature and mostly mononucleated (Snir et al. 2003; Lundy et al. 2013).

The opportunity to generate highly enriched or pure populations of human pluripotent stem cell derived cardiomyocytes is the prerequisite for detailed analysis and further comparison of these cell populations with human heart tissue. Comparison of the transcriptional profiles of enriched cardiomyocytes from human pluripotent cells, fetal and/or adult heart tissue showed similarities concerning cytoskeleton, ion transport and heart development but also distinct differences between the analysed groups, lead to the identification of genes initially not connected to cardiac development (Xu et al. 2009).

Beside the thorough characterization of structural features and gene expression patterns, a comprehensive functional analysis is indispensible prior to any application. Analyses of calcium transients as well as electrophysiological measurements are usually applied for functional characterization of the generated CMs.

Using fluorescent dyes like Fura-2, which build complexes with Ca^{2+}-ions, intracellular calcium transients during the contractile cycle can be analysed in individual CMs or coupled cell clusters. For hESC as well as for hiPSC derived CMs functional RyR (ryanodine receptor) regulated intracellular calcium stores could be demonstrated. In principle, basic proteins for calcium handling are expressed and functional in hPSC derived CMs, however, their function depends on the CM maturation state, e.g. absence of transversal tubules leads to unsynchronized calcium transients in immature hPSC derived CMs (Lieu et al. 2009; Itzhaki et al. 2011b). Sharp electrode assays provide action potential morphologies that allow determining the respective cardiomyocyte subtype (He et al. 2003). Patch clamp analyses on single cells allow to assess the presence and action of specific ion channels (He et al. 2003; Honda et al. 2011) and measurements of extracellular field potentials by microelectrode arrays (MEAs) (Reppel et al. 2005) can provide additional data concerning intracellular coupling and velocity of signal distribution. Different action potential morphologies could be found by these electrophysiology approaches. He et al. (2003) observed nodal, atrial and ventricular like action potential morphologies whereby the last two represented the embryonic phenotype with more depolarized maximum diastolic potentials and a low dV/dt_{max}. By MEA measurements synchronized field potential propagation in a functional syncytium within a beating cluster could be demonstrated (Mehta et al. 2011).

A more detailed functional analysis of the generated CMs is possible through electrophysiological approaches. Furthermore the responsiveness towards cardioactive substances e.g. a chronotrophic response of the generated CMs to isoproterenol and carbachol could be shown (Kehat et al. 2001; He et al. 2003).

Beside the observed similarities between adult or fetal myocardial tissue, or CMs and CMs generated from hPSCs, distinct differences were revealed by these comprehensive analyses. The hPSC derived CMs displayed a rather immature phenotype concerning energy metabolism and resemble a more fetal phenotype concerning the AP morphology (He et al. 2003; Dick et al. 2010).

Applications of Cardiomyocytes Generated from Human Pluripotent Stem Cells

The first report of CM generation from hESCs by Kehat et al. (2001) introduced a variety of envisioned applications like pharmacological testing, cell therapy as well as tissue engineering approaches for these cells. Moreover the generation of patient specific pluripotent cells via reprogramming of somatic cells (Takahashi et al. 2007) and subsequent differentiation to cardiomyocytes (Haase et al. 2009; Zhang et al. 2009) facilitates studying of cardiac defects in cell culture disease models to enable research on mechanism of genetic disorders and subsequent therapy development.

The long QT syndrome, which is characterized by a prolonged QT interval and high risk of sudden cardiac death was the first genetically based cardiac disorder which was recapitulated in a patient specific iPSC model. The established iPSC lines carried a mutation in the KCNQ1 gene that encodes for a voltage gated repolarising potassium channel. CMs generated from these iPSCs showed a prolonged action potential, altered activation and deactivation of the I_{KS} current as well as an abnormal response to catecholamine stimulation when compared to CMs generated from healthy controls (Moretti et al. 2010). A mutation in the KCNH2 gene which also leads to a long QT syndrome could be modelled as well by utilizing this technique (Itzhaki et al. 2011a). The generated CMs showed prolonged action potential duration due to a reduction of the potassium channel I_{Kr}. Development of spontaneous early-after depolarisations (EADs) in the LQTS iPS-CMs provided insights into the

pathogenesis of arrhythmias in the investigated syndrome.

Furthermore, mutations in the PTPN11 gene encoding for a SHP2 phosphatase, which, beside other defects like pulmonary valve stenosis, abnormal genitalia and retardation of growth, also leads to hypertrophic cardiomyopathy could be modelled by establishing iPSC from patients with the so called LEOPARD syndrome (Carvajal-Vergara et al. 2010). Analysis of CMs from these disease specific iPSC lines revealed increased cell size and nuclear localisation of NFATC4 compared to control CMs which are indicative for cardiac hypertrophy. Moreover, utilizing antibody microarray analyses, the authors searched for other unknown molecular effects of the PTPN11 mutation in the LEOPARD-iPSCs and were able to connect one identified upregulated phosphoprotein, pMEK1, upstream kinase of ERK, with increased pERK basal levels known to be associated with LEOPARD syndrome. In addition, failure of ERK activation via bFGF demonstrates that even in the undifferentiated LEOPARD-iPSCs the RAS-MAPK signal transduction is impaired. Further abnormalities in protein phosphorylation could be observed, but more detailed analyses will be required to clarify their contribution to the disease phenotype.

For another hereditary cardiac disorder, hypertrophic cardiomyopathy (HCM), patient-specific iPSCs could be recently generated, which carry a mutation in the MYH7 gene encoding for an argining to histidin substitution (Arg663His). CMs generated from these disease iPSCs recapitulate aspects of the HCM phenotype like cellular enlargement, multinucleation, increased myofibril content and a higher proportion of cells with disorganized sarcomers. Moreover the generated CMs showed dysregulation of Ca^{2+} cycling and increased intracellular Ca^{2+} concentration, which was previously described as key characteristics of HCM. By utilizing CMs generated from HCM-iPSC the authors observed that the irregular calcium transients occur prior to cellular hypertrophy and that pharmacological restoration of the dysregulated Ca^{2+} levels could prevent the development of the HCM phenotype. From these results the authors conclude that the imbalance in Ca^{2+} regulation is a central underlying mechanism of HCM development on single cell level (Lan et al. 2013).

Notably, current human iPSC-based disease models have still limitations, for instance the typically immature state of iPSC-derived cardiomyocytes or the lack of 3-dimensional tissue interactions of native myocardium. Nevertheless, disease specific iPSCs represent exciting versatile tools for elucidating the molecular mechanisms and corresponding phenotypes of genetically based diseases.

Apart from the opportunity to generate patient specific iPSCs for disease modelling hPCS derived CMs could be utilized for drug screening and safety pharmacology approaches. During the first phase of drug development, the most potent compounds are typically identified among several hundred thousands of candidates using cost-effective high throughput assays with limited data quality, for example the dofetilide binding assay or rubidium efflux assay. Whereas even in this early phase of drug screening, higher data qualities would be desirable, such assays are especially unsuitable for detailed characterization of pharmacodynamic properties and potential undesired side effects. In most cases, safety pharmacology studies therefore require the use of sophisticated, labor (and cost) intensive *in vitro* assays with higher predictivity, such as Langendorf heart, or most expensive, animal experiments in dogs or monkeys.

One major problem of all cardiac *in vitro* assays is the cell source. Human cardiomyocytes would be optimal; however, these were so far not available as myocyte-derived tumor cell lines and adult primary cardiomyocytes lose proliferation potential. As an alternative, existing assays use Xenopus oocytes or human tumor cell lines genetically modified to express hERG channels, or primary cardiomyocytes prepared from hearts of other species, for example dogs (Dick et al. 2010). However, the phenotype of these cell sources is far from being able to closely mirror the function of human cardiomyocytes.

To overcome the limitations of the currently used *in vitro* test systems e.g. due to species differences, low throughput or lack of complex

channel interactions, cardiotoxicity testing based on CMs derived from human pluripotent stem cells is considered a suitable *in vitro* tool (Guo et al. 2011). Mehta et al. (2011) demonstrated for CMs generated from virus free hiPSCs by MEA recordings predicted responses for pharmacological active drugs like isoprenaline, verapamil or E-4031. Likewise Guo et al. (2011) were able to show compound specific effects of 28 cardiac active substances on CMs generated from hiPSCs. Meanwhile over 40 cardioactive compounds were investigated for their predicted pharmacological effect on hPSC derived CMs with various analytical methods like patch clam, MEA or calcium transients (Dick et al. 2010). Moreover engineered heart tissues (EHT) composed of HESC derived CMs and fibrin based matrix in a 24 well approach could be utilized for preclinical toxicity testing (Hansen et al. 2010). Utilization of a miniaturized bioreactor for in situ assessment of bioartificial cardiac tissues allows for detailed monitoring of cardiac constructs during tissue development and stimulation by e.g. stretch or cardioactive substances (Kensah et al. 2011).

Improved high throughput *in vitro* screening assays based on these first reports could help to reduce costs of drug screening by preventing late stage drug attrition or withdrawals of prescription drugs from clinical use due to unexpected secondary effects. Furthermore the possibility to screen large molecule libraries for specific effects at a very early stage could improve drug development significantly.

Beside these *in vitro* applications of hPSC derived CMs, the clinical use of these cells for myocardial tissue engineering or direct cell injections is another conceivable application. Myocardial infarction leads to irreversible cell loss and scar formation, which result in impaired cardiac function, cardiac hypertrophy and finally heart failure. The only end stage therapy options are currently organ transplantation or ventricular assist devices. Alternative therapeutic concepts of heart repair either based on direct cell injection, or on transplantation of *in vitro* engineered tissue constructs are urgently required to overcome the shortage of donor organs.

Notably, however, a multitude of recent experimental and clinical studies applying various adult stem cells resulted in no or minor functional improvement much likely due to paracrine effects, only. Despite sporadic myocyte formation significant generation of *de novo* myocardium could not be demonstrated (Ptaszek et al. 2012). Obviously, most concepts of cell transplantation face severe limitations with respect to cell survival and functional integration. In case of cardiac cell therapy, functional coupling and formation of well organized myocardium appears to be difficult to achieve. Direct injection of cells to improve heart function was investigated in different animal models with a variety of cell sources including pluripotent stem cells from mouse and human undifferentiated as well as differentiated to cardiac progenitors or cardiomyocytes. Independent from cell type or application method (intramyocardial or intracoronary injection) only up to 5% of the cells remained in the heart, and significant integration as well as long term survival could not be shown (Nunes et al. 2011). Also, and although survival and cardiovascular *in vitro* differentiation was demonstrated, injection of iPS-derived cardiovascular progenitors did not result in formation of structured myocardium (Mauritz et al. 2011). On the other hand, however, very recent data may indicate light at the end of the tunnel: Recently, applying sodium iodide symporter transgenic hiPSCs, vascular differentiation and long-term engraftment of hiPSCs in a large-animal model of myocardial infarction could be demonstrated for the first time (Templin et al. 2012). Moreover, the formation of relatively large electrically coupled and well structured muscle islands from injected hPSC-derived myocytes as demonstrated in a guinea pig model of MI (Shiba et al. 2012) may represent a true breakthrough in cardiac cell therapy.

Transplantation of *in vitro* engineered heart tissue (Zimmermann et al. 2006) may be an alternative approach to overcome the above limitations. Based on biocompatible or biodegradable materials and living cells, tissue constructs can be engineered *in vitro* either by seeding cells on matrices or mixing of soluble matrix components and cells.

By combining porous, biocompatible scaffolds and hESCs derived CMs with endothelial cells and murine embryonic fibroblasts tissue constructs could be generated which were implanted into healthy rat hearts (Lesman et al. 2010).

More recently Tulloch et al. (2011) described engineered human myocardium based on collagen gel and CMs derived from hESCs and hiPSCs supported by human endothelial and marrow stromal cells as well as mouse embryonic fibroblasts. These constructs could be shown to engraft after transplantation onto rat myocardium and to be connected to the host vasculature 1 week after transplantation (Tulloch et al. 2011). Furthermore the generation of engineered cell sheets consisting of hiPSC derived CMs and subsequent transplantation in a porcine ischemic cardiomyopathy model could improve cardiac function (Kawamura et al. 2012). Notably, we have recently shown that hiPSCs with their ESC-like capacity for proliferation and differentiation, enable the generation of functional bioartificial cardiac tissue (BCT) that develops contractile forces almost similar to native myocardium (Kensah et al. 2012). Further developments led to comparable constructs based on defined human and partially synthetic matrix components, which might facilitate clinical applications in the future (Dahlmann et al. 2012).

Of course, prior to clinical application there are still many hurdles to be overcome. These include the need for proper vascularisation and the elucidation and reduction of risks associated with the recently observed chromosomal abnormalities that become apparent after reprogramming and PSC expansion (Lund et al. 2012). However, it is now foreseeable that the development of induced pluripotent stem cells with their almost unlimited potential for proliferation and differentiation can finally lead to novel therapeutically applicable concepts of cell-based heart repair.

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Characteristics of Satellite Cells and Multipotent Adult Stem Cells in the Skeletal Muscle

6

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Contents

Abstract

Under resting conditions skeletal muscle is a stable tissue with a low turnover rate. Upon injury it has the ability to undergo regeneration, preventing the loss of muscle mass and function. Recent research demonstrated that satellite cells, the stem cells of adult skeletal muscle, are indispensible for muscle regeneration. Satellite cells are generated during embryonic development and persist in adult muscle. They are characterized by the expression of Pax7 and their location underneath the basal lamina of muscle fibers. In resting skeletal muscle satellite cells are mostly quiescent. They become activated during regeneration and fuse to each other generating new fibers or repair damaged ones. Satellite cells are a heterogeneous population based on the expression of two transcription factors, Myf5 and MyoD. This chapter gives an overview on satellite cell function, their heterogeneity, regulation and embryonic origin.

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Introduction

Skeletal muscle serves a multitude of functions in the organism including voluntary locomotion, postural behaviour as well as breathing. It exhibits a remarkable ability to adapt to physiological demands such as growth, training and injury. Regeneration of skeletal muscle after injury is dependant on satellite cells (Lepper et al. 2011; Sambasivan et al. 2011), a small population of cells residing underneath the basal lamina of muscle fibers (Mauro 1961). Originally, satellite cells were characterized by electron microscopy based on their location between the plasma membrane and the surrounding basal lamina of muscle fibers. Studies from recent years have demonstrated that satellite cells are a heterogeneous population (Kuang et al. 2008). In resting adult skeletal muscle satellite cells are quiescent, but become activated after injury, re-enter the cell cycle and differentiate to form nascent myofibers (Bentzinger et al. 2010).

Identification of Satellite Cells

Morphological features of quiescent satellite cells include a high nuclear-to-cytoplasm ratio, few organelles and a smaller nucleus with a high amount of heterochromatin when compared to the nuclei of adjacent fibers. In normal adult muscle satellite cells are mitotically quiescent and express Pax7, CXCR4 (CXC motif receptor R-4), α7-integrin, M-Cadherin and CD34 among other markers like syndecan-3, syndecan-4, c-met and VCAM (vascular cell adhesion molecule 1) (Bentzinger et al. 2010).

Developmental Myogenesis and the Origin of Satellite Cells

Several studies suggest that satellite cells are derived from the same embryonic structure that gives rise to the muscle they are localized in. The majority of skeletal muscle in vertebrates, with the exception of the head muscles, is derived from the somites. This section describes the embryonic development of skeletal muscle in the trunk and the limbs. These muscles are derived from segmental mesodermal structures, the somites, that develop from the anterior end of the pre-somitic mesoderm on either side of the neural tube (Bryson-Richardson and Currie 2008; Buckingham and Vincent 2009). The somites then differentiate along the dorso-ventral axis giving rise to the dorsally located epithelial dermomyotome and the ventrally located mesenchymal sclerotome. The dermomyotome develops into the dermis and the skeletal muscle of trunk and limbs while the sclerotome forms cartilage and bone (Parker et al. 2003).

The transcription factors Pax3 and Pax7 characterize the myogenic precursor cells (MPC) in the dermomyotome. As development proceeds, expression of Pax3 is downregulated and satellite cells, marked by the expression of Pax7, arise (Bentzinger et al. 2010). However, in some muscles Pax3 continues to be transcribed, for example the diaphragm. The first satellite cells are generated in mice around ED15.5, when Pax3/Pax7 positive cells are aligning with nascent myotubes and taking up a sublaminar position (Relaix et al. 2005).

Although Pax7 and Pax3 genes are paralogs with almost identical amino acid sequences and partially overlapping expression patterns during mouse embryogenesis, they bare different developmental functions. Pax3 has an essential role in regulating the developmental program of *MyoD*-dependent migratory myoblasts whereas Pax7 plays an important role in myogenic specification (Bentzinger et al. 2012). Analysis of Pax7 and Pax3 null mutations in mice indicates that they are required for the development of a number of distinct cell lineages and appear to have nonredundant roles in myogenesis (Wang and Rudnicki 2012).

The expression of the transcription factors Myf5 and MyoD accompanies the determination of myogenic precursors into replicative myoblasts which can subsequently exit from the cell cycle to become terminally differentiated myocytes (Fig. 6.1). These terminally differentiated myocytes express Myogenin and MRF4 that are, like Myf5 and MyoD, members of the basic-helix-loop-helix transcription factor family

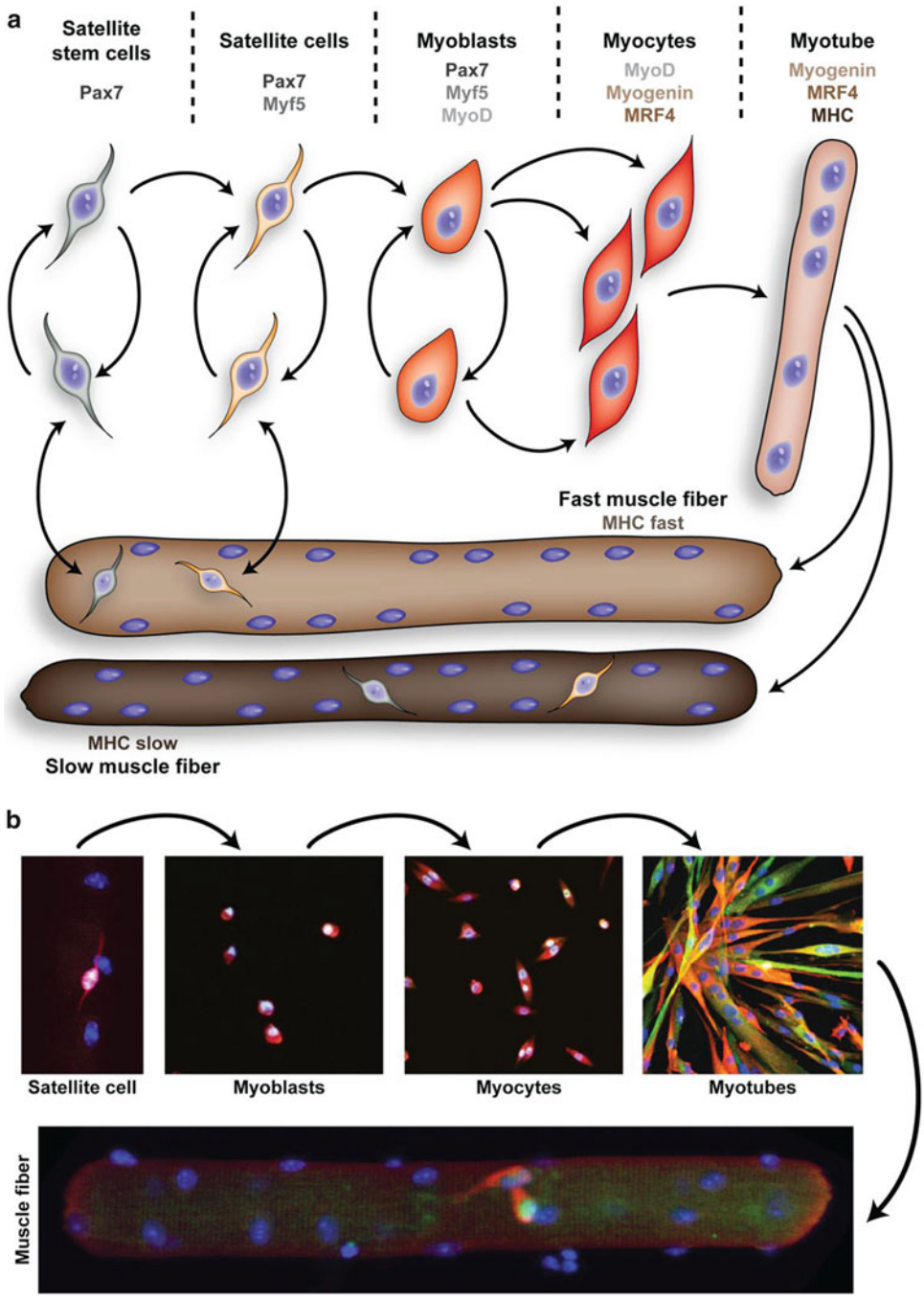

Fig. 6.1 Regulation of the developmental program of satellite cells. (**a**) **Schematic illustrating the myogenic lineage progression**. Satellite stem cells are marked by the expression of Pax7, but have never expressed Myf5 in their developmental history. Satellite stem cells maintain the satellite cell pool by generating satellite cells expressing Myf5 while also undergoing self-renewal. Upon muscle injury satellite cells become myoblasts through the upregulation of MyoD. Once differentiation is initiated Pax7 is downregulated and Myogenin and MRF4 are upregulated. This leads to the conversion of myoblasts into fusion competent myocytes. Subsequently myocytes will fuse to form syncitial myotubes which start to express genes coding for sarcomeric proteins such as MHC. These myotubes will then mature into fully developed and innervated fibers that are marked by proteins typical for the respective fiber type (e.g. MHC fast or slow). (**b**) **Fluorescence microscopy images representative of myogenic lineage progression demonstrating morphological changes during linage progression**

(Parker et al. 2003). Subsequently, myocytes upregulate myosin heavy chain (MHC) and fuse to each other thereby generating multinucleated myotubes which eventually mature into contractile muscle fibers.

Adult skeletal muscle contains different muscle fibers with different fiber type compositions that can be generally categorized into slow and fast contracting ones. Diversification of muscle fibers into distinct types is observed early in development with the initial phase being independent of neural input. In mice, all muscle fibers express embryonic MHC and slow MHCI before ED16. Some of these fibers lose expression of slow MHCI and acquire expression of neonatal MHC. Fast and slow fibers arise from these two populations of primary fibers. Concomitant with this process secondary fibers are generated which either express embryonic MHC or neonatal MHC but no slow MHC. In mice, skeletal muscle is still immature at birth and the fiber type profile changes further during postnatal development. Subsequently, embryonic and neonatal MHC expression is mostly replaced by MHC2A (fast oxidative). This is followed by the upregulation of all fast isoform MHC genes leading to the expression of MHC2A (fast oxidative), fast MHC2X (fast intermediate) and MHC2B (fast glycolytic) in specific fiber subpopulations. In fast contracting murine muscles, such as the tibialis anterior muscle, slow MHCI positive fibers disappear while in slow contracting muscles such as the soleus MHC2A positive fibers transform into slow MHCI expressing fibers. These changes in perinatal fiber composition are critically controlled by the maturation of neuromuscular junctions, mechanical load imposed on neonatal limb muscles and changes in the level of thyroid hormone (Schiaffino and Reggiani 2011). Skeletal muscle has a very high capacity to adapt to physiological demands, e.g. increased exercise. Fiber type composition and volume of the muscle can be influenced by exercise. Hypertrophy of skeletal muscle is characterized by an increase in the size of muscle fibers. This form of adaptation involves satellite cells which under physiological conditions fuse to growing muscle fibers to maintain the size of the myonuclear domain. Moreover, the amount of satellite cells differs between muscle groups. Generally slow contracting muscles contain more satellite cells.

Satellite Cells Are a Heterogeneous Population

The satellite cell population is heterogeneous based on the expression of cell surface markers and expression levels of Myf5. It has been shown that around a fifth of satellite cells display slower proliferation kinetics and are resistant to differentiation. A recent study described the use of a Pax7-GFP live reporter which suggested that satellite cells are heterogeneous based on the expression level of Pax7 (Rocheteau et al. 2012). The authors proposed that Pax7 high expressing satellite cells are more undifferentiated than Pax7 low expressing cells and can distribute their chromatids asymmetrically.

Genetic analysis using the Cre-LoxP system (R26R-YFP/Myf5-Cre) found that approximately 10% of Pax7 positive satellite cells have never expressed Myf5 demonstrated by the absence of yellow fluorescent protein (YFP) expression. However, Kuang et al. (2007) observed up-regulation of Myf5 and induction of YFP expression in satellite daughter cells. This study revealed that $Pax7^+/YFP^-$ satellite cells represent a rare stem cell population within the satellite cell pool, while $Pax7^+/YFP^+$ satellite cells, which make up the majority of satellite cells, are committed myogenic progenitors. During muscle regeneration, both $Pax7^+/YFP^+$ as well as $Pax7^+/YFP^-$ satellite cells proliferate and undergo planar divisions generating two identical daughter cells that both remain either $Pax7^+/YFP^+$ or $Pax7^+/YFP^-$ (Fig. 6.2). Strikingly, apical-basal divisions, with the polarity of cell division at right angles to the basal lamina, were typically asymmetrical, with the basal cell remaining YFP^- and the apical cell up-regulating YFP.

The $Pax7^+/YFP^-$ satellite stem cells presumably represent a lineage continuum of the embryonic Pax3/Pax7 positive cells which did not express any of the MRFs (myogenic regulatory factor) (Fig. 6.2; Kuang et al. 2007). Activated

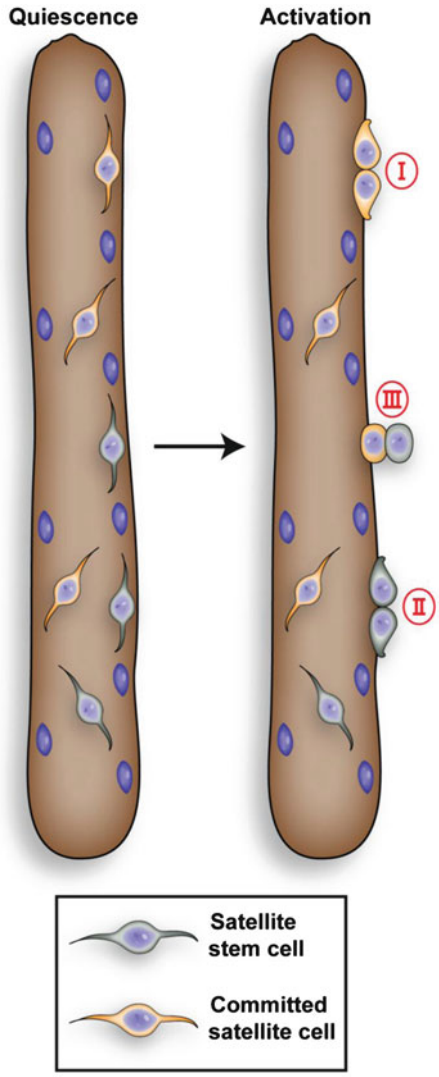

Fig. 6.2 The satellite cell population is heterogeneous. Upon activation satellite stem cells and committed progenitor cells can undergo symmetric divisions (I and II) with the orientation of the division parallel to the muscle fiber (planar). Satellite stem cells can also undergo asymmetric divisions (III) with the division perpendicular to the muscle fiber (apical-basal). This division gives rise to a committed daughter cell and a self-renewing satellite stem cell. Satellite stem cell, which never expressed myf5, comprise about 10% of total satellite cells

$Pax7^+/YFP^+$ satellite cells then upregulate MyoD and start proliferating thereby generating myoblasts (Kuang et al. 2008). Expression of MyoD leads to the upregulation of myogenin, a downstream transcription factor responsible for the transcription of genes involved in terminal differentiation (Bentzinger et al. 2012). Recently Le Grand et al. (2009) showed that Wnt7a drives the symmetric expansion of satellite cells through the planar cell polarity pathway. Wnt7a induces the polarized distribution of the planar cell polarity effector Vangl2, thereby controlling the homeostatic level of satellite stem cells as well as the regenerative potential of the muscle. Another signaling pathway that has been implicated in the control of satellite cell numbers is the Notch signaling pathway. Repression of Notch signaling results in a reduced tissue content of satellite cells due to inhibition of satellite stem cell renewal (Conboy and Rando 2002). The fine regulation of satellite cell self-renewal is essential for maintaining the integrity of the skeletal muscle. Stimulation of the number of symmetric satellite stem cell divisions through application of specific drugs or biologics could potentially increase the regenerative capacity of skeletal muscle and might well be a potential treatment for muscle wasting diseases such as Duchenne muscular dystrophy.

Lineage Progression During Adult Myogenesis

The number of satellite cells decreases after birth. In newborn mice, satellite cells account for 30% of sublaminar muscle nuclei, this is followed by a decrease to less than 5% in 2-month old animals (Wang and Rudnicki 2012). Although the number of satellite cells is decreased in aged muscle, their intrinsic myogenic potential and self-renewal capacity remains unaltered (Day et al. 2010). Muscle grafts from old mice into a young host resulted in a robust activation and regeneration of grafted cells, while satellite cells from young mice performed poorly when transplanted into an old host suggesting that the satellite cell niche is substantially different between old and young mice (Kuang et al. 2008).

The mechanisms regulating satellite cell-lineage progression in the adult share a striking similarity with embryonic myogenesis (Fig. 6.1). It has been observed that during satellite cell activation in the adult a subset of $Pax7^+/MyoD^+$ myoblasts

downregulate MyoD, withdraw from the cell cycle and return to quiescence. This subset comprises the, so called, reserve cells. Pax7 expression induces proliferation of myoblasts and delays their differentiation through regulation of MyoD expression (Olguin et al. 2007). Pax7 expression itself is affected by expression of myogenin leading to its downregulation through negative feedback during differentiation (Olguin et al. 2007). Recent analyses revealed that Pax7 not only regulates MyoD expression but also Myf5 expression in satellite cell derived myoblasts by binding to a region upstream of the Myf5 gene (Bentzinger et al. 2012).

The transcription factors MyoD and Myf5, which are both expressed after activation of satellite cells, seem to have specific roles in adult myogenesis. MyoD is required for the predetermination of myoblasts to differentiate, while Myf5 regulates their proliferation and homeostasis (Wang and Rudnicki 2012). Despite the fact that MyoD and Myf5 can compensate for their loss during embryogenesis they cannot compensate for the loss of each other during adult muscle regeneration.

The expression of myogenin and MRF4 follows the one of Myf5 and MyoD. Expression of myogenin and MRF4 is necessary and sufficient for the formation of myotubes and myofibers (Bentzinger et al. 2012). Neither myogenin nor MRF4 are involved in satellite development or satellite cell maintenance.

Satellite Cells Are Indispensable for Muscle Regeneration

Several experiments illustrated the ability of satellite cells to contribute to regeneration of skeletal muscle. Recent experiments using diphtheria toxin based ablation of satellite cells now clearly demonstrate their necessity for regeneration. When Pax7 positive cells were ablated in two different mouse models (Pax7DTR/+ and Pax7+/CE; R26ReGFP-DTA/lacZ), skeletal muscle failed to regenerate (Lepper et al. 2011; Sambasivan et al. 2011). Instead of nascent myofibers the researchers found adipose tissue infiltrating the damaged muscles. This phenotype is reminiscent of the phenotype of constitutively Pax7 deficient mice (Seale et al. 2000). These experiments suggest that satellite cells are the only contributors to regeneration of skeletal muscle. Since skeletal muscle has a very low turnover rate, satellite cells are mostly quiescent in resting muscle. However, stimuli such as injury or exercise can activate satellite cells. Hence, mice with ablated satellite cells do not display an overt phenotype unless challenged by injury.

Requirement of Pax7 for Satellite Cell Function

Pax7 plays a crucial role in satellite cell function as demonstrated by the phenotype of Pax7 deficient mice (Kuang et al. 2006; Seale et al. 2000). Pax7 deficient mice display a severe phenotype harbouring a depletion of the satellite cell pool in the early postnatal period, most animals die during the first weeks of life (Seale et al. 2000). Muscles from these mice are not able to regenerate following injury demonstrating that Pax7 expression is essential for satellite cell homeostasis in juvenile mice (Kuang et al. 2006; Seale et al. 2000). If Pax7 is inactivated in the adult muscle using inducible Cre-mice combined with a floxed Pax7 allele then mutant satellite cells have been shown to be uncompromised in the regenerating muscle (Lepper et al. 2009). These experiments suggest that satellite cells in juvenile muscles depend on Pax7 but do not require this factor in mature adult muscle. On the contrary, a recent publication suggests that Pax7 is required for regulation of expansion and differentiation of satellite cells not only during neonatal but also adult myogenesis (von Maltzahn et al. 2013).

The Satellite Cell and Its Niche

The satellite cell "niche" is a term used to describe the local microenvironment of satellite cells in muscle. This microenvironment is responsible for the identity and function of the satellite cell as a stem cell. Satellite cells are located underneath the basal lamina and are in contact with myofibers, innervating motor neurons, inflammatory cells and the microvasculature. Important components

of the satellite cell niche are the host muscle fiber that transmits mechanical and chemical signals to the satellite cell, the basal lamina and the microvasculature. The basal lamina consists of the ECM (extracelluar matrix) containing laminins, collagen and proteoglycans.

Evidence for the importance of the microenvironment for satellite cell function arises from studies transplanting satellite cells on myofibers (Hall et al. 2010) and transplantations of satellite cells from old mice into young hosts as well as parabiosis experiments (Kuang et al. 2008). The transplantation of single fibers with their containing satellite cells is much more efficient than the transplantation of isolated cells. Moreover, transplantation of satellite cells from old donors into young recipients resulted in a remarkable myogenic capacity of the transplanted cells. However, when satellite cells from young donors were transplanted into old recipients the transplantation success was low. Through parabiosis experiments it was demonstrated that exposure of old mice to serum from young mice increased the regenerative capacity of 'old' satellite cells. These experiments demonstrate that satellite cells are strongly influenced by their microenvironment.

Regulation of Satellite Cell Proliferation and Differentiation

The balance between proliferation and differentiation of satellite cells following injury is crucial for the homeostasis of adult muscle. Imbalance between proliferation and differentiation might lead to exhaustion of the satellite cell pool as suggested in the case of muscular dystrophy. In adult skeletal muscle a wide array of signaling molecules control satellite cell activation, proliferation and their return to quiescence. These molecules include: Wnt, Notch, Shh (sonic hedgehog).

Satellite Cells and Wnt Signaling

Wnt signaling is involved in regulation of proliferation, polarity and cell fate determination during embryogenesis and homeostasis of adult tissue. Traditionally, Wnt signalling is divided in canonical and non-canonical signaling pathways. Canonical Wnt signaling pathways involve beta-catenin mediated gene activation while non-canonical Wnt signaling includes the PCP (planar cell polarity) pathway and calcium dependent mechanisms. The role of canonical Wnt signaling in adult myogenesis is still discussed controversially. While Otto et al. (2008) found that over expression of canonical Wnt ligands, such as Wnt3a, induces satellite cell proliferation during regeneration of adult skeletal muscle. Brack et al. (2008) demonstrated that a temporal switch from Notch to canonical Wnt signaling is essential for differentiation upon injury. Non-canonical Wnt signaling is responsible for the expansion of the $Pax7^{+}/YFP^{-}$ satellite stem cell pool mediated through activation of the PCP signaling pathway by Wnt7a (Le Grand et al. 2009). Expansion of the $Pax7^{+}/YFP^{-}$ satellite stem cell pool through Wnt7a has been shown to remarkably increase the regenerative potential of injured skeletal muscle.

Satellite Cells and Notch Signaling

Another major regulator of satellite cell function is Notch signaling. Inhibition of Notch signaling prevents satellite cell proliferation and self renewal (Kuang et al. 2008). On the other hand, increased Notch signaling leads to an enhanced regenerative capacity of aged skeletal muscle (Kuang et al. 2008). Furthermore, Notch signaling seems to be involved in asymmetric division of satellite cells. Evidence for this comes from experiments demonstrating the asymmetric distribution of Numb, a Notch inhibitor, in dividing satellite cells. In asymmetric divisions Numb was only detected in the daughter cell that underwent differentiation (Conboy and Rando 2002).

Regulation of Satellite Cells Through Growth Factors

IGF1 (insulin growth factor 1) is upregulated during regeneration of skeletal muscle and promotes proliferation and differentiation. However, an IGF1 splice variant termed MGF

(mechano growth factor) promotes proliferation of myoblasts and inhibits differentiation *in vitro*. Other growth factors facilitating proliferation and inhibiting differentiation are HGF (hepatocyte growth factor) or FGF (fibroblast growth factor), in particular FGF-2 and FGF-6. Recent studies demonstrated a dose-dependent requirement for cultured myoblasts for TGFbeta. TGFbeta has also been implicated in muscle fibrosis through stimulation of differentiation of myoblasts into a fibrogenic cell type (Bentzinger et al. 2010).

In addition to the aforementioned factors, satellite cell quiescence and differentiation are regulated by miRNAs. Cheung et al. (2012) demonstrated that regulation of quiescence is positively mediated through mi-489 while differentiation of satellite cells is mediated through miR-206 and -1 (Chen et al. 2010).

Isolation of Satellite Cells

Pure populations of satellite cells can be easily isolated by FACS (Fluorescence activated cell sorting), a method in which single cells are analyzed in a fluid stream. By using fluorescently labelled antibodies directed against surface markers of satellite cells it is possible to discriminate between satellite cells and other mononucleated cells in adult skeletal muscle, such as hematopoietic cells. Satellite cells do not express a unique cell surface marker, therefore a combination of positive and negative markers has to be utilized to obtain a pure population of satellite cells. Positive cell surface markers for satellite cells include α7-integrin, CXCR4, V-CAM-1 or CD34 (Bentzinger et al. 2010). The isolation of satellite cells, based on cell surface markers, allows for the isolation of satellite cells from various species including mice and humans.

In mice, in addition to using cell surface markers, high numbers of pure satellite cells can be obtained by FACS of samples from genetically modified reporter mice. The advantage of this technique is that there is no effect on satellite cells based on binding to surface receptors. Commonly used genetically modified mouse lines that are used for the isolation of satellite cells are nestin-GFP, Pax7-zsgreen, $Myf5^{nlacZ}$. Cre mouse lines crossed with a ROSA reporter allele are also useful for the generation of identifiable satellite cells, for example R26R-YFP interbred with MyoD-cre, Pax3-cre or Myf5-cre. If using cre-mediated activation of the ROSA locus one has to be aware that all cells which have previously or are currently expressing the cre allele will be labelled. This can lead to contamination with cell types other than satellite cells after FACS.

Other methods for isolating satellite cells include the, so called, preplating technique (Relaix et al. 2005). Briefly, satellite cells are obtained through enzymatic digestion of the muscle, fast adhering cells such as fibroblasts can be removed by short term cultivation on non-coated culture dishes. Cells which do not adhere readily to non-coated culture dishes comprise high numbers of satellite cells while other cell types such as fibroblasts are removed by this method. Upon culture the satellite cells become myoblasts and lose certain characteristics of satellite cells. Therefore, this method results in high yields of satellite cell derived myoblasts but is not suitable to obtain real satellite cells.

Assessment of Satellite Cell Function

An elegant way to investigate the ability of satellite cells to undergo activation and differentiation in their niche is the culture of live single myofibers with their respective satellite cells. Single fibers can be cultivated under floating conditions for several days and the activation and differentiation potential of the satellite cells can be "visualized" by immunofluorescent staining for myogenic markers, such as MyoD or myogenin. Satellite cells can be easily transfected and extracellular ligands such as growth factors can be applied readily.

Another way to assess the functionality of satellite cells is to injure the muscle by myotoxins (e.g. notexin or cardiotoxin), Barium chloride or overuse by eccentric treadmill running. Injection of myotoxins induces degeneration followed by

an inflammatory response and activation of satellite cells, generally during the first day after injury. Proliferation of satellite cells peaks around day 3 after injury, this is followed by the formation of newly generated myotubes at day 5–6 after injury. Combining induced muscle injury together with lineage tracing of satellite cells is a commonly used experimental paradigm to follow the fate of satellite cells and their progeny.

Therapeutic Potential of Satellite Cells

Satellite cell transplantation is considered to be a therapeutic option to restore or enhance the regenerative potential of diseased skeletal muscle. The first report that cultured myoblasts were able to restore dystrophin expression in mdx mice (a mouse model for Duchenne muscular dystrophy) resulted in great enthusiasm within the scientific community. Since allogenic satellite cell/myoblast transplants have to be used for this purpose (autologous transplants would not be able to restore dystrophin expression) the largest issue to overcome is immune rejection of the graft. Other challenges include low survival rates, poor self-renewal and migration of the donor cells after injection into the diseased muscle, especially since systemic delivery of satellite cells is not possible.

Advantages of using satellite cells for cell based therapies include their high abundance, accessibility, established culture conditions for satellite cell derived myoblasts, their ability to self-renew and undergo myogenic differentiation as well as the fact that pure populations can be isolated using FACS. On the other hand, there are also disadvantages of cell-based therapies for muscle diseases. These include, in addition to immune rejection, the fact that they are not suitable for systemic delivery, have a poor migration capacity and survival after transplantation and reduced myogenic potential after culture *in vitro*. For successful transplantation it is necessary to transplant large numbers of satellite cells. These can only be obtained by culturing these cells. As discussed earlier, satellite cells become myoblasts through *in vitro* culture. Myoblasts display a significantly lower myogenic potential than freshly isolated satellite cells and lose the ability of repopulating the satellite cell niche.

Hope for the therapeutic use of satellite cells arises from experiments which demonstrate that even very small numbers of satellite cells – when transplanted together with their niche – can result in robust muscle repair (Hall et al. 2010). A study by Sacco et al. (2008) demonstrated that a single transplanted satellite cell is able to proliferate substantially and to contribute to muscle fibers.

Atypical Muscle Stem Cells

It has been demonstrated that stem cell populations other than satellite cells can undergo myogenic differentiation. Often, their ability to undergo myogenic differentiation is dependent on injury or coculture with myoblasts. Stem cell populations which correspond to these criteria are: pericytes, mesoangioblasts, side population cells and CD45+/Sca1+ cells. All of these cell types share that only low numbers undergo myogenic commitment. Recently, an additional stem cell type was described during postnatal myogenesis, the PW1+ interstitial cells (PICs). These cells are not derived from a satellite cell lineage, however, they do show Pax7 dependent myogenic potential and participate in myogenesis (Mitchell et al. 2010).

Although these atypical muscle stem cells can participate in myogenesis they are not sufficient to promote regeneration or replenishment of the satellite cell pool in satellite cell depleted muscle. This might be due to their low myogenic potential or the lack of paracrine signals emitted from the satellite cells which recruit these atypical muscle stem cells during regeneration.

Concluding Remarks

It has been just over 50 years since the satellite cell was first described. Ever since then research has conclusively demonstrated that

this cell type is indispensible for regeneration of skeletal muscle. The elaborate genetic networks controlling myogenesis during development and in the adult have begun to be elucidated. Moreover, it has been discovered that the adult satellite cell pool is heterogeneous with a subpopulation that has more stem-like characteristics. Future insights into the molecular regulation of satellite cells will hopefully make these cells an option for cell-based treatment of damaged or diseased muscle.

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Part III

Dendritic Cells

Notch Signaling in Differentiation and Function of Dendritic Cells

7

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Contents

Abstract

Hematopoietic stem cells give rise to multiple lineages of mature cells. This process is governed by a tightly controlled signaling network, regulated by cytokines and the direct interaction between progenitor cells and bone marrow stroma. Notch signaling represents one of the major pathways activated during the direct interaction between hematopoietic progenitor cells and bone marrow stroma. In this review, we discuss the recent findings that shed light on the critical role of Notch in the differentiation and function of dendritic cells, and its impact on immune responses.

Introduction: A Complex Network of Dendritic Cell Progenitors and Precursors

Dendritic cells (DCs) are a critical element of an adaptive immunity, responsible for the acquisition and presentation of antigens as well as for the regulation of immune responses to various physiological and pathological stimuli. Several major subsets of DCs are currently recognized in mice: interferon-producing plasmacytoid DC (pDC) and two main subsets of conventional DCs (cDCs): $CD8\alpha^{+}CD205^{+}$ and $CD8\alpha^{-}33D1^{+}$ cells. As in all other hematopoietic cells, DCs differentiate in bone marrow (BM). Their terminal differentiation and activation takes place in the periphery. DCs and other hematopoietic cells share one common ancestor, the hematopoietic stem cell (HSC).

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In mouse BM, several committed DC precursors have been identified. Lin^- $CD11c^-$ MHC II^- $CD117^+$ $CX_3CR_1^+$, an early hematopoietic progenitor, has been shown to have the potential to differentiate into macrophages, splenic $CD8\alpha^-$ and $CD8\alpha^+$ cDCs *in vivo* (Fogg et al. 2006). These progenitors, termed macrophage-dendritic cell progenitors (MDPs), represent ~0.5% of BM nucleated cells. They cannot differentiate into T, B, NK, erythroid, megakaryocytic, pDCs or polymorphonuclear neutrophil (PMN) cells. This pathway of DC differentiation is distinct from the generation of DCs from monocytes; as the ability of BM monocytes to generate steady-state $CD8\alpha^-$cDCs is considerably less than that of MDPs. Most of these monocytes differentiate into $CD8\alpha^+$cDCs. While this finding suggests that macrophages and DCs have a common progenitor, it does not imply that MDPs are the sole progenitors for DCs. A specific common DC progenitor (CDPs), with $Lin^-CD117^{int}Flt3^+$M-$CSFR^+CD11c^-$ phenotype in the BM, has been described by Onai et al. (2007). CDPs can differentiate into pDCs and cDCs but not into other cell lineages. CDPs and MDPs are distinct DC progenitors, because the *in vitro* differentiation of CDP is Flt3L-dependent; whereas, that of MDPs is M-CSF or GM-CSF – dependent. Precursors expressing CD11c, but not MHC II and CD117, have been identified in BM based on their expression of B220 (Diao et al. 2004). The $B220^+$ cDCs precursors are able to differentiate into pDCs and cDCs, while $B220^-$cDC precursors are only capable of producing cDCs.

It is generally accepted that DC precursors develop in BM and migrate into different tissues, where they differentiate into mature cDCs. pDCs are readily detectable in BM; whereas, only a few fully developed cDCs are found in BM (reviewed in Shortman and Naik 2007; Svensson and Kaye 2006). DC development is regulated by the local microenvironment. This includes chemokines, cytokines, cell-cell crosstalk and cell-extracellular matrix (ECM) interaction. These extracellular stimuli are transmitted into cells, *via* receptors on the cell membrane of DC precursors, and affect the transcriptional programs which regulate cell differentiation.

Bone Marrow Stromal Cells and DC Development

It is well established that BM stroma plays a major role in DC development. Stromal cells are capable of forming interstitial tissues and complex organ structures; and, therefore, play a crucial role in development and tissue homeostasis (reviewed in Svensson and Kaye 2006). Stromal cells are comprised of a heterogeneous population of mesenchymal cells: including fibroblasts, adipocytes, epithelial cells and endothelial cells, as well as some cells of hematopoietic origin, such as macrophages. In addition to their role in supporting hematopoiesis, stromal cells also have an immunomodulatory property.

The influence of stromal cells on the proliferation and/or differentiation of T cells, B cells and DCs is well documented (Ngo et al. 2001; Svensson et al. 2004; Tang et al. 2006; Tse et al. 2003; Zhang et al. 2004). Stromal cells play a role in DC development, not only in the BM microenvironment, but also in the spleen (Despars and O'Neill 2006; O'Neill et al. 2004; Svensson et al. 2004; Tang et al. 2006; Zhang et al. 2004). Increasing evidence shows that stromal cells might provide an indirect regulatory control over DC function and differentiation (reviewed in Svensson and Kaye 2006). This could be achieved by the regulation of extracellular matrix (ECM), which is deposited by stromal cells, and is important for cell migration and tissue structure. Mice lacking one ECM component, SPARC (secreted protein, acidic and rich in cysteine), showed more migration of Langerhans cells (LCs) from the skin into draining LNs (Sangaletti et al. 2005). Another ECM component, thymic stromal lymphopoietin (TSLP), secreted by Hassall's corpuscle (groups of thymic epithelial cells), stimulates human thymic $CD11c^+$DCs to express high levels of CD80 and CD86 (Watanabe et al. 2005). TSLP-modified $CD11c^+$DCs are able to induce $CD4^+CD8^-CD25^-$ T cells to differentiate into $CD4^+CD25^+Foxp3^+$ regulatory T cells (Watanabe et al. 2005).

It is believed that DC differentiation depends on the direct physical interaction between HSCs/HPCs and stromal cells, as well as on the presence

of cytokines. The nature of cytokines, necessary for the development of DCs *in vitro,* has been well documented. They include SCF, Flt3L, IL-3, IL-4, IL-6, IL-7, GM-CSF, TNF-α and TGF-β (reviewed in Despars and O'Neill 2004); (Heinz et al. 2006). GM-CSF is often used to generate DCs *in vitro;* but mice, deficient in GM-CSF or its receptor, retain the ability to generate lymphoid-resident DCs (Vremec et al. 1997). Some stromal cell lines, which can support DC development *in vitro,* also lack GM-CSF expression (Despars et al. 2004). Hence, GM-CSF is thought to induce differentiation of DCs from monocytes under inflammatory conditions, rather than to be essential for steady-state development (reviewed in (Shortman and Naik 2007). On the contrary, Flt3L is crucial for steady-state pDC and cDC development. $Flt3L^{-/-}$ mice have fewer $CD8\alpha^{-}$ and $CD8\alpha^{+}$cDCs in spleen, LN and thymus compared to wild type mice, probably due to disruption of HPC differentiation (McKenna et al. 2000). Conversely, administration of Flt3L results in the expansion of DCs *in vivo* (Maraskovsky et al. 1996, 2000). There are some ligands for nuclear receptors, such as all-*trans* retinoic acid (ATRA), which can induce monocyte-derived DCs to form mucosa-type DCs (Saurer et al. 2007).

In contrast to the vast knowledge of the effect of cytokines on DC differentiation, only a few reports describe the direct cell-cell contacts between HSCs/HPCs and stromal cells involved in DC development. Recent studies have provided compelling evidence supporting an important role of Notch signaling in DC differentiation.

Main Characteristics of Notch Family of Transcriptional Regulators and Receptors

The Notch family of transcriptional regulators is highly conserved. Notch signals influence multiple processes that govern normal morphogenesis, including lineage specification among progenitor cells, programmed cell death, and cellular proliferation. The Notch receptors family includes four members (Notch-1–4). Each member is a large, single heterodimeric receptor comprised of noncovalently associated extracellular (ECN), transmembrane (TMN), and intracellular (ICN) subunits. ECN contains a 29–36 tandem epidermal growth factor (EGF)-like repeats, in which repeats 11, and 12 responsible for ligand binding. Following EGF-like repeats is a negative regulatory region (NRR), responsible for preventing receptor activation in the absence of ligands. The intracellular region contains a RAM (RBPjκ association module) domain with conserved motif that binds the transcriptional repressor (CSL/CBF-1), a series of cdc10/ankyrin repeats (ANK domain) that are involved in protein-protein interactions with CSL/CBF-1 and other polypeptides, and a C-terminal PEST sequence responsible for the degradation of the ICN signal. At present, two major Notch ligand families, Delta (Dll) and Jagged (Jag), have been described. Canonical DSL ligands are composed of three connected motifs: N-terminal DSL (Delta/Serrate/LAG-2), DOS (Delta/OSM-11-like proteins), both participating in receptor binding, and EGF repeats. In addition, non-canonical ligands like contactins, MAGPs and DNER have been found in the central nervous system or in culture cells; however, their function remains largely unclear (D'Souza et al. 2008). Notch signaling is initiated by the binding of the extracellular domain of Notch to a Notch ligand. Multiple lines of evidence pointed to a model in which the intracellular domain of Notch (ICN) translocates to the nucleus in a ligand-dependent fashion (Kopan and Goate 2000). Ligand/receptor interactions initiate two successive proteolytic cleavages within the TMN subunit. The protease responsible for the first cleavage (S2 cleavage), which occurs just external about 12 amino acid to the transmembrane domain, is an ADAM metalloprotease. Subsequently, a second cleavage (S3/S4 cleavage), within the transmembrane domain, releases ICN. This cleavage requires the function of two different classes of transmembrane proteins, γ-secretase presenilins and nicastrin (Yu et al. 2000), which represent two components of a multisubunit complex that processes a number of transmembrane proteins in addition to Notch.

The Intracellular Notch translocates to the nucleus; where it interacts with a transcriptional repressor CSL, also known as CBF-1 (RBP-J). In the absence of ICN, CSL/CBF-1 acts as a transcriptional repressor on Notch target genes, forming a complex with other transcriptional corepressor (CoR) and histone deacetilase-1 (HDAC-1). The binding of ICN with CSL displaces co-repressor complexes, thereby derepressing transcription from promoters with CSL binding elements. In addition, the ankyrin repeats and C-terminal transcriptional activation domains of ICN recruit several different transcriptional coactivators, providing an additional stimulus for transcription (Allman et al. 2002; Artavanis-Tsakonas et al. 1999). One functionally conserved protein that may act as a Notch-specific coactivator is mastermind, an adaptor molecule that stabilizes CSL/ICN interaction and potentiates ICN stimulation of transcription from CSL-sensitive promoters. The more variable sequences C-terminal of the ankyrin repeats of Notch1 have been shown to interact with the general transcriptional coactivators p300, PCAF, histone acetyltransterase (HAT) and GCN5. Importantly, all four mammalian Notch receptors bind and activate CBF-1, leading to transactivation. The CSL centered repressor complexes on different target genes vary in components of the complexes and CSL binding arrangements, which regulate gene expression. Targets of ICN/CSL signals, in mammals, include genes of the Hairy/Enhancer of Split (HES) family, which encode bHLH-type transcription factors, as well as the recently described HRT/HERP genes. Other ICN/CSL targets, that may explain the effects of Notch on cell cycle kinetics in certain contexts, are cyclin D1 and p21. Notch signaling is also regulated by other cellular proteins including Deltex, Nrarp (Notch-regulated ankyrin-repeat protein) and MINT (Msx2-interacting nuclear target protein). A more detailed description of Notch structure and function is provided in recent reviews (Fortini 2009; Kopan and Ilagan 2009; Miele 2006; Osborne and Minter 2007).

Notch Signaling and Dendritic Cell Differentiation

During last 10 years, clear evidence emerged demonstrating the important role of Notch signaling in DC development. We have previously shown that Notch-1 is necessary for DC development. The differentiation of DC was significantly impaired in Notch-1 anti-sense mice that have approximately half of the normal level of Notch-1 in HPC (Cheng et al. 2001). These findings were further confirmed in an experimental model of DC differentiation of embryonic stem (ES) cells. ES cells, lacking Notch-1, had a dramatically reduced capacity to generate DCs (Cheng et al. 2003). Different results were obtained by Radtke et al. They generated *notch-1* conditional knockout mice, using the Lox-Cre system, and demonstrated that T cell development was blocked in these mice (Petrasch et al. 2000). However, the number of thymic DCs, cDCs, and LC was normal. Most recently, the work from Feyerabend has shown that pro-T cells, residing in thymus, could convert to thymic DCs, after deletion of Notch-1 at the stage of pro-T cells. However, this conversion, from pro-T to DCs, did not increase the overall number of thymic DCs (Feyerabend et al. 2009). It appears that Notch signaling may play a role of switch between pro-T and thymic DCs. This notion was further confirmed in a Dll4-Notch signaling blockade model. Conditional deletion of nicastrin, a component of γ-secretase, resulted in an accumulation of a population of double negative pro-T, a two to fivefold increase of $CD11c^{+}B220^{+}/PDCA^{+}$ pDC, $CD11c^{+}B220^{-}$ cDC and $CD11c^{+}$MHC II^{lo} immature DC. In addition, regulatory T cells significantly increased as well. The increase was not reflected in the absolute number, because of a severe reduction of total number of cells in thymus. Genetic inactivation of Dll4 resulted in a sevenfold increase in the frequency of DCs and a fivefold increase of Treg (Billiard et al. 2012). The more general evidence that Notch signaling is necessary for DC differentiation came from studies using mice with

DC-specific Notch signaling depletion. Caton et al. used a conditional deletion of RBP-J in BM cells. Since RBP-J is an essential mediator of signaling, by all Notch receptors, this eliminated potential redundancy in the effects of different members of the Notch family. A substantial reduction in the presence of cDCs in the spleens of knockout mice was found. This decrease affected, primarily, the $CD8^-$ DC subset in marginal zone of spleen (Caton et al. 2007). In reconstituted chimeras, RBP-$J^{-/-}$ donor-derived DCs in spleen were reduced to the same extent as in the RBP-$J^{-/-}$ conditional knockout mice, and a noticeable increase of pDC was observed. Thus, it appears that Notch signaling controls the homeostasis of $CD8^-$DC and plays an inhibitory role for pDC in spleen (Caton et al. 2007). The authors suggested that this DC loss might not necessarily reflect an impaired DC lineage commitment. RBP-J–deficient bone marrow gave rise to all DC subsets. An essentially similar phenotype was caused by RBP-J deletion in the bone marrow hematopoietic progenitors (*Mx1*-Cre) and in committed DCs (*CD11c*-Cre). Using a DC-specific Notch 1 or Notch 2 deletion, the same lab has determined that Notch 2, but not Notch 1, deletion reduced $CD8^-CD11b^+$ DC subset in spleen. In addition, a significant decrease in the proportion and absolute number of $CD11c^+$MHC II^+ DC and $CD8^+$ DC were found in spleen; whereas, $CD8^-CD11b^-$cell population was significantly increased. It was consistent with a defect at late stage differentiation, caused by Notch 2 deletion. The same observation was also found in the DC specific (*Itgax-cre*-induced) expression of a dominant negative human MAML1, in which Notch signaling was blocked. This leads to a depletion of $CD8^-CD11b^+$ DCs in spleen and $CD103^+CD11b^+$ DCs in the *lamina proria* of intestine (Lewis et al. 2011). This claim was further supported by a study in which Sekine et al. demonstrated that the blockade of 4 Notch ligands, including Jagged 1, Jagged 2, Dll1 and Dll4, by antibodies in mice, reduced $CD8^-$DCs by half of the normal amount in the spleen (Sekine et al. 2009).

Effect of Different Notch Ligands on DC Differentiation

Notch signaling is usually trigged by Notch ligands from adjacent cells, but it also could be inhibited by the ligands expressed on the same cell (de Celis and Bray 1997; Sprinzak et al. 2010). Dll3 has been shown to function as a Notch antagonist rather than a potent activator (Geffers et al. 2007; Ladi et al. 2005). In mammalian cells, Notch ligands bind to the receptors with no preference; for example, Jag1 and Jag2 are able to bind to Notch 1, Notch2, and Notch 3 (Butler et al. 2010; Yan et al. 2001). However, the avidity of different ligands binding to receptors is different (Van de Walle et al. 2011).

As a primary organ for hematopoiesis, the bone marrow expresses various Notch ligands. Jag1 is widely distributed on BM cells (Dallas et al. 2007). It has been identified at lower levels on hematopoietic precursor cells (Sprinzak et al. 2010), and at a higher level on mature hematopoietic cells such as mast cells, megakaryocytes, B cells and macrophages (Sprinzak et al. 2010). This suggests the important role of Jag1 in the regulation of late stage of hematopoiesis. Jag1 was also highly expressed by BM stromal cells (Glittenberg et al. 2006; Sprinzak et al. 2010), including endothelial cells (de Celis and Bray 1997), osteoblasts (Esler et al. 2000), osteoclasts (Fukushima et al. 2008), and bone-marrow derived macrophages (Daskalaki et al. 2011; Sprinzak et al. 2010). In contrast, Jag2 has been identified in ST-HSCs and LT-HSCs (Tsai et al. 2000), suggesting a role in maintenance of the progenitor pool (Van de Walle et al. 2011) selectively expressed by BM stromal cells. Endothelial cells (Tsai et al. 2000), but not fibroblasts or macrophages (Daskalaki et al. 2011), have also been proven to be rich in the expression of Jag2; although its expression was mostly intracellular (Choorapoikayil et al. 2012). Jag2 has also been detected in osteoblasts (Esler et al. 2000) rather than osteoclasts (Fukushima et al. 2008). Dll1 has been found in BM cells including endothelial cells, osteoblasts

and macrophages; while Dll4 has been detected in endothelial cells. The expression of all Notch ligands are below the detection limit in the fresh human BM MSCs (Nwabo Kamdje et al. 2011); however, some studies have shown Jag1 is a dominant Notch ligand in human BM MSCs (Liotta et al. 2008). The distribution of Notch ligands, and their level of expression in the BM, are tightly associated with their distinct roles in hematopoiesis, including DC differentiation.

Although Jagged and Delta can activate Notch signaling through RBP-J, it appears that their effect on DC differentiation is quite different. Incubation of HPC on fibroblasts, expressing Dll1, induced DC differentiation; whereas, Jag1 expressing fibroblasts had an opposite effect. They inhibited DC differentiation and, instead, promoted the accumulation of immature myeloid cells (Cheng et al. 2007). The distinct effect of the two ligands may reflect their physiological function in the body. The bone marrow environment is conducive for the generation of precursors for macrophages and DCs. About 40% of mouse bone marrow cells are $Gr1^+CD11b^+$ immature progenitor cells. In contrast, in spleens, the environment promotes differentiation of mature myeloid cells. As a result, less than 5% of splenocytes are $Gr1^+CD11b^+$ cells. The expression pattern of Notch ligands in bone marrow and splenic stroma cells is different. Bone marrow stroma expresses a substantially higher level of Jag1 than Dll1; whereas, splenic stroma has a substantially higher level of Delta-1 expression than Jag1 (Cheng et al. 2007).

Dll1 could be involved in the loss of $CD8^-$DCs, as observed in Caton's study (Caton et al. 2007). $CD8^-$DCs are located in the marginal zone of the spleens, the compartment shown to contain Dll1-expressing cells. $CD8^-$DCs in the marginal zone were found to reside in close contact with Dll1-expressing cells, which are typically non hematopoietic stromal cell types such as reticular fibroblasts and/or endothelium of the marginal sinus (Caton et al. 2007; Sekine et al. 2009). However, the blockade of Dll1, alone, by antibody in mice or Dll1 conditional knockout mice, was not enough to mimic the $CD8^-$DC loss, as seen in RBP-$J^{-/-}$ mice. Treatment with antibodies against four Notch ligands: Jag1, Jag2, Dll1, and Dll4 could reproduce the results as in the RBP-$J^{-/-}$ mice; but the excluded Dll1 had no effect. Moreover, any other ligand antibody, combined with anti-Dll1, would significantly reduce $CD8^-$DCs. It suggests that Dll1 could be the key ligand for maintaining $CD8^-$DC in spleen (Sekine et al. 2009).

Dll1 has been shown to have a stimulatory effect on the production of LC from human $CD14^+$ blood monocytes, in concert with GM-CSF and TGF-β (Hoshino et al. 2005). Dll1 is also able to enhance the *in vitro* differentiation of $CD34^+$ $CD38^-$human cord blood HPCs to $CD14^-CD1a^+$DCs (Yamamura et al. 2007). In a culture system containing IL-6, $CD34^+CD38^-$human cord blood HPC differentiate into $CD14^+$ monocytes and $CD15^+$ granulocytes. Dll1 shifted cell differentiation towards $CD14^-CD1a^+$ DCs. These cells expressed DC-related markers: CD11c, CD80, CD86 and HLA-DR (Yamamura et al. 2007). Ohishi et al. have also shown that Dll1 promotes DC differentiation. Monocyte precursors from blood can differentiate to macrophages and DCs, depending on the cytokine provided. GM-CSF supported macrophage differentiation. Immobilized Dll1 induced the differentiation of DCs, with characteristics similar to those observed in immature DCs, derived from monocytes cultured with GM-CSF and IL-4 (Ohishi et al. 2001). The active form of Notch-1 increases the IL-4 production through RBP-J binding to IL-4 promoter region (Amsen et al. 2004). This may help to explain the observed effect in the shift in DC differentiation induced by Dll1. The mechanism of the opposite effect of Jagged-1 on DC differentiation remains unclear. It is possible that this effect could be mediated by the RBP-J independent pathway, since RBP-J was equally activated by both ligands (Cheng et al. 2007). Dll1 may affect DC differentiation, indirectly, by influencing other signaling pathways. It was reported that Dll1 promoted DC differentiation through activation of the canonical Wingless (Wnt) pathway (Zhou et al. 2009). The activation of the Wnt pathway, in Notch-1-deficient embryonic stem cells, restored DC

differentiation; which indicates that Wnt signaling is downstream of the Notch pathway in regulating DC differentiation. Notch signaling activated the Wnt pathway in HPCs, *via* the expression of multiple members of the Frizzled family of Wnt receptors, which was directly regulated by the CSL(RPB-Jκ) transcription factor (Zhou et al. 2009).

In addition to the effect on cDC, several reports described the possible effect of Notch ligands on pDC differentiation. Oliver et al. reported that Notch signaling, *via* one of the Notch ligands, Dll1, promoted differentiation of pDC (Olivier et al. 2006). Human $CD34^+$ HPC and bone marrow CLP were cultured on Dll1-expressing OP9 stroma, in the presence of Flt-3 ligand and IL-7. These conditions provided for the differentiation of BDCA-$2^+CD123^+CD4^+CD11c^-$cells, with complete characteristics of pDC including morphology, expression of toll-like receptor-9 (TLR9), pre-T mRNAs, and secretion of IFN-α in response to CpG (Olivier et al. 2006). Dll1 enhanced the numbers of pDC, by promoting the differentiation rather than the affecting cell proliferation. The inhibition of Notch signaling, by a γ-secretase inhibitor, blocked pDC development. A significant increase in pDC, in this experimental system, was observed at the expense of B cell development (Olivier et al. 2006). The T cell fate was not clear, because they could not be generated under those experimental conditions. However, the function of Dll1 on pDC differentiation is controversial. In a different study, stromal cells expressing Dll1 promoted the differentiation of $CD34^+CD1a^-$thymic progenitor cells to $CD4^+CD8^+$ T cells and blocked pDC development. In that study, the activation of Notch signaling, through Dll1, controlled the two alternative pathways of cell differentiation by preferentially activating GATA-3, a critical transcriptional factor for T-cell development, and inhibiting the ETS family member Spi-B, a key regulator of pDC development. The Notch1-induced block in pDC development can be relieved through the ectopic expression of Spi-B (Dontje et al. 2006). In a mouse system, the overexpression of Dll1 in OP9 stromal cells completely abrogates the pDC differentiation from $Lin^-CD117^{hi}Sca\text{-}1^+$ early lymphoid progenitors (Pelayo et al. 2005). Inactivation of Notch-1 (Ferrero et al. 2002; Radtke et al. 2000), through the Mx-Cre recombinase, did not affect the development of pDC; suggesting that this receptor is not essential for differentiation of these cells. In contrast, RBP-J deficient mice have shown an increased level of pDC (Caton et al. 2007); suggesting that Notch signaling may play an inhibitory role in the development of these cells. In a recent study, OP9 cells, expressing Jag2, have been shown to differentiate human HPCs in culture to $CD34^+CD7^+$ T-/NK-cell progenitors, similar to the effect of Dll1. When a γ-secretase inhibitor was added, the Jag2 effect was reversed; and the differentiation to $CD4^+CD14^+$ monocytes and pDCs was recovered (Van de Walle et al. 2011).

The effect of Dll4 on DC development has been studied, mostly in thymus. Dll4 has been found to play a critical role in the homeostasis of Treg and thymic DC. The blockade of Dll4 skewed the differentiation of $CD4^-CD8^-CD117^+CD44^+CD25$- T cell progenitors towards immature thymic DCs, which subsequently promoted natural Treg cell development (Billiard et al. 2012). A similar result was achieved and showed that Notch1-Dll4 signaling mediated the blockade of cDC and pDC differentiation from progenitor T cells (Feyerabend et al. 2009).

In contrast to its other members, Jag2 has been poorly investigated in DC development. Jag2, similar to Dll1 and Dll4, has been shown to induce the T lineage differentiation at the expense of B cell, monocytic cell and pDC differentiation in the human HSC-OP9 coculture system (Van de Walle et al. 2011).

Thus, most of the experiments with "gain of function," where Notch signaling is activated by Dll1, demonstrated the induction of the differentiation of pDCs. In contrast, most of the experiments with "loss of function," based on knockout mice, showed either lack of effect or inhibition of pDC. It is possible that redundancy in the function of Notch receptors may contribute to the results of these experiments. However, it is likely that the lack of Notch-1 or RBP-J, in knockout mice, may result in the activation of RBP-J independent pathways of

Notch signaling, which may have opposite functional consequences. The nature of these effects remains to be elucidated.

Controversial Role of Notch Signaling in DC Development

Based on a wealth of information accumulated to date regarding the possible role of Notch signaling in DC development, it is proposed that Notch may be a major factor regulating DC differentiation (Campos-Ortega and Knust 1990; Cheng et al. 2007). However, evidence pointed out the possible role of Notch signaling as a tissues-specific regulator of DC differentiation. The homeostasis of splenic $CD8\alpha^{-}$DC, but not other subsets, was disrupted in the RBP-J deficient mice, in which canonical Notch signaling was completely blocked (Selkoe and Kopan 2003). Moreover, the Notch 2-mediated signaling is crucial for the development of splenic $Esam^{+}$ $CD8^{+}CD11b^{+}$ DC and the intestinal $CD103^{+}$ $CD11b^{+}$ DC; but other DC subsets in the same organ are not affected by Notch signaling (Le Gall et al. 2008).

So this raises a question as to why the effects of Notch signaling on cell development and differentiation is so diverse. There is evidence suggesting some potential mechanisms that underlie the divergence in Notch activation by the various Notch ligands. One of the important determinants is the differential efficiencies of Notch ligands in binding to notch receptors. In a binding assay using K562 cells expressing different ligands, Dll4 displayed the strongest binding strength to Notch1-Fc, followed by Jag2, Dll1, and Jag1, respectively (Van de Walle et al. 2011). Each Notch ligand retains a different level of activation strength that is not correlated with the equivalent binding efficiency. Dll4 is the strongest Notch1 activator, followed by Dll1, Jag2, and Jag1, respectively. However, there was no substantial difference in the signal strength hierarchy when signals occurred, exclusively, through Notch 2. Moreover, densities of Notch ligands cause a quantitative difference of signaling and, thus, result in diverse cellular outcomes. Lower densities of Dll1 enhance murine BM $Lin^{-}Sca\text{-}1^{+}CD117^{+}$ cells to differentiate into $Sca\text{-}1^{+}CD117^{+}$ cells, $Thy1^{+}CD25^{+}$ T precursors and $B220^{+}CD43^{-/lo}$ B precursors; while the higher densities of Dll1 promote the generation $Sca\text{-}1^{+}CD117^{+}$ cells and $Thy1^{+}CD25^{+}$ T precursors, but retard the development of B precursors (Dallas et al. 2005). However, the differential expression of the ligands and receptors cannot completely explain the observed distinct signaling activity. The regulation of post-translational modifications and membrane trafficking have been studied as important alternative mechanisms that control active ligand-receptor interactions.

The Notch receptor can be modified by various posttranslational modifications, including the addition of fucose residues by protein O-fucosyltransferase 1 (Pofut1) to the EGF-like repeats. This fucosylation is initially thought to be essential for the Notch signaling; however, latter studies showed non-fucosylated Notch receptors are also capable of binding to ligands and transducing signals. The further glycosylation can be mediated by the protein Fringe family b-1,3-N-acetyl-glucosaminyltransferases. There are three members of Fringe proteins: Lunatic, Manic and Radical. The glycosylation, regulated by Fringe genes, is also a key to explaining the diversity of Notch activation induced by different ligands (Benedito et al. 2009; Kato et al. 2010; Van de Walle et al. 2011). The glycosylation of Notch 1, by Fringe protein, results in a stronger activation by Dll1, probably by enhancing the affinity of Notch binding to the ligands. However, Lunatic and Manic fringe protein have an opposite effect on Jag1 by preventing the effective Notch proteolysis required for activation of downstream signaling events (Yang et al. 2005). In contrast to Jag1, Jag2 induced Notch signaling was slightly increased by Lunatic fringe-modified Notch1; suggesting Jag2 might be acting as non-classical Serrate-like Notch 1 ligands (Van de Walle et al. 2011). Dll4 are able to strengthen Notch activation in the Manic Fringe-modified Notch and suppress the endothelial tip cell formation and sprouting; in contrast to Jag1, in which activity is weak (Benedito et al. 2009). In addition,

some RBP-J-independent non-canonical Notch signaling pathway (Le Gall et al. 2008), ligand endocytosis (Daskalaki et al. 2011; Glittenberg et al. 2006; Hansson et al. 2010; Meloty-Kapella et al. 2012) and oligomerization of ligand and receptor may also result in the divergence of Notch functions (Ahimou et al. 2004). However, at a physiological level, cells usually expressed a combination of Notch ligands, rather than sole ligand, suggesting the complexity of Notch activation by the different ligands.

Conclusions

The wealth of available information clearly demonstrates an essential role of Notch signaling in the regulation of DC differentiation and function. Notch is involved in DC differentiation, *via* interaction with stromal cells, DC activation, as well as in DC-mediated T-cell activation. However, the nature of this regulation remains largely unclear; because of a multitude of rather contradictory results describing the effect of individual members of the Notch family and Notch ligands. These results are not surprising, since the Notch effect is highly dependent on the environment and type of the cells where activation is taking place. Elucidation of the exact nature of the Notch effects on DC development and function is arduous task, but it is necessary for understanding the function of the immune system in physiological and pathological conditions.

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8 Suppression of Differentiation and Maturation of Dendritic Cells: Stem Cells from Different Sources Vary in Their Effect

Barbara Kronsteiner and Susanne Wolbank

Contents

Abstract

Mesenchymal stem cells (MSC) are multipotent progenitor cells with the ability to modulate and suppress immune responses *in vitro* and *in vivo*. One of their immunoregulatory properties is the suppression of dendritic cell (DC) differentiation, maturation and effector functions thus indirectly affecting T-cell priming and polarization. It has been shown that MSC from different sources and species exert varying immunoregulatory effects on DC *in vitro*. These findings demonstrate that careful consideration should be taken when choosing a source of MSC for cellular therapy.

Introduction

Mesenchymal stem cells (MSC) are multipotent cells, which can be isolated from various sources including bone marrow, adipose tissue, placenta and others. Under appropriate *in vitro* conditions MSC have the potential to differentiate towards multiple cell lineages including the adipogenic, osteogenic and chondrogenic lineage (Bajada et al. 2008; Bassi et al. 2011). Since the first findings that MSC confer antiproliferative signals towards stimulated T cells, they have been implicated in the suppression of natural killer cells, cytotoxic T cells, the modulation of B cell functions, the inhibition of dendritic cell (DC) differentiation and the expansion of regulatory T cells (Treg) (Weil et al. 2011). Thus, they are promising candidates for cell therapy and tissue engineering

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and have already been proven clinically relevant for the treatment of graft versus host disease and autoimmune diseases (Tolar et al. 2010).

One of the immunomodulatory phenomena associated with MSC is their ability to suppress differentiation and maturation of DC. It has been demonstrated that MSC from human and mouse bone marrow (BM-MSC) as well as human amniotic tissue are able to inhibit generation, function and migration of monocyte- and $CD34^+$-derived DC, which we will review in depth in the following sections (Beyth et al. 2005; Nauta et al. 2006; Chen et al. 2007; Djouad et al. 2007; Jung et al. 2007; Ramasamy et al. 2007; English et al. 2008; Magatti et al. 2009; Spaggiari et al. 2009; Chiesa et al. 2011; Kronsteiner et al. 2011). Interestingly, not all MSC populations show equal immunomodulatory properties towards DC *in vitro*. This chapter aims at providing a review on the current knowledge about MSC-DC interactions and will present a comparison of the suppressive effect of human MSC from adipose tissue and amnion towards DC.

Dendritic Cells

DC are important initiators and modulators of immune responses. As sentinels of the immune system, they are strategically distributed throughout the body. They reside as immature cells in most tissues and specialize in capturing and processing antigen (Ag) to bind peptides via major histocompatibility complex (Banchereau and Steinman 1998; Banchereau et al. 2000). Once they encounter an Ag and receive a so called "danger signal", for instance in the form of an innate stimulus conveyed by pathogen associated molecular patterns from viruses, bacteria, fungi or protozoa (Reis e Sousa 2004), they express high levels of co-stimulatory (CD80, CD86) and activation molecules (CD83, CD40) and migrate to the lymphoid organs. This maturation process finally leads to the complete transition of DC from antigen-capturing to antigen-presenting cells. Mature DC (mDC) down-regulate their capability for Ag-uptake and gain the ability to prime T-cells and initiate the polarization of the immune response (Banchereau et al. 2000). The cytokine profile secreted by DC critically influences their ability to induce polarization of T-cells into Th1- and Th2-cells mediated by interleukin (IL)-12 and IL-4, respectively (Banchereau and Steinman 1998). Furthermore, it has an impact on tolerance induction. mDC are characterized by high expression of pro-inflammatory cytokines including IL-12p40 and p70, tumor necrosis factor (TNF)-α, IL-1β, IL-6 and nitric oxide (Lutz and Schuler 2002).

In transplantation and contact allergy, DC have been implicated in the induction of both immunity and tolerance. Through their ability to capture and present self-Ag DC are able to induce tolerance in T-cells (Banchereau and Steinman 1998). Three types of DC maturation stages either involved in tolerance or immunity have been described; immature, tissue resident DC inducing T-cell anergy; semi-mature, steady state migratory DC inducing $CD4^+IL\text{-}10^+$ Treg; and fully mature DC eliciting immunity. Crosstalk of DC with Th-cells by CD40-CD40L interactions might be involved in the switch of DC from tolerance to immunity (Lutz and Schuler 2002).

Mesenchymal Stem Cells Suppress Dendritic Cell Differentiation

It is well established that *in vitro* culture of peripheral blood monocytes and bone marrow derived progenitors with recombinant interleukin-4 (rIL-4) and recombinant granulocyte macrophage – colony stimulating factor (rGM-CSF) generates immature DC (iDC), characterized by upregulation of the pan DC marker CD11c and resulting in downregulation of the monocyte marker CD14 as well as upregulation of the DC marker CD1a in case of peripheral blood monocytes. Maturation of *in vitro* generated iDC is typically achieved by stimulation with lipopolysaccharide (LPS) or TNF-α causing upregulation of markers involved in co-stimulation (CD80, CD86), antigen-presentation (HLA-DR), and cell adhesion (CD54) as well as *de novo* expression of activation markers (CD83, CD40). While immature DC continuously take up Ag by endocytosis during the steady state (Lutz and Schuler 2002), mature DC are characterized by decreased endocytotic capacity.

It has been found that MSC interfere with DC-differentiation and -maturation of monocytes as well as $CD34^+$ fetal and adult hematopoietic progenitor cells by inhibiting upregulation of CD1a and other markers important in antigen-presentation, activation and maturation (HLA-DR, CD80, CD83, CD40) (Jiang et al. 2005; Nauta et al. 2006; Li et al. 2008; Magatti et al. 2009; Lai et al. 2010; Kronsteiner et al. 2011). This effect was reversible upon removal of MSC from co-culture (Jiang et al. 2005; Li et al. 2008).

Monocytes differentiated into DC in the presence of MSC are characterized by suppressed protein production and retention in the G_0 phase of the cell-cycle without detectable signs of apoptosis (Ramasamy et al. 2007; Magatti et al. 2009). Co-cultured monocyte-derived cells also down-regulate cyclin D2, $p27^{Kip1}$, cdk4 expression (Ramasamy et al. 2007), TNF-α as well as IL-12 secretion (Magatti et al. 2009; Spaggiari et al. 2009; Kronsteiner et al. 2011). Furthermore they are characterized by increased IL-10 expression (Li et al. 2008).

Mesenchymal Stem Cells Alter Effector Dendritic Cell Functions

MSC-derived DC show impaired T-cell stimulatory capacity (English et al. 2008; Li et al. 2008; Magatti et al. 2009; Spaggiari et al. 2009) and even induce generation of T-cells exerting alloantigen-specific regulatory activity (Li et al. 2008). Chiesa et al. (2011) have demonstrated that MSC impair crosspresentation by DC *in vitro*. When co- culturing CD8+ T cells with ovalbumin (OVA) pulsed DC which were previously co-cultured with MSC, induction of the early activation marker CD69 on CD8+ T cells failed and proliferation was strongly suppressed. This inability of DC to process soluble OVA is caused by MSC induced downregulation of the proteasome subunit MB-1 and immunoproteasome subunit LMP-10 (Chiesa et al. 2011).

Furthermore, MSC interfere with Toll like receptor (TLR) 4 induced activation of DC by inhibiting MAPK signaling downstream of MyD88, thus inhibiting IL-12 production, a hallmark of effector DC. Overall, MSC cause an accumulation of immature DC by interfering with their activation and antigen processing capacity. The relevance of MSC's immunosuppressive action on DC has also been demonstrated in an *in vivo* mouse model of adoptive transfer with OVA specific DO.11.10 transgenic naive CD4+ T cells and subcutaneously injected OVA pulsed DC. Migratory properties of *in vitro* MSC-conditioned DC from the site of injection were decreased *in vivo*. Furthermore, intravenous administration of MSC suppressed the capacity of *in vivo* MSC-conditioned DC to prime OVA specific CD4+ T cells and rapidly blocked migration of DC to the lymph nodes. Reduced numbers of CD11c+DC characterized by decreased expression of chemokine receptor CCR7 and CD49d were present in lymph nodes, thus demonstrating the potential of MSC to confer their immunomodulatory action after injection *in vivo* (Chiesa et al. 2011).

Species Specific Differences in MSC Mediated Suppression of Dendritic Cells

Several research groups have demonstrated that human BM-MSC do not affect maturation of already fully differentiated iDC (Li et al. 2008; Magatti et al. 2009; Spaggiari et al. 2009). In contrast, others showed that murine BM-MSC suppressed maturation of TNF-α as well as LPS-stimulated iDC and inhibited their migratory properties as seen by reduction of CCR7 expression and inhibition of E-cadherin downregulation (Jung et al. 2007; English et al. 2008). These contradictory findings indicate possible species dependent differences in MSC-mediated immunomodulation.

Mesenchymal Stem Cells from Amnion and Adipose Tissue Differentially Affect Dendritic Cells

A study comparing the effect of human MSC from adipose tissue (ASC) and human amniotic MSC (hAMSC) on differentiation and maturation of DC has demonstrated different degrees of immunomodulation (Kronsteiner et al. 2011). In a cell-contact independent co-culture system

hAMSC could completely abrogate generation of mDC phenotype as characterized by failure to acquire and up-regulate lineage-specific (CD1a) and co-stimulatory molecules. In contrast, ASC only moderately inhibited generation of DC and only partly retained them in a less mature state as seen by reduced CD86 and HLA-DR surface density. High donor variability was observed in ASC co-cultures whereas co-cultures with hAMSC derived from different donors resulted in consistent inhibitory properties. Surface markers including CD54, CD49d and CD44 involved in promoting cell-adhesion and -migration have been shown to be partly down-regulated and endocytosis levels were elevated on monocyte-derived cells from both co-cultures indicating properties of iDC. Interestingly, expression of IL-12 was only strongly suppressed in hAMSC co-cultures, thus possibly influencing their potential to generate a Th1 mediated immune response (Kronsteiner et al. 2011). All co-cultures were characterized by a highly elevated IL-1β level, which is involved in the activation of MSC mediated immunosuppression. In particular, $CD14^+$ monocytes have been shown to activate BM-MSC via release of IL-1β to secrete inhibitory molecules, which further led to inhibition of alloreactive T-cells (Groh et al. 2005). Both ASC and hAMSC co-cultures showed high levels of the anti-inflammatory cytokine IL-10 (Kronsteiner et al. 2011). Increased IL-10 secretion was also detected in conditioned media of BM-MSC co-cultured with PBMC, monocytes, or both $CD4^+$ T-cells and monocytes (Beyth et al. 2005). The inhibitory effect of BM-MSC on T-cell responses was only partially reverted by blocking IL-10 activity, indicating that IL-10 possibly works in concert with other immunoinhibitory factors released by MSC-conditioned antigen presenting cells. Autocrine IL-10 has been reported to limit the maturation of monocyte-derived DC characterized by inhibition of IL-12 and TNF-α, thus also impairing their capacity to initiate Th1 responses (Corinti et al. 2001). TNF-α and IL-10 are known key regulators of the Th1/Th2 balance (Kaur et al. 2009), thus the ratio of both cytokines has implications on the polarization of T cells. Co-cultures with all hAMSC donors and only those ASC donors with the most efficient capacity to suppress DC differentiation, were characterized by a TNF-α/IL-10 below 1. In contrast, co-cultures with ASC donors exerting lowest suppressive capacity, showed a high TNF-α to IL-10 ratio thus possibly skewing T cell polarization in different directions (Kronsteiner et al. 2011).

ASC and hAMSC constitutively produced IL-6 and in co-cultures even elevated levels were detected (Kronsteiner et al. 2011). This cytokine has already been proven to be at least partially involved in the mechanism of BM-MSC mediated DC suppression *in vitro* (Djouad et al. 2007; Magatti et al. 2009). Prostaglandin E_2 (PGE_2) has been assigned an important role in the mechanistic effect of MSC regarding suppression of DC differentiation (Chen et al. 2007; Spaggiari et al. 2009). In line with the above demonstrated differences in the effect of hAMSC and ASC on DC differentiation, high levels of PGE_2 were found in all hAMSC co-cultures but only in those ASC co-cultures, where generation of mDC was suppressed efficiently (Kronsteiner et al. 2011).

Overall, these results demonstrate that MSC isolated from different sources do not have the same potential to inhibit generation of mDC. While hAMSC show high suppressive capacity, ASC only exert limited inhibitory properties and high donor variation (Kronsteiner et al. 2011). This observed superiority of hAMSC does not seem to be associated with their early developmental stage, since human adult BM-MSC (Jiang et al. 2005; Nauta et al. 2006; Ramasamy et al. 2007) and human amniotic mesenchymal tissue cells both efficiently suppress DC generation. In contrast, it has been reported that the presence of umbilical cord blood derived MSC (unrestricted somatic stem cells, USSC) does not suppress the acquisition of an iDC phenotype by stimulated monocytes. Interestingly, co-culture of iDC with USSC in the absence of a maturation signal even increases the expression of maturation markers CD80 and CD83 on DC (van den Berk et al. 2009).

Fully Differentiated Cells Share Immunosuppressive Properties of Mesenchymal Stem Cells

Recent studies have demonstrated that the antiproliferative effects of MSC on T-cells are not exclusive to multipotent cells, but a characteristic shared by all stromal cells (Jones et al. 2007). Astonishingly, even fully differentiated renal tubular epithelial cells (RTEC) have been reported to modulate T-cell responses *in vitro* (de Haij et al. 2005). It has been shown that even primary RTEC interfere with the generation and maturation of DC which may possibly rely on the secretion of sHLA-G. Their effect on DC is similar to hAMSC and even superior compared to the suppressive potential of ASC (Kronsteiner et al. 2011).

Mechanisms of Mesenchymal Stem Cell-Dendritic Cell Interaction

MSC exert their inhibitory action on monocytes and $CD34^+$ cells in cell-cell contact as well as transwell co-cultures, where cell populations are physically separated by a semipermeable membrane (Jiang et al. 2005; Nauta et al. 2006; Spaggiari et al. 2009). While Li et al. (Li et al. 2008) observed only partial inhibition of DC differentiation in case of transwell co-cultures, Nauta et al. (2006) demonstrated that MSC are equally efficient under both culture conditions. Others have demonstrated that MSC act by a contact dependent induction of regulatory APC (Beyth et al. 2005) through IL-10 secretion and STAT-3 signalling (Gur-Wahnon et al. 2007).

While some research groups generate DC in the presence of MSC and then assess their effect on phenotypic and functional properties, others focus on the ability of MSC to interfere with the activation of already differentiated iDC. In case of the latter experimental setup Aldinucci et al. (Aldinucci et al. 2010) have demonstrated that only DC activated with LPS while in cell-cell contact with human MSC exhibited reduced stimulatory activity towards CD4+ T cells. In contrast, Chiesa et al. (Chiesa et al. 2011) conducted an experiment where iDC were activated with LPS in the presence of conditioned media from LPS-treated or non-treated murine MSC cultures. They demonstrated that the suppression of DC activation *in vitro* is not only dependent on soluble factor but also enhanced when using media from MSC-LPS cultures. These differences might be in part due to species-specific properties of MSC.

Furthermore, it has been proposed that MSC – at least human MSC – might exert their immunoregulatory properties through a contact-dependent inhibition of the immune synapse formation between DC and T cells (Aldinucci et al. 2010). Overall, these findings demonstrate that MSC from different sources and species show varying effects on DC generation and function. Therefore, careful consideration should be taken when choosing a source of MSC for cellular therapy and thorough characterization of their immunomodulatory properties has to be performed.

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9 Hypertensive Emergencies in Children After Stem Cell Transplantation: Care in Selecting Hypotensive Drugs

Dagmar G. Horn and Georg Hempel

Contents

Abstract

Hypertensive emergencies in children are rare, but associated with a significant risk of morbidity and mortality. These situations make an immediate, and especially a controlled reduction of blood pressure necessary. In children after stem cell transplantation hypertension is usually secondary to a renal impairment caused by the treatment with cyclosporine and tacrolimus. Due to the lack of adequate published experience with the used hypotensive drugs, and the lack of corresponding guidelines for an effective management of these situations, the actual treatment options in children only vary slightly from those in adults, and are primarily based on small retrospective clinical trials, and case reports.

All drugs have their strengths and weaknesses, which should be taken into consideration by the prescriber, and be balanced against the personal experience. Because of their mechanism of action and a potential nephroprotective effect the calcium channel blockers may be particularly suitable in hypertensive emergencies with a renal etiology. Especially nicardipine appears to be the hypotensive agent of first choice, but for an evaluation about its safety in paediatric use the published studies and case reports are not sufficient. Clevidipine may play a major role in the future because of its promising pharmacokinetic and safety profile, but there is a need for further trials concerning its use in children.

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Introduction

Today one of the most serious late complications of allogeneic stem cell transplantation remains to be the graft versus host disease (GVHD). Up to a minimum of 100 days following stem cell transplantation, approximately 50% of patients will experience some degree of chronic GVHD. An optimized immunosuppression is one important part of successful stem cell transplantation to prevent GVHD.

The calcineurin inhibitors cyclosporine and tacrolimus have become standard agents for immunosuppression and the treatment of GVHD. Cyclosporine and tacrolimus cause vasoconstriction of the afferent and efferent glomerular arterioles, leading to a reduction in renal blood flow and glomerular filtration rate (GFR), and often result in hypertension (Chandar and Zilleruelo 2011). Up to now, the exact mechanism inducing vasoconstriction is unexplained, but some possible reasons are discussed in recent publications, including a reduced production of prostaglandins, an increased sympathetic tonus, a transformation of growth factor beta-1, and an upregulation of oxidative stress, resulting in endothelial dysfunction. In some rare cases, hypertension can manifest as a hypertensive emergency.

Severe elevations in blood pressure can be classified as hypertensive urgencies, which are associated with elevations in blood pressure, but excludes the symptoms of end-organ injury, and hypertensive emergencies. A hypertensive emergency has been defined as elevation of both systolic and diastolic blood pressure with acute end-organ damage. The height of blood pressure in hypertensive emergencies is the subject of controversial discussion in the published literature, including blood pressure greater than the 99th percentile for age and sex, and blood pressure increase greater than 15 or 30 mmHg above the 95th percentile for age and sex. Independent of absolute blood pressure values, the common factor in all reports that determines the need for immediate therapy is the evidence of impending or progressive end-organ damage. The organs most likely to sustain damage in these situations are the central nervous system, the heart, the eyes, and the kidneys (Hari and Sinha 2011).

In children, hypertensive emergencies most commonly present with signs and symptoms of hypertensive encephalopathy, which may manifest as an onset of headache, nausea, and vomiting followed by severe headache, confusion, visual changes, stupor, somnolence, seizure, focal neurologic deficits, and coma. Hypertensive encephalopathy can result in cerebral infarction or hemorrhage in children. However, this appears with a higher occurrence in adults. Further examples of end-organ damages are acute myocardial infarction, acute left ventricular failure with pulmonary edema, unstable angina pectoris, dissecting aortic aneurysma, or eclampsia.

The differentiation between hypertensive emergencies and hypertensive urgencies is extremely important in the clinical setting, as the presence or lack of end-organ damage will dictate the urgency and aggressiveness of treatment. The rapid reduction and control of blood pressure is essential in hypertensive emergencies to avoid further end-organ damage or even death, while in hypertensive urgencies the target pressure can be achieved in up to 48 h after its first presentation, with minimal or no change in outcome. The need to lower the blood pressure rapidly to avoid end-organ damage must be balanced against a too rapid reduction in blood pressure with organ hypoperfusion leading to further end-organ damage (Chandar and Zilleruelo 2011). A constant cerebral blood flow during changes in blood pressure is ensured by an autoregulatory arteriolar constriction or dilatation of cerebral vessels, a mechanism with upper limits. In profound hypertension, the loss of this autoregulatory mechanism by the cerebral vasculature makes rapid blood pressure reduction even more harmful as the blood vessels in the brain are not capable of adjusting to too rapid changes in blood flood. The greatest risk thereby is cerebral ischemia, as well as myocardial infarction or blindness.

In current publications, there are different opinions about how fast blood pressure should be lowered in a hypertensive emergency. Some publications recommend a reduction of blood pressure by 10–15% over a period of 30–90 min, others suggest

a decrease of more than 25% over a period of 1 h up to a greater timeframe of 2–3 h or 8 h, followed by a further gradual reduction in blood pressure over the next 26–48 h (Horn et al. 2011).

Although cases of hypertensive emergencies are rare, it is necessary to scrutinize the treatment options, and the available drugs in the forefront. For an optimal adjustment of variable blood pressure levels, children with hypertensive emergencies should be treated inside the monitored setting of a paediatric intensive care unit to prevent steep declines of blood pressure that may have significant morbidity or mortality. The used drugs should be titratable, intravenously applicable, hypotensive, and well tolerated.

The drugs commonly used in hypertensive emergencies are listed below. Furthermore, some key properties of the drugs and a short overview of today's available literature a tentative appraisal about their suitability in hypertensive emergencies in children after stem cell transplantation is made. The recommended paediatric doses, important adverse effects, and contraindications are summarized in two tables.

Sodium Nitroprusside

The most commonly used intravenous agent in hypertensive emergencies is sodium nitroprusside. After being metabolized to nitric oxide it causes direct arterial and venous dilatation. It decreases both afterload and preload and increases cardiac output. Sodium nitroprusside acts within seconds of administration. The duration of action is 1–2 min and it possesses a very short plasma half-life of about 2–4 min, making it easy to adjust treatment if blood pressure is thought to be too low. Due to its potency, blood pressure should be monitored constantly with an arterial line when using this agent.

Erythrocytes metabolize sodium nitroprusside to toxic cyanide. The rhodanase in the liver converts cyanide into thiocyanate, which is excreted renally. Infusions at high doses and over a long duration of up to 48 h and above may cause the accumulation of cyanide and/or thiocyanate. An additional impairment of liver or kidney function increases the risk of accumulation and intoxication. High levels of cyanide or thiocyanate may result in metabolic acidosis, altered mental status, and clinical deterioration. In some cases monitoring of thiocyanate blood levels may be necessary (treatment with high doses and/or for more than 48 h or renal impairment), but monitoring still remains controversial. The range of cyanide levels associated with signs and symptoms of toxicity (>0.5 mg/L) has never been validated in paediatrics and other adverse effects can occur in the absence of elevated cyanide or thiocyanate levels (Thomas et al. 2009). Sodium nitroprusside doses of 5 μg/kg/min or more should be avoided. If the use of sodium nitroprusside is unavoidable, a simultaneous infusion of thiosulfate can provide sulphur necessary to prevent cyanide accumulation, provided that liver function is not impaired.

The important side effects of sodium nitroprusside are shown in Table 9.1. Because of the decrease of cerebral blood flow with increase of intracranial pressure at the same time, sodium nitroprusside is contraindicated in patients with reduced cerebral perfusion. It should be avoided in patients with coarctation of the aorta and atrioventricular shunts.

The rapid onset of action and its short half-life makes sodium nitroprusside a valuable drug in most cases of hypertensive emergencies, but in those with a renal etiology it appears less adequate. The risk of an additional deterioration of renal function can not be compensated by a simultaneous infusion of thiosulfate, because children after stem cell transplantation tend to ascites and fluid overload. In those patients, sodium nitroprusside is the last resort in treatment. It should be used only for short periods of time and be limited to situations where no other suitable drugs are available.

Beta-Adrenergic Receptor Antagonists

Labetalol hydrochloride is a widely used selective alpha$_1$- and non selective beta-adrenergic receptor antagonist. It can be administered either

Table 9.1 Important adverse effects, and contra indications of the drugs (Adapted from Horn et al. 2011)

Drug	Important adverse effects/contra indications
Sodium nitroprusside	Hypotension, reflex tachycardia, metabolic acidosis, weakness, psychosis, headache, thyroid suppression, nausea, sweating, cyanide and thiocyanate toxicity
	Infusions at high doses and over a long duration, up to 48 or 72 h, may cause accumulation of toxic cyanide and/or thiocyanate (which may in metabolic acidosis, altered mental status, and clinical deterioration), and should be avoided
	An additional impairment of liver or kidney increases the risk of accumulation and intoxication, and necessitates dose adjustment.
	Contraindicated in patients with reduced cerebral perfusion
	Should be avoided in patients with coarction of the aorta and atrioventricular shunts
Labetalol hydrochloride	Bradycardia, bronchospasm, atrioventricular block, worsening of heart failure, ventricular arrhythmia, prolonged hypotension, increased risk of hyperkalaemia, worsening of renal function, hepatocellular necrosis, and chronic active hepatitis;
	Should be avoided in patients with asthma, chronic obstructive lung disease, heart failure, bradycardia, or greater than first-degree heart block
Esmolol	Hypoglycemia, hypotension, nausea, vomiting, depression, phlebitis, bronchospasm, heart block, bradycardia, and negative inotropic effect;
	Contraindicated in patients with cardiogenic shock, heart block, severe asthma, or chronic obstructive lung disease
Metoprolol	Conformable to those of labetalol
Urapidil	Severe hypotension, dizziness, nausea, giddiness, headache, fatigue, and palpitations
Hydralazine	Reflex tachycardia, increase in cardiac contractility, sodium and water retention, headache, elevation of intracranial pressure, lupus erythematosus, vasculitis, glomerulonephritis, and aggravation of angina;
	Contraindicated in patients with coronary artery disease, dissecting aortic aneurysm, cerebral vascular accidents, cerebral edema and encephalopathy
Diazoxide	Tachycardia, heart failure, pulmonary hypertension, respiratory failure, fluid retention, hyperglycemia, hirsutism, seizure
Enalapril	Prolonged hypotension, renal failure (especially in neonates), neutropenia, hyperkalaemia, hypoglycaemia, febrile convulsion, bronchopneumonia, bronchial obstruction, and exacerbation of asthma;
	Contraindicated in patients with bilateral renal artery stenosis
Clonidine	Orthostasis, bradycardia, atrioventricular block, significant sedation, dizziness, fatigue, constipation, anorexia, and arrhythmia
Fenoldopam	Hypotension, tachycardia, t-wave flattening, angina, arterial fibrillation, flatter;
	Should be avoided in patients at risk for intraocular hypertension and intracranial hypertension
Nifedipine	Symptomatic hypotension, reflex tachycardia, arrhythmias, bone marrow suppression, alteration in mental status, and oxygen desaturation;
	The combination of cyclosporine and nifedipine may increase the incidence as well as the severity of gingival overgrowth
	Should be avoided in patients with acute CNS injury
Nicardipine	Conformable to those of nifedipine
Clevidipine	Conformable to those of nifedipine

orally, as a continuous infusion or as an intravenous bolus injection. Due to its antagonistic effects on alpha$_1$-receptors, it leads to vasodilatation and the beta-adrenoceptor effects result in a decreased myocardial contractility. Labetalol hydrochloride has approximately no effect on cardiac output. It reduces the systemic vascular resistance without reducing peripheral blood flow. The heart rate is either maintained or slightly reduced. Intracranial pressure is unaffected.

The inactive metabolites are excreted in urine, feces, and bile. The lipohilic drug labetalol is metabolized via glucuronidation. As glucuronidation pathways are not fully matured in infants and small children (Benedetti and Baltes 2003), labetalol achieve sufficient concentrations at lower doses in this population. As metabolism is the main route of excretion of labetalol, renal failure has no significant effect on plasma half-life, clearance, or apparent volume of distribution. Labetalol hydrochloride has a relatively slow onset of action and reaches its peak effect of hypotension in about 10 min. Hypotension can last for up to 4 h. Besides the most common side effects, labetalol hydrochloride may increase the risk of hyperkalaemia and acute renal failure may occur in cases associated with overdoses. Recent literature report partially fatal hepatocellular necrosis and chronic active hepatitis associated with the use of labetalol. It led to its withdrawal from sale in Germany in the early 1990s.

Published information about the administration of labetalol hydrochloride in paediatrics is scant, although it is widely used. In one retrospective chart review (Thomas et al. 2011) continuous infusions of labetalol hydrochloride, nicardipine and sodium nitroprusside were compared in 27 paediatric patients aged less than 24 months concerning their efficacy and safety. Fifeteen patients received labetalol, six received nicardipine, and four received nitroprusside. They found no significant difference of a continuous infusion of labetalol in efficacy and adverse effects compared with nicardipine and nitroprusside. Labetalol produced a mean blood pressure lowering effect of at least 20% in all blood pressure measurements within 8 h. Blood pressure reduction was sufficient up to doses of 0.59 mg/kg/h, with a little benefit a higher doses. Despite the wide use of labetalol hydrochloride in paediatrics, actual publications could not rule out the possibility of worsening renal function and an additional injury of the liver by this drug. Due to this risk, it appears not suitable to apply labetalol in critically ill children.

Esmolol is a pure cardioselective $beta_1$-blocker. It decreases blood pressure by reducing heart rate and myocardial contractility, and thus cardiac output. It has no direct vasodilatory effect, and is particularly beneficial when there is associated tachycardia (Chandar and Zilleruelo 2011). The important traits of esmolol are the very short onset of action after intravenous application and its short half-life making therapy easy to control. It acts within 60 s or less and due to its fast metabolism, hypotension can last only for 10–20 min. Up to now, no clear dose-effect relationship has been published. Esmolol metabolism does not depend on renal or hepatic function and is based on intracytoplasmic red blood cell esterases via rapid hydrolysis of ester linkage. This makes the drug potentially well-suited for critically ill patients with multiorgan failure (Flynn and Tullus 2009), but particular care is necessary in any prerequisite that causes anaemia, because a prolonged half-life of esmolol can occur. Esmolol can cause congestive heart failure, bradycardia, and bronchospasm. Its contraindications are those expected from a beta-adrenergic blocker. The data on paediatric use of esmolol are mainly based on small clinical trials and case reports. In the US, a paediatric trial was completed, but no paediatric labelling has been approved by the FDA (Flynn and Tullus 2009). This still induces some doubts about its suitability.

Like esmolol, metoprolol is a selective $beta_1$-blocker decreasing heart rate, myocardial contractility, and cardiac output without any direct venous dilation. The peak effect after intravenous application occurs within 10 min after administration. Compared to esmolol it has a longer half-life of about 3–5 h and undergoes an extensive hepatic metabolism via cytochrome P450 isoenzyme 2D6, entailing the capability of essential drug-drug interactions. The side effects of metoprolol are similar to those observed with of labetalol and esmolol.

In current publications, there is only one case report about the paediatric use of metoprolol in a hypertensive emergency in a 12-year old female (Liesemer and Mullen 2009). The girl received intravenous metoprolol 2.5 mg 3 times at intervals of 5 min, resulting in a decline in heart rate and blood pressure, and subsequent resolution of electrocardiogram changes. Apart from this, no

further published experiences with paediatric use of intravenous metoprolol can be found. The tenuous data on paediatric use of intravenous metoprolol, its long half-life and its metabolism through CYP 2D6 makes its use in hypertensive emergencies at least questionable. Other drugs should be preferred in this indication.

Urapidil

Urapidil reduces blood pressure by decreasing vascular resistance, mainly due to its postsynaptic $alpha_1$-adrenoceptor antagonism. It inhibits the vasoconstrictive action of catecholamines and has an antagonistic effect on central 5-hydroxytryptamine $(5\text{-HT})_{1A}$-receptors. This explains the absence of a sympathetic nervous system response like reflex tachycardia. Urapidil has no effect on intracranial pressure. It acts in about 3–10 min. The hypotensive effect lasts for a minimum of 30 min up to 6 h. Urapidil has a half-life of about 5 h and undergoes an extensive hepatic metabolism. It does not affect lipid or glucose metabolism, nor does it impair renal function.

Despite the wide use of urapidil both in adults and children, the published facts about the paediatric use are extremely limited. Only one clinical trial concerning its use for the treatment of acute hypertensive crises in 19 infants and children can be found (Schöber et al. 1984). In all cases, a prompt decrease in systolic, mean and diastolic blood pressure was achieved within the first 15 min and the effect lasted throughout the whole time of continuous venous infusion of urapidil. The authors report a slow increase in serum potassium and a decrease in serum sodium being significant only after at least of 12 h of therapy. There was no change in heart rate and urine volume.

The wide use of urapidil especially in Germany seems to be based on its good tolerance and the experiences of the attending physicians. The long half-life of 5 h, and especially the scarce published data about the paediatric use still raises questions about its suitability. Without further publications about its controllability and safety in children no recommendation can be made.

Direct-Acting Vasodilators

Hydralazine is a direct vasodilator of arteriolar smooth muscle that decreases systemic blood pressure. It can be administered either as intravenous bolus or intramuscular injection. The mechanism of action is still unexplained, but consists most likely in an alteration of intracellular calcium metabolism, leading to an interference with the calcium movements within the vascular smooth muscle that are responsible for initiating or maintaining the contractility. Hydralazine has an onset of action that occurs within 5–30 min after intravenous administration. Hypotension can last for up to 12 h. The use of hydralazine is accompanied by potential side effects. It stimulates the central nervous system which causes reflex tachycardia and an increase in cardiac contractility. Besides others the activation of the renin-angiotensin-aldosteron system, sodium and water retention, elevation of intracranial pressure and drug-induced lupus erythematosus, vasculitis and glomerulonephritis has been reported.

The published data about the use of hydralazine in children is scant, most likely because the drug is used primarily in pregnant woman with preeclampsia. The extremely long half-life can result in an unpredictable effect on blood pressure, limiting its controllability. This fact and also the possibility of significant adverse effects make its use in critical ill children doubtful at any rate.

Diazoxide is another direct-acting vasodilator. It is not only used in the treatment of hypertension, but also in the treatment of hypoglycaemia. It acts by increasing the permeability of the vascular smooth muscle cell membrane to potassium ions. Diazoxide is structurally similar to thiazide diuretics but the drug possesses no diuretic properties. With multiple doses it is even more likely to increase plasma volume by sodium and water retention resulting from increased reabsorption at the proximal tubules. Diazoxide acts within 10 min after intravenous application and reaches its peak of hypotensive effect after approximately 30 min. As well as with hydralazine, reduction of blood pressure can maintain for up to 12 h.

Plasma half-life of diazoxide ranges within 20–30 h and is explained by extensive renal tubular reabsorption and high plasma protein binding of about 90%. In patients with renal impairment, an adjustment of doses is required. Severe adverse effects are related to the use of diazoxide (Table 9.2). Until now, the mechanism of the potentially occurring heart failure remains uncertain, but it may be caused by direct toxic effect on the myocardium. Thus far, no correlation between the dosage of diazoxide and the severity of fluid retention or heart failure has been proved.

Few clinical trials and case reports on the paediatric use of diazoxide in hypertensive emergencies have been published, because diazoxide is mainly used as a hypotensive agent in pregnant woman or in the treatment of hyperinsulinaemic hypoglycaemia.

Regarding to its long half-life, which limits its controllability, and the risk of the occurrence of potentially life-threatening adverse effects, the use of diazoxide in hypertensive emergencies can not be recommended.

Enalapril

The angiotensin-converting enzyme (ACE) inhibitor enalapril is a prodrug that is metabolized *in vivo* to the active form enalaprilat by various esterases. Enalaprilat produces vasodilation, and decreases vascular resistance. Excretion of enalaprilat is primarily renal. Therefore, renal impairment results in a significant accumulation of enalaprilat and necessitates dose reduction. Enalapril has a slow onset of action, which may limit its use during a hypertensive emergency. A hypotensive effect can be seen in 10–60 min after injection and lasts for up to 12 h. Apart from other adverse effects, especially in neonates enalapril may cause acute renal failure (Flynn and Tullus 2009). Some clinical trials and case reports on the paediatric use of enalapril can be found. In most of the publications patients were treated due to of chronic hypertension, without any sign of end-organ damage, resulting in the use of tablets or capsules of enalapril, rather than the use of the intravenous form. Therefore, the facts about the use of intravenous enalapril in hypertensive emergencies are limited. The paediatric use of enalapril in hypertensive emergencies can not be recommended, because the hypotensive effect is unpredictable and comparatively uncontrollable, especially in cases with a renal aetiology.

Clonidine

The lipid-soluble imidazolidine derivate clonidine is an alpha$_2$-adrenergic receptor agonist. It lowers blood pressure, and has also sedative properties. The exact mechanism of action of clonidine is complex and unexplained. It may act on the central nervous system by direct stimulation of the alpha neuronal adrenergic receptors, and another role may play endothelial-derived nitric oxide (NO). The sedative effects of clonidine may be explained by the stimulation of alpha$_2$-adrenergic receptors in the locus coerulens, by augmented release of gamma-aminobutyric acid (GABA), and by interaction with serotonergic and opioid receptor systems. Clonidine metabolism does not depend on hepatic function or the cytochrome P 450 enzyme system, and instead follows minor pathways. Approximately 50% of the intravenous administered clonidine is excreted unchanged in urine without prior metabolism.

Clonidine has an onset of action of about 5 min after injection, and about 15–30 min after oral application. The hypotensive effect can last for up to 8 h. Based on actual publications, no clear recommendation on dose of intravenous clonidine can be made. Some authors report no effect on blood pressure with doses between 0.2 and 2.0 μg/kg/h, while other report on doses between 0.625 and 1.25 μg/kg/h that are already able to induce a reduction in blood pressure. So far, a plasma concentration dependent blood pressure effect has not been proven. 18–36 h after discontinuation of oral, intravenous, and transdermal use of clonidine signs of sympathetic hyperactivity may occur which may lead to withdrawal effects, including tachycardia, tremors, sweating, and rebound hypertension. The sudden withdrawal of the drug can result in severe

Table 9.2 Recommended paediatric doses of the drugs used in hypertensive emergencies (Modified from Horn et al. 2011)

Drug	Recommended paediatric dose	Need for dosage adjustment
Sodium nitroprusside	Starting dose 0.3–0.5 μg/kg/min	Renal failure: (+)
	Can be titrated up to 8–10 μg/kg/min	Hepatic insufficiency: (+)
		During dialysis: (–)
Labetalol hydrochloride	Bolus dose 0.2–1.0 mg/kg/dose (with a maximum of 40 mg/dose)	Renal failure: (–)
	Followed by continuous infusion of 0.25–4.0 mg/kg/h	Hepatic insufficiency: (+)
		During dialysis: (–)
Esmolol	Loading dose of 500–700 μg/kg/min over 1 min	Renal failure: (–)
	Maintenance dose of 20–1,000 μg/kg/min	Hepatic insufficiency: (–)
		During dialysis: (–)
Metoprolol	Bolus 0.1 mg/kg (maximum dose of 5.0 mg) administered up to 3 times at 5 min intervals	Renal failure: (–)
	Followed by a continuous infusion of 1–5 μg/kg/min	Hepatic insufficiency: (+)
		During dialysis: (–)
Urapidil	Starting dose of 1–14 mg/kg/h (2 mg/kg/h in children up to 6 years of age)	Renal failure: (+)
	Can be reduced to maintenance dose of 0.2–3.3 mg/kg/h (1 mg/kg/h in children up to 6 years of age)	Hepatic insufficiency: (+)
		During dialysis: no information
Hydralazine	0.1–0.2 mg/kg/dose (with a maximum of 20 mg/dose) every 4–6 h	Renal failure: (+)
		Hepatic insufficiency: (–)
		During dialysis: (–)
Diazoxide	1–3 mg/kg/dose (with a maximum of 150 mg/dose), repeated at intervals of 5–10 min	Renal failure: (+)
		Hepatic insufficiency: (–)
		During dialysis: (–)
Enalapril	5–10 μg/kg/dose every 8–24 h (with a maximum of 1.25 mg/day)	Renal failure: (+) (p.o.)
		Hepatic insufficiency: (+)
		During dialysis: (+)
Clonidine	1–2 μg/kg every 6 h (with a maximum of 0.8 mg/day) (p.o.)	Renal failure: (–)
		Hepatic insufficiency: (–)
	0.2–2.0 μg/kg/h (i.v.)	During dialysis: (–)
Fenoldopam	0.2–0.8 μg/kg/min	Renal failure: (–)
		Hepatic insufficiency: (–)
		During dialysis: (–)
Nifedipine	0.1–0.6 mg/kg (with a maximum of 20 mg/dose) every 4–6 h (p.o.)	Renal failure: (–)
		Hepatic insufficiency: (+)
	0.2–1.0 μg/kg/min (i.v.)	During dialysis: (–)
Nicardipine	0.5–5.0 μg/kg/min	Renal failure: (+)
		Hepatic insufficiency: (+)
		During dialysis: (–)
Clevidipine	Starting dose of 0.5–1 μg/kg/min	Renal failure: no information
	Followed by continuous infusion of 0.5–3 μg/kg/min (Towe and Tobias 2010)	Hepatic insufficiency: no information During dialysis: no information

rebound hypertension. It has to be reduced gradually. The concomitant use of a beta-blocker can increase the susceptibility of withdrawal effects and should be avoided.

The few publications about the use of intravenous clonidine for the treatment of hypertensive emergencies report its use only in adults. Attributable to its relative long half-life, and its unclear Pk/PD relationship, the hypotensive effect of clonidine is unpredictable and entails the risk of unnecessary overdoses.

Fenoldopam

A newer agent is the vasodilator and selective dopamine$_1$ receptor agonist fenoldopam. It is a dopamine D_1-like receptor agonist that also binds to α_2-adrenoceptors, but not to other vascular receptor. It lowers peripheral vascular resistance and has no direct effect on cardiac contractility, but cardiac output may increase because of a decrease in afterload. Fenoldopam increases renal blood flow and urinary flow and induces natriuresis. The drug causes no significant change in glomerular filtration rate. It undergoes a fast and extensive metabolism by the liver, independent of the cytochrome P450 enzymes. The nontoxic metabolites are mainly excreted in the urine and partially in the feces. The hypotensive effect occurs within 5 min after intravenous application, and can last for up to 60 min. It has an elimination half-life of approximately 10 min. After continuous infusion of fenoldopam for greater than 48 h, tolerance can occur which may lead to a reduced effectiveness. Compared to the potency in adults, fenoldopam showed a relatively poor efficacy in children. Fenoldopam can cause a dose-dependent increases in intraocular pressure. It should be avoided in patients at risk for intraocular and intracranial hypertension. The intravenous application form of fenoldopam contains sodium metabisulfate that may cause acute allergic reactions in patients with potential sulfite sensitivity.

Published experience about its use in children is limited. By now, there is only one published clinical trial and few case reports. Hammer et al. (2008) reported on 77 children aged 1 month to 12 years of age undergoing surgical procedures requiring controlled hypotension. The authors noticed that fenoldopam at doses 0.8–1.2 μg/kg/min is able to reduce blood pressure significantly, and doses between 1.0 and 1.2 μg/kg/min resulted in continued control of blood pressure. Furthermore, it is noteworthy that the effective dose range was significantly higher in children undergoing anaesthesia and surgery than labelled in adults, while pharmacokinetic and side effect profiles were similar in children and adults.

The short elimination half-life makes fenoldopam good controllable, but concerning its efficacy in children there are still some open questions. And the determining disadvantage of fenoldopam may be the appearance of a tolerance after infusions for a period of longer than 48 h, because in some cases of hypertensive emergencies continuous infusions over days are necessary to control blood pressure. Fenoldopam may be a promising alternative to other drugs for short-term therapy. Based on actual publications a recommendation can not be made. Additional experiences on its use in children are warranted.

Calcium Channel Blockers

Calcium channel blockers (CCB) primarily cause dilatation of the afferent arteriole in the glomerulus, and also have a small effect on the efferent arteriole. As a result, the glomerular filtration rate (GFR), and the renal blood flow increases. Due to the decrease of renal vascular resistance, the excretion of sodium and water is increased. Hereby the renal perfusion and function are maintained. In addition to their hemodynamic effects CCB may be able to suppress mesangial cell proliferation and attenuate mesangial entrapment of macromolecules. They may modulate gene transcriptions involved in proinflammatory changes (Cuschieri et al. 2002). They may also have a moderate antiproteinuric effect during profound blood pressure decreases (Kloke et al. 1998).

CCB are metabolized in the liver by the cytochrome P450 3A4, and could affect the clearance

of drugs like cyclosporine or tacrolimus, which also share this system for their metabolism.

A protective effect of CCB on graft survival in renal transplant patients treated with cyclosporine or tacrolimus has been suggested by some authors. For example, Harper et al. (1996) reported a significant improvement of initial graft function, rejection frequency and long term graft survival in 147 patients using the combination of oral nifedipine and cyclosporine. Nifedipine may minimise vasoconstrictive cyclosporine nephrotoxicity, allowing a higher maintenance dose of cyclosporine to be used, and reducing the incidence of rejection. On the one hand, Feehally et al. (1987) reported a better renal function in renal transplant patients receiving nifedipine compared with hypertensive patients receiving other hypotensive agents, but on the other hand they found a shorter mean graft survival in those patients treated with nifedipine. They suggested that nifedipine may have a potential value as a cyclosporine nephroprotective agent. No author was able to clarify the exact mechanism of this possible protective effect. Further, it appears to be independent to blood pressure lowering. Other publications did not confirm the beneficial influence of CCB on graft function or survival.

Nifedipine is a first-generation dihydropyridine calcium channel blocker that is indicated for the use in adults with vasospastic and chronic stable angina. In addition it is widely used for the treatment of hypertensive emergencies both in adults and in children. Nifedipine is a potent vasodilator that produces reflex sympathetic stimulation of the heart with an increase in heart rate and cardiac output. It may have a more powerful effect on diastolic than on systolic pressure. Like other CCB's, nifedipine undergoes an extensive hepatic metabolism via the cytochrome P450 3A4 enzyme system, but it appears not to interact with cyclosporine and tacrolimus. Nevertheless rises and falls in drug levels have been seen in some patients. All metabolites are inactive and excreted in the urine within the first 24 h. In addition, to other adverse effects (Table 9.2), the combination of cyclosporine and nifedipine may increase the incidence as well as the severity of gingival overgrowth. Nifedipine can be administered orally (short-acting-nifedipine) or intravenously.

Short-acting nifedipine acts within 5–10 min following oral administration. The peak effect occurs within 30–60 min and can last for up to 8 h. In adults, short-acting nifedipine has been associated with cases of stroke, an increased risk of myocardial infarction, cerebrovascular ischemia, syncope, conduction disturbances, fetal distress, and death. This has lead to a wide abandonment of the use in hypertensive emergencies in adults. Some authors still advocate short-acting nifedipine as a safe agent for use in children with severe hypertension. As an explanation, it is mentioned that the frequency of severe adverse events may be greater in adults because severe hypertension in adults may be associated with a higher incidence of generalized cardiovascular disease. Until now, a common reason for the severe adverse events in adults has not been found. Strong short-term drops in blood pressure followed by increased levels may have a negative impact on the cardiovascular system. Castaneda et al. (2005) noted that the high concentration of nifedipine in the available preparations for oral administration make it difficult to accurately apply small doses of nifedipine. This may increase the risk of incorrect dosage. Consequently, rapid drop in blood pressure could be greater than desired. As a result of this unpredictable magnitude of the antihypertensive effect the risk of severe adverse effects can also increase and may produce end-organ damage from hypoperfusion. They suggested that it may be more appropriate to treat hypertensive emergencies with intravenous agents that can be titrated for a better control of reduction in blood pressure. Flynn (2003) also noted that short-acting nifedipine may be safe in some hypertensive children, but alternative agents that produce more controlled reductions in blood pressure, and that are easier to administer and accurately dose, should probably be chosen for the majority of children with severe hypertension.

Especially in Germany, a continuous infusion of nifedipine is still used in the treatment of hypertensive emergencies both in adults and in children. This may derive from the fact that the

intravenous application of nifedipine acts within 1 min and may compensate the difficulties of accurate dosage. Because of its insolubility in water, the available preparations for intravenous nifedipine contain 18% [V/V] ethanol which implies an additional and above all a preventable risk for critically ill children (Horn et al. 2011). This is aggravated by absolutely no published original data concerning the suitability and safety of intravenous nifedipine in children.

The hypotensive effect of orally applied short-acting nifedipine is unpredictable which is based on the inexactness of dosage. This could be amended with an intravenous application and perhaps the experience of the attending physicians outweigh the missing published experiences about its paediatric use, but the serious adverse effects observed in adults still raise the suspicion about its suitability in critically ill children. The use of nifedipine should be more scrutinized because there may be alternative drugs with a better safety profile.

Nicardipine is a second-generation dihydropyridine calcium channel antagonist. It is structurally related to nifedipine but it differs from nifedipine by having a tertiary amine structure added to the ester side chain at position three of the hydropyridine ring and a nitro group moved to the meta position of the phenyl ring. Therefore, nicardipine is about 100 times more water soluble and more stable towards light than nifedipine (Curran et al. 2006). Compared to nifedipine, nicardipine has a higher selectivity for L-type calcium channels in vascular smooth muscle than in cardiac myocytes and possesses a strong cerebral and coronary vasodilatory activity. Nicardipine has minimal inotropic cardiac effects and shows no significant venodilatory action. Its hypotensive effect appears to be greater in hypertensive patients than in healthy normotensive volunteers (Curran et al. 2006). Nicardipine is primarily metabolized in the liver through the cytochrome P450 enzyme system. Oxidation is mainly mediated by human CYP3A4, CYP2C8, and CYP2D6. Thus nicardipine can be a relatively potent competitive inhibitor of these CYP enzymes. Nicardipine can cause a higher increase in cyclosporine and tacrolimus blood levels than nifedipine, and raises the risks for toxicity. Hooper et al. (2011) reported on the interaction between tacrolimus and intravenous nicardipine in the treatment of post-kidney transplant in two cases and in an analysis of 2,068 kidney transplantations. Adverse effects of tacrolimus were threefold more likely in paediatric patients treated with intravenous nicardipine than in those treated with other continuous intravenous antihypertensive drugs. A close monitoring of cylclosporine and tacrolimus blood levels is recommended to avoid overdose and toxicity. Nicardipine may also interact with many clinically used drugs reported as a specific substrate for CYP2D6. Despite the fact that it is principally metabolized by the liver, a lower clearance has been reported in patients with renal impairment.

Nicardipine has a rapid onset of action. The hypotensive effect occurs within 1 min after application and can last for up to 3 h. In addition to its side effects, nicardipine is able to produce a statistically significant increase in intracranial pressure and should be used with caution in patients with space-occupying brain lesions. An abrupt withdrawal of the drug may cause rebound angina and hypertension.

The use of intravenous nicardipine in severe hypertension in adults is based on a wide range of publications, but there are only some case reports and small clinical trials about the use of nicardipine in the treatment of hypertensive emergencies in children (Horn et al. 2011). In these publications the number of reported cases is low and the clinical trials have either a small number of patients or they are retrospective. Treluyer et al. (1993) reported about the use of intravenous nicardipine in 14 children with severe hypertension. In all patients, nicardipine was effective and save. No side effects were seen with the mean cardiac frequencies not varying significantly. Gouyon et al. (1997) came to similar results. They reported about the use of intravenous nicardipine in eight hypertensive preterm infants. In all cases nicardipine was effective without hypotension or other clinical side effects. Levene et al. (1990) reported on their experience with intravenous nicardipine in four severely asphyxiated fullterm infants at high risk for adverse outcome

and had abnormal cerebral Doppler haemodynamic studies. In all patients, the heart rate increased. In two infants, they noticed a sudden and marked fall in mean arterial blood pressure (MAP), together with severe impairment of skin blood-flow and a concurrent fall in cerebral blood-flow velocity. In these cases of collapse, the serum levels of nicardipine were ≤40 ng/ml, so apparently this reaction was not due to toxic levels. The authors supposed that it may be possible that a negative inotropic effect together with peripheral vasodilatation, have caused sudden failure of peripheral blood-flow, and underlined that infants suffering from severe birth asphyxia may have compromised myocardial function, which may be an additional factor in the acute failure of cardiac output in the presence of drug-induced vasodilatation. In their opinion, the use of nicardipine, and possibly other calcium channel antagonists, in asphyxiated newborn infants should be only attempted if blood pressure is carefully monitored. Milou et al. (2000) reported on the use of intravenous nicardipine in 20 neonates (15 preterm) with systemic hypertension. In all patients the systolic blood pressure significantly decreased after 3–48 h of nicardipine treatment. They noticed no hypotension. Heart rate, water urinary excretion and plasma levels of sodium, potassium, urea and creatinine did not vary significantly through the first 2 days of treatment. Other clinical side effects like edema, flushing or seizures were not observed. Flynn et al. (2001) reviewed retrospectively the medical records of 29 children with hypertensive emergencies treated with intravenous nicardipine. In all patients nicardipine effectively lowered blood pressure. Only a small number of adverse effects were seen during the treatment (including tachycardia, flushing, palpitations, and hypotension), most of them were relatively minimal and had no adverse impact on the patients 'clinical status'. They also turned out that smaller patients required higher doses of nicardipine on a per-kilogram body weight basis than larger ones. An additional antihypertensive effect has not been seen above 4 μg/kg/min. In their opinion nicardipine appeared to be a safe and effective agent for the management of severe hypertension.

There are also some retrospective clinical trials and case reports about the use of nicardipine for controlled hypotension during some surgical interventions. In all reported circumstances nicardipine was effective for the control of perioperative hypotension, and the given dosages between 1 and 10 μg/kg/min were also well tolerated. The noticed side effects (including limited increase of heart rate, prolonged duration of action, and hypotension) had no outcome on the patients' clinical courses.

Clevidipine is an ultrashort-acting dihydropyridine calcium channel antagonist for intravenous control of blood pressure. It possesses selectivity for arteriolar dilatation without changing heart rate, central venous pressure, pulmonary artery occlusion pressure or cardiac index even with increasing doses. The volume of distribution is small, the clearance is high, and because of the additional ester link of the structure, there is a rapid hydrolysis by esterases in arterial blood and extravascular tissues to inactive metabolites. Clevidipine has an onset of effect within 2–3 min after continuous infusion. The possibility of a quick hydrolysis of the ester link results in an extremely short half life of approximately 1 min.

The pharmacokinetics of clevidipine may be similar to those of sodium nitroprusside. A comparative study of clevidipine and sodium nitroprusside in adults following coronary artery bypass has shown no significant difference between these two agents in controlling the mean arterial pressure (Powroznyk et al. 2003). The only difference found by the authors was the haemodynamic changes including tachycardia that were less pronounced with clevidipine than with sodium nitroprusside. Aronson et al. (2008) found a significantly higher ($p=0.004$) mortality for sodium nitroprusside-treated patients compared to clevidipine-treated patients. The potential of clinically significant drug-drug interactions of clevidipine or its metabolites via induction and inhibitation of cytochrome P450 is small. Clevidipine and its major metabolite H152/81 have shown a moderate induction of CYP 3A4, but only at concentrations that greatly exceed anticipated therapeutic levels. Beyond that, both clevidipine and H152/81, moderately decrease CYP 2C9 activity (Zhang et al. 2006).

Almost all published studies and case reports are dealing with its use only in adults. Until today, just one retrospective review by Towe and Tobias (2010) concerns the use of clevidipine in ten paediatric patients. The described indications were the control of perioperative hypertension, to provide controlled hypotension during orthopedic surgical procedures, and in one patient to improve distal perfusion during a toe-to-finger implant. The target blood pressure was achieved between 5 and 10 min in all patients, two patients required intermittent doses of metoprolol to control reflex tachycardia. They noted no adverse effects such as excessive hypotension. In three patients receiving clevidipine with propofol the triglyceride levels were obtained.

The fast onset of action, and the short half-life are suitable attributes of clevidipine making it a promising drug for the management of hypertensive emergencies. It appears that it also has a low drug-drug interaction potential, and a good safety profile. For a better evaluation of this promising new agent, further studies about the potential and safety of this agent especially in the management of hypertensive emergencies, and about its use in children are desirable.

In conclusion, all drugs used in hypertensive emergencies have their assets and drawbacks. And all of them may cause serious adverse effects, which are able to further deteriorate the critical state of the paediatric patient. Not surprisingly, the published facts about paediatric use of all drugs are scare and often based on case reports and small, mainly retrospective, clinical trials. In a few of them no data at all on their paediatric use can be found. Corresponding guidelines are lacking. This is aggravated by the fact that the underlying cases regularly have a different clinical history and can barely be compared with one another. In all-probability this unsatisfactory status will remain unchanged in the foreseeable future. And it should not to be forgotten that the experience of the attending physicians is of particular importance in choosing the right drug for the actual situation. For this reason, an evidence-based recommendation can not be made. Hypertensive emergencies in children after stem cell transplantation usually have a renal etiology and for the stated reasons, the calcium channel blocker nicardipine appears to be the drug of first choice. Clevidipine may play a major role in the future, because of its promising pharmacokinetic and safety profile, but there is the need for further studies concerning its use in children. It is essential for the attending paediatrician to evaluate the properties, side effects, possible drug-drug interactions, the barely adequate published data and the personal experiences with the drugs already in the forefront of a hypertensive emergency.

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Decellularized Stem Cell Matrix: A Novel Approach for Autologous Chondrocyte Implantation-Based Cartilage Repair

10

Jingting Li and Ming Pei

Contents

Abstract

Cartilage defects due to injuries of the knee result in more than 200,000 surgical procedures annually. Current conservative and operative treatments prove inadequate at fully repairing/resurfacing the defects. The most promising cell-based therapy, autologous chondrocyte implantation (ACI), has evolved for generations and is successful in young patients though long-term clinical outcomes are not yet known. The major limitations for current ACI are autologous chondrocyte shortage and dedifferentiation during monolayer expansion. Stem cells, especially tissue-specific stem cells, may be able to overcome autologous chondrocyte shortage in future ACI treatment due to their self-renewal, multi-lineage differentiation potentials, and lack of ethical issues. Synovium-derived stem cells have been suggested to be a tissue-specific stem cell for cartilage regeneration due to their excellent chondrogenic capacities both *in vivo* and *in vitro*. For the concern about cell dedifferentiation during expansion, decellularized stem cell matrix could provide an *ex vivo* expansion system to rejuvenate and/or reprogram expanded cells in proliferation and chondrogenic potential. The combination of a tissue-specific stem cell and decellularized stem cell matrix would help provide large-quantity and high-quality cells to improve cartilage regeneration and benefit cartilage repair, which will greatly advance the development of next generation ACI in the near future.

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Introduction

Cartilage functions as a pivotal part of the joint environment. Its unique mechanical properties allow consistent frictionless movement over a lifetime. However, cartilage is a poorly vascularized tissue with limited self-healing ability. Other than trauma and aging, degenerative osteoarthritis is the leading cause of cartilage defects. Cartilage injuries of the knee affect approximately 900,000 Americans annually, resulting in more than 200,000 surgical procedures (Cole et al. 1999). Chondral defects are seen in 34–62% of knee arthroscopies in young patients with a median age of 35 years old (Widuchowski et al. 2007).

Drug therapy and physiotherapeutic interventions at present can improve symptoms of joint dysfunction and relieve pain. Unfortunately, no clinical research studies to date have proven that any drugs have the ability to inhibit cartilage degradation or reverse the degenerative process. Operative treatments repair cartilage defects completely by fixing the detached cartilage fragment or by chondrocyte transplantation and tissue regeneration. Current surgical procedures include debridement/chondroplasty, bone marrow stimulation techniques (i.e., microfracture, drilling), osteochondral autografting/allografting, and cell based therapies using autologous chondrocyte implantation (ACI). Despite substantial differences in the complexity and technical application of each method, the ultimate goals of successful cartilage repair are reducing pain; improving symptoms and long-term function; preventing early osteoarthritis and subsequent total knee replacements; rebuilding hyaline cartilage instead of fibrous tissue; and preventing degeneration. Clinical studies have reported outcomes for each technique according to different lesion sizes and the age of the patients. Though short-term success and a low failure rate have been achieved by generations of ACI, the long-term effect of the procedure and the results of full-thickness defect repair are not clear (Harris et al. 2011).

Fortunately, emerging technologies and the next generation of cartilage tissue engineering provide hope. The major areas that investigators are studying include a tissue-specific cell source, a biocompatible scaffold, and an optimal *in vitro* culture environment with bioactive molecules to promote and control proliferation, cellular differentiation, and maturation. First of all, stem cells, especially adult stem cells with self-renewal and multi-lineage differentiation potentials, are a more accessible cell source with no ethical issues compared to embryonic stem cells. Among the adult stem cells, bone marrow derived stem cells (BMSCs) are the first and most widely investigated and adipose derived stem cells (ASCs) have gained more popularity recently due to ease of collection from liposuction (Hildner et al. 2011). Unfortunately, chondrogenically differentiated BMSCs become hypertrophic and eventually result in endochondral bone formation; ASCs have a limited ability toward chondrogenic differentiation. Recently, synovium-derived stem cells (SDSCs) have been suggested as a tissue-specific stem cell; they possess superior chondrogenic potential both *in vitro* and in certain *in vivo* states (Pei et al. 2008; Jones and Pei 2012). Secondly, extracellular matrix (ECM) is known as a key component of the stem cell niche *in vivo*. It functions as a reservoir for growth factors and provides natural and intrinsic cues to direct the remodeling process during cell differentiation. Decellularized ECM (DECM) has been successfully used as a scaffold in tissues such as adipose and organ regeneration, such as lung, heart, brain, liver, and bladder (Hoshiba et al. 2010). Most recently, decellularized stem cell matrix (DSCM) from SDSCs has been demonstrated to provide a three-dimensional substrate to enhance cell proliferation and chondrogenic potential, possibly by immobilized biomolecular signals. This chapter will focus on causes of cartilage defects, the current status of ACI based cartilage repair, matrix based next generation cartilage repair, and future prospects.

Causes of Cartilage Defects

Osteoarthritis, trauma, and metabolic disorders of the subchondral bone, such as osteonecrosis or osteochondritis dissecans are the primary causes

of cartilage defects (Madry et al. 2011). In 1,000 knee arthroscopies, 61% of the knee joints had cartilage defects; 44% of them were due to osteoarthritis, 28% were due to focal cartilage defects, and 2% were due to osteochondritis dissecans (Hjelle et al. 2002). Osteoarthritis is the most common articular disorder and a highly prevalent disease. It affects more than 20% of American adults and 10% of men older than 60 develop osteoarthritis. The current understanding about osteoarthritis is that it is a disease of the entire joint and not only a pathological degradation of articular cartilage. Inflammation of synovial membrane causes release of chondrotoxic proteins leading to cartilage destruction. Typically, osteoarthritis is characterized by areas of poorly delineated defects. Untreated cartilage defects resulting from trauma or osteochondritis dissecans tend to predispose patients to the development of osteoarthritis.

Aging is the accumulation of changes in a person over time. It affects every organ to a different degree. Aged chondrocytes are less responsive to mechanical and inflammatory stimuli. Their protein secretion has been altered, as evidenced by decreased anabolic activity and increased production of proinflammatory cytokines and matrix-degrading enzymes. The above changes make cartilage more susceptible to damage and can lead to the early onset of osteoarthritis. Age has an influence on cell properties when using ACI (Pestka et al. 2011). Cartilage is vulnerable to traumatic injury; due to its avascular nature, inability to access MSCs, and the irreversible aging process, the ability of cartilage to self-heal is disappointing. Patients with symptomatic cartilage defects often report pain, swelling, joint locking, stiffness, and clicking. Symptoms may cause significant functional impairment, often limiting one's ability to work, play sports, and perform activities of daily living.

Autologous Chondrocyte Implantation Based Cartilage Repair

The first generation of ACI was introduced in 1987 and published in 1994 (Brittberg et al. 1994). The procedure involves harvesting autologous chondrocytes from non-weight bearing aspects of the knee. Chondrocytes are then isolated by collagenase digestion and expanded *in vitro*. During a second surgery, the cartilage defect is debrided up to the healthy edges and covered with an autologous periosteal patch, taken from the medial tibia. Finally, the suspension of healthy autologous cultured chondrocytes is directly injected into the chondral defect under the periosteal patch. Despite significant improvements and positive clinical reports, the adoption of periosteal based ACI (ACI-P) has been limited partly owing to post-operative complications such as cell leaking and periosteal-related hypertrophy.

The second generation of ACI is known as collagen-covered ACI (ACI-C) and is characterized by the application of a bioabsorbable collagen membrane in place of the periosteal flap. The chondrocytes are cultured with collagen membrane for several weeks; the membrane is then cut to the correct size and shape of the cartilage defect. Despite ACI-C showing similar clinical improvements and fewer complications, cutting and repeated manipulation of the seeded membrane may result in the loss of critical chondrocytes. A modified ACI-C technique has been developed in which expanded chondrocytes are applied to the collagen membrane after it has been cut to size, reducing the risk of viable cell loss while retaining the ease and speed of the technique. However, ACI-C still suffers from technical problems such as insufficient mechanical stability, uncertain cell distribution within the defect, and the necessity of an intact cartilage shoulder surrounding the defect.

The third generation of ACI is tissue-engineered matrices seeded with autologous chondrocytes; it is called matrix associated/induced ACI (MACI). Cultured autologous chondrocytes are directly seeded onto biodegradable collagen type I/III membrane or allowed to penetrate into a three-dimensional (3D) scaffold or fleece prior to intra-articular implantation. The cell carrier based MACI technique procedure was recently reviewed by Brittberg (Brittberg 2010). Several commercially available products have been developed and marketed in Europe, such as MACI®, Hyalograft® C, Novocart® 3D, and BioSeed®-C *etc.* (Harris

et al. 2011). The widely adopted MACI® used in routine orthopaedic practice is the only third-generation cell carrier that is currently being evaluated in a randomized, controlled trial to meet European regulations for marketing approval and potentially those of other countries. MACI minimizes donor site morbidity by avoiding the harvest and implantation of a periosteal flap. The 3D cultures that MACI can provide also solve the problem of chondrocyte dedifferentiation during expansion and serve as a barrier to fibroblast invasion.

Scaffolds made of natural biomaterials, such as collagen and hyaluronic acid, can maintain the expression of aggrecan and collagen type II in chondrocytes. For instance, collagen has been intensively studied as a natural polymer with respect to cartilage tissue engineering. Chondrocytes cultured within collagen gels preserve their phenotype and glycosaminoglycan (GAG) production for as long as 6 weeks in culture (Kimura et al. 1984). Matrices and membranes derived from collagen also stimulate chondrocytes to produce new collagen (Zheng et al. 2007). Hyaluronan, a highly concentrated component in the ECM of articular cartilage, is also a good candidate for biodegradable and biocompatible scaffold material. Chondrocytes cultured with chemical cross-linking hyaluronan scaffold express more chondrogenic markers, collagen type II, aggrecan, and less collagen type I (Grigolo et al. 2002). Other natural polymers derived from ECM used in cartilage engineering as 3D culture systems include fibrin glue, alginate, agarose, chitosan, chondroitin sulfate, gelatin, and silk fibroin (Danisovic et al. 2012).

Unfortunately, the use of autologous chondrocytes is fraught with unsolved challenges, such as the loss of chondrocyte markers and dedifferentiation in culture during expansion, potential cell leakage, uneven cell distribution in 3D space, potential donor site morbidity (complications of donor site healing), shortage of chondrocytes due to limited cell number from biopsy, short lifespan of chondrocytes, lack of uniformity of *in vitro* expanded chondrocytes from laboratory to laboratory, and low cell quality from aged patients.

Decellularized Matrix Based Next Generation Autologous Chondrocyte Implantation

Hyaline cartilage is an avascular and aneural compact tissue with abundant ECM. Embedded chondrocytes are not only responsible for the production of basic ECM constituents – collagen type II and proteoglycans-but also play an important role in the maintenance and remodeling of ECM in cartilage. Contemporarily, studies have proven that components of ECM can be beneficial to cartilage regeneration. Extrinsic signaling from the surrounding microenvironment, such as ECM, also exerts control over intrinsic genetic pathways to regulate stem cell self-renewal and multi-lineage differentiation potentials. Decellularized ECM, as a whole, can serve as a 3D structure to provide support with mechanical and biochemical cues. Due to the highly conserved nature of ECM between species, DECM is applicable in tissue-processing methods. In recent years, DECM derived from different tissues has been engineered through similar simple biochemical methods that function as a natural scaffold to support proliferation and differentiation of the recellularized cells. Demineralized bone matrix was first used in the 1960s to induce ectopic bone formation due to the existence of the active osteoinductive ingredient bone morphogenetic protein (BMP) (Urist 2009). Cartilage derived matrix can also serve as a scaffold for cartilage defect repair by inducing BMSC chondrogenesis and neocartilage formation from chondrocytes without exogenous growth factors (Matsumoto et al. 2010). Components of native cartilage ECM can provide the required signals to drive ASCs toward chondrogenesis (Cheng et al. 2009). Lu et al. (2011a, b) also engineered an autologous ECM scaffold by culturing autologous cells in a 3D poly(lactic-co-glycolic acid) mesh template.

Despite the fact that DECM deposited by chondrocytes can delay dedifferentiation of chondrocytes (Hoshiba et al. 2012), recent studies have suggested that the enhancement of proliferation and redifferentiation capacity from

SDSC derived DSCM is more significant compared to expansion on chondrocyte derived DECM (Pei et al. 2011b). More interestingly, DSCM deposited by SDSCs also exhibits a rejuvenating effect on chondrocytes. Replicative senescence and dedifferentiation of chondrocytes were delayed and redifferentiation was enhanced, accompanied by an increased expression of CD90, an MSC marker (Pei and He 2012). In other words, DSCM deposited by SDSCs can provide a tissue-specific microenvironment favoring expanded cell chondrogenesis (He et al. 2009; Li and Pei 2011; Li et al. 2011). Through intra-articular injection of DSCM pretreated SDSCs in a minipig model, Pei et al. (2012) for the first time demonstrated that the repair of partial thickness cartilage defects is superior compared to plastic flask expanded SDSCs, though both SDSC groups were better than the saline group. Human fetal and adult SDSC deposited DSCM were generated to further investigate the rejuvenating mechanisms. Not surprisingly, fetal SDSC derived DSCM has a significantly superior effect over adult SDSCs in terms of enhancing proliferation and restoring chondrogenic differentiation potential (Li et al. 2014). Similarly, senescent human diploid fibroblasts could be restored to a more youthful state through interaction with ECM deposited by younger cells, possibly through delaying telomere shortening (Choi et al. 2011). The rejuvenating effect of younger BMSC derived DSCM was observed both *in vivo* and *in vitro* (Sun et al. 2011). The above literature suggests that the 3D microenvironment of DSCM can profoundly influence cell senescence possibly through mimicking the *in vivo* stem cell niche and providing essential biochemical and biophysical cues for stem cells to behave in a dynamic fashion (Li and Pei 2012). Further investigations are planned. DSCM deposited by SDSCs with nucleus pulposus (NP) cells or SDSCs alone can facilitate expansion and NP-lineage differentiation of SDSCs (He and Pei 2012; Pei et al. 2013). DSCM prepared under hypoxia can benefit NP cell viability and redifferentiation. The above studies have shown that expansion using the DSCM system can provide large-quantity and high-quality cells for cartilage repair and possibly intervertebral disc tissue engineering and regeneration (Pei et al. 2011a, c).

Potential usage of DSCM and tissue-specific stem cells in the next generation of ACI is promising. Tissue-specific DSCM could serve as an excellent *ex vivo* expansion system for either chondrocytes or stem cells to regain and maintain potent proliferative and chondrogenic differentiation capacities. Through the creation of a youthful and natural *ex vivo* microenvironment by DSCM, the senescence of incubated cells could be partially reversed. More investigation, especially *in vivo* studies, is required before the application of DSCM can go further. Clarifying the mechanisms of the role of DSCM in cartilage tissue-engineering could benefit millions of patients suffering from cartilage damage.

Summary and Future Directions

The need for cartilage defect repair is demanding as the aging population grows. The benefits of a cell based technique such as ACI that results in hyaline repair tissue with good integration at the defect is attractive and companies have been investing to support research (Jaklenec et al. 2012). However, modest results from clinical trials show that limitations of ACI, such as poor cell persistence, viability, post-translation, and cell relocation to non-target sites, still exist (Vasiliadis et al. 2010). Long-term evaluation and more *in vivo* studies are needed. Current development of ACI and the next generation of cartilage repair largely depend on progress in the cartilage tissue engineering field. One focus in the next generation of cell-based cartilage tissue engineering and repair is stem cells, especially tissue-specific stem cells (Dhinsa and Adesida 2012; Danisovic et al. 2012). Finding tissue-specific stem cells for cartilage tissue engineering is not accomplished yet though SDSCs have been proposed as a good candidate (Jones and Pei 2012). Biomaterials, which can be applied directly or used as stem cell delivery vehicles, should help elicit and enhance beneficial stem cell responses. Ideally, a tissue-specific DSCM

could be reconstructed to rejuvenate and/or reprogram autologous chondrocytes and stem cells in proliferation and chondrogenic potential, which may be a future direction for the next generation of cartilage engineering.

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Overcoming Radioresistance of Lung Cancer Stem Cells

11

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Contents

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Abstract

Every year lung cancer claims more lives than any other cancer type. Poor outcomes in this disease are linked to the development of resistance to existing treatments, including radiotherapy. Radioresistance in lung cancer may be driven by a population of cancer cells with properties in common with normal stem cells, including limitless self-renewing potential. These "cancer stem cells" are capable of reconstituting tumors following treatment and of seeding metastatic sites. In this chapter, we review the evidence implicating cancer stem cells in the radioresistance of cancers of the lung and other tissues. Strategies for interfering with mechanisms of radioresistance within cancer stem cells are discussed. Therapeutic modifications that augment the effects of radiotherapy on CSCs are likely to improve outcomes for lung cancer patients.

Introduction

Lung cancer is the leading cause of cancer death worldwide. Histological features and clinical behavior distinguish the two major subtypes of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC, which is composed of a variety of subtypes including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, accounts for approximately 85% of lung cancer diagnoses.

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The prognosis for patients with lung cancer is related to clinical stage. Early stage lung cancer is curable with surgery or radiotherapy. Unfortunately, in the majority of patients, cells have already metastasized to lymph nodes or other organs by the time of diagnosis. Treatments for metastatic lung cancer remain inadequate. Despite recent advances in treatment, survival rates for all patients with lung cancer combined remains <20% at 5 years. Therapies that target aberrant receptor tyrosine kinase activity have shown dramatic clinical benefits in a subset of cases, but responses are invariably temporary and relapse occurs usually in less than 1 year. Thus, novel therapeutic strategies are needed to improve the efficacy of treatments and boost survival for this difficult disease.

A growing understanding of stem cells and of mechanisms of cell death and differentiation during development has provided important insights into cancer biology and tumor behavior. Several aspects of stem cell biology are relevant to cancer. First, by virtue of their capacity for continual self-renewal, stem cells in normal adult tissues are likely targets of transformation since they are often the only long lived cells in a tissue. Second, many tumors recapitulate the developmental patterning of normal tissues and analogous to adult stem cells in normal tissues harbor a population of cells ("cancer stem cells", CSCs) with indefinite proliferative potential that can give rise to more differentiated progeny. Third, many of the signaling pathways and gene expression programs active in normal stem cells are also be found among CSCs.

Cellular Hierarchy and Stem Cells in Normal Adult Tissues

The lung is a developmentally complex organ. In part due to this complexity, the identification of somatic epithelial stem cells in the normal adult lung has been challenging. Far better understood are the stem cells of the hematopoietic system (hematopoietic stem cells, HSCs), which serves as a model for other tissues. Hematopoiesis is a tightly regulated process in which a small pool of HSCs continually self-renew while giving rise to the full complement of mature cell types that make up the hematopoietic system (e.g., erythrocytes, granulocytes, macrophages, platelets, and lymphocytes) (Seita and Weissman 2010). HSCs must balance self-renewal and differentiation to ensure homeostatic control of their numbers. HSCs represent less than 0.05% of bone marrow cells and are admixed with a heterogeneous pool of progeny with more limited potential for self-renewal and differentiation.

A similar cellular hierarchy is mirrored in many adult mammalian tissues. However, in the adult lung a linear hierarchy of cellular differentiation is less evident (Rock and Hogan 2011). The respiratory epithelium is composed of heterogeneous cell types, and its organization changes along the proximal-distal axis. Large proximal airways (trachea and bronchi) narrow and branch into intralobar bronchioles. The bronchioles terminate in distal alveoli, which are the sites of gas exchange. The trachea and bronchi are lined by a pseudostratified columnar epithelium, whereas the bronchioles and alveoli are lined by a single layer of cuboidal or simple squamous epithelium, respectively. Ciliated cells, goblet cells, Clara cells, basal cells, and neuroendocrine cells are all found throughout the epithelium of the trachea, bronchi, and bronchioles (Fig. 11.1).

Adult stem cells contribute to the maintenance and repair of mature tissues through (1) self-renewal and (2) the generation of more differentiated progenitor or effector cells. By examining cellular recovery following targeted lung injury, a large number of studies have uncovered a group of progenitor cells with stem cell properties that are important for regenerating the lung epithelium (Rock and Hogan 2011). The prevailing view is that distinct pools of stem or progenitor cells exist along the proximal-distal axis, including separate stem/progenitor compartments in the proximal airways, bronchioles, and alveoli. In a contrasting model, a single stem cell has been proposed to have the capability of self-renewal and differentiation into all epithelial cell types in the lung (Kajstura et al. 2011). The same cells have also been proposed to give rise to endothelium within the pulmonary vasculature, although this finding remains controversial.

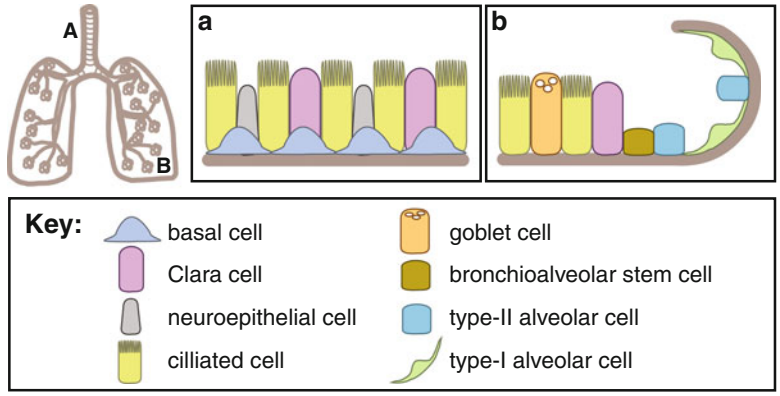

Fig. 11.1 Schematic illustrating the cellular organization in the human adult lung. (**a**) The architecture of the proximal airway epithelium in the trachea, bronchi, and bronchioles. (**b**) The airway epithelium in the distal airways, including the terminal bronchioles (*left, straight*) and alveoli (*right, curved*). The key indicates the diverse cell types of the lung epithelium

Genetic Regulation of Self-Renewal and Implications for Cancer

Stem cell numbers in normal tissues are tightly regulated, whereas in many tumors there is an expansion of cells with stem-like properties. Activation of known oncogenes can disrupt the normal homeostatic regulation of (1) cell death and/or (2) cell differentiation. For example, *Bcl-2* overexpression within HSCs results in reduced cell death through inhibition of apoptosis (Domen et al. 2000), and *c-myc* overexpression prevents the differentiation of HSCs (Prochownik and Kukowska 1986). These studies illustrate how stem cell numbers during tumorigenesis are affected by an imbalance in the regulatory networks governing self-renewal.

Many of the signaling pathways implicated in the regulation of self-renewal in normal tissues are emerging as key players in tumorigenesis. Classic examples are the *Wnt*, *Notch* and *Sonic hedgehog* (*Shh*) pathways. *Wnt* encompasses a large family of secreted proteins that bind to receptors to activate β-catenin. Wnt/β-catenin signaling plays a pivotal role in the self-renewal of normal stem cells and in the oncogenic progression of many human cancers (Holland et al. 2013). The Notch family of receptors controls development and differentiation processes in many tissues. Aberrant Notch signaling is a common occurrence in cancer, and Notch inhibition is now being explored as a promising therapeutic strategy for solid cancers as well as leukemias (Purow 2012). Shh is a secreted molecule that binds to the *Patched* family of receptors. Shh signaling regulates self-renewal in a number of normal tissues. Mutations in *Patched-1* are observed in basal-cell carcinoma and medulloblastoma, and pharmacological inhibition of the Shh pathway has recently shown great promise for treatment of these diseases (Rudin et al. 2009; Sekulic et al. 2012). Taken together, these observations indicate that stem cells and cancer cells often rely on similar self-renewal pathways. Further inquiry will be necessary to exploit these pathways specifically in cancer cells without adversely affecting normal developmental programs.

Stem Cells and Tumorigenesis

Studies on the cellular hierarchy in the normal tissues have direct implications for tumorigenesis and the cellular origin of cancer. Because stem cells possess properties that allow for perpetual self-renewal and survival, these cells may be most capable of giving rise to malignant clones (Reya et al. 2001). Indeed, this phenomenon has

been demonstrated in mouse models of multiple tumor types, including in adenocarcinomas of the colon, breast, and prostate (Visvader 2011).

Distinct progenitor cell populations are thought to be capable of giving rise to subtypes of lung cancer. Lung adenocarcinoma may originate from type-II alveolar cells (Xu et al. 2012) or from bronchioalveolar stem cells situated at the bronchiolar-alveolar junction (Kim et al. 2005). Squamous cell carcinoma has been proposed to arise from tracheal basal cell progenitors (Sutherland and Berns 2010). Small cell lung cancer can arise from pulmonary neuroendocrine cells in experimental systems (Sutherland et al. 2011). Future investigations harnessing lineage tracing techniques in mouse models of lung cancer (Rawlins et al. 2009), will be needed to fully characterize the oncogenic potential of individual populations of normal stem and progenitor cells in the adult lung.

Cancer Stem Cell Theory and Lung Cancer

CSCs are malignant cells capable of self-renewal and differentiation into progeny within tumors. Owing to these properties, surviving CSCs within primary tumors or metastases can reconstitute an entire tumor following treatment or upon transplantation into immunodeficient mice (Reya et al. 2001). The first unequivocal demonstration of CSCs was in acute myeloid leukemia (Lapidot et al. 1994), and the presence of CSCs in solid cancer was first shown for breast adenocarcinoma (Al-Hajj et al. 2003). CSCs have since been identified in many different tumor types (Nguyen et al. 2012).

A number of methods have been proposed to enrich for cellular fractions containing cells with stem-like properties in lung cancer. Most of these methods are designed to prospectively identify cells that are more likely to undergo clonogenic growth under defined laboratory conditions. They discriminate between cells based on physical or biochemical properties, such as Hoechst dye efflux (Ho et al. 2007) and Aldefluor assays (Jiang et al. 2009), or based on cell surface expression of specific proteins, such as CD133 (Eramo et al. 2008) or CD166 (Zhang et al. 2012).

There are practical limitations of these assays that have important ramifications for the CSC theory. First, the degree of overlap between cellular populations identified by different assays is not well characterized. It has been demonstrated that the proper CSC enrichment strategy likely depends on the histologic or genetic subtype of NSCLC (Curtis et al. 2010). This presents obvious challenges to the application of the CSC theory to the study of inherently heterogeneous diseases such as lung cancer. Additionally, the defining property of a CSC is the ability to give rise to a new tumor that contains more CSCs as well as the more differentiated non-tumorigenic cancer cells that make up the bulk of most tumors. In light of this, rigorous studies of CSCs in NSCLC must continue to functionally evaluate CSC populations and must include in vivo assays rather than relying on surrogate markers or tissue culture assays.

The Role of Radiotherapy in the Treatment of Lung Cancer

The role of radiotherapy in the treatment of NSCLC depends primarily on stage at diagnosis.

For early-stage NSCLC, the standard-of-care treatment involves resection of the primary tumor by lobectomy with simultaneous dissection of hilar and mediastinal lymph nodes. In cases where no lymph node spread is detected at diagnosis, >60% of patients may be cured by surgery alone. However, up to one third of early stage NSCLC patients are not surgical candidates. For these patients, high-dose-per-fraction stereotactic ablative radiotherapy (SABR), also called stereotactic body radiotherapy, can achieve similar rates of cure (Senan et al. 2011). For unresectable disease that has spread to the lymph nodes in the chest, external beam radiotherapy is delivered with conventional fractionation (e.g., 2 Gy per fraction for ~30 fractions) either alone or with concurrent platinum-based chemotherapy.

Radioresistance is commonly seen with conventionally fractionated radiotherapy. Local recurrence is detected clinically in up to half of cases. Thus, the identification of novel strategies for sensitizing NSCLC cells to the effects of ionizing radiation—without increasing treatment-related morbidity—will be important for improving the outcomes for these patients. For patients treated with SABR, however, local control is approximately 90% (Senan et al. 2011). The predominant mode of failure for these patients is within lymph nodes and/or distant sites, suggesting that improved outcomes in this group of patients will require identification of individuals at high risk of disease spread who would likely benefit from treatment intensification, for example by addition of adjuvant chemotherapy or immunotherapy.

Radioresistance in Cancer

Resistance to radiotherapy is an important clinical problem because of its impact on patient survival and subsequent treatment options. The primary cytotoxic effect of therapeutic ionizing radiation (IR) is DNA damage. Double strand breaks (DSBs) are the most lethal form of DNA damage caused by IR because these are the most difficult for the cell to accurately repair. The most common type of radiotherapy in use today for lung cancer involves treatment using photons (X-rays). Photons are primarily indirectly ionizing, meaning that the damage they inflict is a result of the production of free radical reactive oxygen species (ROS) within cells.

The degree of radioresistance varies across tissue types. Contributing to this heterogeneity are the diversity of cellular architecture within an organ and the availability of molecular oxygen supplied by the vasculature. In addition, many cell intrinsic factors influence resistance to IR. Cells produce a group of antioxidant enzymes that neutralize ROS (Kobayashi and Suda 2012). The expression of these ROS scavengers has been shown to affect resistance to IR and cytotoxic chemotherapy in human cancers, presumably as a result of reduced levels of DNA damage. Once DNA damage has occurred, the ability of a cell to sense and repair it depends on the type of DNA lesion, the phase of the cell cycle, and the expression of repair machinery. In the case of catastrophic DNA damage that cannot be accurately repaired, the p53 pathway normally triggers apoptosis; however, in cancer deranged p53 signaling and/or other pro-survival genetic lesions can lead to an inability for cells to die appropriately (Helton and Chen 2007; Smith et al. 2010). Genomic instability resulting from such checkpoint failure can lead to higher mutation rates and rapid selection of resistant cells. Thus, there is a multistep progression toward cell death as a result of IR, and each step presents an opportunity for a cancerous cell to intervene and to survive treatment.

Mechanisms of Radioresistance in CSCs

Tumor recurrence following radiotherapy can have dire consequences. Retreatment presents significant challenges and the potential for increased toxicity. Moreover, the risk of metastasis generally rises once a tumor recurs. Importantly, radioresistant cells that lack the capacity for self-renewal would not be as problematic as resistant clones that have this capacity and are thereby able to initiating regrowth of a tumor (Reya et al. 2001).

Recent findings have confirmed that CSCs from multiple tumor types including lung cancer are programmed to preferentially survive the effects of chemotherapy and radiotherapy (Diehn et al. 2009a; Jiang et al. 2009). Mechanisms of CSC resistance to IR have begun to be uncovered in human cancers and mouse models (Morrison et al. 2011) (Fig. 11.2). Both cell-intrinsic and microenvironment-dependent mechanisms have been proposed. Cell-intrinsic mechanisms include slower progression through the cell cycle, increased expression of ROS scavenging enzymes, enhanced sensing and repair of DNA damage, and pro-survival signaling.

The quiescent state of some stem cells and CSCs may render them relatively resistant to IR since they are less likely to traverse mitosis,

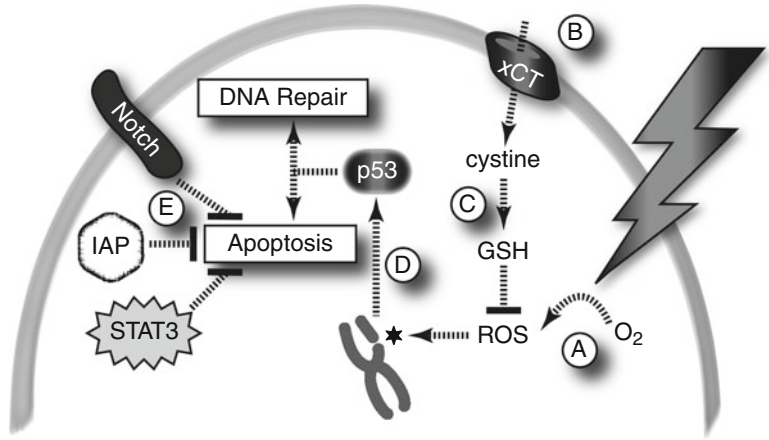

Fig. 11.2 Mechanisms of radioresistance in CSCs. (*A*) *Hypoxia:* CSCs in hypoxic microenvironments may be resistant to IR by virtue of the relative paucity of molecular oxygen from which to derive ROS. (*B, C*) *ROS scavenging:* CSCs import cystine and produce high levels of enzymes necessary to convert it into GSH, a potent ROS scavenger. (*D*) *Checkpoint signaling:* DNA DSBs that result from IR/ROS activate p53 through ATM, CHK1, and CHK2 signaling; these pathways may be more active in CSCs. (*E*) *Survival signaling:* Pro-survival signals, including XIAP, STAT3, and the Notch pathway, are activated in CSCs and lead to inhibition of apoptosis

the most radiosensitive phase of the cell cycle. However, CSCs in some tumor types have been shown to cycle at similar rates to other tumor cells (Al-Hajj et al. 2003). Radioresistance of CSCs could also be explained by lower levels of ROS as compared to non-CSC tumor cells as a result of higher levels of glutathione (GSH) and other ROS scavengers in those cells (Diehn et al. 2009b; Ishimoto et al. 2011). DNA repair in response to IR may be more robust in CSCs as a result of robust checkpoint signaling. CSCs in glioma preferentially repair DNA DSBs following IR in a manner dependent upon the Chk1/Chk2 checkpoint kinases (Bao et al. 2006). Similarly, in a mouse model of medullosblastoma, nestin-positive CSC-like cells preferentially undergo IR-induced p53-dependent cell cycle arrest and recovery, whereas nestin-negative tumor cells are more likely to undergo p53-dependent apoptotis (Hambardzumyan et al. 2008). Thus, CSCs in multiple tumor types appear to be protected against catastrophic DNA damage through a combination of ROS scavenging and checkpoint activation leading to enhanced repair of DSBs.

Recent advances have also highlighted the contributions of the tumor microenvironment to CSC radioresistance. Dysfunctional tumor vasculature and unrestrained cancer cell proliferation contribute to pockets of hypoxia where radioresistant CSCs may arise. Hypoxia is detrimental to effective cell killing by IR due to reduced ROS and correlates with radioresistance and treatment failure in human cancer (Brizel et al. 1999). In response to hypoxia, hypoxia-inducible factors (HIFs) drive gene expression programs that promote self-renewal and survival of glioma CSCs (Li et al. 2009). With these data in mind, there has been interest in sensitizing tumors to radiation through increased tissue oxygenation (Overgaard 2011), but the effect of such maneuvers on the CSC populations in clinical samples is not known.

Radioresistance in Lung CSCs

Several of the mechanisms discussed above have been shown to contribute to radioresistance of CSCs or CSC-like cells in lung cancer. For example, ROS scavengers are expressed at higher levels within CD133-positive NSCLC cells (Salnikov et al. 2010), and augmented checkpoint signaling may contribute to reduced apoptosis in response to IR (Lundholm et al. 2013).

With regards to the microenvironment, direct measurements with oxygen electrodes have revealed that even small lung tumors are hypoxic, and the degree of hypoxia correlates with prognosis (Le et al. 2006).

Taken together, CSCs from various tumor types utilize multiple and perhaps distinct strategies for avoiding cell death in response to IR. Even within a single tumor, genetic heterogeneity and varied microenvironments provide opportunities for CSCs with different properties to arise (Odoux et al. 2008). Thus, for reproducible clinical benefits to be observed with novel radiosensitizers directed at CSCs, multiple radioresistance mechanisms may need to be targeted simultaneously.

Targeting CSCs in Lung Cancer

Efforts to translate the CSC hypothesis into clinical practice will drive future advances in the management of lung cancer. Strategies for interfering with resistance mechanisms of CSCs, or for otherwise leading to their selective inhibition, are discussed below.

Promoting Apoptosis in CSCs

Recent evidence supports the view that CSCs in some tumor types depend on a blockade of apoptosis pathways in order to survive the effects of therapeutic radiation (Hambardzumyan et al. 2008). The Inhibitor of Apoptosis Protein (IAP) family, key regulators of apoptosis in cancer cells, has been implicated in radioresistance of multiple tumor types including lung cancer (LaCasse et al. 2008). XIAP inhibitors block clonigenic survival of glioma CSC-like cells in vitro (Vellanki et al. 2009). Importantly, XIAP inhibitors have also been shown to increase radiosensitivity in a NSCLC cell line (Cao et al. 2004). STAT3, like XIAP, promotes cell survival in cancer. Recent reports have shown promise for a strategy of radiosensitizing CSCs with STAT3 inhibitors (Hsu et al. 2011). Further studies are needed to evaluate the effects of XIAP and STAT3 inhibition on the radiosensitivity of normal tissues.

Promoting Differentiation in CSCs

CSCs are capable of self-renewal and differentiation. Driving cellular differentiation may be an effective strategy for limiting the degree of self-renewal and potential for tumor expansion and spread. All-trans retinoid acid (ATRA) has potent effects on cellular differentiation. Preclinical investigations have demonstrated an inhibitory effect on CSCs from glioma and head and neck squamous cell carcinoma (Ning et al. 2013).

Inhibiting Self Renewal in CSCs

In addition to strategies for promoting cellular death or differentiation, signaling pathways involved in self-renewal of normal and cancer stem cells are being targeted for therapeutic gain. Clinical investigations are ongoing for Wnt, Notch, and Hedgehog inhibitors in solid tumors (Morrison et al. 2011). Inhibition of telomere reverse transcriptase (TERT) is another promising therapeutic strategy for preventing unrestrained self-renewal of CSCs (Ning et al. 2013). For all of these inhibitors, their ability to sensitize stem cells and CSCs to IR will need to be investigated.

Targeting CSC Cell Surface Proteins

Selective inhibition of CSCs through cell surface markers is an area of active investigation. Targeting of c-kit and CD133 has been investigated in lung cancer and glioma, respectively. In some NSCLC cell lines, a sub-population of CSC-like c-kit-positive cells are resistant to cisplatin but are effectively inhibited by the c-kit inhibitor, imatinib (Levina et al. 2010). Importantly, the combination of cisplatin with either imatinib or anti-SCF antibodies (SCF: stem cell factor, the c-kit ligand) had additive effects on growth inhibition. Whether additive effects are also seen with radiotherapy is not known. In glioblastoma multiforme, in which CD133 is thought to label CSCs (Singh et al. 2004), Wang et al. designed an antibody-conjugated cytotoxin that is activated by near-infrared laser light (Wang et al. 2011). The result was targeted

killing of CD133-positive cells in vitro. While not immediately translatable to clinical practice, these proof-of-concept experiments suggest that the combination of antibody-based therapies with radiotherapy could result in eradication of CSCs.

Hypofractionated Radiotherapy and Tumor Hypoxia

Conventional radiotherapy is ineffective for eliminating some CSCs. SABR and hypofractionated radiotherapy may overcome some of the limitations of conventional radiotherapy due to additional mechanisms of cell killing seen at higher per-fraction doses, including stimulation of a potent cytotoxic antitumor inflammatory response (Lee et al. 2009) and acute endothelial disruption (Garcia-Barros et al. 2003). However, hypofractionation limits the number of opportunities for tumor cell reoxygenation during a course of radiotherapy, which potentially contributes to radioresistance. The addition of the hypoxic cell radiosensitizer etanidazole to SABR has been proposed as a strategy to more effectively eliminate hypoxic CSCs and to improve the therapeutic ratio of SABR for NSCLC (Brown et al. 2010). In support of this strategy, modification of hypoxia in other hypoxic tumor types appears to produce greater clinical benefits when larger fraction sizes are used (Overgaard 2011).

Particle Therapy for Hypoxic Tumors

Radiotherapy for lung cancer is primarily delivered using megavoltage photons. Photons exert most of their effect on DNA damage indirectly via generation of ROS due to their low linear energy transfer (LET) value. Over the past decade the number of clinical centers offering proton-based radiotherapy has increased dramatically. Protons and other heavy particles, which have higher LET values, offer a theoretical advantage over photons for killing CSCs or other cells with high expression of ROS scavengers. Likewise, protons may be more effective at eliminating CSCs within hypoxic microenvironments. In support of this hypothesis, CSC-like cells have demonstrated increased radiosensitivity to high-LET radiation as compared with photons in lung and colon cancer cell lines (Moncharmont et al. 2012). Future studies are needed to confirm in vivo the observation that high-LET radiation may provide enhanced killing of CSCs.

Combining CSC-Targeted Therapies with Radiotherapy

Radiotherapy is an integral component of the management of NSCLC. Resistance to IR limits the efficacy of both conventional radiotherapy and SABR, and overcoming this resistance with novel therapies will be important for improving clinical outcomes. A new generation of anticancer therapies specifically targeting CSCs could someday play an important role in improving the efficacy of radiotherapy for NSCLC. A principle challenge for translating CSC-targeted therapies into clinical practice will be to limit toxicities associated with treatment. Therefore, optimal combinations of CSC-targeted therapies with radiotherapy will need to be empirically tested.

Sensitization of CSCs to IR

A common strategy employed clinically for lung cancer to improve outcomes with radiotherapy is to concurrently administer a radiosensitizing agent, such as a platinum-containing drug. The goal of this strategy is, in part, to make IR-dependent killing of cancer cells more effective. Likewise, inhibitors of free radical scavenging pathways, XIAP, or STAT3 may be able to potentiate the cytotoxic effects of radiotherapy by eliminating a mechanism that some CSCs employ for escaping cell death after IR. While such therapeutic combinations are likely to have synergistic activity against CSCs, normal adult stem cells within the radiotherapy field may also be at risk. Thus, as novel CSC-directed radiosensitizers are investigated, the consequences of injury to normal tissues will require careful evaluation.

IR-Independent Inhibition of CSCs

An alternative strategy for combining novel therapies with radiotherapy is to select agents that act independently of IR to kill the radioresistant CSCs. Agents that promote CSC differentiation or inhibit self renewal would be good candidates. This strategy would likely limit the risk of toxicity within the radiotherapy field but could potentially have more systemic adverse effects by affecting the self renewal properties of stem cells throughout the body.

Future Directions

The notion of CSCs as the driving force behind tumor growth, metastasis, and treatment resistance presents a new vantage point for the war on cancer. The key to harnessing this recent conceptual framework for developing novel cancer therapeutics will be to target CSCs without significant toxicity from harming the pool of normal adult stem cells. The optimal approach for combining CSC-directed therapies with existing anticancer treatments will need to be evaluated in prospective clinical trials that include mechanisms for careful monitoring of toxicity and survivorship. To some extent, existing anticancer treatments such as radiotherapy may be improved by modifications that augment their effect on CSC survival.

This effort would benefit from more robust methods for identifying CSC populations in vivo within primary tumors. Current methods that rely on the expression of a single cell surface marker or enzyme are unlikely to be reproducible between tumors, or even within subclones of a single tumor. Lineage tracing experiments in mouse models of NSCLC will be an important tool for pre-clinical identification and characterization of different stem- and CSC-specific markers. In addition, single-cell gene expression and genetic analyses are more likely to capture the true cellular heterogeneity within primary tumor specimens and allow for better characterization of tumorigenic cell populations (Dalerba et al. 2011). Together, these advances will increase the power of downstream analyses on CSCs and comparisons with other cells within tumors and with normal adult stem cells. Such comparisons will be crucial in order to exploit differences between these cell populations with novel CSC-based therapies.

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Part IV

Regenerative Medicine

Phenotypic Correction of Murine Hemophilia A Using Cell-Based Therapy

12

Neelam Yadav

Contents

Abstract

Hemophilia A (HA) is caused by mutations within the factor VIII (FVIII) gene, which leads to depleted protein production and inefficient blood clotting. Current therapies include fixes-dose FVIII prophylaxis, factor VIII replacement therapies, and most recently, gene therapy. Several attempts at gene therapy have failed for various reasons-including immune rejection. Liver is the primary site of FVIII synthesis; however, the specific cell types responsible for its synthesis remain controversial. Several reports have demonstrated the capacity of bone marrow stem cells (BMSCs) to transdifferentiate into hepatocytes and liver sinusoidal endothelial cells (LSECs). These findings created enormous interest because they uncovered a new property of BMCs and opened the possibility that these cells could be used in the treatment of liver injury and acute or chronic liver failure. We propose that the severity of the bleeding disorder could be ameliorated by partial replacement of mutated liver cells with healthy cells in HA mice. Our study showed that BM-derived hepatocytes and endothelial cells can synthesize FVIII in liver and correct bleeding phenotype in HA mice. Thus, BM-stem cell therapy is a potential alternative approach to managing HA.

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Introduction

Hemophilia A (HA) is an X-linked recessive bleeding disorder that results from a deficiency of antihemophilic factor VIII (FVIII). The incidence of HA is 1 in 5,000 males, constituting about 80% of all hemophilia cases, and manifests in mild to severe disease, depending on the relative expression of functional FVIII. Severe HA patients have 1% or less of normal plasma FVIII activity and spontaneously bleed. Patients with 1–5% of normal activity have less severe bleeding, and patients with 5–25% of normal activity usually bleed only with surgery or trauma. The clinical manifestation of this disease is unpredictable, recurrent, and spontaneous bleeding in various areas, including soft tissues, major joints and occasionally in internal organs. The standard treatment options for HA are either on-demand or prophylactic therapy with plasma-derived or recombinant human FVIII. The therapeutic use of this purified factor can be a potential biohazard due to blood-borne pathogens, and is ineffective due to the formation of inhibitors. Moreover, the life-long requirement for replacement therapy can have a significant economic impact on patients. Gene therapy has the potential to provide life-long correction of the bleeding disorder in animal models but clinical trials have not conclusively shown long-term therapeutic benefits in treating hemophilia B (HB) (Manno et al. 2003, 2006). This was primarily due to a cell-mediated immune response against the adeno-associated virus (AAV) capsid protein that causes a decline in FIX activity (Manno et al. 2006). Another concern associated with the use of viral vectors is the transient elevation of liver transaminases in response to the vectors. Therefore, alternate therapeutic options need to be developed.

The FVIII gene has been identified in different tissues of mammals. Using RNase protection analysis, Wion et al. (1985) showed that FVIII mRNA is expressed in the liver, purified hepatocytes, the spleen and lymph nodes. Pancreas, kidney and muscle also showed low-level expression of FVIII mRNA (Wion et al. 1985).

Liver perfusion and orthotopic liver transplantation studies in both animals and humans have suggested that the primary site of FVIII synthesis is the liver (Owen et al. 1979). Various lines of evidence indicate that, within the liver, hepatocytes are the major FVIII-producing cells (Owen et al. 1979; Zelechowska et al. 1985). Thus, cell based therapy using isolated hepatocytes has been proposed as an attractive approach to treat clotting disorders. The therapeutic effectiveness of human hepatocytes transplanted under the kidney capsules of mice has been demonstrated (Ohashi et al. 2005). In addition to hepatocytes, sinusoidal endothelial cells have been shown as a cellular site of FVIII synthesis in mice (Do et al. 1999).

Based on the above studies, either hepatocytes or LSECs could be candidate cells for the therapeutic intervention of HA, but these treatments require cadavers or partially hepatectomised liver tissue from allogenic sources. A cell-based therapy can become successful if the graft is easily obtained. Numerous studies have demonstrated that bone marrow (BM) stem cells differentiate into hepatocytes in rodents (Mallet et al. 2002; Wang et al. 2003; Jang et al. 2004; Khurana and Mukhopadhyay 2007). Experimental findings from our and other groups heighten the scope for exploitation of this novel phenomenon of adult stem cells plasticity for the treatment of HA disease (Yadav et al. 2009, 2012; Follenzi et al. 2012). It has been presumed that BM-stem cell therapy not only helps for the synthesis of missing protein, it may protect liver from hepatocellular carcinoma or other adverse pathological changes of the liver. This review discusses the pathophysiology of HA disease, their treatment limitations, and finally proposed BM-stem cell therapy to treat the same.

Pathophysiology of the Disease

The pathogenesis of HA is linked to deficiency of FVIII coagulant protein also known as antihemophilic factor (AHF). The gene for FVIII is located on the long arm of chromosome X, within the Xq28 region. The gene (*F8C*) is unusually large, representing 186 kb of the X chromosome. It comprises 26 exons and 25 introns. Mature FVIII contains 2,332 amino acids. Approximately,

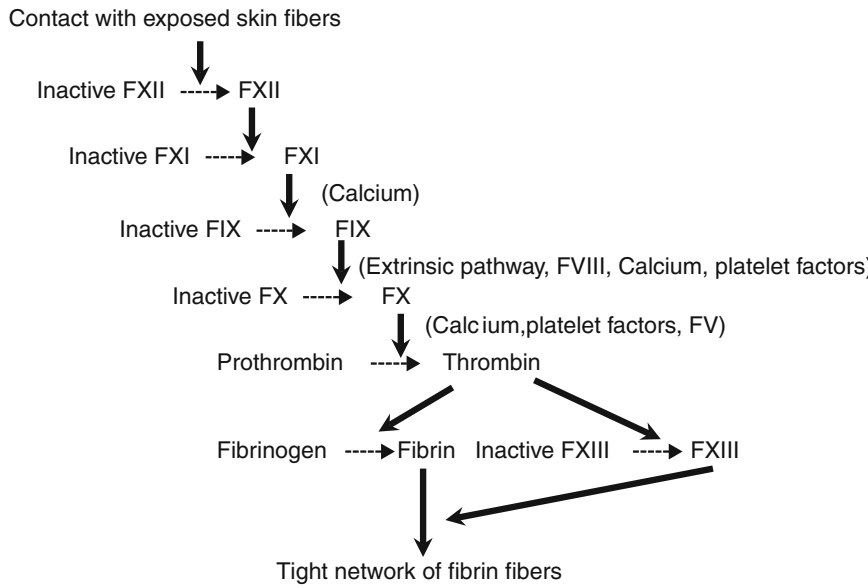

Fig. 12.1 Blood Coagulation Cascade: In the blood coagulation cascade factors VIII and IX play an important role in clot formation. The activated factors stimulate the conversion of other factors into their active state and so on, until fibrin fibers form a tight meshwork. Items marked by parentheses are also responsible for the activation step of indicated factors (Figure adapted from Germann and Stanfield (2005))

40% of cases of severe FVIII deficiency arise from a large inversion that disrupts the FVIII gene. Deletions, insertions, and point mutations account for the remaining 50–60% of HA defects. Human FVIII is a heterodimeric protein composed of an 80 kDa light chain and a 90–200 kDa heavy chain and circulates in the plasma complexed to von Willebrand factor (vWF). Extensive biochemical analysis has demonstrated that a 100-kDa portion of the heavy chain, termed the B domain, is not necessary for FVIII procoagulant activity and that B domain-deleted (BDD) FVIII has biochemical and functional properties similar to those of wild-type FVIII (VandenDriessche et al. 1999).

The process of blood coagulation occurs through a series of activating steps whereby one enzyme activates another enzyme that then activates another enzyme and so on (Fig. 12.1). FVIII acts in conjunction with factor IXa, phospholipids, and Ca^{+2} to activate factor X in coagulation cascade. Understanding the sequence of coagulation assists in deducing the correct treatment options for patients with a missing link in the sequence. Coagulating factors are produced in the liver by hepatocytes. The end products of the coagulation cascade are polymers of fibrin, which prevent blood loss upon injury by associating with aggregates of platelet cells. Factor VIII deficiency, dysfunctional FVIII, or FVIII inhibitors lead to the disruption of the normal intrinsic coagulation cascade, resulting in spontaneous hemorrhage and/or excessive hemorrhage in response to trauma. Symptoms of hemophilia can range from mild to severe depending on the amount of clotting factors present in blood. In general, patients with hemophilia bleed for a longer period of time than healthy individuals. Common symptoms include large bruises, spontaneous bleeding from gums or nose, tightness in joints, and blood in emesis or feces. Severe symptoms of hemophilia include bleeding in the joints, brain, or internal organs; profuse bleeding after injury or surgery; and anemia, which can lead to shock and death. Bleeding in the joints can develop into swelling that, over time, breaks down cartilage and bone and can progress to chronic joint pain and immobility.

Approximately, 30% of patients with severe HA develop alloantibody inhibitors that can neutralize FVIII. These inhibitors are typically immunoglobulin G (IgG), predominantly of the IgG4 subclass that do not fix complement and do not result in the end-organ damage observed with circulating immune complexes. They neutralize the coagulant effects of replacement therapy.

Present Treatments and Limitations

Hemophilias are genetic bleeding disorders for which there are still no cures. Current available treatments for hemophilia disease and their limitations are shown in Table 12.1. It may be noted that these mode of treatments do not offer cure from the ailment, only the disease associated adverse consequences are minimized. Three primary types of treatment methods are reported for patients with hemophilias A and B: plasma-derived products, recombinant factors, and gene therapy, as well as the therapies for patients who develop inhibitors: activated Prothrombin complex concentrates, recombinant factor VIIa, and immunosuppressive or immuno-tolerance inducing treatments.

Replacement therapy using plasma-derived concentrates or recombinant FVIII administered after bleeding episodes is the current mode of therapy for HA. Although prophylaxis with protein has been shown to significantly reduce spontaneous bleeds, treatment efficacy is limited by the short half-life of FVIII *in vivo*, high production

Table 12.1 Present methods and their limitations for the treatment Hemophilia

Treatment Options	Effectiveness	Limitations
Plasma-derived products	Generally effective	Reinjection necessary
	Widely available	Risk of pathogen transfer
	Cost effective	Formation of inhibitors
Recombinant factors	Generally effective	Longer recovery than plasma-derived factors
	Safer than plasma-derived products	Expensive than plasma-derived product
		Difficult to manufacture
		Formation of inhibitors (in some patients)
Gene Therapy	Theoretically promising therapy	Mutagenesis of inserted gene
		Expensive
		Surgical process may be necessary
		Formation of inhibitors (in some patients)
		Limited use in human subjects
aPCC for patient with inhibitors	Generally effective (not as effective as recombinant factor)	Possible resistant to treatment
		Risk of pathogenic infection
		Expensive due to reinjection
	No immune response	Risk of liver damage and cardiovascular problem
Recombinant VIIa for patient with inhibitors	Generally effective	Possible resistant to treatment
	No immune response	Other side effects
		Cardiovascular problems
Immunotherapy for patients with inhibitors	Generally effective	Strenuous treatment
	No immune response	Possible susceptibility to pathogen
		Infusion accompanied by side effects
		Expensive

costs, repeated intravenous administrations, and development of host antibodies to the therapeutic protein. The choice between the two most common therapies, plasma-derived products or recombinant FVIII or FIX, is still a dilemma for clinicians involved in the care of patients with hemophilia.

Gene therapy appears promising in treating hemophilia as the disease is caused by a single gene defect and a small increase in gene products could essentially transform a severe form of hemophilia into a mild one (Gan et al. 2006). Clinical studies show that about 20% of HA patients develop inhibitors to treatment (High 2006) and that these patients are difficult to treat (Mannucci 2003). The main concern of gene therapy is safety, immune response, germ line transmission, and insertional oncogenesis.

Liver transplantation cures HA, demonstrating that the liver is a major site of FVIII synthesis. Hepatocytes, LSECs or both have been proposed as site of FVIII synthesis. The main limiting factor for liver or hepatocyte transplantation is the availability of donor liver and immune-rejection. Further, sometimes repeated transplantation is required to maintain the therapeutic effects of the foreign tissue, which may augment the problem of unavailability of liver/hepatocytes.

Transdifferentiation on Bone Marrow Cells into Liver Cells

Transdifferentiation is direct conversion of one lineage committed progenitor/stem cells into mature cells of another lineage without dedifferentiating into repertoire of cells in earlier developmental stage. This new property of adult stem cells (ASCs) created enormous interest and thus opens up the possibility for the treatment of acute and chronic liver diseases. Animal studies have shown that different types of stem cells can regenerate liver. Among them, embryonic stem (ES) cells, induced-pluripotent stem (iPS) cells, and mesenchymal stem cells (MSCs) are most important. Whether stem cells should be directly transplanted in the liver before or after *in vitro* differentiation into hepatocyte-like cells will govern by their type and pathological conditions of the liver. ES and iPS cells often give rise to teratomas, if injected as such. Therefore, it is recommended that hepatic differentiation should be carried out prior to the transplantation of cells.

Transdifferentiation Process and Liver Regeneration

Petersen et al. (1999), for the first time, demonstrated that BM-cells can differentiate into hepatocytes. In his study, BM chimera was established in female rats using congenic male cells, and the liver was subsequently injured by administering 2-acetyl-aminofluorene (2-AAF) and carbon tetrachloride (CCl_4). The oval cells isolated from liver were found to be Y-chromosome bearing immuno-phenotypically resembled with hematopoietic stem cells (HSCs) (Petersen et al. 1999).

Wang et al. (2002) demonstrated that HSCs can transdifferentiate into hepatocytes without liver injury. This finding has major significance for understanding the mechanism driving the transdifferentiation process. It suggested that differentiation of BM stem cells and other hepatic stem cells (e.g. oval cells) into hepatocytes may differ significantly. Oval cells differentiate into hepatocytes only when the proliferation of the latter is impaired, whereas BM stem cells can differentiation into hepatocytes in undamaged liver (Wang et al. 2002). It was reported that a strong selective pressure is required to achieve therapeutic levels of liver regeneration by BM-derived hepatocytes (Mallet et al. 2002). Whenever, the precise stimuli that lead to transdifferentiation are defined, it should be possible to improve the engraftment of these cells into the liver parenchyma and consequently enhance the level and speed of repopulation. Even BM-derived stem cells are considered as a potential alternate source of liver cells and having a major advantage over hepatocytes because of their shortage, for the time being it is believed that hepatocytes are the best option for cell-based therapies in case of liver. It was shown that using normal adult hepatocytes about 50% repopulation of fumarylacetoacetate hydrolase ($FAH^{-/-}$) mice can be

achieved after 3 weeks of transplantation (Wang et al. 2002). In contrast, the same degree of repopulation was not achieved until 22 weeks after BM stem cells transplantation. Despite the efficiency of transplantation of adult hepatocytes, significant level of repopulation can be achieved only when a method of selection for the transplanted cells is applied. Perhaps, the transplantation of fetal hepatocytes may be another useful option. In contrast to adult hepatocytes, these cells proliferate for longer period and can repopulate a normal liver without selection pressure (Shafritz and Dabeva 2002).

Jang et al. (2004) showed that HSCs were converted into CK18 expressing hepatocytes within 48 h of co-culture in the presence of damaged liver. They used a trans-well apparatus to separate HSCs from injured liver tissue. Besides, several past studies showed that under specific conditions, BM cells are potential to differentiate into hepatocytes and thus play a major role in liver regeneration. In a mouse model, embryonic day 10.5, $CD45^+$ cells were found potential to differentiate into functional hepatocytes (Khurana and Mukhopadhyay 2008a). More interestingly, it was also found that BM-derived cells can transdifferentiate into hepatocyte-like cells *in vitro* in the presence of damaged liver serum (Khurana and Mukhopadhyay 2007, 2008b). The study also showed that a subpopulation of BM cells can migrate to the damaged tissue. *In vitro* studies demonstrated that the differentiation process is accompanied with the nuclear localization of hepatocyte nuclear factor (HNF)-4α in oncostatin M (OSM) dependent manner (Khurana and Mukhopadhyay 2010). OSM was shown to be critical in development of fetal liver and in liver regeneration following CCl_4-injury.

There are two different types of stem cells present in the BM: MSCs and HSCs. HSCs give rise to all lineages of blood cells. Khurana and Mukhopadhyay (2008b) showed in an *ex vivo* model for transdifferentiation of HSCs into functional hepatocytes, which engrafted in normal liver. This finding offers an alternative strategy for treating many liver ailments using autologous BM cells, hence avoiding immunosuppressive drugs. In another study, it showed that lineage-depleted oncostatin M receptor β-expressing ($Lin^-OSMR\beta^+$) mouse BM cells can directly differentiate into hepatocytes in a hepatic differentiation culture system (Khurana and Mukhopadhyay 2010). The transdifferentiation of BM cells into hepatocytes has created enormous interest in applying this process to the development of cellular medicine for degenerative and genetic diseases.

Cell-Based Therapy

Cell-based therapy using isolated primary hepatocytes (Ohashi et al. 2005; Tatsumi et al. 2008) or LSECs (Do et al. 1999; Kumaran et al. 2005; Follenzi et al. 2008) is suggested to treat clotting disorders. Factor VIII or IX are synthesized in the liver before it enters into circulation. Cell therapy is an achievable goal for HA because replacement of even 5% FVIII activity converts the disease to a mild form. Second, insights have been obtained in transplanting healthy cells by relevant methods into appropriate organs. Cell therapy rather than whole organ transplantation has advantages because cells from a single organ could be used for multiple individuals, isolated cells could be expanded, and cells could be derived from stem cells. Moreover, outcomes of cell therapy in HA could be readily determined by FVIII activity assays in blood samples. Third, exciting progress has been made in understanding where FVIII is made in the body.

Recent evidence showed that liver transplantation corrected HA; liver is recognized as the primary site of FVIII synthesis with immunohistochemical staining showing FVIII expression in both hepatocytes and LSECs. Transplantation of LSECs in peritoneal cavity corrected the phenotype of HA mice (Kumaran et al. 2005), indicating LSECs are capable of synthesizing functional FVIII protein in the liver, whereas transplantation of hepatocytes did not correct murine hemophilia (Fig. 12.2). We hypothesized that the partial replacement of mutated liver cells by healthy cells in HA mice could manage the severity of the bleeding disorder. We have shown

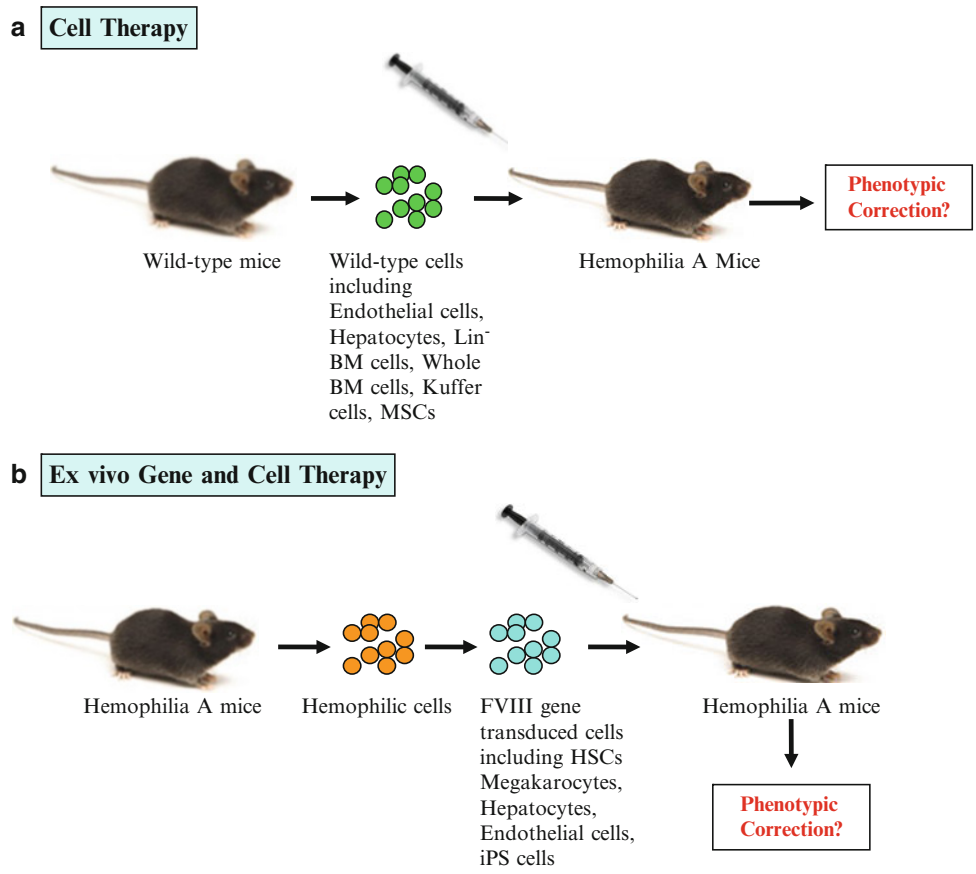

Fig. 12.2 Phenotypic correction of hemophilia A in mice after adoptive transfer of cells isolated from wild-type mice or FVIII gene-transduced cells isolated from hemophilia A mice. (**a**) Illustration of adoptive cell therapy. (**b**) Illustration of *ex vivo* gene and cell therapy

that lineage depleted (Lin^-) BMCs can correct the HA phenotype in mice by producing active FVIII protein (Yadav et al. 2009, 2012). Recently, Follenzi and colleagues demonstrate that transplantation of BMCs into HA mice partially restore FVIII production and protected HA mice from bleeding challenge (Follenzi et al. 2012). Whole bone-marrow cells were freshly isolated from wild-type mice and transplanted into recipient HA mice (Fig. 12.2). A majority of the treated mice (~70%) had average 8–12% circulating FVIII activity and survived bleeding challenge with correction of hemophilia. In recipient mice, the donor BM-derived mononuclear cells and mesenchymal stromal cells (MSCs) contributed to major FVIII gene expression and activity. Subsequently, it was also demonstrated that transplantation of Kuffer cells (KCs) or mesenchymal stem cells respectively isolated from wild-type mice (Fig. 12.2) diminished the mortality of recipient HA mice from excessive bleeding, indicating that these cells indeed produce a significant amount of functional FVIII.

Furthermore, previous *ex vivo* gene therapy studies using gene transfer vectors targeting HSCs, megakaryocytes, endothelial progenitor cells, and iPS cells achieved therapeutic levels of FVIII gene expression (Petrus et al. 2010) (Fig. 12.2). Identification of BM-derived cell types that contribute to the production of biologically functional FVIII will increase the therapeutic potential of the BM cells in adoptive cell therapy as well as *ex vivo* and *in vivo* gene therapy for the treatment of hemophilia.

Preclinical Studies for Hemophilia

Gene therapy can be use for the treatment of hemophilia A and B diseases, as stable expression of coagulation FVIII and IX may correct the bleeding diathesis. Hepatic gene delivery using vectors derived from adeno-associated virus (AAV) resulted in therapeutic but transient functional clotting FIX expression levels in severe HB patients. Lentiviral vectors are being explored for *in-vivo* hepatic gene delivery and for *ex-vivo* transduction of HSCs. This resulted in stable correction of the bleeding diathesis in hemophilic mice. Finally, nonviral vectors derived from transposons result in sustained clotting-factor expression in rodent models.

Small and large animal models of HA and HB diseases are available for preclinical testing. Different Phase I clinical trials were initiated for the treatment of haemophilia by gene transfer (Roth et al. 2001; Manno et al. 2003; Powell et al. 2003). Several different gene delivery systems were used in these trials, including a retroviral vector, an adenoviral vector, two different adeno-associated viral vectors, and a non-viral gene delivery method. VandenDriessche et al. (1999) also investigated an alternative approach to target dividing hepatocytes to infuse retroviral vectors into neonates, whose hepatocytes are naturally undergoing rapid cell division to fully correct FVIII deficiency in a murine model of HA. This approach was successfully extended to the canine model of HB by Xu et al. (2003), who achieved up to 3.5% of normal FIX activity levels following retroviral vector transduction of neonatal HB dogs. Long-term expression of clotting factors has been successfully achieved in large animal models of haemophilia using multiple gene transfer strategies, but these findings have not yet been translated into success in patients.

Earlier, we have reported therapeutic potential of BM-derived hepatocytes in phenotype correction of FVIII$^{-/-}$ mice. Uncommitted BM cells (Lin^{-}) were transplanted in the recipients whose liver were perturbed by administration of acetaminophen. Quantitative analyses of donor-derived hepatic and endothelial cells were carried out by flowcytometry. Approximately, 85% of GFP^{+} cells expressed albumin, whereas vWF was expressed in 4–8% of donor-derived cells (Yadav et al. 2009). Combining results of flowcytometry and immuno-histochemistry suggested that engrafted BM cells can differentiate into hepatocytes and LSECs, as shown in Fig. 12.3 (Yadav et al. 2009, 2012). The phenotypic correction in HA mice was determined by activated prothromboplastin time (aPTT) assay and tail-clip challenge experiment. The plasma FVIII activity in FVIII$^{-/-}$ mice was $0.7 \pm 0.25\%$ of wild type mouse, which was increased to $15.7 \pm 3.2\%$, $26.4 \pm 9.4\%$, and $19.1 \pm 5.1\%$ in transplanted mice after 5, 12, and 18 months of transplantation, respectively. The survival rate in transplanted mice was increased from 23 to 80% in tail-clip challenge experiment. In contrast, the majority of FVIII$^{-/-}$ mice did not survive more than 20 h of tail-clip (Yadav et al. 2009). Most interestingly, 9 of the 9 transplanted mice examined between 5 and 18 months of the study confirmed that BM cell therapy did not cause the formation of FVIII inhibitors. Overall, these findings were in agreement with the maintenance of secretory function of BM-derived liver cells and the ability of these cells to supply active FVIII in the diseased mice.

Problems and Controversies in BM Stem Cell Plasticity

Bone marrow stem cells therapy has opens exciting possibilities for the therapeutic use of the cells in the management of hepatopathic patients. These cells are easily obtained from living donors BM/peripheral blood, or umbilical cord blood, or other sources of adult stem cells. Allogenic, unrelated cells may pose problem of immune rejection. It can be overcome by concomitant replacement of both hematopoietic and hepatic systems from the same donor, which will induce immunological tolerance, preventing or reducing the risk of rejection (Sakamoto et al. 1999). Moreover, patients affected by inborn metabolism errors, their own HSCs could represent an easily accessible vehicle to deliver therapeutic

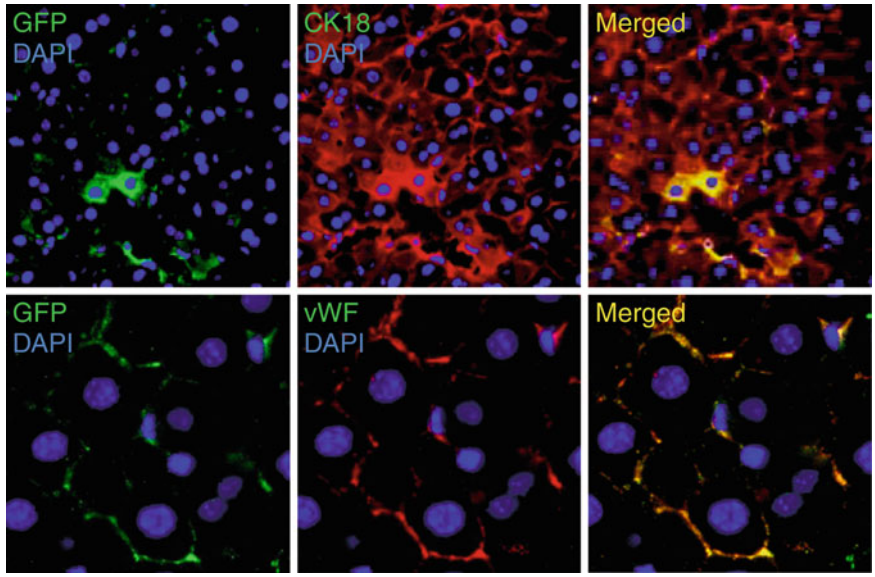

Fig. 12.3 Transdifferentiation of BM-derived stem cells into hepatocytes and liver sinusoidal endothelial cells in FVIII-KO mouse liver. Liver was perturbed by administering acetaminophen prior to the transplantation of Lin^- BM cells. Five months after transplantation, liver cryosections were stained with either anti-GFP and anti-CK18, or anti-GFP and anti-vWF antibodies. Representative images show expression of CK18 (hepatic marker) or vWF (endothelial marker) in BM-derived (GFP^+) cells. Magnifications: ×200 (*top panel*); ×600 (*bottom panel*)

genes to the liver, which overcome the problem of immune-rejection. The plasticity of adult stem cells represents an exciting challenge, but a number of issues have still to be addressed, these are graft versus host disease in allogenic setup and long time of hepatic transdifferentiation. Besides, there are some conceptual issues that may limit BM-stem cells therapy in the clinic, these are: (a) fibrogenic effect of BM-derived cells, (b) identification of competent cells and the differentiating microenvironment, (c) route of delivery of the cells, (d) fusion between donors with the recipient hepatocytes.

Fusion of cells may increase the risk of malignant transformation of the transplanted cells, which imposes a careful evaluation and longer follow-up periods for assessing the safety and efficacy of the therapy. Many reports have shown that the correction of liver disease is caused by fusion of donor BM cells with recipient hepatocytes, rather than direct differentiation (Wang et al. 2003; Vassilopoulos et al. 2003). However, we and others have shown that fusion is rarely occurs in liver regeneration by BM-derived stem cells (Jang et al. 2004; Yadav et al. 2009). In $FVIII^{-/-}$ liver perturbed mouse model, the results of fluorescent *in situ* hybridization and immunocytochemical analyses suggested that BM-derived cells can directly differentiate into hepatocytes (Yadav et al. 2009). Cell fusion was found to be a normal process during development. In adults, cell fusion used to take place at a low frequency in some tissues. Fusion of BM cells is not only seen in case of hepatocytes, but also observed in neurons and cardiomyocytes (Alvarez-Dolado et al. 2003). In these cases, the nuclear materials of the two partners undergo fusion in which polyploid fusion partner dominates the final outcome of the fused cells. The fused heterokaryons were reported to undergo ploidy reduction to become diploid hepatic cells (Wang et al. 2003; Duncan et al. 2009). Even though it is now widely accepted that transdifferentiation phenomenon is real, many questions still remain. This controversy demonstrate the challenges of studying adult stem cells and suggests that additional research using adult stem cells is necessary to understand their full potential as future therapies.

Summary

Hemophilia treatments are readily available in developed countries, however it is estimated that about 70% of the people with this disease worldwide are undiagnosed or under-treated. Treatment of hemophilias are difficult because patients need repeated infusion of missing coagulation factors, some patients develop inhibitors to the infused factors, and gene therapy is still not suitable for treatment. Cell-based therapies using isolated primary hepatocytes or LSECs are suggested to treat clotting disorders. Most recently, BM-derived liver cells, monocytes/macrophages and mesenchymal stromal cells can synthesize FVIII and correct bleeding phenotype in HA.

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Improved Renal Revascularization Outcomes in Pigs Using Stem Cells

13

Alfonso Eirin, Behzad Ebrahimi, and Lilach O. Lerman

Contents

Abstract

The incidence of atherosclerotic renal artery stenosis (ARAS) rises as the age of the population increases, and is accompanied with an increased risk for cardiovascular morbidity and mortality. Although the available data are limited and in part conflicting, they suggest renal angioplasty offers no additive benefit for preservation of renal function compared with antihypertensive therapy alone, which establishes the need for new therapeutic interventions in addition to revascularization to avoid consequences of disease progression. The therapeutic potential of endothelial progenitor cells (EPC) and mesenchymal stem cells (MSC) demonstrated in cardiovascular tissues motivated development of novel treatments in a number of kidney diseases. The purpose of this chapter is to provide a concise overview of the main characteristics of EPC and MSC and to summarize published data regarding their role in renal repair in experimental ARAS.

Introduction

Atherosclerotic renal artery stenosis (ARAS) remains the most common primary disease of the renal arteries and is being diagnosed with increasing frequency in individuals older than 65 years of age (Hansen et al. 2002). Importantly, ARAS is associated with impaired renal function and loss of kidney mass, being responsible for approximately 16% of new patients entering

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dialysis programs in the United States (US Renal Data System 1997). Apart from the risk for progression to end-stage renal disease, the high risk for cardiovascular events represents the major cause for morbidity and mortality in this population. For example, large follow-up studies showed higher relative risk even in patients with low-grade ARAS for long-term cardiovascular events compared to essential hypertensive subjects (Dechering et al. 2010). Similarly, prospective observational studies have identified a significant association between ARAS and coronary artery disease, cardiac dysfunction, and cerebrovascular disease (Chrysochou and Kalra 2009), underscoring the need for effective strategies to reduce associated risk and improve the prognosis of ARAS patients.

For many years it has been recognized that activation of the renin-angiotensin-aldosterone system plays a critical role in the pathophysiology of ARAS. Thus, antihypertensive therapy with angiotensin-converting-enzyme inhibitors (ACEi) or Angiotensin II receptor blockers (ARBs) has become the mainstay for the treatment of these patients. However, renal revascularization, most commonly performed by percutaneous transluminal renal angioplasty and stenting (PTRA) has been until recently a popular powerful treatment option in ARAS. Technical success rates for renal artery stent placement have been reported as high as 95% (Rundback et al. 1998) although some significant procedure-related complications have been described. However, the clinical benefit of revascularization for the treatment of ARAS raises considerable debate and data from large randomized controlled clinical trials have failed to identify major benefits from renal angioplasty for the preservation of renal function compared to medical therapy alone (Bax et al. 2009; Wheatley et al. 2009). In agreement, we have previously shown in swine ARAS that treatment with PTRA restores blood pressure, but fails to improve tubulointerstitial injury, microvascular rarefaction, and renal function in the stenotic kidney (Eirin et al. 2011). Taken together, these observations emphasize the need for more effective therapies in conjunction with PTRA to preserve renal structure and function, and improve outcomes after renal revascularization.

In recent years, experimental and clinical studies have illustrated the enormous potential therapeutic value of stem cells and progenitor cells in several diseases. Indeed, stem cell transplantation, which consists of the delivery of autologous or allogeneic stem cells in a diseased organism, has grown rapidly. Among the different types of stem cells described in the literature, circulating endothelial progenitor cells (EPC) and mesenchymal stem cells (MSC) possess unique abilities to provide reparative treatments for kidney diseases. The regenerative properties of EPC and MSC place them as new and exciting treatment approaches to restore cellular integrity and improve renal outcomes after revascularization. This chapter focuses on the role of EPC and MSC transplantation in combination with renal revascularization and discusses their place in the spectrum of current therapies for ARAS.

Endothelial Progenitor Cells

First described in 1997 (Asahara et al. 1997), bone marrow-derived circulating EPC are well recognized for their ability to promote blood vessel repair and neovascularization in ischemic tissues. These cells are characterized by the expression of both haemopoietic stem cell markers (CD34) and endothelial markers such as kinase insert domain receptor (KDR), von Willebrand factor, VE cadherin, or CD146. However, more immature haemopoietic stem cell markers such as CD133 have been used for their characterization ('early EPC'). These cells can be isolated from mononuclear cell fraction of peripheral blood and quantified by either flow cytometry (number of circulating cells positive for CD34/KDR) or colony-forming units in culture media (George et al. 2006).

The release of EPC from the bone marrow is regulated by a variety of growth factors. For example, studies from our group (Chade et al. 2010) identified release of key homing signals, stromal cell-derived factor (SDF)-1 and stem cell factor (SCF), that serve for EPC mobilization

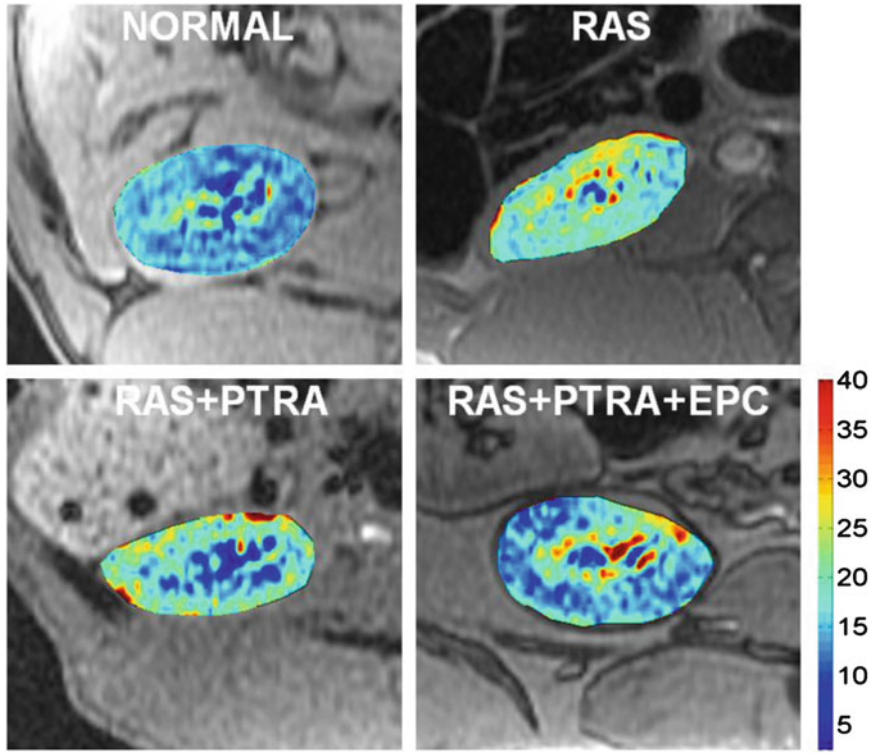

Fig. 13.1 BOLD parametric map in normal, RAS, RAS+PTRA, and RAS+PTRA+EPC pigs 10 weeks after RAS and 4 weeks after revascularization. Stenotic RAS kidneys show greater distribution of cortical green and yellow colors, suggesting hypoxia, which was slightly attenuated by PTRA, but markedly improved by PTRA+EPC

from bone marrow to the site of injury in a swine model of ARAS. Furthermore, we have recently shown that the post-stenotic human kidney retains CD34+/KDR+ EPC in parallel to the release of several inflammatory mediators and homing signals (including SDF-1and SCF), suggesting that these mediators are released by the injured tissue to attract and retain EPC to promote repair and attenuate loss of renal function in ARAS (Eirin et al. 2012a).

Considerable evidence from experimental studies shows that EPC rapidly mobilize after ischemia, conferring renoprotection in response to acute kidney injury (Kwon et al. 2010; Patschan et al. 2006). In line with these observations, we have previously shown in a swine model of non-atherosclerotic RAS that a single intrarenal infusion of autologous EPC restored the hemodynamics and function of the stenotic kidney, by normalizing renal blood flow (RBF) and glomerular filtration rate (GFR) (Chade et al. 2009). In addition, administration of EPC improved angiogenesis, preserved intrarenal microvascular architecture, and attenuated tubulo-interstitial fibrosis beyond the stenotic lesion, underscoring the feasibility and potential for EPC to rescue the kidney in chronic renovascular disease. Similarly, we further showed that intrarenal delivery of autologous EPC, in addition to PTRA, restored medullary oxygen-dependent tubular function, assessed by changes in deoxyhemoglobin after furosemide using blood oxygen level-dependent magnetic resonance imaging (BOLD-MRI) (Ebrahimi et al. 2012a) (Fig. 13.1). Notably, treatment with PTRA+EPC reduced fibrosis and inflammation, supporting EPC as an adjunct therapy to preserve renal outcomes in RAS after PTRA (Ebrahimi et al. 2012b).

There are several explanations for the potential mechanisms of renoprotection exerted by EPC. Considerable evidence shows that EPC

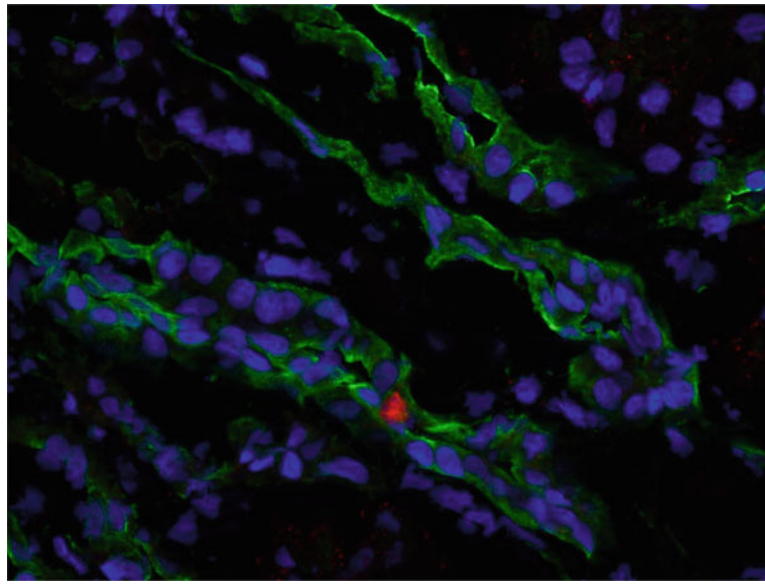

Fig. 13.2 Representative fluorescence of CM-DiI (*red*, 40×) EPC and cytokeratin (*green*) stained in the stenotic kidney 4 weeks after administration. *Blue*: DAPI nuclear stain. Injected EPC were incorporated into renal tubules

have the ability to engraft the damaged tissues. In line with these observations, we have previously shown in our swine model of ARAS that CM-DiI labeled EPC engrafted in medullary tubular structures 4 weeks after revascularization (Fig. 13.2) (Ebrahimi et al. 2012b). Furthermore, by using double fluorescence of CM-DiI and CD31 or cytokeratin staining, we were able to demonstrate that an important percentage of the injected EPC engrafted into blood vessels and tubules, assuming endothelial and tubular features (positive double staining with CD31 and cytokeratin) (Chade et al. 2009).

Previous studies have shown that the beneficial effect of EPC is mediated not only by their capacity for engraftment into damaged tissues, but also by transient secretion of vascular growth factors within this region (Rehman et al. 2003). In agreement, our experiments demonstrated that EPC isolated from both normal and RAS animals secrete vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) in culture media (Chade et al. 2009). Augmented availability of these potent angiogenic factors may have influenced the adjacent parenchyma, stimulating capillary proliferation and neovascularization.

Finally, recent data suggest that the regenerative effects of EPC on ischemic tissues could be attributed to the release of microvesicles. These small particles derived from the endosomal compartment play a key role in cell-to-cell communication by transferring proteins or RNA between cells (Deregibus et al. 2007). Cantaluppi and colleagues recently demonstrated in a rat model of ischemia reperfusion injury that microvesicles released from EPC exerted micro RNA-mediated protective effects characterized by a significant decrease in serum creatinine levels and by improvement in histological signs of microvascular and tubular injury (Cantaluppi et al. 2012). These observations suggest that micro-RNA shuttled by microvesicles may also contribute to their regenerative potential.

Mesenchymal Stem Cells

In contrast to EPC that need to be isolated from peripheral blood, MSC can be isolated from a variety of tissues, such as adipose tissue and bone marrow. This heterogeneous population of stromal cells has the ability to differentiate into a

broad spectrum of cell types, including fibroblasts, epithelial cells, osteoblasts, chondroblasts, and adipocytes (Asanuma et al. 2010). Among the markers expressed by MSC are mesenchymal markers (CD44, CD90, and CD105), but not endothelial (CD31) or inflammatory (CD14 and CD45) markers. Furthermore, their immunomodulatory and tissue-trophic properties that promote tissue repair and decrease inflammation (Marigo and Dazzi 2011), make MSC unique candidates to protect the damaged kidney.

The homing potential of circulating MSC is primarily regulated by their adhesion to the endothelium (Potapova et al. 2009), yet MSC migration to the site of injury appears to be modulated by a variety of angiogenic and inflammatory cytokines such as VEGF, transforming growth factor (TGF)-β, interleukin (IL)-8, neurotrophin-3, tumor necrosis factor (TNF)-α, platelet-derived growth factor (PDGF), and SDF-1 (Schichor et al. 2006). Once MSC reach the damaged tissue, they secrete several bioactive molecules, such as cytokines, antioxidant, proangiogenic, and trophic factors, and impair migration and proliferation of inflammatory cells, stimulating repair and limiting the immune response (Charbord 2010).

Previous data from experimental rodent models of acute renal failure have shown that administration of MSC accelerated tubular proliferation, restoring renal structure and function (Morigi et al. 2004). Similarly, studies in animal models of ischemia/reperfusion injury uncovered a protective effect of MSC achieved by stimulating renal parenchymal regeneration (Morigi et al. 2004). In agreement, we have shown in swine ARAS that intra-renal administration of adipose-tissue derived MSC improved renal function and structure after revascularization and reduced oxidative stress, apoptosis, fibrosis, inflammation, and microvascular remodeling in the stenotic kidney (Eirin et al. 2012b). The protective effect of MSC in our study was partly mediated by improvement of microvascular remodeling (Fig. 13.3) and cortical reduction of oxidative stress and inflammation beyond the stenotic lesion via paracrine mechanisms. In support of this concept, we have shown that MSC secreted VEGF and TNF-α in the culture media. Nevertheless, 4 weeks after their infusion, MSC were detected in renal tissue sections, mostly at the interstitium, confirming their potential to engraft the damaged tissue.

Endothelial Progenitor Cells or Mesenchymal Stem Cells?

Based on the data presented above, it is reasonable to speculate on which of these treatment approaches would confer more efficient renoprotection in ARAS. Although is evident that MSC possess several advantages (easier to obtain, immunomodulatory properties, potent paracrine mechanisms), EPC might enhance neovascularization more efficiently in than MSC (Aguirre et al. 2010). However, a recent study from our group demonstrated that EPC and MSC induced comparable restoration of the renal microcirculation in RAS, although by employing different mechanisms (Zhu et al. 2012). While EPC showed direct pro-angiogenic potency achieved by upregulation the expression of VEGF and its receptors, MSC induced a substantial decrease in apoptosis and endoplasmic reticulum stress-induced apoptosis by reducing the expression of caspase-3 and C/EBP homologous protein (CHOP) in tubular cells through mechanisms involving cell contact. Therefore, the mechanisms of action and pathophysiology of these regenerative strategies may need to be considered during selection of cell-based therapies to improve the damaged kidney.

Safety and Future Strategies

Several clinical studies have shown that EPC and MSC can be well tolerated in patients with cardiovascular diseases and have an excellent safety record (Mathiasen et al. 2009; Wang et al. 2007). Likewise, in our studies, we have not observed any evidence of cellular rejection, micro-infarcts, or tumors masses in tissue sections from cell-treated pigs up to 12 weeks after injection (Chade et al. 2009, 2010; Ebrahimi et al. 2012b; Eirin et al. 2012b; Zhu et al. 2012). However, the

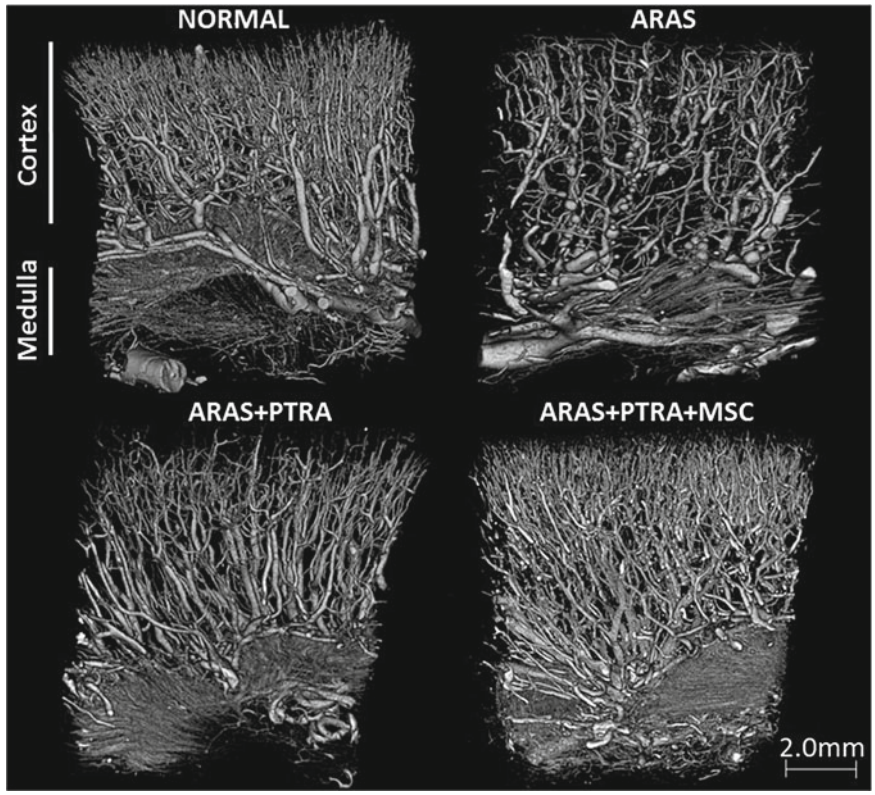

Fig. 13.3 Representative micro-CT 3D images (18 μm size voxels) of the stenotic-kidney showing improved microvascular architecture in ARAS animals treated with PTRA+MSC

ability to MSC to suppress the immune response and to promote tumor growth (Tolar et al. 2007) warrants long-term follow-up studies to determine the safety of MSC therapy.

Increasing evidence supports that modulation of ex-vivo culture conditions might potentiate the protective effects of cell-based approaches. For example, Peterson and colleagues demonstrated the beneficial effect of hypoxia preconditioning preserving MSC viability and function, through preservation of oxidant status (Peterson et al. 2011). Similar results were obtained after preconditioning with melatonin (Mias et al. 2008) or kallikrein (Hagiwara et al. 2008). Furthermore, manipulation of MSC to overexpress factors that increase their homing, adhesion, differentiation, or potency may also enhance their therapeutic potential (Chavakis et al. 2010). Results from these studies offer additional promise as novel therapies for improving MSC-induced renal protection.

Conclusion

The evidence available from a large number of studies identifies a potential for cell-based therapies for preservation of renal structure and function, alone or in conjunction to PTRA in experimental atherosclerotic renovascular disease. These studies suggested that the protective effects of EPC and MSC could be due to their ability to engraft the damaged tubules as well as secrete paracrine factors. No doubt, more experimental and clinical data, and appropriate guidelines are needed to define more precisely how to treat ARAS patients with either EPC or MSC.

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14 Generation of Autologous Multipotent Endothelial-Like Cells from Lipoaspirates of Human Adipose-Derived Stem Cells and Polymer Microarrays Technology: Potential Cardiovascular Regeneration

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Abstract

Endothelial progenitor cells (EPCs) are a small population capable of self-renewal which participate actively in vasculogenesis, angiogenesis and arteriogenesis. Blood-derived EPCs or bone marrow (BM)-derived stem cells feature several drawbacks as far as their clinical utility is concerned, such as the extremely small number of circulating EPCs in the bloodstream and the low availability and harvesting difficulties of BM-derived stem cells. In contrast, human adipose-derived stem cells (hASCs) can be isolated in a greater number through a safe non-invasive liposuction procedure. We have developed a new approach of easy-to-derive large number of multipotent endothelial-like cells (ME-LCs) from human adipose tissue in culture for long periods. ME-LCs displayed increased expression levels of endothelial and hematopoietic lineage markers and EPC markers. Moreover, they formed tube-like structures when grown on 2-D coated Matrigel™ surfaces, secreted increased levels of SDF-1 and showed the ability to migrate attracted by cytokines. Importantly, ME-LCs retained the capacity to differentiate into cardiomyocyte-like cells.

An emerging bioengineering research is the development of synthetic biopolymer matrices as defined environments for EPC growth.

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A family of biopolymers capable of promoting adhesion and differentiation of human EPC was identified and used to coat a 3-D scaffolds for the generation of blood vessels *in vitro*. We showed a notable difference in the process of vascularization between the scaffolds coated with biopolymers than the gold standard matrix support. These results suggested a possible application of such biopolymers for remedying to ischemic injury allowing the endothelialization of artificial endoluminal vessel of intravascular prosthesis devices.

Introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide. According to 2008 data from the World Health Organization died from this cause 17.3 million people, being 7.3 million due to heart attacks and 6.2 million to cerebrovascular accidents (Mendis et al. 2011).

Main strategies developed for healing or relief these CVD are based primarily on the use of pharmacological or surgical approaches. However, the discovery in 1997 of an adult population of CD34+/KDR+ hematopoietic progenitor cells, with ability to differentiate into endothelial cells *ex vivo* (Asahara et al. 1997), and the subsequent observation that these circulating endothelial progenitor cells (EPCs) could be derived from bone marrow (BM), have opened the possibility to use these cells, as promising candidates, in cell therapy for CVDs (Asahara et al. 2011).

Different studies show that the number, migratory activity and functionality of EPCs are significantly lower in subjects with hypercholesterolemia, hypertension or diabetes as well as smokers and elderly people (Asahara et al. 2011; Sen et al. 2011). In addition, it has been demonstrated an increase of circulating EPCs in the early stages after ischemic cardiopathy, after a cerebrovascular accident, or in a severe traumatic brain injury (Sen et al. 2011).

Currently, there are three main strategies for the clinical application of EPCs in CVD treatment:

(i) The development of treatments to improve EPCs mobilization, proliferation and/or functionality (Sen et al. 2011).

(ii) Cell transplantation, in fact several clinical trials based on EPCs transplantation have already be conducted providing evidence of their feasibility, safety and utility for the treatment of these pathologies (Asahara et al. 2011; Sen et al. 2011).

(iii) Tissue engineering through the implantation of biomaterials such as nanofibers, alginate or cardiac extracellular matrix (ECM) scaffolds to create a suitable environment for regeneration of damaged tissue, which is known as *in situ* tissue engineering. Another variant is based on the generation of tissue structures *in vitro*, where cells are growth on the biomaterial structures, and are subsequently implanted into the damaged tissue. For example, a two dimensional structure that mimic cardiac tissue characteristic have been generated and attached to the patient's beating heart without the need to use sutures or adhesives (Eschenhagen et al. 2012).

Although the use of EPCs to treat CVD seems to be a promising approach, there are some problems for their clinical use. EPCs are difficult to isolate from blood, due the reduced number of this cell population in the blood, and from BM due the invasiveness and discomfort of the procedure. In contrast, human adipose-derived stem cells (hASCs) can be easily isolated from lipoaspirates. hASCs can be expanded in culture and differentiate into different cell types, including endothelial cells, in a controllable and reproducible manner, and can be safely and effectively transplanted into an autologous or allogeneic host. Furthermore, the clinical potential of hASCs has been proved to be similar to the potential of BM derived mesenchymal stem cell (BM-MSC) (Strioga et al. 2012).

The term mesenchymal stem cells (MSCs) encompasses a heterogeneous group of plastic-adherent multipotent cells that share their capacity for self-renewal and differentiation into mesenchymal lineage cells, including bone, cartilage and adipose tissue. Moreover, MSCs are capable of differentiating into other cell types of mesodermal origin, such as muscle cells, endothelial cells or cardiomyocytes.

These cells reside *in vivo* in the vascular pericytes population present around the capillaries

and microvessels. In addition, MSCs show a transcriptome and a surface phenotype similar to pericytes, suggesting that they originate from pericytes. In normal physiological conditions MSCs appear as quiescent pericytes and may respond to tissue injury and contribute to the repair and regeneration. Likewise, it has been also postulated that MSCs may be related with the tunica adventitia of arteries and veins, supporting the notion that natural niche of the MSCs is the perivascular region (Corselli et al. 2012).

To facilitate the MSC characterization the International Society for Cellular Therapy (ISCT) proposed three minimum criteria for identification: (i) must be plastic-adherent, (ii) the cell population must submit expression levels ≥95% of CD105, CD73 and CD90, and lack of expression of the CD45, CD34, CD14 or CD11b, CD79α, CD19 or HLA class II markers, and (iii) the cells must be able to differentiate *in vitro* into several cell types under differentiation conditions.

Mesenchymal stem cells are used in regenerative medicine mainly based on their ability to differentiate into multiple cell types and their specific production capacity of soluble factors that promote the regeneration of damaged tissue by various mechanisms. 5-azacytidine (5-aza) has been proved to be a potent agent to induce *in vitro* differentiation of MSCs toward a cardiac phenotype. The combination of several factors such as insulin, dexamethasone and ascorbic acid have been also employed in various assays for cardiac differentiation of human MSCs (Kuraitis et al. 2011). In addition, co-culture of MSCs with cardiomyocytes and the use of cell extracts obtained from human cardiomyocytes are other successfully strategies to obtain differentiated cardiomyocytes from MSCs (Peran et al. 2011).

Recent studies demonstrated that MSCs are involved in postnatal vascularization, especially during ischemia of tissues and tumor development. It has been identified the signaling mechanism which vascular endothelial growth factor (VEGF) regulates mobilization, recruitment to sites of neovascularization and differentiation of MSCs into vascular cells. Although MSCs do not express VEGF receptors (VEGFR), they present many platelets derived growth factor receptors (PDGFR) which are stimulated by VEGF (Ball et al. 2010).

Mesenchymal stem cell differentiation toward vascular-like cells has been shown *in vitro* using medium supplemented with certain factors. Especially, VEGF induces endothelial differentiation of MSCs, with the ability to form capillary-like structures and to express endothelial lineage specific markers (Ball et al. 2010). Another methodologies consist in the co-culture of MSCs with mature endothelial cells (ECs) or under hypoxic conditions.

Endothelial Progenitor Cells: Characterization and Differentiation

Endothelial progenitor cells are a small population of circulating endothelial lineage cells found in the peripheral blood. These cells are poorly differentiated, are capable of self-renewal and actively participate in vasculogenesis, angiogenesis and arteriogenesis that occur during postnatal neovascularization, both physiological and pathological processes (Asahara et al. 2011). Upon vascular injury, EPCs are mobilized from the BM, by cytokine secretion such as vascular VEGF and SDF-1, in order to migrate and regenerate the damaged tissue (George et al. 2011; Kim and von Recum 2008).

Endothelial progenitor cells were identified for the first time by Asahara et al. (1997), calling them "angioblasts", and postulated a possible common origin with the hematopoietic stem cells (HSCs) (Asahara et al. 1997). There are different theories about the origin of EPCs and its relation with HSC: (i) the hemangioblast model, which proposes the existence of a common progenitor endothelial and hematopoietic, called hemangioblast, which would be originated from the mesodermal germ layer (ii) the hemogenic endothelium model, which postulates that the endothelium itself can undergo asymmetric divisions and originate both HSCs and EPCs, which would be released into the peripheral blood; (iii) or the hybrid model, which defends the existence of a hemangioblast that originate so HSCs as a hemogenic endothelium, which, in turn, serve as

a source for the generation of both EPCs and HSCs (Bautch 2011).

The characterization of EPCs is still controversial and, at present, there is no consensus with respect to phenotype of this cell population. EPCs were first isolated from human peripheral blood, based on their ability to express the surface markers CD34, and grown in culture surfaces coated with fibronectin to promote its adhesion (Asahara et al. 1997). One of the most important aspects to consider when identifying the EPCs is to distinguish them from the ECs, as both have a similar phenotype, and from the HSCs, that show some similarities due to their common origin. In this sense, like in HSCs, EPCs can be characterized as a population of cells expressing the surface markers CD34, CD133, CXCR4, KDR, Tie-2, FLT1 and CD105, among others (Asahara et al. 2011; Kim and von Recum 2008); but, unlike them, EPCs show low expression or a complete loss of the hematopoietic antigen CD45 and express endothelial cell-specific markers such as VE-cadherin and CD146. In addition to these markers, also the endothelial lineage is usually determined by the binding capacity to certain lectins such as agglutinin-1 of Ulex europaeus (UEA-1), the uptake of acetylated low density lipoprotein (Ac-LDL), or the intracellular expression of vWF (Kim and von Recum 2008).

Although these cells have been identified in BM, peripheral blood or umbilical cord; however, recent studies reveal that they can also be obtained from organs such as the liver, intestine, spleen, adipose tissue or the walls of blood vessels. Currently it is postulated that EPCs might be divided into two main categories depending on their origin: (i) hematopoietic EPCs derived from the BM and, in turn, can stimulate various cellular components of the blood causing a heterogeneous cell population composed of colony forming EPCs, no colony forming EPCs, myeloid EPCs and angiogenic cells, (ii) and non-hematopoietic EPCs, which are not related to HSCs and derive from different tissues or blood vessels from organs (Asahara et al. 2011).

It has been shown that the non-hematopoietic EPC type, known as endothelial outgrowth cells, do not originate from hematopoietic precursors CD45+ or CD133+. It has also been demonstrated that EPCs mobilized from organs, such as the liver or gut, to the blood derive from precursor cells with c-KIT+/CD45− phenotype residents in these tissues (Asahara et al. 2011). All these studies show the difficulty to establish a standard phenotypic characterization of EPCs, because it can be affected by both the origin and their maturation state.

The maturation of EPCs towards a differentiated lineage shows that during this process there is an increase of CD31, VE-cadherin and vWF expression, while occurs a progressive loss of expression in CD133 and CD34 antigens (Schatteman et al. 2007). It has also been determined that TIE-2 or CCR7 genes stop expressing, while DLL4 and CXCR4 gene expression is maintained and CDK2 gene increases (Igreja et al. 2008). This allows distinguishing different EPCs populations according to their state of differentiation, which are known as early EPCs and late EPCs (George et al. 2011).

One of the main features of the EPCs is their ability to EC differentiation. In this regard, it is known that VEGF, in addition to mediate mobilization and expansion of EPCs, is the main inducer of endothelial differentiation. This differentiation is favored *in vitro* using scaffolds or culture surfaces coated by an ECM (Mooney and Vandenburgh 2008). Studies also demonstrate that the *in vitro* application of a shear stress, defined as the force per unit area that the blood flow exerts on the vascular wall, causes the endothelial differentiation of EPCs. It has been observed that the application of this force to cultures suppresses the expression of genes encoding CD34 and CD133, and at the same time increases the mRNA expression of the genes coding for CD31 and vWF (Suzuki et al. 2012).

Besides the ability to differentiate EPCs to ECs, some studies indicate that these cells are capable of transdifferentiate to smooth muscle cells through a process of endothelial-mesenchymal transition (EndMT) when treated with TGF-β1 (Imamura et al. 2010). Furthermore, there is also evidence that EPCs might express cardiac markers *in vitro* by co-culturing with cardiomyocytes. Also, *in vivo*

experimental assays have demonstrated that EPCs can transdifferentiate into cardiomyocytes and participate in cardiac tissue regeneration after injury (Feng et al. 2012).

Regarding the relationship of EPCs with cardiomyopathy and vascular disease, studies showed that the number of circulating EPCs is inversely correlated with cardiovascular risk and they can be used as a prognostic marker of potential cardiovascular events (Sen et al. 2011). When a vascular accident occurs there is a decreased flow of oxygen in the tissue, that is detected by ECs through a protein prolyl hydroxylase domain (PHD) which hydroxylate different hypoxia inducible factors (HIF). These cells produce HIF-1α which in turn induces the production of factors such as VEGF and SDF-1 cytokines. The binding of SDF-1 to its receptor CXCR4 causes activation of ERK signaling pathway of phosphatidylinositol 3 kinase (PI3K)/AKT and increased eNOS expression, which are responsible for the migration of EPCs to ischemic site (George et al. 2011).

Furthermore, the main function of VEGF is to induce proliferation and differentiation of EPCs to EC in neovascularization sites. Other factors such as granulocyte-colony stimulating factor (G-CSF) or the granulocyte-macrophage colony stimulating factor (GM-CSF) also mediate migration of EPC to sites of new vessel formation (George et al. 2011). Moreover, EPCs migrating to these tissues do not always participate in the formation of blood vessels, but may also be resident in the interstitial tissue, releasing cytokines and pro-angiogenic growth factors such as VEGF, ANG-1, SDF or HGF 1. These cytokines act in a paracrine manner to promote local angiogenesis, proliferation and mobilization of ECs into the living tissues to restore homeostasis (Asahara et al. 2011).

Human Adipose-Derived Stem Cells

From all possible sources to obtain MSCs in the adult, the adipose tissue has attracted more interest since its discovery earlier this century. The plastic-adherent multipotent cells in this tissue have received different nomenclatures such as processed lipoaspirates cells, stromal cells derived from adipose tissue, fat-derived MSCs or human multipotent adipose-derived stem cells. So in 2004 the International Society Fat Applied technology agreed to use the "human adipose-derived stem cells" (hASCs) term to define this cell population.

These cells show certain advantages that make them more attractive from the point of view of potential clinical application respect to those obtain from BM: (i) adipose tissue is abundant and is found in all individuals; (ii) hASCs can be isolated in a greater number, up to billions of cells, through a safe non-invasive routinely liposuction procedure under local anesthesia; (iii) from the enzymatic disintegration is possible to obtain stromal vascular fraction containing different cell types including hASC, fibroblasts, endothelial cells, pericytes, smooth muscle cells and other circulating cells such as immune system cells and HSCs. The hASCs are isolated based on their capacity for adhesion to the culture plastic surface, showing a fibroblastic morphology when grow *in vitro*. These hASCs can also be expanded in culture and differentiated into different cell types, including ECs, in a controllable and reproducible manner, and can be safely and effectively transplanted into an autologous or allogeneic host (Strioga et al. 2012). Therefore, hASCs might be a promising source of progenitor cells that could be used for regeneration of vascular and/or heart lesions. Also, hASCs secrete multiple potentially synergistic pro-angiogenic growth factors including VEGF, HGF and chemokine SDF-1 which are likely to play a pivotal role for the hASC-mediated angiogenesis.

These cells meet the minimum requirements set by the ISCT to be considered MSCs, although they show some differences in their immunophenotype, proliferative capacity, differentiation potential, their transcriptome, proteome and their immunomodulatory activity compared to those obtained from bone marrow, being partly due to differences in the protocols used for isolation and cultivation (Strioga et al. 2012).

The procedure to isolate hASCs from adipose tissue begins with several washed using phosphate-buffered saline (PBS) to remove, as far as possible,

the blood present in the sample. Then, it is digested with type I collagenase and after centrifugation it can be obtained a high density cell pellet without mature adipocytes which is plated on flask cultures. The characterization of these cells shows expression of CD105 (>99%), CD90 (>90%) and CD73 (>99%) surface markers and a lacked expression for both hematopoietic and EC markers CD45, CD34, CD133, CXCR4 and KDR (Marchal et al. 2012).

Multipotent Endothelial-Like Cells: Isolation and Expansion

Recently, our group has developed a new approach of easy-to-derive large number of ME-LC through a series of consecutive stages (Marchal et al. 2012). Firstly, hASCs were cultured in DMEM-FBS (stage I), and then, culture medium was replaced by serum-free medium for 3 weeks (stage II), which resulted in the appearance of sphere cluster formations (SCF) that increased in number and size throughout the subsequent culture stages. After that SCF were cultured in SFO3 medium (stage III) composed by RPMI-1640: DMEM: F12, 0.1% bovine serum albumin (BSA) and 2-mercaptoethanol and in EC medium (stage IV) composed by endothelial basal medium-2 (EBM-2, Lonza) containing 5% FBS, and human recombinant vascular endothelial growth factor (VEGF), hydrocortisone, human recombinant epidermal growth factor (rhEGF), human recombinant long R insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, human recombinant basic fibroblast growth factor (rhFGF) and gentamicin sulfate-amphotericin-B EGM-2 (Fig. 14.1). Previously, Hirashima et al. (2003) demonstrated that a chemically defined serum-free culture system, including 2-mecarptoethanol, have the ability to support the proliferation of endothelial cells and their progenitors from mesoderm cells (Hirashima et al. 2003). Cells cultured in stage III and IV were considered as ME-LC because they increased the expression of EPC and hematopoietic markers (CD34, CD133, KDR, CXCR4, and CD45). The coexistence of hematopoietic and endothelial markers in the ME-LC is indicative of a phenotype resembling early vascular progenitors since it has been reported the existence of the hemangioblast, capable of giving rise to both hematopoiesis and vascular endothelium (Bautch 2011). In contrast to these cells, HUVEC mature endothelial cells were negative for CD34 and CD133 progenitor markers and strongly positive for CD31. Both CD34 and CD133 antigens are lost upon differentiation of endothelial progenitors to endothelium (Schatteman et al. 2007).

High levels of expression of the MSC markers CD105, CD73 and CD90 were observed throughout the different stages of the ME-LC isolation procedure. Interestingly, the CD90 MSC marker, which expression is practically absent in HUVEC, was the marker which expression most significantly decreased when ME-LC were cultured in SOF3 medium (stage III), suggesting endothelial differentiation. In agreement, it has been shown a loss of CD90 antigen expression on MSCs by angiogenic stimulation *in vitro* induce angiogenic differentiation without experimenting a complete endothelial maduration (Marchal et al. 2012).

All together, these data suggest that ME-LC isolated from hASCs cultures express markers resembling a vascular progenitor phenotype. Gene expression profile confirmed the endothelial progenitor phenotype with the expression and maintenance of specific genes involved in self-renewal, cell cycle promotion and antiapoptotic such as has been recently showed in cord blood (CB)-derived EPC. CXCR4 and CD133 vascular genes were expressed at similar levels throughout the several culture stages. Nevertheless, genes expressed in differentiated EPCs such as *Cdk2* and *Flt-1* (Igreja et al. 2008) showed a weak expression level in ME-LC cultured in SOF3 medium. Another interesting result was the disappearance of *Tie-2* gene expression, only detected at stage I, which has been previously reported as an angiogenic factor clearly induced in the differentiated ECs (Furuhata et al. 2007). In addition to endothelial cells, Tie2 is expressed in a subpopulation of HSC being, in part, responsible of maintaining a quiescent state in the bone marrow niche.

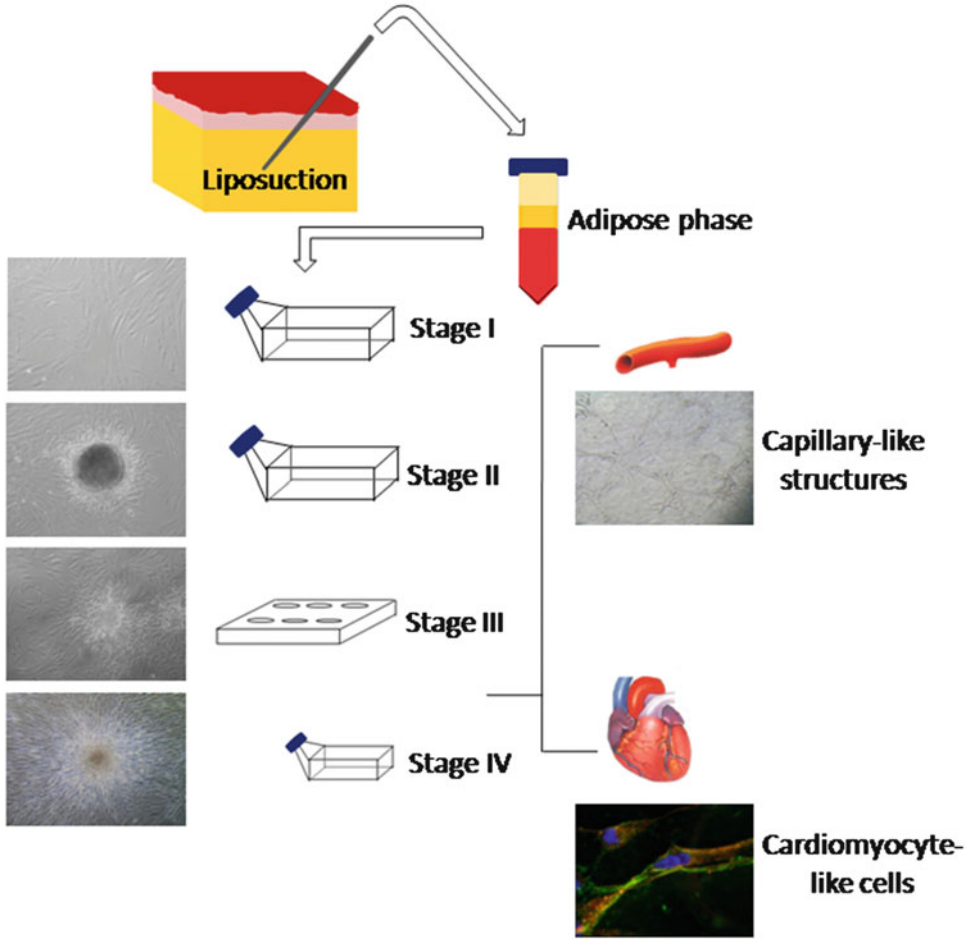

Fig. 14.1 Culture and differentiation of ME-LCs. hASCs were obtained from liposuction procedure. These cells were cultured along different stages in diverse media: DMEM-FBS (stage I), DMEM free-serum (stage II), SFO3 medium (stage III) and EC medium (stage IV). Cells obtained from stage III and stage IV were considered ME-LCs. These cells are capable to form capillary-like structures similar in comparison with the data rendered by HUVEC and differentiated to cardiomyocyte-like cells with the acquisition of a cardiogenic phenotype and the expression of cardiomyocyte-specific markers such as troponin T (*red*) and desmin (*green*)

Ability of Multipotent Endothelial-Like Cells to Enhance Functional Capillary-Like Structures Formation

The ability to form capillaries of ME-LCs was assayed using cells obtained from each stage and were cutured independently on Matrigel™ plates and EGM-2 medium. Cells from cultures at stage I and II were not able to form any capillaries over a 7 days period. On the other hand, cells previously grown in SFO3 or EGM-2 (stages III and IV, respectively) displayed a large number of capillary-like structures as early as 4 h after being seeded and the appearance of capillary tube-like structures increased overtime. After 7 days in culture a well-established cellular network was present in all cell cultures. By stage IV, the number of capillary-like structures was similar in comparison with the data rendered by HUVEC, used as positive control (Marchal et al. 2012).

Recent studies have been shown that VEGF stimulation of HUVEC induces DLL4

expression which reduced vessel sprout length in a 3D tubulogenesis assay, confirming that DLL4 signaling inhibits angiogenesis. DLL4 expression seems to acts as a switch blocking endothelial cell proliferation and allowing induction of a more mature differentiated phenotype (Harrington et al. 2008). Isolated ME-LCs showed a down regulation of this ligand, which correlated with the early formation of large number of capillary-like structures and the late formation of a vascular network.

Moreover, ME-LCs showed an increased secretion of SDF-1 in comparison with hASCs, even when they were seeded for 2 weeks on Matrigel™. In addition, the role of SDF-1 to induce migration of CD133+/CD34+/KDR+ cells has been shown (Peichev et al. 2000). Results demonstrated how the ME-LCs, which express the SDF-1 receptor CXCR4, were able to migrate towards a SDF-1 gradient (Marchal et al. 2012). These data suggest the potential homing of ME-LCs to sites of vascular injury for tissue repair.

Ability of Multipotent Endothelial-Like Cells to Differentiate into a Cardiac Phenotype

The potential of ME-LCs to differentiated toward a cardiomyocyte phenotype following exposure to 5-aza has been tested. These cells were seeded on Matrigel™-coated plate in EGM-2 medium. This culture medium was replaced 2 weeks later by EBM-2 containing 5–10 or 15 μM of 5-aza for 24–48 h and changed back to EGM-2. Then these cells are cultured for 3–4 weeks.

Following exposure to 5-aza ME-LCs retained the capacity to differentiate into cardiomyocyte-like cells with the acquisition of a cardiogenic phenotype and the expression of cardiomyocyte-specific markers. Morphological changes consisted in the appearance of wide, branching and multinucleated cells resembling cardiac muscle cells. The expression of typical cardiomyocyte markers such as troponin-T and α-sarcomeric actinin and the presence of a desmin filaments network, which take part in regulating the mesodermal specification into cardiomyocytes (Fig. 14.1) (Labovsky et al. 2010), support the cardiomyocyte differentiation potential of ME-LCs.

Polymer Microarrays – Powerful Technology to Identify New Synthetic Matrices for Endothelial Progenitor Cells

Since the discovery of circulating ECs in peripheral blood a decade ago (Asahara et al. 1997), regenerative stem-cell-based therapeutic strategies have aimed at both developing novel therapies and improving current treatments by restoring ischemic tissues (Chan and Mooney 2008). Traditional methods of direct cell infusion or injection (which represent the most commonly utilized cell delivery strategy in the clinic – for example, BM transplants) often lead to big issues such as massive cell death, extremely poor homing/engraftment efficiency to targeted tissues and loss of control over the fate of transplanted cells (Karp and Leng Teo 2009). The introduction of biomaterial scaffolds as cell carriers provide a solution to these problems, due to their templating capability for guiding the formation of new tissue and for promoting engraftment within the host (Eschenhagen et al. 2012; Hench and Polak 2002). An emerging area of bioengineering research is, therefore, the development of synthetic biopolymer matrices as defined environments for EPC growth, providing sites of adhesion together with signals that can control EPC propagation and synchronize their differentiation (Mooney and Vandenburgh 2008).

This chapter describes a high throughput screening of polymer microarrays (Pernagallo and Diaz-Mochon 2011) for the identification of new polymer matrices which contain the necessary signals that not only promote adhesion and propagation of EPCs but also, synchronize their endothelial specialization. Due to the complexity and diversity of biocompatibility testing, it has been difficult to find a universal method which permits high throughput determination of *in vitro*

properties. It is impossible to theoretically predict cellular response, and thus every time a new material is generated it is essential to test its biocompatibility using cells with which it will come into contact. To address this issue the development of a new system of screening, based on microarray technology has been developed. Polymer microarrays accelerate the process to find new biomaterials while minimizing expenditures regarding time and money. Furthermore, as these polymers are synthetic animal-free materials which can be manufactured to GMP standards they overcome one of the biggest issues, in terms of regulation and human health safety, of current industry standards, such as Matrigel™, which have animal-source origins. Therefore, they can provide clinically useful materials for the construction of extra-corporeal devices and facilitate novel studies in modern vascular surgery.

Pre-formed and fully characterized polymer libraries were printed using a robotic printer onto agarose coated glass-slide following a defined xy pattern (Pernagallo and Diaz-Mochon 2011). These arrays were then used to identify new substrates for attachment, promotion, and propagation of EPCs which at the same time would keep cell functionality (Fig. 14.2 a, b).

Cord blood (CB) products (50 mL) were aspirated from umbilical placental veins from normal caesarean deliveries and collected into heparin (Pernagallo et al 2012). Mononuclear cells (MNCs) were isolated by buoyant density centrifugation and cultured over 3 weeks in endothelial basal medium-2 (EBM-2). By that time, spindle-shaped EPCs started to emerge. These cells express a number of endothelial markers comparable to a mature EC and human umbilical vein endothelial cells (HUVECs) and they are able to form new blood vessels in Matrigel™ (Skovseth et al. 2007). Thereafter cells were passaged until incubation with the polymer microarrays. As control HUVECs, which are specialized ECs, were also maintained in cell culture. At that point EPCs and HUVECs were removed from their substrate and plated onto two identical polymer microarrays containing 345 polyurethanes and polyacrylates, printed in quadruplicate given rise to 1,345 feature arrays. After 3 days of culturing, under standard cell culture conditions, cells were fixed and stained with a nuclear stain (Hoechst 33342), and conjugated monoclonal mouse anti-human CD31-PE antibody (Fig. 14.2c, d). CD31 (Anti-PECAM-1) recognizes the platelet/endothelial cell adhesion molecule-1 (PECAM-1), a 130–140-kDa single-chain integral membrane glycoprotein that is a member of the immunoglobulin gene superfamily (Pinter et al. 1997). The CD31 antigen is expressed on ECs, functions as a vascular cell adhesion molecule and is involved in the process of leukocyte migration through the intercellular junctions of vascular ECs (Delisser et al. 1997). Following fixing and staining, arrays were scanned using a High Content Screening Platform (Nikon 50i fluorescence microscope (×20 objective) with an automated XYZ stage, equipped with the Pathfinder™ software package – IMSTAR) fit with two fluorescence channels (DAPI and Cy3). Cell numbers on spots were determined by analyzing images captured using DAPI channel (Hoechst 33342ex/em 355/465 nm) while endothelial phenotype was assessed via Cy3 channel (R-PE ex/em 545/575 nm).

Primary screening identified 6 polyurethanes and 12 polyacrylates that supported attachment of EPCs providing over 150 cells/mm^2. After looking at initial data with more detail it was found out that 3 out of those initial 18 hits (3AA7, 8g7 and 9g7) provided to be excellent substrates, with over 200 cells/mm^2, for both EPCs and HUVECs. These results supported the idea that 3AA7, 8g7 and 9g7, as well as enabling high cell adhesion could enhance endothelial maturation of EPCs towards specialized cells such as HUVECs. In order to study the endothelial function in further detail and confirm the results of these experiments, the three selected polymers (3AA7, 8g7 and 9g7) were scaled-up and spin-coated onto glass cover slips which were then used to grow both EPCs and HUVECs for flow cytometry analysis. EPCs and HUVECs cultured on polymer – coated surfaces, expressed endothelial cells surface markers vWF, VEGFR2 (also referred to as Flk-1 or KDR), endothelial nitric oxide, synthase (eNOS), CD31, CD34, CD105 and CD146 but not the hematopoietic cell surface

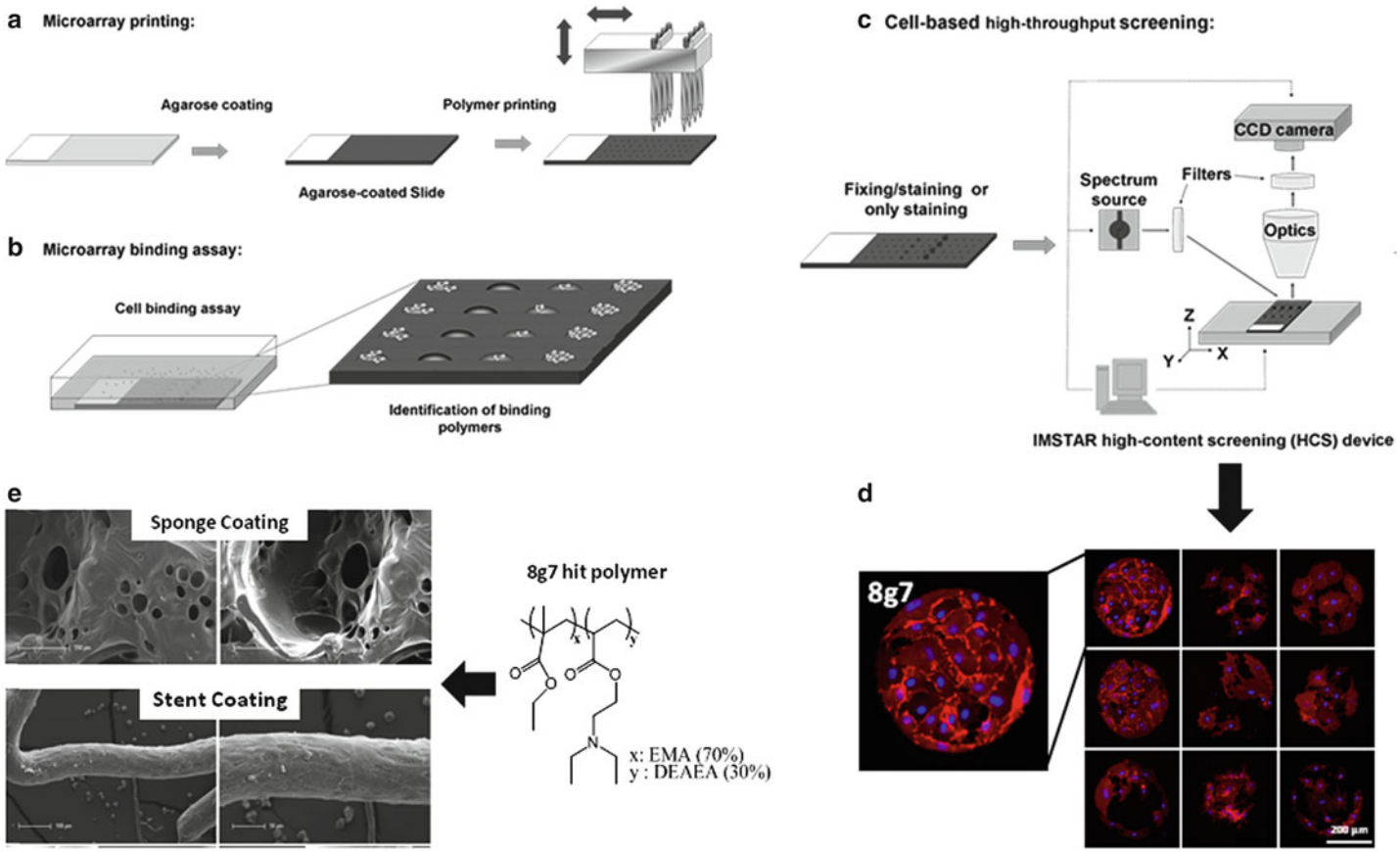

Fig. 14.2 Finding novel synthetic materials compatible with endothelial progenitors cells and their direct application for coating extra-corporeal devices. (**a**) Polymer microarray preparation; (**b**) cell-based microarray binding assay; (**c**) cell-based high-content screening; (**d**) fluorescence images from a polymer microarray incubated with EPCs and stained with Hoechst 33342 and CD31-PE antibody and (**e**) extra-corporal devices – sponges and stent – coated with a polymer – 8g7(see *insert*) – identified via polymer microarray approach (**a**–**d**) incubated with EPCs

antigens CD45. Therefore, flow cytometry results confirmed HTS data, thus demonstrating that new substrates, identified via polymer microarray approach, enhance specialization of EPCs (Pernagallo et al. 2012).

Generation of Coated Devices to Make Them Biocompatible with Endothelial Progenitor Cells

Further studies were undertaken by cultivating EPCs on pieces of sponges coated with a solution of 8g7 (Pernagallo et al. 2012). The ultimate aim of studying sponge as a cell scaffold was to generate functional 3-D tissues outside of the organism, which might then be implanted into the organism. In an ideal scenario 8g7 coated sponges would have the ability to adhere EPCs in a *in vitro* fashion before being implanted into animals so that they can either promote the growth and the specialization of those cells previously anchored to the sponge as well as recruit distant BM-derived progenitor cells for restoring vascularization at the implanted scaffold/host tissue interface. One cm^3 sponges were dip-coated in a 1% w/w solution of 8g7 in THF and further sponges were also dip-coated in the current gold standard, Growth Factor Reduced Matrigel™ (GFR-MG). Polymer coated sponge showed a smoother uniformly coated layer allowing a friendlier environment for cells to grow. The coated sponge was impregnated with either EPCs or HUVECs in complete EBM-2 medium that supports endothelial cultivation. After overnight incubation, cells were fixed and SEM analysis carried out. SEM images clearly showed that only sponges pre-coated with polymer allowed tube formation while sponges pre-coated with GFR-MG lacked tube formation features (Fig. 14.2d). It was also clear that by increasing number of EPCs adhered to the 8g7 sponge, a major release of both cytoprotective and pro-angiogenic factors in a paracrine manner to promote the survival and proliferation of endothelial cells happened (Gnecchi et al. 2008). The functional 3-D tissue generated *in vitro* was tested for its ability to promote cell growth and recruit distant BM-derived progenitor cells for promoting engraftment with the host, after implantation. Therefore, sponges were pre-treated with 8g7 and loaded with EPCs *in vitro*. Following initial incubation, these sponges were implanted into animals. Each animal had a EPCs-loaded 8g7 impregnated sponges on one side and a control EPCs loaded native sponge on the other. Twenty days after implantation, mice were sacrificed and sponges were excised and analyzed for vessel formation. Sponges were divided into three pieces and analyzed as follows: SEM imaging; Chalkley count and hemoglobin assay (Pernagallo et al. 2012).

After implantation in mice, the sponges impregnated with 8g7 promoted better engraftment within the host, showing clearly the formation of a vascular network in contrast with the blank (uncoated) sponge. Notably, uncoated sponge resulted in a poor and incomplete engraftment within the host, showing the native sponge without cells at several points. It seems evident that the large number of EPCs adhered to the 8g7 sponge, allowed not only a large quantity of local cell growth but also made possible a major release of cytokines which recruited distant BM-derived progenitor cells that effectively mediated vascularization at the implanted scaffold/host tissue interface. Histological analysis revealed that significantly increased numbers of vessels formed on the polymer coated sponge compared with the control (sponge uncoated). One part of the sponge was fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with haemaoxylin/eosin for identification of blood vessels. Sponge vessel density was determined by using the mean of triplicate Chalkley counts on two sections per sponge. Histology showed vascularization by a number of blood vessels but in polymer coated sponges had significantly more blood vessels compared to non polymer coated sponges. A further study focused on measuring hemoglobin levels of the sample. This is an excellent method of determining the frequency of blood vessels, as they are proportional to the blood hemoglobin concentration. Polymer coated sponges had significantly more hemoglobin, displaying ~twofold increase compared to uncoated polymer sponges.

A clear application was the coating of stent used commonly to treat ischemia related pathologies. A notable step forward in the treatment of ischemia related pathologies occurred in the1990s with the introduction of metal scaffolds, stents, capable of giving the stenotic vessel the necessary mechanical support to avoid collapse (Fanelli and Aronoff 1990). The introduction into medical practice of such devices revolutionized the field of interventional cardiology, bringing notable improvements in prevention of restenosis. In spite of the fact that stents are capable of eliminating elastic recoil of the stenotic vessel, however, their implantation and the consequent trauma to the vessel walls initiates an excessive proliferation of neointima and, therefore, an in-stent restenosis.

An initial negative reaction following implant of the stent is due to the raised thrombogenicity of the metal link of which it is made. Therefore, a first intuitive approach is aimed at improving the biocompatibility of the implant through there coating of the stent with polymer film (Tepe et al. 2006).

In order to study adhesion of endothelial outgrowth cell to the polymer-coated stent, 8g7 was used (Pernagallo et al. 2012). A coronary stent was dip-coated in the 8g7. The control was a coronary stent in its native form. EPCs were removed from their substrate and re-incubated onto both, a 8g7-coated stent or native stent and cultured over-night in conditions that support EC survival. In order to assess cellular adhesion and the utility of 8g7, following incubation cells on both stents were fixed and SEM performed to asses cell morphology. EPCs maintained on the uncoated stent showed poor cell attachment, whereas EPCs maintained on the 8g7-coated stent exhibited a consistent adhesion with around 80% of confluence (Fig. 14.2d). These data exemplify the value of 8g7 and EPCs in an alternative setting for improvement in angioplasty therapy.

Conclusions

In summary, these data indicate that subcutaneous adipose tissue may be a useful source of autologous ME-LCs with the capacity to maintain their vascular progenitor properties. Future studies should explore further the functional *in vitro* and *in vivo* mechanisms of ME-LCs along with their therapeutic potential, since these data suggest that these cells may prove to be a valuable tool for vascular and cardiac repair.

Moreover, results presented in this chapter suggest a possible application *in vivo* of polymer technology as a potential method for remedying ischemic injury. Artificial vessel prostheses generated *in vitro* coated with 8g7 was implanted in mice with a notable difference in the re-endothelialization in contrast to the native sponge. The reason for this is unclear but might have been due to the fact that there was already a large quantity of cells growing on the sponge. Alternatively, it might have been caused by the fact that those cells attracted BM-derived progenitor cells through the release of cytokines. The capacity of 8g7 to bind EPCs suggests the possibility of using this polymer for coating stents. These cells might be isolated from blood from the patient and used to adhere and cover the stent pre-coated with the selected polymer *in vitro*. At this point the stent could be reintroduced into the patient avoiding the problems mentioned above. It was demonstrated that a stent can easily be coated with 8g7, and, as a consequence of cell adhesion, an EPCs layer was generated *in vitro*. This method might, therefore, offer a future means of reducing the process of thrombogenesis and in-stent restenosis and improving angioplasty therapy. Finally, actually studies are ongoing to analyze the ability of 8g7 to bind ME-LCs obtained from lipoaspirates which could have interesting clinical application.

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Adipose-Derived Stem Cells as a Novel Tool for Future Regenerative Medicine

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Abstract

Stem cell-based therapies for the repair and regeneration of damaged tissues and organs offer a paradigm shift that may provide alternative therapeutic solutions for a number of diseases. The use of either embryonic stem cells or induced pluripotent stem cells in clinical situations may be still limited due to cell behaviors, ethical considerations, and genetic manipulation, even though these cells are theoretically highly beneficial. Adipose-derived stem cells (ASCs) seem to be an ideal population of stem cells for practical regenerative medicine since they are plentiful, of autologous tissue origin and thus non-immunogenic, and are more available due to minimal ethical considerations. Furthermore, recent basic research and preclinical studies have revealed that the use of ASCs in regenerative medicine is not limited to mesodermal components, but extends to both ectodermal and endodermal tissues and organs, even though ASCs are mesodermal in origin. This chapter will describe the biology of ASCs and their proliferation and differentiation capacities, and will summarize the current preclinical and clinical data from a variety of medical fields on the use of ASCs in regenerative medicine.

Introduction

Promising bioengineering technologies such as tissue engineering, which is an interdisciplinary field involving physicians, engineers and scientists,

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may provide novel tools for reconstructive surgery. A number of life-threatening conditions, such organ failure, tissue loss due to trauma, cancer, or congenital structural anomalies, can be treated by current clinical procedures/surgical strategies that include organ transplantation, autologous tissue transfer, and the use of artificial materials. However, these treatments have potential limitations, including organ shortages, donor site morbidity in harvesting autologous tissues, allergic reactions, and immune rejection. Recent developments in the emerging field of stem cell science, stem cell-associated growth factors, and regenerative medicine may allow the use of stem cells to repair tissue damage and, eventually, to replace organs.

A stem cell is characterized by its ability to self-renew and to differentiate along multiple lineage pathways. Candidate stem cells include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells. ESCs are capable of extensive self-renewal and expansion, and have the potential to differentiate into any type of somatic tissue. These traits make human ESCs promising for future use in regenerative medicine. The iPSCs are derived from differentiated cells such as skin fibroblasts and appear to have the same potential and properties as ESCs; however, their generation is not dependent upon a source of embryos. As such, although the therapeutic potential of both ESCs and iPSCs is enormous due to their self-renewal and pluripotent features, there are limitations to their practical use such as the regulation of teratoma formation, ethical considerations, and immune responses to ESCs, as well as difficulties with genetic manipulation of iPSCs. By contrast, adult stem cells are, by their nature, immunocompatible, and there are no ethical concerns related to their use.

Multipotent mesenchymal stem cells (MSCs) are non-hematopoietic cells of mesodermal origin that are present in a number of postnatal and adult organs and connective tissues. To date, MSCs with characteristics similar to bone marrow-derived MSCs have been isolated from different tissue sources including trabecular bone, periosteum, synovial membrane, skeletal muscle, skin, pericytes, peripheral blood, deciduous teeth, periodontal ligament and umbilical cord. Although the stem cell populations derived from these sources are variable, common problems include limited amounts of harvested tissues and low numbers of harvested cells. With such a limited supply of stem cells, *ex vivo* expansion or further manipulation would be required to obtain a sufficient quantity of cells for clinical applications, and the efficacy and safety of such procedures would require verification before they could be used clinically.

Adipose tissue is present in abundance in many stores throughout the body, where it plays a major role in energy balance and displays strong endocrine functions. The multipotent stem cells within adipose tissue, termed adipose-derived stem cells (ASCs) (Zuk et al. 2001), are one of the most promising stem cell populations since human adipose tissue is ubiquitous and easily obtained in large quantities with little donor site morbidity or patient discomfort. Therefore, the use of autologous ASCs both as research tools and for cellular therapeutics is feasible, and ASCs are both safe and efficacious, as shown by preclinical and clinical studies of injury and disease. To date, the biology of ASCs has been described, preclinical studies of the use of ASCs in regenerative medicine have been performed, and the efficacy of ASCs has been determined in several clinical trials. Therefore, this chapter will highlight recent progress in the preclinical and clinical application of ASCs in various medical fields, as well as summarize our understanding of ASC biology, and discuss future directions for the use of ASCs with particular reference to the field of regenerative medicine.

Localization and Isolation of ASCs

Adipose tissue is a highly complex tissue consisting of mature adipocytes, which constitute more than 90% of the tissue volume, and a stromal vascular fraction (SVF), which includes preadipocytes, vascular smooth muscle cells, endothelial cells, fibroblasts, resident monocytes/macrophages, lymphocytes and ASCs. Many of the characteristics of ASCs may differ according

to the location of the harvested adipose tissue. The density of stem cells in adipose tissue is also variable and is a function of location, tissue type, and species. In white adipose tissue, ASC yields are greater in subcutaneous deposits compared to visceral fat, with the highest concentration of ASCs in arm adipose tissue deposits. ASCs from inguinal adipose tissue deposits exhibit the greatest plasticity (Prunet-Marcassus et al. 2006). ASCs are also found within brown adipose tissue deposits distributed within white fat deposits, and these ASCs readily undergo skeletal myogenic differentiation.

Stem cell yields are greater from adipose tissue than from other stem cell reservoirs, which is another significant factor in their suitability for use in regenerative medicine. Approximately 1×10^7 adipose stromal/stem cells can be routinely isolated from 300 ml of lipoaspirate with greater than 95% purity (Boquest et al. 2006). The average frequency of ASCs in processed lipoaspirate is 2% of nucleated cells, and the yield of ASCs is approximately 5,000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, compared with estimates of approximately 100–1,000 CFU-F per milliliter of bone marrow.

Subcutaneous adipose tissue can be easily obtained under local anesthesia. Current methods for isolating ASCs rely on collagenase digestion followed by centrifugal separation to isolate the SVF from primary adipocytes. The ASCs exhibit a fibroblast-like morphology and lack the intracellular lipid droplets observed in adipocytes. Isolated ASCs are typically expanded in monolayer culture on standard tissue culture plastics with a basal medium containing 10% fetal bovine serum.

Cellular Characteristics of ASCs

Freshly-isolated SVF cells are a heterogeneous cell population that includes putative ASCs (CD31−, CD34+/−, CD45−, CD90+, CD105−, and CD146−), vascular smooth muscle cells or pericytes (CD31−, CD34+/−, CD45−, CD90+, CD105−, and CD146+), endothelial progenitor cells (CD31+, CD34+, CD45−, CD90+, CD105−, and CD146+), and hematopoietic cells (CD45+) in uncultured conditions (Zimmerlin et al. 2010). In addition, freshly-isolated SVF cells and early passage ASCs express higher levels of CD117 (c-kit), HLA-DR, and stem cell-associated markers such as CD34, and lower levels of stromal cell markers such as CD13, CD29 (β1 integrin), CD44, CD63, CD73, CD90, CD105 and CD166 compared to ASCs from later passages (Bailey et al. 2010). While the consequences of the decrease in CD34 expression in later passage ASCs are not clear, there is at least one study demonstrating that CD34 expression can be maintained during 20 weeks of culture (Yoshimura et al. 2006). In addition, several studies have shown that CD34+ ASCs have a greater proliferative capacity, while CD34- ASCs have greater plasticity.

As indicated above, ASCs share many cell surface markers with pericytes and bone marrow-MSCs. In addition to those mentioned above, the pericyte markers expressed by ASCs include smooth muscle β-actin, platelet-derived growth factor receptor-β (PDGF-β), and neuro-glial proteoglycan 2 (NG2), while the markers shared by ASCs and MSCs include CD13, CD29, CD44, CD58 and CD166. The exact location of ASCs within adipose tissue remains elusive; however, several studies have suggested that ASCs may exist within the perivascular tissue since they express cell surface antigens that are similar to pericytes.

Culturing of ASCs eventually results in the appearance of a relatively homogenous population of mesodermal or MSCs. However, the different techniques used for harvesting ASCs greatly affect the proportions of various cell types that accompany them. Furthermore, cell culture conditions markedly affect ASC gene expression profiles; particularly important are the medium used and the mechanophysiological environment (e.g., three-dimensional culture, the imposition of mechanical force on the cells, and the degree of oxygenation).

ASCs exhibit elevated telomerase activity. Although the activity is lower than that in cancer cell lines, the level of telomerase activity indicates

that ASCs have the capacity for self-renewal and proliferation (Jeon et al. 2011). Finally, Puissant et al. reported that human ASCs lack HLA-DR expression and have immunosuppressive properties (Puissant et al. 2005). Based on such findings, preliminary clinical data demonstrated that severe steroid-refractory acute graft-versus-host disease (GVHD) could be treated with human ASCs from HLA-mismatched donors.

Proliferation Capacity of ASCs

The proliferative capacity of ASCs is greater than that of bone marrow-derived MSCs. The doubling time of ASCs during the logarithmic phase of growth ranges from 40 to 120 h, and depends on donor age, tissue type (white or brown adipose tissue) and location (subcutaneous or visceral), harvesting technique, culture conditions, plating density, and media formulations. The younger the donor, the greater the proliferation and cell adhesion of the ASCs. With repeated passaging, the cells gradually lose their proliferative capacity. Cell senescence of ASCs, based on β-galactosidase activity, is similar to that of bone marrow-derived MSCs.

The proliferation of ASCs can be stimulated by application of individual growth factors such as fibroblast growth factor (FGF-2), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), or tumour necrosis factor α (TNF-α). Particularly, FGF-2 is an effective growth-stimulating factor and is required for the long-term propagation and self-renewal of ASCs via the extracellular signal-related kinase 1/2 (ERK1/2) signalling pathway (Zaragosi et al. 2006). The proliferation of ASCs can be also stimulated by PDGF via activation of Jun amino-terminal kinase (JNK) and by Oncostatin M via activation of the microtubule-associated protein kinase/extracellular signal-regulated kinase and the JAK3/STAT1 pathways. Proliferation of ASCs is also enhanced by multiple growth factors, which can include any of the single growth factors mentioned above supplemented by thrombin-activated platelet-rich plasma, human platelet lysate and human thrombin (Kakudo et al. 2008).

ASCs are generally considered to be stable in long-term culture. ASCs that underwent more than 100 population doublings retained a normal diploid karyotype (Rodriguez et al. 2004). One report suggested that human ASCs undergo malignant transformation when passaged for more than 4 months, but recent reports show that spontaneous transformation of MSCs may be due to cross-contamination with malignant cell lines such as fibrosarcoma and osteosarcoma lines. Since the issue of spontaneous ASC transformation is still controversial, further experiments and discussion are required, and careful manipulation of ASCs and long-term observation of patients after clinical use of ASCs are essential.

Differentiation Potential of ASCs to Various Lineages

Numerous reports have demonstrated that ASCs possess the potential to differentiate into a variety of cell lineages both *in vitro* and *in vivo*. Although ASCs are obviously of mesodermal origin, it is now clear that they can differentiate not only into mesodermal lineages, but also into ectodermal and endodermal lineages.

ASCs can differentiate into many mesodermal lineages, including adipogenic, osteogenic, chondrogenic, myogenic, cardiomyogenic, angiogenic, tenogenic and periodontogenic lineages, and tissue regeneration studies with suitable scaffolds and growth factors in an appropriate external environment have been conducted. Survival of fat tissue grafts is limited and, thus, is associated with inconsistent clinical outcomes. The transplanted fat graft loses volume over time due to tissue resorption that can result in the loss of 20–90% of the original graft volume. Graft loss is mainly attributed to a lack of vascularization within the new tissue, leading to cell necrosis. Recently, the use of grafts containing ASCs and aspirated fat, called cell-assisted lipotransfer (CAL), was reported (Matsumoto et al. 2006). In theory, this type of approach could accomplish several desired effects, including (1) direct differentiation of ASCs into adipocytes as a reservoir for adipose tissue turnover; (2) direct differentiation of ASCs into

endothelial cells and, subsequently, an increase in blood supply to the grafted fat tissue, thereby decreasing the rate of graft resorption; (3) release of angiogenic growth factors by ASCs and induction of angiogenesis; (4) protection of the graft from ischemic reperfusion injury by ASCs; and (5) acceleration of wound healing at the recipient site.

There have been a number of publications on ASC-based bone engineering and regeneration since the first report showing ASC-driven osteogenesis *in vivo* (Cowan et al. 2004). Different reports have examined various features of bone regeneration, including different scaffolds and methods for osteogenic induction. Currently, several scaffolds are being used in preclinical bone engineering studies, including hydroxyapatite, beta-tricalcium phosphate (β-TCP), polylactide (PLA), polyglycolide (PGA), poly (lactic-co-glycolic) acid (PLGA), anorganic bovine bone (ABB), and composite scaffolds. Exogenous administration of bone morphogenetic protein-2 (BMP-2) is also promising for bone tissue engineering and regeneration.

Cartilage has unique histological characteristics due to its abundant extracellular matrix, large water content, and low cell density. These characteristics allow restoration of the cartilage after compression or extension following tissue deformation. In articular cartilage, chondrocytes and adjacent connective tissue cells receive stresses, including high hydrostatic pressure, distortional stress, and changes in osmotic pressure due to weight bearing and joint loading. These stresses alter the production of cartilage-specific proteoglycans (aggrecan), collagen type II, and hyaluronan. Various procedures have been developed using MSCs and cell culture technology to generate cartilage *in vitro*. The pellet culture technique results in the formation of 3-dimensional structures and mimics pre-cartilage condensation, which is as important as cell-cell interactions among chondrocytes in preventing dedifferentiation. Although it is still difficult to construct 3-dimensional neo-cartilage from ASCs, both *in vitro* and *in vivo*, direct transplantation of ASCs, pre-induced toward chondrogenesis, into a cartilage defect area such as an articular surface or an intervertebral disc, has resulted in cartilage regeneration as evidenced by the expression of type II collagen and aggrecan (Ganey et al. 2009).

Cell-based therapies and regenerative medicine approaches in the cardiovascular field are highly sought after since heart disease is still the leading cause of death in humans. To address this problem, numerous studies have investigated the potential of using either the SVF or ASCs to treat acute myocardial infarctions and chronic heart failure using animal models (Bai et al. 2010). Methods for administering ASCs into the heart can be divided into three categories: direct injection into the cardiac wall, intracoronary administration using a catheter, and patch grafting of an ASC sheet onto cardiac muscle. Regardless of the administration method, functional evaluation after an ASC engraftment generally shows improved thickness of the left ventricle anterior wall, and improvement of the left ventricle ejection fraction and cardiac output compared to the control group. ASCs secrete significant quantities of angiogenic and anti-apoptotic factors, including hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), which led to a series of *in vivo* studies focused on evaluation of their therapeutic potential. Recently, human ASCs were shown to be superior to human MSCs in mediating angiogenesis in a mouse ischemic hind limb model (Kim et al. 2007).

There have been a few studies describing the differentiation of ASCs to ectodermal lineage cells. Monolayer cultures of ASCs treated with retinoic acid express the epithelial marker cytokeratin 18, while ACS cultured on a fibrin matrix in the presence of EGF express the epithelial markers E-cadherin and cytokeratin 8. ASCs can also differentiate into neuronal or neuronal precursor cells, both morphologically and functionally, under appropriate culture conditions. Several interesting preclinical studies have been conducted, in particular, for the treatment of stroke and spinal cord injury. Following a stroke due to brain ischemia or hemorrhage, the intravenous administration of ASCs resulted in both functional and histological improvement, as confirmed by a reduction in brain water content, brain atrophy and glial proliferation in a

hemorrhagic stroke model (Ikegame et al. 2011). In addition, a recent study revealed that when ASCs are administered intravenously in a spinal cord injury model, they migrate to the injured spinal cord, partially differentiate into neurons and oligodendrocytes, and eventually restore locomotor functions (Kang et al. 2006).

ASCs also differentiate into endodermal lineage cells. Several reports have revealed that ASCs have the potential to differentiate into hepatocytes as evidenced by the presence of HGF and FGF-1 and 4 (Banas et al. 2009). In theory, ASCs could be used to reduce liver inflammation and treat liver fibrosis by differentiating directly into hepatocytes or by secreting factors such as angiogenic, anti-apoptotic, anti-inflammatory and anti-fibrotic factors. In addition to hepatic differentiation, the exposure of ASCs to nicotinamide, activin-A, exendin-4, HGF and pentagastrin resulted in the production of pancreatic-like ASCs that were capable of insulin, glucagon and somatostatin secretion. Pancreatic duodenal homeobox-1 (PDX-1) is a transcription factor expressed in beta and delta cells of the islets of Langerhans and in dispersed endocrine cells of the duodenum. Previous reports have shown that ASCs can differentiate into insulin-producing cells *in vitro* when they are either transfected with a PDX-1 expression vector or cultured under a specific differentiation protocol (Chandra et al. 2009). Transplantation of such differentiated ASCs into streptozotocin-induced diabetic animals reduced blood glucose levels and increased glucose tolerance, suggesting that ASCs are a suitable candidate for future diabetes mellitus cell-replacement therapies.

The Role of Soluble Factors from ASCs in Regenerative Medicine

Analyses of the soluble factors released from human ASCs have revealed that, at relatively early passages, cultured ASCs secrete HGF, VEGF, transforming growth factor (TGF)-β, IGF-1, basic fibroblast growth factor (bFGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, interleukins 6, 7, 8, and 11, adiponectin, angiotensin, cathepsin D, pentraxin, pregnancy zone protein, retinol-binding protein, and CXCL12 (Salgado et al. 2010). Moreover, the soluble factors secreted by ASCs can be modulated by exposure to different agents; for example, HGF expression is increased after the cells have been exposed to bFGF, EGF, or ascorbic acid. ASCs, once transplanted into tissues/organs *in vivo*, secrete several growth factors in specific conditions, which can provide paracrine stimulation to the host tissues/organs being regenerated. Thus, when ASCs are transplanted into inflammatory or ischemic regions, they may actively secrete these growth factors, thereby significantly promoting wound healing and tissue repair.

Immunomodulatory Effects

One potential application of ASCs involves harnessing their immunomodulatory effects to treat allergic disorders, autoimmune diseases, and GVHD (Cho and Roh 2010). In a rat allergic rhinitis model, for example, intravenously-injected ASCs migrated to the surface of the nasal mucosa, inhibited eosinophilic inflammation, modulated T-cell activity, and eventually reduced the allergic symptoms. At the immunoglobulin level, IgE and the IgG1/IgG2a ratio, representing the Th2 immune response, were significantly decreased by intravenous administration of ASCs, while IgG2a levels, which indicate the Th1 immune response, were significantly increased by ASCs. In addition to allergic rhinitis, collagen-induced arthritis in DBA/1 mice was also reduced by the systemic administration of ASCs through a similar mechanism involving the suppression of effector T-cells. Bone marrow-derived MSCs can ameliorate steroid-resistant severe GVHD after hematopoietic stem cell transplantation. Similarly, ASCs can ameliorate steroid-resistant severe GVHD after hematopoietic stem cell transplantation (Fang et al. 2007). However, since none of the preclinical studies examining the effects of ASCs on GVHD have been published, further studies are needed to examine the mechanisms and to determine the long-term effects of such treatment.

Other Therapeutic Applications of ASCs

Wound healing is a complex process that involves the coordinated efforts of many types of cells and the cytokines they release. Recent breakthroughs in understanding the roles played by ASCs in wound healing and tissue regeneration have provided new options for treating difficult wounds. Preclinical studies in a murine model have shown that the topical administration of autologous ASCs in a type I collagen sponge matrix accelerated the healing of diabetic ulcers in an animal model (Nambu et al. 2009). The healing mechanism may be due to the release of several growth factors, including VEGF and HGF, and to subsequent angiogenesis and proliferation of keratinocytes or dermal fibroblasts since there is no direct evidence that ASCs differentiate into epithelial cells or dermal fibroblasts *in vivo*. In addition to wound healing, ASCs injected into a skin flap can extend its survival both directly, by differentiating into endothelial cells, and indirectly, by promoting angiogenesis via secretion of angiogenic growth factors (Lu et al. 2008).

Because current therapeutic options are limited, periodontal tissue regeneration using autologous stem cells may be promising as a future cell-based therapy for periodontal disease. Tobita et al. have reported that a mixture of ASCs and platelet-rich plasma (PRP) implanted into a periodontal defect induced almost complete regeneration, not only of the cementum, the surface of the dentin, and the alveolar bone, but also of the periodontal ligament, which is the most difficult component to regenerate in both murine and canine models (Tobita et al. 2008).

Clinical Trials Using ASCs

The first-in-man clinical trial of ASC-mediated tissue repair was presented as a case report that described the repair of a calvarial bone defect with autologous ASCs in a child (Lendeckel et al. 2004). A 7-year-old girl, who exhibited wide calvarial defects after a severe head injury with multifragment calvarial fractures, received decompressive craniectomy for refractory intracranial hypertension followed by resection of the re-implanted bone due to chronic infection. Thereafter, the patient underwent reconstructive surgery where not only autologous cancellous bone from her ilium but also ASCs were simultaneously engrafted onto the critical calvarial defect. Recently, maxillary bone that can tolerate dental implants was successfully engineered using autologous ASCs with β-TCP and BMP-2 (Mesimaki et al. 2009).

In the fields of plastic, reconstructive, and aesthetic surgery, adipose tissue engineering and regeneration holds great promise for breast reconstruction post-mastectomy, breast augmentation for cosmetic purposes, soft tissue augmentation, and the improvement of contour deformities due to trauma, cancer excision, and congenital anomalies. Recently, a soft tissue augmentation procedure using ASCs combined with lipoaspirate, a technique called CAL, was developed. In clinical trials, CAL has been used in breast reconstruction after partial mastectomy, in breast augmentation for cosmetic purposes, and in the correction of hemifacial atrophy (Yoshimura et al. 2008). Thus far, there are several hundred cases where CAL has been performed for breast augmentation, and the resorption rate was estimated to be 20–40%.

Based on preclinical studies using ASCs in wound healing, synthetic dermal substitutes containing ASCs have been applied clinically for wound bed preparation. Exposure to radiation causes cellular damage, and the histopathology of radiation injury is progressive obliterative endarteritis, which leads to severe tissue ischemia. Several studies have demonstrated the beneficial effects of human MSCs, including ASCs, on radiation-induced complications (Akita et al. 2010). Rigotti et al. examined the use of autologous lipoaspirates in the treatment of tissue damaged by post-mastectomy radiotherapy (Rigotti et al. 2007). In this study, the lipoaspirate helped to regenerate healthy tissue at the damaged sites, resulting in improved healing in 19 of 20 patients. Here, ASCs were not isolated and used to enrich the lipoaspirate. However, based

on current understanding of ASC biology, it is likely that the beneficial effects of the lipoaspirate injections were due to the SVF cells, rather than to the mature adipocytes. Furthermore, in patients suffering from Crohn's disease, as well as in non-Crohn's disease patients, complex fistulas were healed following direct injection of ASCs and a fibrin glue sealant into the digestive tract wall, and no adverse effects were observed (Garcia-Olmo et al. 2009).

Many other clinical trials using ASCs for different purposes can be found in public clinical trial registries (Mizuno et al. 2012). Some results have been published and further insight into ASC biology and potential therapeutic uses awaits the publication of the results of additional trials.

Safety Concerns and Future Directions for ASCs in Regenerative Medicine

Generally, the use of adult stem cells, including ASCs, in cell-based therapy is considered safer and more practical than either ESCs or iPSCs, as adult stem cells are autologous, immunocompatible, and do not elicit any ethical controversies or involve genetic manipulations. Another issue to be addressed with the use of ASCs is cellular transformation. The long-term culture of MSCs prior to use results in significant changes in cell cycle kinetics, decreases in telomerase activity, and karyotype abnormalities. These changes increase the potential risk of transformation to malignant cells (Izadpanah et al. 2008). However, compared with other sources of stem cells, it is unlikely that there will be a need for long-term culture for ASC expansion *in vitro*, as adipose tissue is plentiful and ASCs can be harvested in larger quantities than other adult stem cell populations. Secondly, there is still a lack of information on the optimal dose of ASCs in targeting any disorders. In addition, the timing, frequency and route of administration of ASCs still remain elusive since ASCs administered intravenously are likely trapped in the lung microcirculation. Longer follow-up animal experiments and multi-center randomized clinical trials will be necessary to clarify such controversies in the future.

During the past decade, both preclinical studies and clinical trials using ASCs have been conducted, from cardiovascular research to applications for corneal diseases. ASCs are classified as adult multipotent stem cells and, as such, their multipotency is limited compared with ESCs and iPSCs. In addition, relatively few clinical trials in a limited number of areas have been conducted to assess the therapeutic potential of ASCs, compared with the large number of published preclinical studies. However, ASCs have practical advantages in clinical medicine because adipose tissue, the primary source of ASCs, is abundant and easy to obtain with few donor site morbidities. Further preclinical and clinical studies are needed to determine whether ASC-based therapies can fulfil expectations and be used successfully to treat disorders for which current medical and surgical therapies are either ineffective or impractical.

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Repairing the Stroke-Damaged Brain: From Neural Stem Cells to Tissue Engineering

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Michel Modo and Andre Massensini

Contents

Abstract

Stroke remains the main cause of adult disability with only very limited treatment options. Cell therapy is gradually emerging from the bench to the bedside, but the specific conditions under which administered cells can be effective remain to be defined. This translation needs to be seen in the context of endogenous repair processes which to some extent involve endogenous neural stem cells which reside in the sub-ependymal zone bordering the lateral ventricles and the sub-granular zone of the hippocampus, as well as local changes involving astrocytes, microglia and endothelial cells. The integration of cells with biomaterials also opens the opportunity for considering a replacement of lost tissue that leaves a cavity in the infarct area. Together, these approaches present new perspectives to repair some damage caused by stroke and eventually to improve outcome of patients for a better quality of life.

Introduction

Stroke is a devastating brain injury affecting approximately 795,000 Americans every year, but survival rates in the past decade improved dramatically (20.7%) due to faster access to medical centres and the development of acute stroke centres, specialized in stabilizing patients (Go et al. 2013). Still, stroke remains the main cause of adult disability in industrialized nations

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and its incidence is rapidly increasing in developing countries. Annual costs in the US alone exceed $74 billion and by 2050 are estimated to be around $2.3 trillion, due to an increase in our aging population, as well as unhealthy sedentary lifestyles (Stepanova et al. 2013).

Disappointingly, very little progress has been achieved to treat stroke once it occurred. As stroke is caused by a blockage or rupture of a blood vessel, the longer this vessel remains occluded, the more brain cells will die (i.e. time is brain). Providing an early re-perfusion of the blood vessel therefore has the greatest impact in preserving brain tissue. Indeed, within the first 6 h after stroke, in 3% of patients, thrombolysis can achieve a re-perfusion of the ischemic tissue and significantly reduce the impact of damage. Although beyond this time window neuroprotection of dying neurons was efficacious in preclinical models of stroke, none of these have succeeded in clinical trials. Crucially, the size of the therapeutic effect dramatically decreases with a delay in treatment with very little benefit seen beyond 24 h after infarction (Saver et al. 2013).

Despite the dim outlook on pharmacological interventions, patients gradually show some improvements after stroke over a period of approximately 12 weeks (Carmichael 2003). This spontaneous recovery occurs mostly due to a resolution of edema, unmasking of dormant pathways and diaschisis. During this chronic phase (>12 weeks), the only known efficacious therapy consists of rehabilitation (Cumberland Consensus Working et al. 2009). It remains a matter of debate though if rehabilitation facilitates brain plasticity or if improved outcomes are mostly a product of adaptation, a learning process where the patient learns to solve the same task in a new way (Carmichael 2003). Consequently, survivors remain often very severely impaired and unable to care for themselves. Stroke therefore remains the only major disease without an effective treatment. Considering the human and economic cost, there is, nevertheless, a dire need to develop efficacious strategies that improve outcome after stroke.

Endogenous Neural Stem Cell Response

Approximately two thirds of all strokes affect the middle cerebral artery. Occlusion of this artery in the circle of Willis leads to neurobiological changes (i.e. cell loss, destruction of the tissue matrix, macrophage infiltration) in anatomical structures, such as the striatum (caudate-putamen in primates), as well as the cortex. In animals, this type of damage is achieved by middle cerebral artery occlusion (MCAo) and affords detailed molecular, histological and behavioral analyses. Using these animal models, it is hence possible to describe the cascade of processes that are induced by the blockage of blood vessels in stroke.

Early after stroke, a glial response encompasses a large area of the infarcted region (Fig. 16.1a), but gradually damaged neuronal cells will die (Alfieri et al. 2011). Eventually, not only will neurons and glial cells be cleared from the infarcted regions, but also blood vessels and the extracellular matrix that maintains cells in a tissue structure are degraded. These consequences leave areas of infarction to be completely cleared of tissue, hence producing a cavity that is filled with extracellular fluid (ECF). Nevertheless, it is important to recognise that the peri-infarct area also has undergone changes that result in heterogeneous sub-divisions of anatomical regions (Katsman et al. 2003). Surrounding the tissue cavity, a glial scar is formed that seals off this area and provides a structural support to the weakened peri-infarct tissue. Within the peri-infarct tissue, sub-divisions of tissue are characterized by a dramatic pan-neuronal loss, although tissue structure is maintained. Nevertheless, selective neuronal loss of GABAergic DARPP-32+ neurons is also evident in less severely affected regions (Zoli et al. 1997). In some cases, overlapping or anatomically distinct peri-infarct areas have a virulent astrogliosis (i.e. activated/reactive astrocytes) that commonly extends over larger areas of tissues, reaching even into the sub-ependymal zone (SEZ) (Kazanis et al. 2013).

The SEZ stretches along the ependymal cell layer that separates brain tissue from the lateral ventricles (LV), where the choroid plexus is producing cerebrospinal fluid (CSF) (Kazanis 2009). Occlusion of the MCA affects the SEZ by tissue astrocytosis extending into the SEZ, as well as actually tissue loss extending to the ventricles in the more extreme cases (Fig. 16.1b). In either case, it is not uncommon that there are structural changes occurring in and around the SEZ that affect its normal physiological role, notably the continuous production of olfactory neurons (Danilov et al. 2012). In brain development, neurons are generated in the centre of the brain, around the lateral ventricles in a region known as the Sub-Ventricular Zone (SVZ), that gradually evolves into the SEZ. During brain development, neurons born in the SVZ migrate outwards along radial glia to form anatomically distinct brain regions. As this process completes, the SEZ nevertheless, retains its ability to generate neuroblasts (A cells) from transit amplifying cells (C cells). Transit amplifying cells are a progenitor type of cell that is derived from neural stem cells (B cells). Together with capillaries, these cells are forming the neural stem cell niche, a specialized form of a neurovascular unit (NVU). In the normal brain, these neuroblasts will migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they will replace olfactory neurons (Kazanis 2009).

However, a disturbance to the SEZ in stroke leads to a shift in its activity from the ventral to the dorsal region, where the ventricular horn is dramatically increasing the number of new neurons being formed (Smith et al. 2012). A variety of strategies have been pursued to increase this endogenous neurogenesis. Especially the use of secreted factors, such as betacellulin, have been very efficient in increasing the proliferation of endogenous NSCs. After a stroke, a significant amount of neuroblasts from the SEZ are diverted away from the RMS towards the damaged striatum where they migrate alongside blood vessels using stromal cell-derived factor-1 (SDF-1) (Cui et al. 2013). There is evidence that these neuroblast-derived cells express DARPP-32, potentially suggesting that these cells replace lost GABAergic output neurons in the striatum and herewith lay the foundation for some of the observed spontaneous recovery. Nevertheless, DARPP-32 is also known to be expressed on olfactory progenitor cells (Perez and Lewis 1992). It remains hence unclear if endogenous neurogenesis after stroke indeed produces striatal neurons or if this population reflects olfactory neurons that have gone astray. Importantly, very few of these newly formed neurons survive in the peri-infarct area, hence questioning their capability to truly replace lost neurons (Thored et al. 2006). Even if these endogenous neuroblasts do not replace lost neurons, they can still significantly affect the fate of the peri-infarct tissue.

Neural stem cells and neuroblasts are known to secrete a variety of growth factors that can rescue damaged neurons (e.g. brain-derived neurotrophic factor, BDNF), but also can influence endothelial cells along which these are migrating. Indeed, certain peri-infarct areas are showing a robust increase in small arteries (Beck et al. 2000). It remains unclear how these vessels are formed, if they are the product of a sprouting angiogenic stimulus or the result of intussusception. Vasculogenesis, a third process of generating new blood vessels, is thought to only occur during development. A neovascular response of peri-infarct tissue is a physiological response to a lack of sufficient nutrient being available to support tissue needs. As a tissue is undergoing hypoxia, hypoxia-induced factor-1 α (HIF-1 α) is upregulated (Alfieri et al. 2011). This in turn engenders a molecular cascade that involves neuronal, as well as vascular elements to dedifferentiate endothelial cells in the vascular bed and promote their proliferation, as well as sprouting outgrowth. A process that has been extensively studied in brain tumors and this sprouting angiogenesis is dependent on high levels of vascular endothelial growth factor-A (VEGF-A) (Ribatti and Crivellato 2012). Intussusception (i.e. parting of a blood vessel), in contrast, is less dependent on VEGF-A, but is induced by peri-vascular cells protruding through the lumen, while depositing extracellular matrix to create a gap in between two vessels. In both cases, signalling of neuroblasts from the SEZ can influence

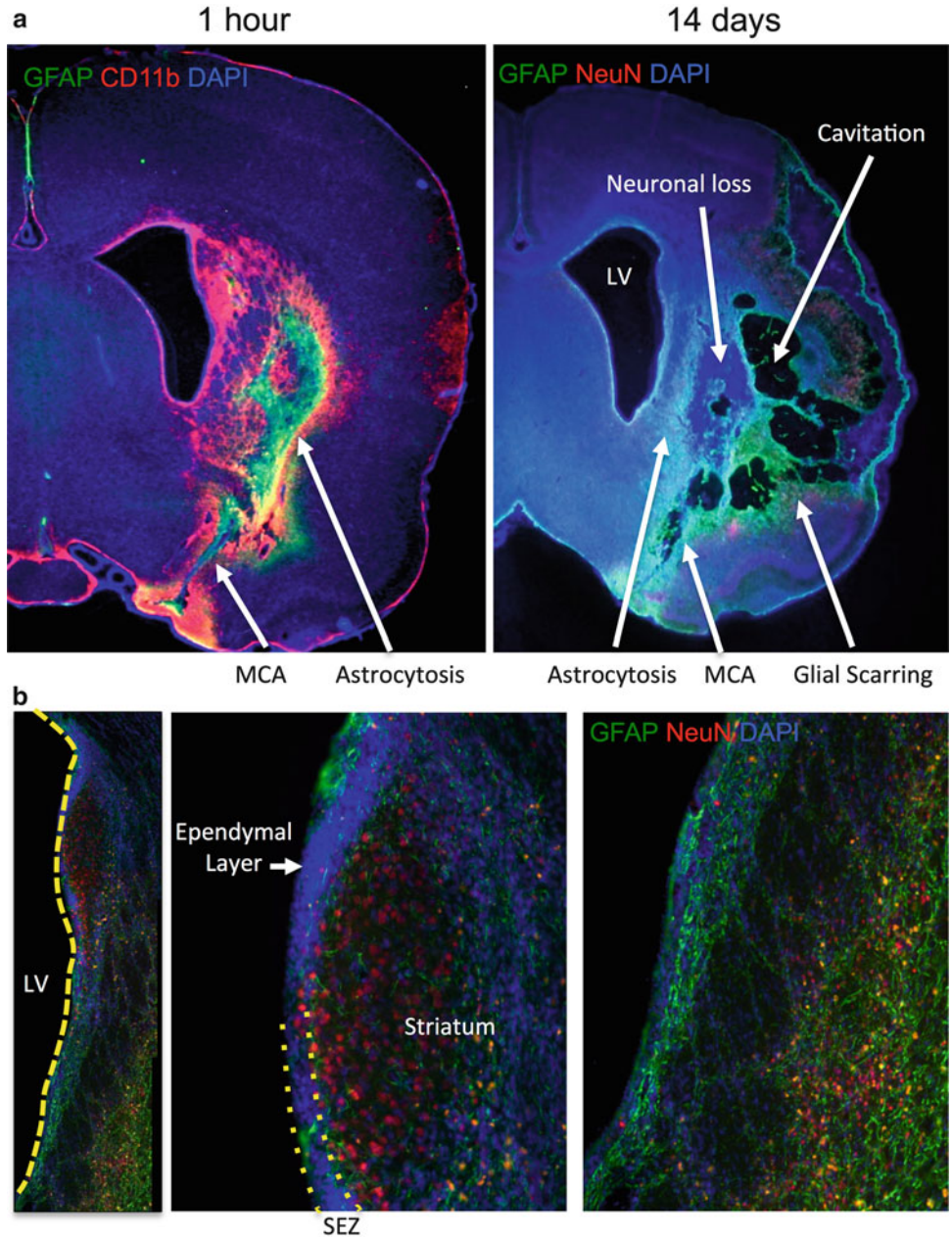

Fig. 16.1 Endogenous neural stem cells and stroke. (**a**) Stroke invokes a neurobiological response in the affected tissue that is diverse by involving different types of cells, such as astrocytes (glial fibrillary acid protein-positive cells) and microglia (CD11b+ cells), even within 1 h post-stroke. These cells delineate the affected area after occlusion of the middle cerebral artery (*MCA*) that supply blood to the striatum and medial portion of the neocortex. However, the extent of damage, as well as the neurobiological response evolves over time. Eventually severely affected areas will loose all cells and supportive extracellular matrix to produce a tissue cavity. The peri-infarct area surrounding the cavity is a heterogeneous environment sub-divided by different responses to the ischemic insult. In some areas, astrocytosis is predominant, whereas in others there is total loss of neurons (NeuN+ cells) and in others there is some angiogenesis. Commonly astrocytosis caused by the insult extents through large parts of the remaining striatum and reaches to the lateral ventricles (*LV*) indicating that these neurobiological changes also affect the site of endogenous neurogenesis, the sub-ependymal zone (*SEZ*). (**b**) As the name implies, the SEZ is situated next to the ependymal cell layer that separates the "brain" from the cerebrospinal fluid (*CSF*) in the lateral ventricles. In the case of stroke, where astrocytosis, or in severe cases even the damage itself, extents to the LV, the activity of the SEZ which represents the endogenous neural stem cell niche is altered. Specifically, neurogenic activity is shifted dorsally and results in a dramatic increase in neural progenitors being present in the ventricular horn, where these migrate along the corpus callosum or blood vessels to invade the damaged striatum (Smith et al. 2012)

neovascularization (Thored et al. 2007). However, at present there is no evidence that peri-infarct areas with neovascularization are preferentially invaded by endogenous neuroblasts. Moreover, it remains unclear if neurogenesis is indeed causally linked to improvements of behaviour (Thored et al. 2006) or if it is merely an epiphenomenon.

Transplantation of Neural Stem Cells

Spontaneous recovery and the improvements observed with neurorehabilitation, nevertheless, are limited and most patients remain severely impaired. Although increasing endogenous neural stem cells production and integration could provide greater improvements in the future, it is important to also consider if exogenous supplementation of new cells in the peri-infarct areas could exceed the outcome observed after spontaneous improvements.

Over the past two decades, developing brain tissue has been a source of interest for transplantation. Due to the specific timing of neurogenesis and gliogenesis in the developing brain, it is possible to harvest brain tissue from fetuses that is predisposed to develop into neurons or astrocytes. Importantly, neurogenic tissue will also produce astrocytes, but material collected later in development has a lower propensity to produce neurons (Rosser et al. 2003). Tissue from earlier developmental periods harbors the risk of tumor formation due to the cells being pluri-potent (i.e. can produce multiple organs) rather than multipotent (i.e. multiple cells from the same organ). Therefore derivation of the primary material will be essential to characterize its neurobiological properties and how these lead to the survival and integration of the materials, as well as behavioral improvements.

Early studies using fetal tissue pieces containing a heterogeneous mix of partially differentiated neurons/astrocytes, neural stem cells (NSCs), progenitors, endothelial cells (ECs), as well as microglia. Little attention was also given to the specific developing brain regions from which these were derived. From the rat, tissue was typically derived between E14-E16 that is considered the neurogenic period, which corresponds to week 8–10 during human development. Initial experiments were encouraging with tissue pieces surviving and demonstrating some connectivity between host and graft in terms of vasculature, as well as axonal projections. Injections into the lesion cavity resulted in some improvements of behavioral functions, but this was influenced by the time of transplantation, as well as the age of the donor (Grabowski et al. 1994). It was generally thought that these improvements were due to functional integration of graft tissue. However, there was evidence that if immunosuppression was not optimal there was a risk of an immune rejection. Two key elements in fetal tissue grafts were thought to induce a host immune response: (1) Endothelial cells from the graft established functional connections with the host vasculature and hence exposed foreign Major Histocompatibility Complex (MHC) molecules to immune cells travelling in the circulating blood (akin to solid organ grafts) and (2) Microglia contained within fetal tissue were highly immunogenic and could even mount a graft-versus-host response. A further consideration in fetal tissue grafts was the poor survival that was thought to be due to partially differentiated cells undergoing apoptosis after implantation (Emgard et al. 2003). Neural stem cells within these tissue pieces were hence thought to potentially produce differentiated cells upon implantation without incurring a major risk of an immune response.

Isolation of neural stem cells and the generation of cells lines was hence first achieved in rodent cells (Marone et al. 1995), but rapidly followed by the generation of human neural stem cell lines (Villa et al. 2000). Although it is possible to derive primary human neural stem cells for implantation, their in vitro propagation is limited, as with an increasing number of passaging the cells gradually shift phenotype which can lead to senescence (so called Hayflick phenomenon) or changes in their differentiation profile. Controlling their proliferation and differentiation is therefore advantageous to develop NSCs into a large scale population of cells which could be used to treat a large number of patients, as would be required for stroke. One way to achieve this is

to conditionally immortalize cells. Immortalization of cells leads to their continued proliferation, but does not provide a reliable control over their differentiation. In contrast, conditional immortalization defines the conditions under which cells proliferate and differentiate, hence providing control over cell fate and improving the safety of the cell line. Moreover, this conditional immortalization provides greater stability to the cell lines in terms of maintenance of specific phenotypes and hence allows definition of quality control procedures.

A further important change in the application of both primary cells and cell lines in stroke is that these are no longer implanted as a piece of tissue, but individual cells are suspended in a vehicle that serves to inject cells. Injection of suspension grafts by themselves into the lesion cavity, however, does not lead to the formation of a tissue. Instead injected cells migrate into host tissue and are therefore preferentially injected into the peri-infarct tissue to facilitate their integration in host tissue. Although a variety of peri-infarct injection sites have been used to successfully improve outcome with neural stem cells, intracerebroventricular injection of cells leads to poor survival and no improvement in outcome (Smith et al. 2012). Importantly, human neural stem cells show very little migration away from the injection site (Smith et al. 2012), whereas mouse neural stem cells even migrate from one hemisphere to another to improve outcome (Modo et al. 2002). Although these experiments provide evidence that certain sites will not be conducive to improve behavioral outcome, the diversity of injection sites peri-infarct with recovery suggest that the specific peri-infarct location might not be as important. It currently remains unclear if a single injection could be sufficient to improve outcome irrespective of peri-infarct location or if multiple injections covering the peri-infarct region would still improve outcome.

The topology of the lesion further carries significance in the observed deficits, as well as the observed recovery after peri-infarct injections (Smith et al. 2012). Occlusion of the middle cerebral artery affects a vast territory of brain tissue. Functional deficits and the resulting behavioral impairments are hence a consequence of which anatomical regions are affected by the stroke (Crum et al. 2013). Consequently, implantation of cells peri-infarct is likely to affect functions that are subserved by these peri-infarct regions or those that are connected. The topological reach of the stroke therefore is an important modulator of the efficacy of neural stem cell efficacy (Smith et al. 2012). Although initial studies considered recovery to be mediated through neuronal replacement, increasing evidence suggest that the more important mechanisms are astrocytic differentiation that can lead to a scavenging of excitotoxic glutamate, modulation of microglia activity and the resulting inflammatory response, as well trophic factor support to dying neurons. Interestingly, an interaction of neural stem cells with host vasculature can potentially dramatically influence these mechanisms. For instance, blocking of the effects of vascular endothelial growth factor from human neural stem cells significantly affects behavioral recovery (Horie et al. 2011). Nevertheless, establishing mechanisms of recovery is extremely challenging as multiple signalling interactions occur and affect different physiological processes (Drago et al. 2013). Interfering with one of these can affect the others and hence complicate the investigation of their specific involvement in recovery.

Nevertheless, translation of cell therapy for stroke is not dependent on the identification of a mechanisms of recovery (Chopp et al. 2009). Although mechanism of recovery are essential to ensure optimal efficacy and potentially improving efficacy, clinical translation does not require a detailed understanding of how a therapy works, but is contingent on proven efficacy and safety. To date, three clinical trials have been conducted for intracerebral cell injections in stroke. The first clinical translation applied a human teratocarcinoma cell line that produced post-mitotic cholinergic neurons upon treatment with retinoic acid. Preclinical studies indicated a dose-dependent efficacy of these cells in a rodent model of stroke and there was no evidence of ill-effects (Borlongan et al. 1998). A phase I clinical trial showed no ill-effects of the cells or the stereotactic surgery (Kondziolka et al. 2000), but a phase

II clinical trial only revealed minor improvements (Stilley et al. 2004) with post-mitotic cell survival beyond 27 months and a relationship between peri-infarct glucose uptake and motor performance. No further trials were conducted using this cell line. Due to the shortage, logistical difficulties, as well as the ethical concerns regarding the use human fetal tissue material (Rosser et al. 2003), porcine-derived tissue was considered as an alternative source for transplant material. However, a clinical trial using porcine CNS fetal tissue was abandoned after two patients developed side-effects (worsening motor deficits and seizures) (Savitz et al. 2005). More recently, the first clinical trial using a human neural stem cell line that was conditionally immortalized using the c-myc under tamoxifan control completed a phase I clinical trial without any ill-effects being apparent after 12 months post-injection (Sinden et al. 2012). Extensive preclinical testing preceded this translation (Smith et al. 2012), as well as the development of a large cell bank that can be used to treat a significant number of patients using the same "cell product", facilitating quality control and ensuring that each patient receives the same treatment. Still, phase II trials will need to establish if indeed this approach can successfully improve outcome. The preclinical development, as well as the clinical translation, provides a framework for future approaches.

Tissue Engineering Using Neural Stem Cells and Biomaterials

Even if peri-infarct neural stem cell implantation and/or endogenous neurogenesis will result in clinically relevant efficacy, these approaches do not replace the lost tissue and hence a large tissue cavity remains. To allow neural stem cells to be retained within the cavity requires the use of biomaterials that provide a structural support (Bible et al. 2009b). Bioscaffolding has been extensively used in other organ tissues and even in the spinal cord. Nevertheless, there is a general lack of studies in the brain (Orive et al. 2009). Tissue engineering in the brain faces two major technical hurdles (Bible et al. 2009a): (1) Delivery of the material needs access through the skull. This can either be using a minimal approach of delivery through a drill hole or by means of a craniectomy. A craniectomy is very invasive, changes intracranial pressure and is a potential source for opportunistic CNS infections. Injections through a Burr hole in the skull are hence favorable, but require the material to fit through a thin needle. (2) Delivery of a scaffold requires knowledge regarding the location and volume of tissue to be replaced. Injection of a large volume of material inside existing brain tissue will lead to a dramatic increase in pressure and cause tissue damage. It is therefore essential to use non-invasive imaging, such as Magnetic Resonance Imaging (MRI), to provide surgical planning and guide the injection of the material for in situ tissue engineering (Bible et al. 2009a).

Key features of biomaterial for tissue engineering are that these are biocompatible and biodegradable (Orive et al. 2009). Biocompatibility refers to the material's properties and how these interact with the tissue in which these are implanted. Ideally, the host tissue will not recognize the biomaterial as a foreign object to which it invokes a foreign body reaction that leads to glial scarring. In contrast, biodegradability denotes the property of material to slowly decompose in response to biological activity (e.g. hydrolysis or enzymatic activity). Biodegradation can lead to the material being decomposed into constituent parts that are not biocompatible. It is therefore essential that biocompatibility be evaluated also in biodegradation conditions, ideally in vivo. It is important here to note that for regulators, the combination of cells and biomaterials will fall under the regulations concerning biologicals and hence necessitate in vivo testing, as it is difficult to foresee the biological effects of the combined products purely from in vitro assays (Lee et al. 2010). A myriad of biomaterials have been developed, but only a small proportion of these have been evaluated for CNS applications (Pakulska et al. 2012).

For tissue engineering in stroke, incorporation of neural stem cells with biomaterials can either be achieved by attaching cells to the biomaterials in the form of microparticles or they can be mixed

with the liquid form of the material before it solidifies into a gel. Attachment of NSCs to, for instance, poly-l-lactic-glyoclic acid (PLGA) microparticles afforded the targeted injection of these into the lesion cavity and within 1 week a primitive de novo tissue formed (Bible et al. 2009b) (Fig. 16.2a). Solid microparticles, however, create a significant amount of voided space that is not covered by cells. The use of hydrogels is therefore advantageous as it can cover the complete cavity (Fig. 16.2b). Moreover, cell incorporation is technically less challenging than cell attachment to microparticles (Bible et al. 2009a). Hydrogels can be generated from different materials, including molecules derived from the extracellular matrix (ECM). Injection of NSCs in an ECM bioscaffold indicated good coverage of the lesion and extensive survival of human cells within the stroke-damaged rat brain (Bible et al. 2012a) (Fig. 16.2c). Instead of using de-cellularized tissue as the basis for an ECM bioscaffold, it is also possible to generate hydrogels using specific ECM molecules, such as hyaluronan. Nevertheless, merely replacing the neural and structural component of a tissue is insufficient to create a new functional tissue.

The brain is organized into different overlapping units of organization. One of these is functional cell assemblies that underlie behavioral function (within and in-between anatomical regions), but also a physiological interdependence of cells that is defined by the neurovascular unit (NVU). The NVU is composed of a neural (neurons, astrocytes, oligodendrocytes) and vascular component (endothelial cells, pericytes). The vascular component is required to guarantee the survival of the neural component and in the case of stroke, if the physiological supply of nutrients through the vasculature is blocked, the neural component cannot meet its metabolic demands. To create a de novo tissue, a functional vasculature is therefore a *conditio sine qua non* to afford the long-term survival of cells (Bible et al. 2009b, 2012a). In the short-term, when diffusion barriers within the new tissue have not been established, the ECF is sufficient to maintain cell survival, but as the tissue structure is developing, nutrients can no longer flow to implanted cells. Attracting a vascular supply to the lesion cavity is therefore essential for the success of in situ tissue engineering. Endothelial cells within an established blood vessel can, nevertheless, be induced to create a neovasculature by stimulating these using appropriate factors (Liman and Endres 2012). VEGF-A is one of these factors that are considered angiogenic. Secretion of VEGF from, for instance, PLGA microparticles seeded with NSCs, was adept at attracting endothelial cells into this newly forming tissue (Bible et al. 2012b). There was evidence of a neovasculature developing in parts of the tissue, but there was also evidence of overproliferation of endothelial cells and areas without any endothelial cell invasion. One of the challenges for future in situ tissue engineering in the brain is the diverse microenvironments present within the lesion cavity, as well as the peri-infarct area and how these affect the interaction with biomaterials and cells inside the cavity. For instance, peri-infarct regions with evidence of angiogenesis are likely to be primed to invade a newly forming tissue, whereas the opposite site of the same lesion the attraction of a vasculature might have to overcome anti-angiogenic signals (e.g. thrombospondin-1). It is therefore unclear at present to what degree current biomaterials can be responsive to their implanted environment to shape an appropriate response. “Smart” or “conditional” materials that secrete particular factors in certain environments, but not in others, might hence be required to provide the optimal conditions for in situ tissue engineering.

Conclusion

Great strides have been achieved in improving the survival of patients suffering from stroke. An ever-greater number of survivors suffer from long-term disability. Unfortunately, therapeutic interventions for stroke have been extremely limited despite a significant research and translational effort. Nevertheless, in the past two decades the discovery of an endogenous neural stem cell

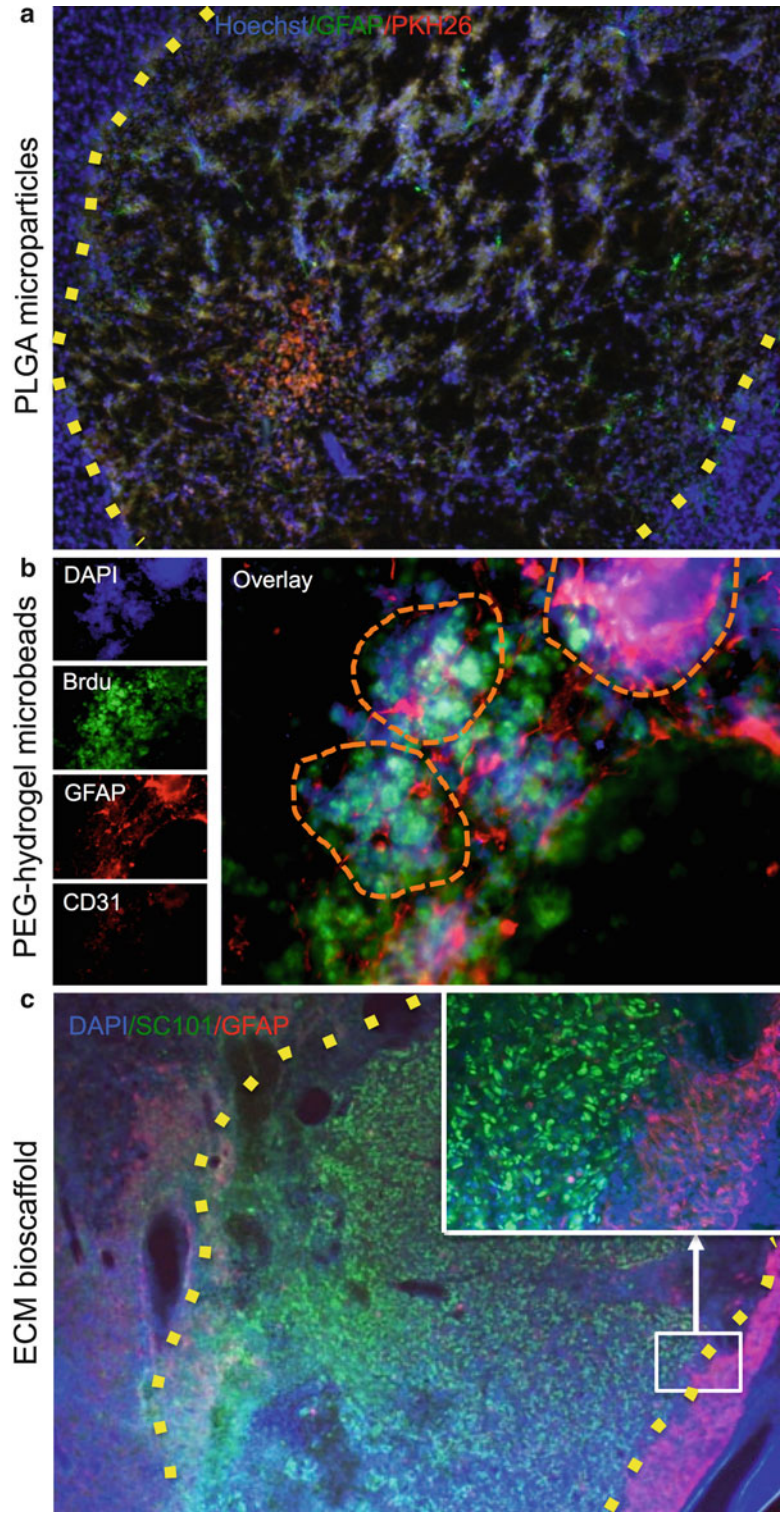

Fig. 16.2 Tissue engineering in stroke. (**a**) Poly-L-Lactic-Glycolic (*PLGA*) microparticles seeded with neural stem cells were implanted under MRI-guidance into the stroke cavity and within 7 days created a primitive de novo tissue (Bible et al. 2009b). However, solid PLGA microparticles created a significant amount of voided space and hence alternative biomaterials that incorporate neural stem cells rather than require their attachment are interesting alternatives. (**b**) During the formation of microbeads made out of poly(ethylene glycol) (*PEG*) hydrogel, neural stem cells can be incorporated and implanted through a Hamilton syringe into the stroke-cavity based on MRI coordinates. NSCs can degrade the hydrogels and exit these to interact with the host brain. (**c**) In contrast to these preformed beads, de-cellularized extracellular matrix can be mixed with human neural stem cells prior to injection and form a hydrogel inside the cavity that will adapt to the topology of the lesion. Excellent cell survival and coverage of the lesion is seen using this approach (Bible et al. 2012a)

niche provides hope that these cells can be exploited to improve the spontaneous recovery typically observed within the first couple of months post-stroke. Further supplementing endogenous repair strategies with implanted neural stem cells is a potential therapeutic avenue that is likely to define the next decade of clinical trials in stroke. It is hoped that the multitude of mechanisms of repair these cells invoke, as well as their local delivery and application to chronic stroke will overcome some of the shortcomings of pharmacological approaches. Still, making acute neuroprotection work will have the most significant impact on reducing overall disability and therefore a continuous effort will be needed to ensure that this eventually will find an efficacious path to the bedside. However, the challenge to approach the final frontier, to replace lost tissue, has merely started. It is already evident that neural stem cells by themselves are insufficient to achieve tissue regeneration, but that at a minimum these require scaffolding support by biomaterials. In all likelihood further complexity and sophistication is required to generate a vascular supply to de novo tissue, as well as positional specification. Yet, these current efforts demonstrate that novel treatment options involving regenerative medicine approaches are on the horizon and hopefully will provide new hope to patients suffering from stroke.

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Part V

General Applications

Epithelial Plasticity Regulation by MicroRNAs

17

Antonio Díaz-López and Amparo Cano

Contents

Abstract

Embryonic development requires the acquisition of a mesenchymal migratory phenotype by some epithelial cells in strictly spatio-temporal regulated fashion during organ formation. Interestingly, this epithelial-to mesenchymal transition (EMT) process is aberrantly activated during tumor progression. The reverse mesenchymal-to epithelial transition (MET) process is, nevertheless, required for development of metastatic foci, implying a highly dynamic regulation of epithelial plasticity. The expression of different transcription factors (called EMT-TFs) activates this reversible complex program although its regulation remains partially elusive. Recently, non-coding microRNAs have emerged as novel regulators of cell differentiation by targeting EMT-TFs and other molecules involved in tumor progression. In this chapter, we will discuss the different mechanisms used by these microRNAs to regulate epithelial plasticity of tumor cells during tumor progression and metastasis. We will pay particular attention to two negative feedback pathways involving miR-200/ZEB1 and p53/miR-34/SNAIL1 that clearly shows the plasticity and interconnectivity of the EMT and MET programs. Remarkably, recent studies showed that certain microRNAs might be used in the diagnostic and treatment of cancer providing new therapeutic opportunities to combat tumor progression and increase patient survival.

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Introduction

Epithelial to mesenchymal transition (EMT) is characterized by the acquisition of mesenchymal properties by epithelial cells. EMT involves the loss of cell-cell adhesion and apico-basal polarity and the gain of mesenchymal markers associated to an increase of the migratory/invasive cell properties. In fact, EMT implies the functional loss of E-cadherin, a master regulator of cell-cell interactions, and this is considered a hallmark of the EMT process. Importantly, the process might be reversible in an equivalent program called mesenchymal to epithelial transition (MET). Interestingly, several rounds of EMT/MET occur in embryogenesis during the formation of organs and tissues and aberrant EMT appears in pathological processes such as tumor progression and fibrosis (Thiery et al. 2009). In particular, activation of an EMT process facilitates cell invasion and spreading of cancer cells and might be involved in other properties of tumor cells like cell survival, chemoresistance and acquisition of stemness (Thiery et al. 2009). Interestingly, distant metastasis often reproduce the epithelial histological pattern of the primary tumors implying that the process is highly dynamic and that a MET process is required for establishment of overt metastasis at distant sites (Polyak and Weinberg 2009). Thus, understanding the biological mechanisms of epithelial plasticity underlying the EMT/MET programs might help to identify therapeutical targets to impair metastasis formation and ultimately increase patient survival.

Several EMT transcription factors (presently called EMT-TFs) have been described as inducers of the EMT program; they include members of the Snail (SNAIL1/2), the basic helix-loop-helix (bHLH) [TCF3 (also known as E47), E2-2 and TWIST1/2] and the ZEB family (ZEB1/2) that act as direct or indirect repressors of *E-cadherin* (Peinado et al. 2007). Apart from those classical EMT-TFs, expression of other transcription factors like the Winged helix/Forkhead (FoxC2), Homeobox (Goosecoid) and Kruppel like (KLF8) factors or other regulatory molecules like lysyl oxidase-like, LOXL2 and LOXL3, also initiate an EMT process (Peinado et al. 2007; Thiery et al. 2009). In fact, the expression of the classical EMT-TFs is related to tumor progression and poor prognosis in multiple cancer pathologies (Peinado et al. 2007; Yang and Weinberg 2008; Thiery et al. 2009). Whether all these EMT-TFs work independently or in coordinated networks is still partially elusive although several recent reports suggest a hierarchical and cooperative regulation providing robustness to the system (reviewed in Nieto and Cano 2012).

There is a plethora of signaling pathways that trigger EMT in both embryonic development and in normal and transformed cell lines. Signaling pathways including TGFβ superfamily, Wnts, Notch, EGF, HGF, FGF, or hypoxia, trigger EMT by increasing the levels of one or more EMT-TFs (Yang and Weinberg 2008; Thiery et al. 2009; Nieto and Cano 2012). In addition to transcriptional regulation, EMT-TFs are subject to multiple post-transciptional and post-translational regulatory pathways, including nuclear localization, phosphorylation or ubiquitinylation. For example, phosphorylation in different serine residues of Snail1 are required for nuclear transport, while GSK3β, a key player of the Wnt pathway, promotes Snail1 phosphorylation leading to both Snail1 nuclear export and degradation by the proteasome machinery reducing Snail1 protein levels and subsequently its repressive activity. An opposite effect has been reported for LOXL2, a lysyl oxidase member, interacting with the K98 and K137 residues of SNAIL1 and increasing protein stability, and for several activating phosphorylations of Snail1 (Nieto and Cano 2012).

Recently, several EMT-TFs have been identified as targets of specific miRNAs that emerge as a novel post-transcriptional regulatory pathway of the EMT process and epithelial plasticity. In the present chapter we will describe the role of miRNAs in the regulation of epithelial plasticity emphasizing the importance of the miRNAs in the regulation of EMT-TFs in cancer and how the cells regulate those specific miRNAs in order to assure stability to the system. As we will describe, aberrant homeostasis of the network is found in tumor pathologies facilitating cell spreading and metastasis.

MicroRNAs: An Overview

MicroRNAs (miRNAs) are conserved non-coding small RNAs (~22 nt pair nucleotides) that control gene expression by binding to the 3′ untranslated region (3′UTR) of the targeted mRNA promoting their degradation or impairing their translation. Thus, miRNAs are a clear example of the emerging view that non-coding RNAs (ncRNAs) regulate biological processes in a protein-independent fashion. The study of the miRNAs began in the early nineteens of the last century when Ambros and Ruvkun discovered that *lin-4*, a gene known to control *C. elegans* development, does not code for a protein but for a pair of small RNAs of 22 and 60 nucleotides in length; the longer one was predicted to fold into a stem loop structure and being the precursor of the shorter one. Further studies demonstrated that *lin-4* binds to the 3′UTR sequence of the *lin-14* mRNA mediating repression of the gene (Lee et al. 1993). Over the last years, hundreds of these tiny miRNAs and their targets have been identified controlling such different biological pathways as developmental timing, cell death, proliferation, haematopoiesis and more recently epithelial plasticity (Brabletz and Brabletz 2010; Pasquinelli 2012).

MiRNAs are transcribed by RNA polymerase II as ~80 nucleotide transcripts that adopt an RNA stem-loop structure which is part of a several hundred nucleotides long miRNA pro-precursor termed primary miRNA (pri-miRNA) that might contain several miRNA precursors. Later, each pri-mRNA is cleaved by the Drosha complex releasing each single miRNA precursor (pre-miRNA) consisting of the RNA hairpin loop structure of approximately 70–80 nucleotides. After active transportation to the cytoplasm accompanied by exportin 5 (XPO5), pre-miRNA are activated by the RNase III enzyme Dicer that removes the loop region from the pre-miRNAs resulting in a dsRNA. One of that strands of the pre-miRNA is bound by Argonaute to form a miRNA-induced silencing complex (miRISC), which specifically targets the mRNA to be regulated. Specificity of the miRNA is given by the "seed" sequence present in the 5′ of the miRNAs at 2–7 positions but additional complementarity can be found by extensive pairing at the 3′ sequence. Although miRNAs eventually regulate protein levels, different mechanisms of repression have been reported. In plants, miRNAs mainly form near-perfect matches to the coding sequence of the mRNA enabling the cleavage and repression of the target gene. In contrast, in animals, partial pairing between a miRNA and the mRNA target site, usually at 3′UTR, results in reduced protein expression through a variety of mechanisms that involve mRNA degradation and/or translational repression (Pasquinelli 2012). Indeed, the absence of perfect pairing provides an excellent platform to amplify the number of target mRNAs that can be regulated by a single miRNA.

Most miRNAs genes are localized in distant regions from coding genes, implying that they are expressed from independent transcription units. However, 25% of the miRNAs are located within the introns of pre-mRNAs and are likely to be controlled in concert with gene expression rather than by independent regulation. Interestingly, some miRNAs genes are grouped in clusters and transcribed as a multi-cistronic primary transcripts, such as the miR-200 family and others. Extensive steps in the identification of miRNAs have been performed in the last years. As a result, two highly sensitive scoring algorithms have been generated; the miRscan for nematode and vertebrate and the miRseeker for insects; however, the absence of complete pairing found in animals requires the generation of more elaborated and complex algorythms.

miRNAs and Tumor Progression

Tumorigenesis is a multistep process that requires not only the loss of cell proliferation control and anti-apoptotic signals but acquisition of an invasive phenotype facilitating dissemination and distant metastasis. Since the identification of the first miRNAs, multiple pro-tumorigenic or anti-tumorigenic properties of different miRNAs have been identified that directly or indirectly affect

tumor development and progression and, importantly, some of them affect to metastatic dissemination. The diverse biological function of the miRNAs targets provides the possibility to regulate almost any biological function in the organism. Interestingly, almost 50% of the annotated miRNA genes are localized in "fragile sites", unstable chromosome regions often deleted or amplified in cancer. For example, miR-125b-1 is spanned on chromosome 11q24 that is often deleted in breast, lung and ovarian tumors, supporting a role as a tumor suppressor (Calin et al. 2004). Other interesting examples are the miR15a and miR16-1 whose mechanism of action was recently uncovered. Both miRNAs are considered tumor suppressor genes because they negatively regulate *BCL2*, an anti-apoptotic gene overexpressed in leukemia and lymphomas (Cimmino et al. 2005). In this section, we will extensively focus on miRNAs involved in the acquisition of migratory and invasive properties, and therefore, in tumor cell plasticity.

One of the first miRNA families related to tumor progression was the miR-10 family. This group of miRNAs, which contains five members: miR-10a, miR-10b, miR-196a-1, miR-196a-2 and miR-196b, spans a region that it is frequently amplified in melanoma and breast cancer. Mechanistically, miR-10 activates cell invasion by targeting *HOXD10* mRNA, a repressor of the pro-migratory protein RHOC, in breast cancer cells and is associated with the metastatic outcome in breast cancer patients (Ma et al. 2007). Nevertheless, some controversy exists as to whether the amplification of the miR-10 containing region promotes metastasis and alternative models suggesting that miR-10 may facilitate cancer by regulating ribosome biogenesis have also been postulated.

miR-21 is another miRNA strongly upregulated in most solid cancers including breast, colon, lung, pancreas, prostate and ovarian cancer. Additionally, recent studies indicate that miR-21 is also up-regulated in non-solid tumors as leukaemia. Interestingly, several targets of miR-21 have been identified such as *PDCD4*, *RECK*, *TPM1* and *PTEN* mRNAs, some of which have well-known inhibitory effects on cancer detachment, migration and invasion (Krichevsky and Gabriely 2009), key steps on the metastatic process. Moreover, upregulation of miR-21 is correlated with tumor grade in glioma, breast and colorectal cancer and it is proposed as a prognostic marker in these neoplasias. Importantly, miR-21 can be detected in exosomes isolated from blood samples of ovarian and lung cancer patients, facilitating the detection of miRNA levels, and providing an excellent tool that might be applied in clinical studies as a biomarker of patient prognosis, therapeutic response or disease recurrence (Taylor and Gercel-Taylor 2008).

On the other hand, there are also miRNAs that act as tumor suppressors. For example, miR-335 suppresses metastasis and migration by targeting mRNAs of the progenitor cell transcription factor *SOX4* and of the extracellular matrix component *tenascin C*. Loss of miR-335 expression is found in most breast cancer tumors with a poor prognosis outcome, even more sensitive when the analysis includes the expression of miR-126 (Tavazoie et al. 2008). Interestingly, miR-126 has a more general role impacting cell proliferation, adhesion and cell migration targeting the transcripts of pro-angiogenic factor *VEGF* and of the adaptor protein *CRK*. Beyond cancer cells, miR-126 is able to inhibit endothelial recruitment, angiogenesis and colonization at the metastatic site by targeting *IGFBP2*, *PITPNC1*, and *MERTK* mRNAs that facilitate tumor progression (Png et al. 2012).

Recently, miRNAs have emerged as novel regulators of epithelial plasticity providing new key players to explain the underlying mechanisms and the highly dynamism of the EMT/MET processes. In order to regulate cell plasticity by triggering the EMT process, miRNAs can bind either EMT-TFs or EMT-TF regulators. Nevertheless, a novel direct mechanism targeting the tumor suppressor E-cadherin (coded by *CDH1*) has been also reported. Ma et al. (2010) reported that miR-9, a MYC-induced miRNA upregulated in breast cancer cells, mediates E-cadherin downregulation by direct binding to the 3′UTR of *CDH1* mRNA. Interestingly, *in vitro* studies in normal and malignant breast cancer cells showed a significant increase of cell motility and invasiveness after miR-9 overexpression.

Moreover, decreased E-cadherin levels are accompanied by the activation of the β-catenin/TCF pathway which upregulates vascular endothelial growth factor (VEGF) expression and angiogenesis thus facilitating tumor invasion and metastasis. Conversely, miR-9 inactivation reduces the number of metastasis in highly metastasic cells (Ma et al. 2010).

Remarkably, miRNAs are not always direct regulators of EMT-TFs and the opposite regulation is also observed. TWIST, an EMT-TF member of the bHLH family, activates the expression of miR-10b in breast and gliobastoma cancer cells (Ma et al. 2007). Thus, TWIST contributes to cancer progression by a double mechanism, increasing miR-10b levels and participating directly in EMT induction.

Complex Regulatory Circuits Regulate EMT and Epithelial Plasticity

The ZEB/miR-200 Negative Feedback Pathway

The ZEB/miR-200 pathway is the best well-known regulatory pathway controlling epithelial plasticity and it has been thoroughly investigated in relation with EMT in the last years. ZEB family is formed by double zinc-finger and homeobox containing transcription factors involved in transcriptional repression through binding to promoter targets containing E-box elements, such as the *CDH1* promoter. The family includes two proteins; ZEB1 (also known as δEF1 or TCF8) and ZEB2 (also known as SIP1). Interestingly, ZEB family expression in different epithelial models results in a complete EMT accompanied by the loss of cell polarity, cell-cell adhesiveness and strong downregulation of E-cadherin expression (Peinado et al. 2007). Hence, aberrant expression of ZEB family is related to cancer metastasis and poor prognosis in several tumorigenic pathologies (Peinado et al. 2007; Yang and Weinberg 2008; Thiery et al. 2009). Indeed, ZEB1-silencing in xenograft colorrectal models blocks metastasis formation further supporting the role of ZEB1 in tumor progression (Spaderna et al. 2008). Regardless its known role as an EMT regulator, the underlying mechanism of ZEB family regulation was poorly understood until the post-transcriptional regulation by miR-200 family was fully characterized.

miR-200 family is composed of five members in two polycistronic pri-miRNA transcripts present on chromosome 1 (miR-200b, miR-200a and miR-429) and 12 (miR-200c and miR-141). According to the seed sequence two subfamilies can be defined; the first one comprised by miR-200a and miR141, and the second one by miR-200b, miR-200c and miR-429. The first evidence involving the miR-200 family in cell differentiation was reported by expression analysis of a collection of 60 human cancer cell lines included in a drug screening panel of the National Cancer Institute series (NCI60) (Park et al. 2008). Moreover, ectopic expression of miR-200 induces E-cadherin expression and the reorganization of cell-cell adherent junctions. Conversely, miR-200 inhibition results in E-cadherin downregulation and the acquisition of mesenchymal properties. The underlying mechanism of this novel regulation was partially addressed by Park et al. (2008) showing that miR-200 family members target 8 and 7 sites at the 3′UTR of ZEB1 and ZEB2, respectively. In addition, miR-205 was also shown to target ZEB family playing an additive role with miR-200 members. Moreover, miR-200 loss of expression was correlated with decreased E-cadherin in a cohort of metaplastic and ductal breast (Gregory et al. 2008) and ovarian tumors (Park et al. 2008). The existence of a feedback loop was finally rounded by the Brabletz's and Goodall's groups reporting the first evidences that ZEB1 directly represses the expression of the miR-200 family members, miR-141 and miR-200c (Burk et al. 2008), and miR-200a, miR-200b and miR-429 by binding to a promoter region localized 4 kb upstream of miR-200b (Bracken et al. 2008). Hence, the result of the final balance between miR-200 levels and ZEB family members will determine the epithelial or mesenchymal fate of the cell and explain the intratumoral heterogeneity

found in many human tumors (Brabletz and Brabletz 2010).

More recently, a new role of the ZEB/miR-200 loop has emerged, regulation of stemness. miR-200 members regulate the expression of diverse stemness-related genes such as *CD44*, *BMI1* and *c-myc*. Indeed, ZEB1 also represses the expression of miR-183 and miR-203 which target *BMI1* and other stemness factors such as *SOX2* and *KLF4* (Wellner et al. 2009) supporting the link between cell plasticity and stemness properties proposed from previous studies on induction of stemness by EMT-TFs (Mani et al. 2008). Interestingly, the EMT-stemness association is also found *in vivo* in basal like breast tumors, a subtype of breast cancer associated with poor differentiation grade, enriched in numerous EMT and stemness markers (Sarrio et al. 2008). Moreover, EMT and the stemness properties might explain both disseminating and stemness properties of the tumor preventing apoptosis and senescence (Brabletz and Brabletz 2010). In addition, the miR-200 family has also been recently implicated in metastasis colonization by promoting a MET process that involves not only E-cadherin re-expression but also targeting of the secretome pathway (Korpal et al. 2011). Thus, the miR200 family emerges as a crucial regulator of epithelial plasticity and stemness during tumor progression.

Despite the present evidence in favor of the relevant role of miRNA-200 in the regulation of cell plasticity and tumor progression many opened questions still remain unanswered. To mention a few, what is the role of the other EMT-TFs in miRNA regulation? Do they have just a role in providing robustness to the system or have specific functions? Are they also regulated by miRNAs? Do oncogenes or tumor suppressors control cell differentiation by modifying EMT-TFs? Some of these questions have been partially addressed for Snail1 and p53.

The p53-SNAIL1-ZEB Connection

Maybe the most important hallmark in cancer is genome instability and cell cycle dysregulation. P53, known as "the guardian of the genome", has an essential role in maintaining genome stability and activating apoptosis pathways when the former is compromised. Importantly, p53 has a prevalent role in cancer being the most frequently mutated gene in about 50% of human tumors. In addition to its well-known properties on cell cycle regulation a new role in the control of cell differentiation has been recently reported. Kim and colleagues observed that isogenic HTC116 cells in which both p53 alleles have been inactivated by homologous recombination (HTC116-p53$^{-/-}$) express very low E-cadherin levels suggesting a negative role in cell differentiation. Similar conclusions were obtained in A549 non-small lung carcinoma or MCF-7 breast carcinoma cells in p53 silencing studies (Kim et al. 2011a). Moreover, those authors demonstrate that p53 wild type, but not a mutated form, controls the differentiation status by modulating SNAIL1 expression through transcriptional regulation of the miR-34 family members (miR-34a and miR-34b/c), which indeed bind to a conserved site in the *SNAIL1* mRNA 3′UTR and reducing Snail1 protein levels.

As well as a single miRNA might target more than one mRNA, one specific mRNA can be targeted by many miRNAs, thus increasing the complexity of the regulatory circuits controlling epithelial plasticity and stemness. Interestingly, miR-34a mediated downregulation is not limited to *SNAIL1* but also target other EMT-TFs like *SNAIL2* and *ZEB1* and the stemness factors *BMI1*, *CD44*, *CD133* and *c-MYC* (Siemens et al. 2011). Moreover, SNAIL1 and ZEB1 factors negatively regulate the expression of miR-34 in a second differentiation regulatory feedback loop similar to the ZEB/miR-200 one (Siemens et al. 2011). In addition to miR-34, p53 is also able to regulate directly cell differentiation and stemness by controlling miR-183, miR-192 and miR-200 expression (Chang et al. 2011; Kim et al. 2011b). Loss of p53 expression results in decreased expression of miR-200c, the acquisition of a mesenchymal phenotype and expression of the stemness markers *BMI1* and *CD44*. Conversely, miR-200c ectopic expression reverts the mesenchymal and stemness characteristics acquired by p53 loss of function (Chang et al. 2011). This

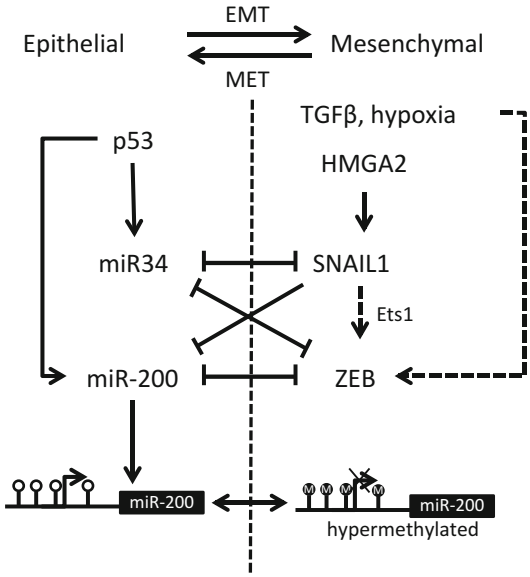

Fig. 17.1 Epithelial plasticity is controlled by two negative feedback pathways involving miR-200 and miR-34 families. P53 activates the expression of the miR-34 and miR-200 families, therefore maintaining low levels of SNAIL1 and ZEB proteins and an epithelial phenotype. Conversely, TGF-β or other cytokines, induces a mesenchymal phenotype by increasing the levels of SNAIL1 and ZEB1 which repress miR-34 and miR-200. Hypermethylation of the miR-200 promoters provides additional regulation of miR-200 expression and thus to the balance between epithelial and mesenchymal cell states. Thus, microRNAs and EMT-TFs regulate epithelial cell plasticity in several negative feedback circuits affected by intracellular and extracellular pathways

association was further validated in a cohort of breast cancer patients with loss of p53 and enhanced expression of EMT and stemness markers. Furthermore, p53 also regulates miR-192 which represses ZEB2 expression (Kim et al. 2011b) providing additional robustness to the system. Thus, p53 controls cell differentiation by at least two distinct miRNAs feedback loops: miR-34/SNAIL1 and miR-200/ZEB1 (Fig. 17.1) as well as additional miRNAs, as miR-192.

On the other hand, in addition to miR200/miR-192 binding, *ZEB2* expression is regulated by an elegant mechanism involving the expression of a natural antisense transcript (NAT) induced by Snail1. Interestingly, the NAT binds to a 5′-UTR ZEB2 intron preventing the splicing of an internal ribosomal entry site (IRES). Thus, the expression of the NAT by Snail1 highly induces the expression of *ZEB2* (Beltran et al. 2008). Remarkably, NAT expression is present in human cancer cells of mesenchymal phenotype, further confirming its biological role in tumor pathologies.

The finding of the p53 regulatory network is especially relevant by describing a novel role for p53 in cell differentiation, in addition to its well-known function in maintenance of genome stability. Together with the interconnections between the different players, it provides further strength and robustness to such key EMT-TFs as SNAIL1 and ZEB in the EMT/MET regulatory network (Fig. 17.1).

Other Regulatory Circuits

In addition to the miR-200/miR-34 circuits other miRNAs can target EMT-TFs regulators in order to control the epithelial or mesenchymal status. A new regulatory loop between miR-203 and SNAIL1 has been determined by bioinformatics analyses although the biological implications are still to be defined (Moes et al. 2012). *SNAIL2* has also been recently described as a direct target of miR-204 and miR-203 in retinal pigment epithelium and breast cancer cells, respectively. Ectopic expression of miR-203 induces apoptosis and block cell cycle and invasion in breast cancer cell lines mediated, at least in part, by *SNAIL2* downregulation (Zhang et al. 2011). In a recent report, miR-214 was also shown to be involved in intrahepatic cholangiocarcinoma metastasis by targeting *TWIST1* (Li et al. 2012).

Other miRNAs control EMT-TFs by indirect mechanisms, among them let-7 miRNAs. The let-7 family is formed by 12 miRNAs members located on 8 different chromosomes in humans. Interestingly, let-7 is regulated both transcriptionally and through pri-miRNA processing. For example, during early embryonic development, let-7 pri-miRNAs are highly expressed, but no mature let-7 miRNA is available, providing a pool of sleeping miRNAs ready to be utilized on cell demand (Thomson et al. 2006). Different roles in cellular differentiation, embryonic

patterning and morphogenesis have been attributed to let-7. Remarkably, similar to miR-200, the let-7 family has not tissue-dependent activity but rather related to the differentiation state of the tissue suggesting that both miRNAs families are central differentiation regulators. In fact, let-7 family levels are downregulated in various cancer pathologies, including lung, colon, ovarian, gastric and melanoma, during tumor progression supporting their central role in epithelial plasticity. Mechanistically, let-7 acts as a tumor suppressor in humans, mainly because it directly binds to the transcripts of multiple genes with oncogenic activity such as *RAS* or the high mobility group *(HMG)A2* mRNAs (Thomson et al. 2006). This latter target provides to let-7 the ability to control EMT indirectly. HMGA2 is required for TGFβ-mediated EMT by regulating *SNAIL1/2*, *TWIST* and *ID2* transcription in NMuMG cells. Indeed, HMAG2 controls EMT by cooperating with the TGF-β/Smad pathway in regulating *SNAIL1* gene expression (Thuault et al. 2008). Thus, several miRNAs regulate direct or indirectly different EMT-TFs providing a fine modulation of these important players of epithelial cell plasticity.

The miRNA biogenesis machinery might also play an important role in tumor progression. For example, the miR-103/107 family controls miRNA biosynthesis by targeting Dicer. As a consequence, several miRNAs, including the miR-200 family, are diminished triggering cell de-differentiation and subsequently facilitating metastasis dissemination in breast cancer cells (Martello et al. 2010). Additionally, mutations or deletions of the argonaute complex including *AGO1*, *AGO3* or *AGO4*, are reported in some tumors as in renal Wilms tumors (Carmell et al. 2002).

MicroRNA Regulation in Cancer

Apart from the direct regulation of several miRNAs by transcriptional regulation described above, epigenetic regulation is also emerging as an important mechanism to control miRNAs involved in modulation of epithelial cell plasticity. Hypermethylation of tumor suppressor genes is a hallmark of cancer pathologies. Since several miRNAs act as tumor suppressor genes their epigenetic regulation has started to be investigated by several independent groups.

Inactivation of miR-34 by CpG methylation is found mainly in prostate tumours (80%) and melanoma (63%) but also in many cancer lines derived from breast, colon, lung and other types of cancer (Vogt et al. 2011). Similar to the miRNAs/EMT-TFs feedback loop, CpG promoter methylation provides an additional control step into the regulation of epithelial plasticity. As discussed above, TGF-β treatment induces a complete EMT in different epithelial cell models. Interestingly, a progressive gain of CpG methylation in the miR-200 family promoter region was observed after TGF-β treatment accompanied with an increase of ZEB protein levels and the loss of epithelial features. Conversely, TGF-β withdrawn resulted in an increase of miR-200 by promoter de-methylation associated with a decrease of ZEB proteins and re-expression of E-cadherin (Davalos et al. 2012). However, prolonged treatment with TGF-β induces the activation of an autocrine TGF-β/ZEB/miR-200 signaling network that induces a permanent methylation of the CpG miR-200 promoter islands locking the plasticity of the system into a mesenchymal state in MDCK cells (Gregory et al. 2011). Epigenetic regulation of the miR-200 family and its relationship with aggressiveness and chemoresistant behavior has been established in different cancer cell types including NSLC, colon and breast carcinomas (Davalos et al. 2012; Wee et al. 2012). In addition, the promoter of miR-203 (targeting SNAIL2) is also hypermethylated in several cancers including hepatocellular, prostate and breast carcinoma (Zhang et al. 2011).

Recently, the role of CpG methylation of two important miR-200 family members, miR-200b-a-429 and miR-200c-141 has been analyzed in detail by Esteller's group showing a dynamic epigenetic regulation associated with an EMT or MET process in cancer progression (Davalos et al. 2012). Thus, a methylated status was correlated with low expression of E-cadherin and the

acquisition of a mesenchymal phenotype, while an unmethylated status was found in epithelial cells (Fig. 17.1). Moreover, gain and loss of function studies of miR-200 further demonstrated the plasticity of the system balancing from an epithelial to a mesenchymal phenotype and vice versa. The biological relevance of the methylation status was also further confirmed by microdissection of human primary colorectal samples. Interestingly, normal epithelial colon mucosa crypts and stroma (mesenchyma) bear unmethylated and methylated miR-200 promoter, respectively, whereas colorectal tumors undergo selective miR-200 hypermethylation of their epithelial component confirming the association between methylation and differentiation status (Davalos et al. 2012). Interestingly, it has been shown recently that the methylation rate of the miR-200 loci is related to the breast cancer subtype and metastasis outcome. Promoter analysis in 93 breast cancers has identified the presence of a novel CpG promoter region in the Hsa-mir-200b cluster mapped 2 kb upstream of the 5′stem loop region whose hypermethylation was associated with loss of either ostrogen or progesterone receptor while hypermethylation of the upstream promoter is associated with lymph node metastasis (Wee et al. 2012).

Diagnostic and Therapeutic Implications

Apart of the biological relevance of miRNAs in cancer, there are also diagnostic and clinical implications. Indeed, several studies have tried to establish miRNAs signatures in order to investigate their potential clinical utility. A macrostudy with 334 human samples including multiple human cancers showed that miRNA profile signature was more accurate to determine the developmental lineage and differentiation state of the tumors than the mRNA profile (Lu et al. 2005). On the other hand, miRNA profiling in endometrial carcinosarcomas showed that several miRNAs, including miR-200, miR203, miR-23b and miR-29c were downregulated in the mesenchymal part of the tumor and thus participating in the maintenance of the dedifferented phenotype and stemness features (Castilla et al. 2011).

The recent finding of the presence of several cancer-related miRNAs in the serum or plasma has opened new strategies for their use as biomarkers allowing to differentiate between the healthy population and patients with a tumor invasive pathology. For example, miR-141 has been recently proposed as a prostate tumor marker since its expression is elevated in the patient's serum. Similar observations have been made for miR-92a and miR-29a in colorectal cancer (reviewed in Zhang and Ma 2012).

Concluding Remarks

The finely regulation between EMT and MET programs is emerging as essential for embryonic development and tumor progression. Recently, miRNAs have emerged as key players of these regulatory circuits by targeting the classical EMT-TFs and establishing regulatory feedback loops between them that in turn can be controlled by key cell keepers as p53. This kind of mechanism provides flexibility and robustness to the cell since a single miRNA might modify different pathways such as cell cycle, apoptosis, stemness and the differentiation program. However, several challenges remain to be elucidated. Why are there so many miRNAs involved in cell differentiation and tumor suppression? Can they be used in the clinic as biomarkers or prognostic markers? Do certain miRNAs can be used to treat cancer pathologies? If so, which step of the metastatic process should be more efficient to target? The central role of miRNAs regulating different targets might simplify the treatment Thus, understanding the biological roles and modifying finely miRNAs levels open new strategies to combat cancer progression.

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Methods in Mathematical Modeling for Stem Cells

18

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Contents

Abstract

As the field of stem cell research advances, there will be an ongoing and increasing need for mathematical models and other quantitative tools which facilitate research and discovery. Our main concern in this chapter is the description of the applications for several available models to this field of stem cell research. Two important examples of such models include the use of hyperbolastic growth models in stem cell proliferation and the hypertabastic survival model in analysis of time until differentiation. In addition we describe a number of other cases where development of mathematical models or quantitative tools may facilitate stem cell research. One additional area of particular concern is in mathematical modeling related to the cancer stem cell hypothesis. The targeting of cancer stem cells has the potential to transform cancer treatment, and we discuss several related issues and directions for mathematical modeling.

Introduction

As stem cells are used with increasing frequency in areas of medical research, medical treatment, and biomedical engineering, we can expect an increasing demand for mathematical tools that can assist practitioners and researchers in these areas. Here we focus on several mathematical models and introduce their applications in various

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aspects of stem cell research. A growth model applied in a previous article of Tabatabai et al. (2011b) to proliferation of stem cells, has proven to give an accurate representation of rates and time course of proliferation for both embryonic and adult stem cells. One of the fundamental areas of research for stem cells is their differentiation, and in this chapter we also propose use of several models for research in this area. The hypertabastic survival model of Tabatabai et al. (2007) has demonstrated versatility and robustness in modeling of time to event data, and we propose use of this model in the study of time to differentiation for stem cells. This model allows for the inclusion of the role of covariates in influencing time to event and can thus be used in studying the influence of external agents in directing the differentiation of stem cells, including factors such as growth factors, oxygen, or retinoic acid. Another primary concern for differentiation is the percentage of the stem cells that differentiate, and this percentage can likewise be influenced by additional covariates. We consider how the hyperbolastic growth models, and particularly the multivariable forms of these models, can be used to model the influence on covariates on the percentage of cells that undergo differentiation.

In addition to the vast potential for use of stem cells in direct medical therapies, one of the promises of stem cells research includes a more thorough understanding of embryonic development and pathologies that may develop during the gestational period. One particular area of interest in embryonic development has been the role of Hes-1 gene expression, from its role in somitogenesis to its role in neural development, morphogenesis, and immune system development. We briefly review a few related models, including the oscillabolastic model introduced by Tabatabai et al. (2012b). The action of Hes-1 affects various aspects of embryonic development as it plays a role in self-renewal and differentiation of stem cells. Consider also that somtiogenesis and the role of Hes-1 in differentiation and self-renewal are only some of the numerous important events in embryonic development, and there is a potential for modeling in many other related areas. We may expect many such models to be produced as the field develops, and issues of signaling and differentiation are likely to play a critical role in this area. Above we have mentioned several separate models related to various stem cell issues, including proliferation, self-renewal, differentiation, and inter-cellular signaling. We also outline a proposal to form integrated model combining these issues, and we are particularly interested to see how parameters representing factors such as time to differentiation, proliferative rate, or related quantities can affect the composition of the larger system of cells generated from the stem cells.

Finally, we explore the role of cancer stem cells in fueling the growth of tumors, as related to the cancer stem cell hypothesis. We briefly describe how the existence of cancer stem cells directly impacts the treatment strategies that should be used in combating these tumors. Existence of stem cells in a tumor affects the growth of the tumor as well as the response to therapies, and it is desirable to design therapies that will target these stem cells. We outline some mathematical modeling we have begun in this area, simulating tumor growth under different assumptions and differing approaches to targeting cancer stem cells. In addition, we consider how biomarkers relating to cancer stem cells can be used within the survival analysis for cancer patients. We apply our recently developed methods of survival analysis to this analysis. We close with a short survey of several important streams of biomathematical research that will interact strongly with the functioning of stem cells. In these areas of cellular signaling, systems biology, and bioinformatics, there is ongoing development of mathematical models and tools, with many important discoveries and breakthroughs to be expected in the coming years. Inclusion of these issues is essential for a more complete understanding of stem cell action, thus we close with this brief hint of what is possible in the years to come with the inclusion of these essential streams of research in the currents of stem cell modeling.

Modeling of Proliferation

One of the essential functions of stem cells is the production of new cells for use in the organism, and our first problem is to find a mathematical model which accurately represents the rate of cellular proliferation. We have selected one model from a group of three hyperbolastic growth models developed in Tabatabai et al. (2005) as flexible models for representation of biological growth. The model H3, short for the hyperbolastic growth model of type III, will be presented below and is in many cases the most accurate of these three hyperbolastic growth models. This model has been particularly useful in representing cases of cellular growth, with highly accurate representations of cancer growth demonstrated in Tabatabai et al. (2005) and Eby et al. (2010), wound healing demonstrated in Tabatabai et al. (2011a), and stem cell proliferation studied in Bursac et al. (2006) and Tabatabai et al. (2011). The H3 model we present below is a representation of self-limiting growth based on the Weibull model, but which provides more flexibility in the shape of the curve, allowing further variation in the growth rate with the time variable.

The growth rate will be given by the nonlinear differential equation

$$\frac{dP(t)}{dt} = \left(M - P(t)\right)\left(\delta\gamma t^{\gamma-1} + \frac{\theta}{\sqrt{1+\theta^2 t^2}}\right), \qquad (18.1)$$

with the initial condition $P(t_0) = P_0$, where M, δ, γ and θ are parameters. We refer to the model (18.1) as the hyperbolastic ordinary differential equation of type III or H3. This rate of growth is a product of one factor representing the distance of the current population from its limiting value M and a second factor including the intrinsic rate δ, an allometric constant γ, and an additional term θ allowing flexibility in growth rate over time. The solution to the Eq. (18.1) is the function

$$P(t) = M - \alpha EXP\left[-\delta t^{\gamma} - \operatorname{arcsinh}(\theta t)\right], \qquad (18.2)$$

where

$$\alpha = \left(M - P_0\right) EXP\left[\delta t_0^{\gamma} + \operatorname{arcsinh}(\theta t_0)\right].$$

We call the function $P(t)$ of Eq. (18.2) the hyperbolastic growth model of type III or simply H3. If necessary, one can introduce shift or delay parameters in this model. The doubling time t for the model H3, in the case where $P_0 < M/2$, is the solution to the equation

$$\ln\frac{M - 2P_0}{\alpha} + \delta t^{\gamma} + \operatorname{arcsinh}(\theta t) = 0.$$

Here we briefly address the biological meaning associated to the parameters M, δ, γ, and θ. The parameter M has the same units as $P(t)$, the number of stem cells, and it represents the limiting value of the size of the population, or the carrying capacity. The parameter δ corresponds to the intrinsic biological growth rate; however the overall rate of growth is jointly determined by all of the parameters δ, γ, and θ. The units of δ is 1/(time)$^{\gamma}$, which in the cases of this paper is 1/(days)$^{\gamma}$. The parameter γ is known as the allometric constant, and a similar parameter occurs in the Weibull model. This parameter is dimensionless. It is sometimes called a statistical shape parameter. Finally, the parameter θ has units 1/(time), in our case 1/(days), and to more fully describe its biological meaning we rewrite the Eq. (18.2) in the form

$$P(t) = M - \frac{\alpha}{\theta t + \sqrt{1+(\theta t)^2}} EXP\left[-\delta t^{\gamma}\right].$$

For θ = 0, the term in front of the exponential reduces to α, and the model reduces to the Weibull growth model. When $\theta \neq 0$, the expression $\alpha(t,\theta) = \frac{\alpha}{\theta t + \sqrt{1+(\theta t)^2}}$ allows this factor to vary with time t, according to this formula and the value of θ. Thus the parameter θ provides variation in the quantity α, which represents a rescaling of the distance between the initial population and the limiting value, allowing adjustment of this growth rate over time. In application of the model a constant value is determined for θ that best represents the growth observed in the data.

In the following paragraphs we apply the hyperbolastic model H3 to some publicly available data on embryonic stem cell growth, found on the NIH website. We consider the cell lines GB01, WA01, and WA07, for which there were 6 days of growth data available, and we display

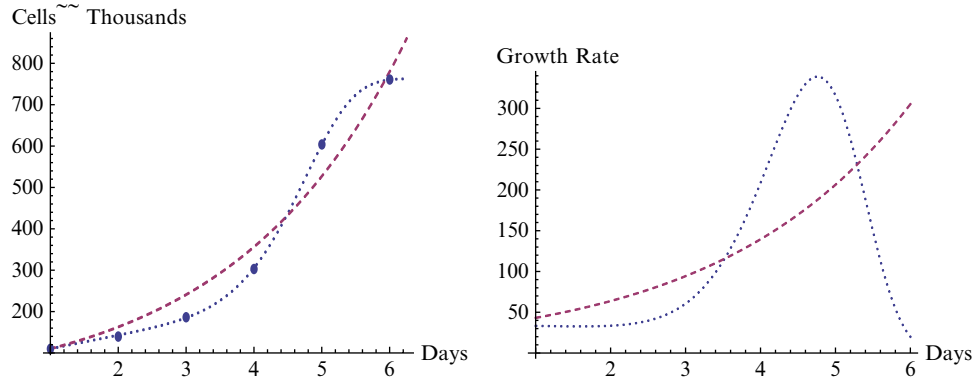

Fig. 18.1 Growth and growth rate for GB01 line, H3 and Sherley models

the difference between H3 and the Sherley model, a commonly used model for cellular growth. Although the parameters in the Sherley model represent the dividing time and the proportion of cells actively proliferating, we see that the estimated growth curves for this model do not fit the data points. See Figs. 18.1 to 18.3. In cases like these the parameter estimates cannot really be considered to be representative of the biological data. In contrast, the H3 model provides a very close fit to the data in all three cases. In addition to the significance of the H3 parameters, as presented above, these explicitly defined functions also allow explicit representation of the rate of growth, as given in each of the figures below. We note that this growth rate, and particularly the location of its maximum, can be investigated for the underlying biological significance.

We begin with a statistical assessment of the accuracy of the two models for these data sets. In each case the H3 is considerably more accurate than the Sherley model, and in two of the three cases it is nearly perfect. For the first case of the BG01 cell line, the parameters for the Sherley model are $\alpha = 1.000$ and $DT = 1.770$, while the parameters for H3 are $M = 762.922$, $\delta = 3.137E\text{-}6$, $\gamma = 7.990$, and $\theta = 0.051$. The H3 model is nearly a perfect fit, with R^2 of 1.000 and RMS of 8.470, while the Sherley model has R^2 of 0.966 and RMS of 3096.170. We observe more in Figs. 18.1, 18.2 and 18.3 how H3 really makes a much better representation of the growth curve, capturing the overall shape. In contrast, the Sherley model does not capture the overall shape of the data and can be expected to magnify the errors if time is extended. For the WA01 cell line of Fig. 18.2, the fit of Sherley is a little better, while the fit of H3 is a little worse, although this difference is probably due to the existence of a misplaced point. The Sherley model use parameters of $\alpha = 1.000$ and $DT = 1.770$, while the parameters for H3 are $M = 10193.018$, $\delta = 4.204E\text{-}9$, $\gamma = 1.769$, and $\theta = 0.069$. With this misplaced point, R^2 for H3 has decreased to 0.994, with an RMS of 180,819.041. In contrast, Sherley has R^2 of 0.981 with an RMS of 282,099.496. A case like this is about as close as Sherley can get to H3, due to the placement of the point, as we discuss further below. Nevertheless H3 still provides a considerably better fit, with just over half the RMS or Sherley. The third case of the WA07 cell line returns to a case with points in standard position, and the H3 has a fit with $R^2 = 0.999$ and $RMS = 17{,}817.688$ with parameters $M = 7635.150$, $\delta = 0.003$, $\gamma = 4.054$, and $\theta = -0.031$, compared to Sherley with $R^2 = 0.955$ and $RMS = 476{,}844.808$ for parameters $\alpha = 0.525$ and $DT = 0.184$. In the Figs. 18.1, 18.2 and 18.3 depicting these models, the dashed curve represents the Sherley model, while the dotted curves represent H3.

We note that in Fig. 18.1 for the BG01 cell line and Fig. 18.3 for the WA07 cell line the growth curve from H3 captures the overall shape of the data and displays only minimal errors in

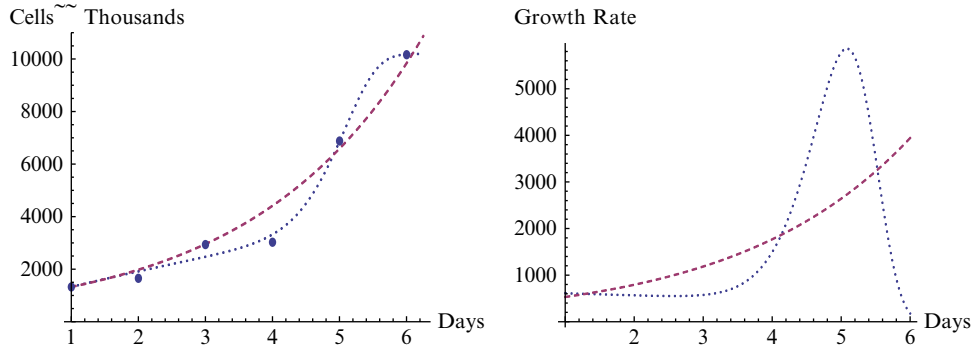

Fig. 18.2 Growth and growth rate for WA01 line, H3 and Sherley models

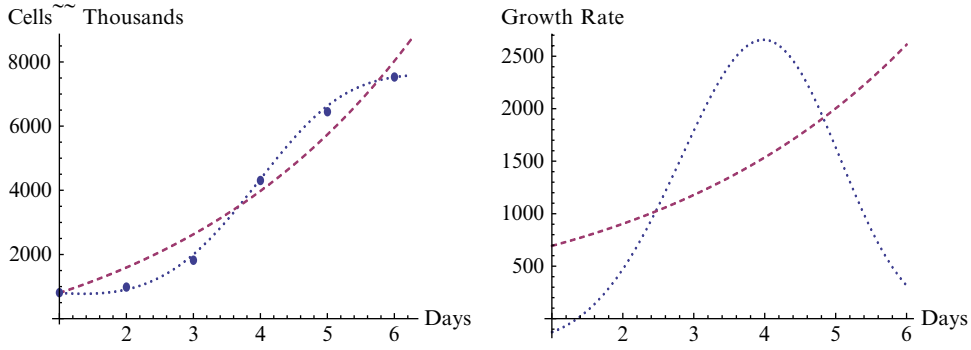

Fig. 18.3 Growth and growth rate for WA07 line, H3 and Sherley models

approximating the points, with each point contacting the curve. This is in marked contrast to the representations by the Sherley model, where the overall shape of the data cannot be represented by the curve and all of the points are at some distance from the curve, yielding considerably more error in approximation. As each of the figures clearly represent self-limiting, or sigmoidal shaped growth, the H3 model clearly better represents the growth, and the increasing growth rate of the Sherley model will lead to even larger errors for extended time.

The case in Fig. 18.2 for growth of the WA01 cell line is interesting because of the unusual pattern in the data for days 3 and 4, where the data shows the number of cells is static, in contrast to the standard pattern of growth. Sherley model is able, by coincidence, to find a close fit to the other data points by nearly neglecting the data point from Day 4. However, clearly this curve still does not represent the overall sigmoidal shape of this or the other data sets. The H3 model cannot attain as high an accuracy as the other cases as the curve straddles the data points for days 2, 3, and 4. But it is still very close, and is clearly the better representation of the two curves, even moreso than can be seen in the differences in R^2 and RMS. Note that with a slightly lower value on day 3 or a slightly higher value on day 4, Fig. 18.2 would fit the same pattern as Figs. 18.1 and 18.3, and the statistical measures of fit would return to the comparable level.

The above examples of applying the H3 model to represent proliferation of embryonic stem cells are representative of the high level of accuracy found in this model. The papers of Bursac et al.

(2006) and Tabatabai et al. (2011b) compare the accuracy of diverse models for the proliferation of the BG01 cell line, demonstrating that H3 indeed provides the best fit, followed closely by the other hyperbolastic models H2 and H1. The paper of Tabatabai et al. (2011b) also demonstrates the accuracy of the hyperbolastic models, particularly H3, in representing the proliferative index for adult stem cells. We assume these models are equally effective in representing proliferation of induced pluripotent stem cells.

We are currently developing the hyperbolastic models H1 to H3 in several further directions. One such direction is the development of robust methods for these hyperbolastic models so they can maintain an accurate representation of the growth curve, even when one or several of the points are displaced. The above discussion allied with the growth curve for the WA01 cell line as depicted in Fig. 18.2 is representative of some of the difficulties that can arise in robustness for such non-linear models, due at least in part to the flexibility in the shape of the curve. In addition we are currently developing these hyperbolastic models for use in longitudinal studies as non-linear hyperbolastic mixed effect models. We also note that a multivariable version of the model already exists and has been applied in Tabatabai et al. (2011b), in Tabatabai et al. (2011a), and in a recent work of the authors modeling phytoplankton growth dynamics. This multivariable version uses a link function and additional parameters to represent the role of additional covariates in accelerating or decelerating the growth rate. One additional topic for continuing development will be in relation between the model parameters and the biological characteristics of the system being modeled. Particularly in cases where these models are used together in a system of differential equations, as in the section on the Integrative Model below, it will be important to tie the model parameters in to the biological characteristics.

Differentiation

Another fundamental property of stem cells is their ability to differentiate into a variety of cell types, as required for use within the organism. The course of differentiation is primarily guided by gene expression and epigenetic regulation, and an important role is also played by intercellular signaling, primarily directed by growth factors and other soluble factors. A more thorough understanding of the means of directing differentiation is desirable and would be beneficial to stem cell therapies. Here we propose use of two mathematical models that can assist in the study of differentiation of stem cells and the role of explanatory variables. The first of these models is a survival model which can be applied to study the time to event in various applications, including survival time, time until maturation, healing time, as well as many others. In the present context we will study time until differentiation of stem cells together with the influence of one or more covariates, such as level of oxygen or differentiating agents, on the differentiation time. One of the main concerns here is a quantitative assessment of the role of such explanatory variables on the differentiation procedure. The key features of the model we use, the hypertabastic survival model introduced in Tabatabai et al. (2007), are the quantification of the roles of one or more covariates and the flexibility of the distribution in assuming various shapes of hazard curves dependent upon the underlying distribution of data.

We begin by defining the hypertabastic distribution function, which is given as follows

$$F(t)=\begin{cases}1-sech\left\{\alpha\left[1-t^{\beta}coth\left(t^{\beta}\right)\right]/\beta\right\} & t>0\\ 0 & t\le 0\end{cases}$$

The hypertabastic probability density function is given by

$$f(t)=\begin{cases}sech\left[W(t)\right]\left[\alpha t^{2\beta-1}csch^{2}\left(t^{\beta}\right)-\alpha t^{\beta-1}coth\left(t^{\beta}\right)\right]tanh\left[W(t)\right] & t>0\\ 0 & t<0\end{cases} \tag{18.3}$$

where $W(t)=\alpha[1-t^{\beta}coth(t^{\beta})]/\beta$, and $\alpha,\beta>0$.

The hypertabastic proportional hazard model has a hazard function of the form

$$h(t \mid x,\theta)=h_0(t)\,g(x \mid \theta) \qquad (18.4)$$

where $h_0(t)$ is the baseline hazard function, given by

$$h_0(t)=\alpha\left[t^{2\beta-1}csch^2\left(t^{\beta}\right)-t^{\beta-1}coth\left(t^{\beta}\right)\right] tanh\left[W(t)\right].$$

We will use $g(x|\theta)=Exp[\Sigma_{k=1}^{p}\theta_k x_k]$. The x_k represent the covariates being studied in the model, and the associated parameters θ_k describe the influence of these covariates.

Similarly the hypertabastic survival function $S(t \mid x,\theta)$ for the proportional hazards model has the form

$$S(t \mid x,\theta)=\left[S_0(t)\right]^{g(x|\theta)} \qquad (18.5)$$

where $S_0(t)$ is the baseline survival function, given by

$$S_0(t)=sech\left\{\alpha\left[1-t^{\beta}coth\left(t^{\beta}\right)\right]/\beta\right\}.$$

In some cases, the accelerated failure survival model may be more appropriate than the proportional hazards model just presented. For the accelerated failure form of the model, see Tabatabai et al. (2007).

We mention that the hypertabastic survival model differs from other parametric survival models in the increased flexibility of the shape taken by it hazard function, which varies considerably with the parameters α and β. See Tabatabai et al. (2007) for illustration of some of the various shapes taken by the hazard function. Simulation of the hypertabastic distribution in comparison to other distributions such as Weibull, log logistic, and log normal have demonstrated in Bursac et al. (2006) that the hypertabastic distribution is flexible to data of all these types and it thus displays a robustness with respect to departure from distribution.

In conclusion we recommend this hypertabastic survival model for use in modeling time to differentiation of stem cells. The inclusion of covariates allows us to study the influence of the level of oxygen or growth factors or other differentiating agents upon the time to differentiation. This will allow a quantitative analysis of factors which accelerate or decelerate the process of differentiation.

The second mathematical model we are proposing for the study of differentiation of stem cells will also yield a quantitative description of the influence of covariates upon the process of differentiation. In this case we are concerned with the percentage of cells which follow a certain (given) path of differentiation, and in particular how this path is affected by covariates such as level of oxygen or levels of growth factors or other soluble factors. Similar to dose response studies, we are curious to make a quantitative assessment of how the level of the differentiating agent affects the percentage of the cells which will follow a prescribed path of differentiation. Such dose response curves follow a sigmoidal curve, with the potential for significant nonlinearities, making the hyperbolastic models, and particularly H3, the ideal candidates for models. We propose use of H3 for this quantitative analysis. Using methods similar to those outlined in the section on Proliferation, it is possible to use H3 to both find a close approximation to the dose response curve as an explicitly defined function and furthermore to analyze the dynamics of this function, including the maximum rate of change.

The scientific program of research to understand or even to control the differentiation function of stem cells is of fundamental importance in the use of stem cells for medical therapies. The two tools presented in this section should help researchers to describe the quantitative effects of covariates in influencing both the time until differentiation and the percentage of cells which follow a given path of differentiation. Deeper biological understanding of the means of directing the differentiation of stem cells will also be a critical area of study. Advances in this area may likely come through deeper understanding of intercellular signaling and of the genomic programming behind the passage to differentiation. See the section "Cancer Stem

Cells and Survival Analysis" for additional ideas in applying the hypertabastic distribution together with gene signatures in the understanding of differentiation. Also see the section "Relations to Genomics, Systems Biology, and Cellular Signaling" for ideas of how tools of bioinformatics may be applied to understand the mechanisms of differentiation. Understanding the means of differentiation of stem cells is critical when producing a specific type of cell for use in a medical therapy. Furthermore the differentiation of the embryonic stem cells in a developing embryo is critical for the proper development, and deeper understanding of these processes is highly interesting to embryologists. In the next section we treat one specific mathematical model of a process which plays a key role in embryonic development.

Embryonic Development and the Role of Hes1

The role of stem cells in both initiating the growth of an individual organism as an embryo and in maintaining and renewing the organism is one of the fundamental areas of study in biology, linking together several essential aspects of the science of life. A delicate balance is held between adult stem cells maintained in the stem cell niche in a state of immortality and the direction of daughter cells along pathways of differentiation to become functional cells with a temporal role in the organism. And in the area of embryonic development where the whole of the organism is produced from one totipotent cell there is an even greater balance and greater level of complexity required as each cell achieves the proper degree of specialization for the development of the organism. This miraculous transformation occurs through the unwinding of genetic programming, as this programming interacts with and directs the development of the embryonic stem cells. The role of stem cells in the development of the embryo gives them a central position in the earliest stages of the development of an organism. It only makes sense that researchers should investigate these areas to more fully understand these vital aspects of life sciences, all with the potential to lead to advances in medicine.

This field of study is vast in the understanding of stem cells within embryonic development, and certainly there is a potential to apply mathematical modeling in many areas of this field. However, we focus on one specific topic as a representative example. One fundamental step in the development of vertebrate embryos is somitogenesis, in which temporal oscillations in gene expression of Hes1 are transformed into the segmentation of the mesoderm, eventually forming the vertebrae, as described in Pourquié (2003). Several mathematical models have been formulated to represent the process of somitogenesis and the role of Hes-1, and we mention briefly the models of Baker et al. (2008) and of Zeiser et al. (2007).

Hes1 is a source of one of the earliest known oscillations in embryonic development, where Hes1 and Hes7 are among the first genes discovered oscillating within the presomitic mesoderm. This oscillation forms the basis for the spatial oscillation which forms the somites, a central event in embryonic development. Furthermore Hes1 has been shown to play a key role in many areas of embryonic development including neural development, blood, heart valves, and aspects of the immune system. The ultradian oscillations of Hes1 are important for signaling within the Notch pathway, a key feature of many areas of embryonic development, including neural, cardiovascular, and endocrine development.

The authors have recently introduced an oscillabolastic model in Tabatabai et al. (2012b) to be used for accurate approximations, including in cases of damped oscillations or growth combined with oscillations. This oscillating model has a growth rate given by

$$\frac{dP(t)}{dt} = \frac{\gamma\theta}{t\sqrt{1+\theta^2 t^2}} + \frac{M - P(t) + \alpha\beta cos(\beta t)}{t} \quad (18.6)$$

for initial condition $P(t_0) = P_0$ and $t > 0$, where α, β, γ, and θ are model parameters and $M = P_0 - \alpha sin(\beta t_0)/t_0 - \gamma \text{arcsinh}(\theta t_0)/t_0$.

If $t_0 = 0$, then

$$M = \lim_{t \to 0^+} \begin{pmatrix} P_0 - \alpha \sin(\beta t)/t \\ -\gamma \text{arcsinh}(\theta t)/t \end{pmatrix} = P_0 - \alpha\beta - \gamma\theta.$$

The solution of the initial value problem (18.3) is

$$P(t) = M + \alpha \sin(\beta t)/t + \gamma \operatorname{arcsinh}(\theta t)/t \qquad (18.7)$$

where $\lim_{t\to\infty} P(t) = M$, and $\lim_{t\to 0^+} P(t) = M + \alpha\beta + \gamma\theta$. Recall that molecular processes such as mRNA transcription, protein transportation, and Hes1 decay have been shown to affect the form of the oscillations of Hes1 gene expression. We note that the oscillabolastic model provides flexibility in the patterns of the oscillations, thus allowing the model to represent oscillations in Hes1 signal intensity.

See Tabatabai et al. (2012b) for more information on the parameters and for some applications of this model. One important application of such a model is in representation of oscillations of gene expression, such as observed in gene expression of NF-κB, p53, or Hes-1. This article of Tabatabai et al. (2012b) demonstrated the good fit of this model for a set of experimental data of Hes-1 gene expression, and it is expected that similar applications can be made to oscillations of these other transcription factors, based on similar factors of time delay in transcription and protein transportation. The application of this model to Hes-1 gene expression has particular relevance here because of the role in embryonic development, as mentioned above. Further discussion of importance/role of Hes1 can be found in our article, Tabatabai et al. (2012b) and the references therein. Recent papers including Jamieson (2010) and Kunisato et al. (2003) address the role of Hes1 in self renewal of various types of stem cells, including hematopoietic and neural stem cells. In the early stages of embryonic development the totipotent embryonic stem cells begin to differentiate into the three germ layers, which later differentiate further in the formation of organ systems. The recent work of Kobayashi and Kageyama (2011) implicates the oscillations of Hes-1 in this heterogeneous differentiation of embryonic stem cells. Notch signaling is also implicated in regulation of the cell cycle and of cellular proliferation, and thus we see Hes-1 plays an integral role throughout many aspects of embryonic development.

We have only touched on several small points within the field of embryonic development, and we mention briefly the potential for mathematical models in many other directions relating to embryonic development as the field develops. The many areas for future study are too numerous to list, but include important issues such as the action of AVE, BMP, Wnt, and other genes in the patterning of the body, the role of capases and the CED and BCL genes in the regulation of apoptosis and the formation of body structures. Also of interest are the signaling and regulation of the earliest differentiation events, including the action of the GATA class of transcription factors. Many others are of interest, but we close by mentioning the role of tumor suppressor genes and the relation of embryonic development to later development of cancer and also the role of methylation of areas of DNA and the changing patterns of DNA methylation during development. The role of signaling and the direction of the course of differentiation may be expected to play an important role within this field.

Integrated Model

The models presented above in the sections modeling of Proliferation and Differentiation give mathematical tools for research in stem cells which give an accurate representation of experimental results, such as the representation of stem cell proliferation rate in the section "Modeling of Proliferation". Although these models deal with various aspects of stem cell research, including proliferation and differentiation, it would be valuable to develop an integrated model for stem cell growth which may be used for understanding of the population of daughter cells descending from a group of stem cells.

Action of stem cells through self-renewal, symmetric and non-symmetric division, and differentiation generate and support a population of cells within a living system. Any loss of cells, shocks to the system, or normal turnover of aging cells will be accommodated within this population through the action of the stem cells and their progeny. We are interested to form an integrated model which considers such a population of cells, formed and maintained by the stem cells and their progeny. This system will include parameters to represent rates of proliferation,

time until proliferation, and course of differentiation for each of the types of cells. The population of cells with growth fueled by the stem cells and their progeny will reach an equilibrium population, and one goal of the model will be the determination of this equilibrium level, as well as an understanding of how it is affected by changes in the system.

The importance of such an integrated model is to model the relation between the stem cells, the precursor cells, and the functional cells of the organism, as well as the functional dependence between these cell types. In part, such an integrated model will allow us to study the issue of how this larger system maintains an equilibrium and how adjustments may be made to maintain homeostasis in response to shocks. Furthermore this integrated model will provide a means to study how this larger living system can be affected by changes in quantities such as the proliferation rate for each cell type, rate of differentiation, the proportion of cells differentiating in various directions, the rate at which certain cells die, the total number of stem cells in the compartment, or other related quantities for the system.

Based on the topics just mentioned, it is clear how this model could be useful in study of related pathologies which could affect some of these quantities. Similar uses of the model could be made in studying effects of stem cell therapies which may likewise affect the above quantities. Such a unified model addresses the interactions between the stem cells, as a source of the other functional cells in the organism, and the larger system. Such a mathematical model can represent how the functional cells of the organism interact with and are affected by the stem cells which are their source. Thus the unified model will be useful for a more thorough understanding of the basic points of proliferation, self-renewal, and differentiation, and their impact on the larger system (organism). In the context of proliferation, it will be interesting to study how the models of proliferation presented above, and their parameters, relate to this unified model and its parameters representing more fundamental processes of stem cells. An integrated model may find relevance in relation to embryonic stem cells and embryonic development. The use of such an integrated model will also be critical in the modeling of action of cancer stem cells and tumor response to treatment discussed below in the section "Cancer Stem Cell Hypothesis and Cancer Treatment".

Note that the concept of stem cell niche is directly related to the issues treated in the integrated model. These local environments in which the stem cells reside also help direct their fate, and as a consequence also the fate of the larger group of cells supported by the stem cells. It is important that the stem cell niche and other important factors including, intercellular signaling, genomic programming, and epigenetics (gene expression) affect the stem cell fate and thus also the other cells in this larger system. Many of the quantities and interactions that go into determining the dynamics of this system will be established by the above means. Although formation of an individual model which includes all of this information would be too complex, it may still be informative to study how factors such as genomics, epigenetics, and cellular signaling affect stem cell action. Study of these issues will be expected to be critical for a deeper understanding in this field and will relate directly to application of such a model. We also take up idea for models related to some of these issues in the remaining sections.

Cancer Stem Cell Hypothesis and Cancer Treatment

The concept of a stem cell first emerged from study and treatment of cancer, and current research has identified cancer cells with stem cell like properties in numerous types of cancers, including leukemia, melanoma, multiple myeloma, and cancers of brain, breast, colon, ovary, pancreas, and prostate. Stem cells were first conceptualized in cancers with a high turnover rate, in particular leukemias in which the high rate of proliferation combined with the immortality of the stem cells produces the conditions in which large numbers of mutations could accumulate, leading to cancer. It is also in these cases where cancer stem cells are best understood and have been most targeted in therapies.

Cancer stem cells are receiving ongoing attention and research, and their existence has been revealed experimentally in numerous cancers, as mentioned above. These cells are differentiated from other tumor cells through differences in surface and receptors on cellular membrane, and may also be observed through the ability to regenerate the tumor. Breast cancer stem cells were originally observed in Al-Hajj et al. (2003) as the small subset of all cancer cells that possessed the ability to form new tumors. The underlying concept in which the self-renewal properties of stem cells and their immortality relate to the growth and invasiveness of a tumor calls for further investigation. Some of the critical properties of tumors faced by oncologists and cancer patients, including high rates of growth or invasiveness, resistance of certain cells to treatment, recurrence of tumors can be explained in the context of the existence of cancer stem cells. We also address briefly the role of cancer stem cells and their self-renewal in the process of metastasis.

The conventional cancer treatment strategies including chemotherapy and radiation therapy focus on decreasing the bulk of tumor cells, whereas a strategy targeting the cancer stem cells which fuel the growth of the tumor could be more effective. Many researchers have proposed that therapies which target cancer stem cells have the potential to revolutionize the approach to cancer treatment. Al-Hajj (2007), Chumsri (2007), and Regenbrecht (2008) discuss approaches such as targeting of these cancer stem cells, disruption of their self-renewal pathways, and therapies that induce the differentiation of such cancer stem cells. The cancer stem cell hypothesis predicts many of the current limitations in cancer therapy, including regrowth of tumor cells after initial reduction, the limited effect of therapies on certain cells, the development of resistance of later generation tumor cells to these therapies, and the existence of especially invasive or aggressive cases of cancers. Instead of indiscriminately applying a therapy equally to all tumor cells with the guiding principle of tumor mass reduction, the strategy of targeting cancer stem cells interfaces directly with developing trends in cancer treatment, including molecular and phenotype characterization, targeting of related pathways, and individualized therapies. The modern perspective introduces the potential to make use of recent advances in biological research in areas such as genomics, signaling, molecular and cellular processes to comprise a more thorough regimen of treatments to this pathology. Increased understanding of and treatment of the tumor cells known as cancer stem cells promises to be an important part of these advances in cancer therapies.

We give a short view of differentiation therapy, a therapy based on the cancer stem cell model which has already proven its efficacy in several important cases. The idea behind differentiation therapy is to induce differentiation of the cancer stem cells into other tumor cells which no longer fuel the growth of the tumor. Instead of self-renewing, the cancer stem cells can be induced, through signaling pathways, to undergo symmetric division and produce two cells that go on to become transit-amplifying cells and later more fully differentiated cells. Early successes inducing differentiation in teratocarcinomas using retinoic acid from Sell and Pierce (1994), and recent successes in treating leukemia, from Sell (2005), provide evidence this intriguing approach to cancer treatment can prove effective. The prospect of directly treating the cancer stem cells can potentially be very powerful, especially in cases otherwise resistant to treatment.

We are currently working on several approaches to mathematical modeling of the cancer stem cell hypothesis and its potential impact for treatment strategies. The first of this group of models focuses primarily on the difference between a treatment strategy which indiscriminately eliminates cells in the goal of overall tumor mass reduction compared with any of several means of targeting the cancer stem cells. We focus primarily on the difference in the patterns of regrowth for the post-treatment tumors, and the corresponding outlook for the patients. We compare a stochastic randomization of which cells are eliminated by the therapy with any of several approaches to target the stem cells. The models for original tumor growth and regrowth are based on the hyperbolastic models

of the section "Modeling of Proliferation", which have a demonstrated accuracy in representing tumor proliferation. In addition we are concerned with the tumor mass as a heterogeneous collection of cells as generated by the tumor stem cells, and we also incorporate relevant aspects of the section on the "Integrative Model". In the sense that the given tumor system is generated by the cancer stem cells within the tumor, any means we can find of targeting these cells will clearly produce an improved treatment strategy. Our goal here is to introduce some basic quantitative models for purpose of comparison. We are also interested to see how actual data for tumor regrowth after treatment compare with the predictions under our models, with various assumptions.

Al-Hajj and Clarke (2004) observed that disruption of the tight genetic control on proliferation of stem cells would lead to a cell with the properties of expansiveness and propensity to metastasize, as found in cancer cells. As metastasis is such significant aspect of cancer progression and plays such a large role determining the survival of patients Tabatabai et al. (2012a), it will be valuable to better understand how metastasis originates and what role may be played by cancer stem cells. On a conceptual level, metastasis may be considered as a system consisting of the underlying cancer stem cells surrounded by other tumor cells they have generated, which has broken away from the larger mass of the tumor to develop in a different area of the body. Clearly the self-renewal properties of the cancer stem cells and their ability to reform the tumor are critical to the metastatic potential. The concept of stem cell niche, which refers to the local environment of the stem cell and its direction of stem cell fate, plays an important role in stem cell transplantation and will also be relevant for metastasis. As metastasis is such a critical issue in progression of and treatment of cancer, we propose to further study the relation to cancer stem cells and their niche. Tumor cells which metastasize must be based on cancer stem cells which are able to reformulate a tumor, and the ability to metastasize relates in some way to a stem cell niche with the versatility to move and be retransplanted in other locations. Further understanding of these issues may provide means to disrupt the metastatic process and, this will be an important subject for modeling.

In addition to the mathematical modeling mentioned above, we are also interested to model aspects of the genesis of cancer and particularly the formation of cancer stem cells. understand whether these cancer stem cells result from mutations to stem cells or whether the mutations to progenitor or other more fully differentiated cells that create the cancer qualities of these cells also induce the stem like property in these cells as part of this transformation. We also mention this aspect of modeling is also interesting in its interaction with the cancer control aspects of the genome, particularly the tumor suppressor protein, p53.

As one final point of modeling, we recall that the cancer stem cell hypothesis helps to explain the increasing resistance of tumor cells to standard therapies such as chemotherapy and radiotherapy, as the tumor cells reconstituted from the cancer stem cells acquire a resistance. We would like to model both the means of this increasing resistance and its effect on treatment. Furthermore we could look at interactions with the newer therapies specifically designed to target the cancer stem cells.

Cancer Stem Cells and Survival Analysis

Although the origin of cancer stem cells is still uncertain, their role in the progression of the tumor, its potential to metastasize, its resistance to treatment, and recurrence after treatment makes these cells of central concern for doctors treating cancer patients. As mentioned above, strategies to treat cancers based on targeting of the cancer stem cells have the potential to revolutionize approaches to cancer treatment. Furthermore, recent studies have suggested the aggressiveness of the cancer and the likelihood for the patient's survival may relate directly to important aspects of the tumor and the cancer stem cells propelling its growth. Recent application of microarrays to measure the level of gene

expression in a tumor has led to the development of biomarkers which show the potential for a drastic improvement in prediction of outcome for cancers. Primarily these biomarkers have been based on distant early metastasis, such as the 70-gene signature of van't Veer et al. (2002), classification by molecular subtype in Sørlie et al. (2001), and phenotype representative of an active or quiescent, as developed in Chang, et al. (2004, 2005b). However, more recently a gene expression signature for cancer survival based on gene expression for self-renewal of the cancer stem cells has been established in Glinsky et al. (2005). This gene signature is based on the common pathways used in self-renewal of stem cells which also contribute to the uncontrolled growth observed in tumors, particularly the MBI-1 pathway. This 11-gene signature relating to the self-renewal capacity of stem cells is found to be effective in predicting metastatic potential, short time to recurrence, and survival in cancer patients. Glinsky (2005) discusses further the role of the genes in this signature in self-renewal of hematopoietic and neural stem cells and the role this may play in metastasis of tumors. This interesting study leaves fertile ground for further exploration into the role of self-renewal pathways in cancer progression, the role of cancer stem cells in metastasis of cancer, and possible interactions with wound healing pathways.

Based on recent work we have completed in survival analysis for cancer patients in relation to gene expression signatures, we would like to apply the hypertabastic survival model to analyze the data for this stem cell self-renewal gene expression signature. The advantage of a parametric model like the hypertabastic model is the ability to analyze the time course of the progression in hazard and decline in survival and the effect of the gene-expression variable on this time course, as we illustrated in Tabatabai et al. (2012a) and a related recent study. Furthermore, this recent study emphasizes the ability to combine several gene expression variables, particularly in cases where they are determined from underlying biological processes which are complementary. A nice extension would be the exploration of how this stem cell self-renewal gene signature will interact with the gene expressions for distant early metastasis, wound healing, and Her2-expression, which were analyzed in Tabatabai et al. (2012a). It would also be particularly interesting to investigate the interaction of this stem cell self-renewal gene signature with the metastasis of the tumor, especially in relation to the physiology of the metastasis process. In the previous section we already mentioned the relation between metastasis and the existence of a stem cell niche which is mobile and can be transplanted to other parts of the body. A more thorough study of the relation between metastasis and the nature of cancer stem cells is warranted, particularly whether any targeted stem cell therapies can either prevent or impede the metastasis process.

Another point of interest would be consideration of how the cancer stem cells and their genetic pathways will react with various treatment strategies. We propose a study comparable to those of J.C. Chang, et al. (2005a) in which gene expression patterns in tumors are assessed in response to treatment from the cancer drug decetaxel. Especially since cancer stem cells have shown to be resistant to standard therapies, it would be valuable to determine what therapies are most effective for these cases and to explore the underlying issues in gene expression.

In conclusion we would like to suggest that more can be done in this area of studying the role of cancer stem cells both in the progression of tumors and in their resistance to treatment. Further attention should be given to the genetic basis in the formation of cancer stem cells and to the regulatory environment allowing for their self-renewal and invasiveness. In addition to the development of biomarkers in these directions, it is particularly relevant to investigate the responsiveness of the cancer stem cells to various treatment strategies and what underlying genetic expression corresponds to successful treatment. As part of this theme of utilization of gene expression patterns and the development of biomarkers, we also pause to mention that the main themes of our earlier sections, the proliferation, differentiation, and self-renewal of stem cells,

can also be profitably studied utilizing these tools of gene expression and the development of biomarkers. For instance, we mention the recent paper of Salasznyk et al. (2005), in which gene expression has been applied to better understand differentiation of stem cells.

The use of gene-expression signatures as biomarkers extends the current theme in biological research wherein current microarray technology and statistical tools are utilized to explore deeper issues in biological function in relation to the genome, genetic expression, and genetic programming. Although the genes selected for use in these signatures do not tell us directly the underlying biological mechanisms, these gene signatures can still confirm (or deny) the central role of certain pathways in the given process. To reverse engineer or more fully detail the underlying genetic pathways is a more complex, but also interesting problem from systems biology. Although this would also be the eventual goal, for the deeper theoretical knowledge and for the larger range of clinical utility, the distance to this goal remains vast. This brings us to the point of the applicability of issues from genomics, epigenetics, systems biology, and cellular signaling within the area of stem cell research. All of these can also be explored through applicable models and tools from mathematics and statistics, as we outline in the next section.

Relations to Genomics, Systems Biology, and Cellular Signaling

A more thorough understanding of the action of stem cells and the underlying biological mechanisms will require a deeper investigation of the role of cellular signaling and of gene expression, and metabolism in these three key areas of proliferation, self-renewal, and differentiation. Further investigations may also explore more fully the role of gene expression and cellular signaling in creation of the cellular niche for stem cells and to what extent these conditions can be reproduced *ex vivo*. Uncovering of these important issues related to the control of stem cell function in a living system is vital to the more complete biological understanding and will also provide more potential and more control in use of stem cells for medical therapies. These essential directions for stem cell research are active areas, with much ongoing research. We have just observed some examples of how use of genomics plays a role in treatment of cancer from the perspective of cancer stem cells. A deeper understanding of these underlying biological events will allow for more control of these processes and a greater effectiveness of the associated therapies. These problems are clearly at a high level of complexity, as is evident from biological experience in working in the areas of the genome, gene expression, and understanding of cellular signaling. The complexity of the mathematical models for these processes is also sure to be at a high level, especially considering the number of genes, number of players, and complexity of interactions. However the hope is that mathematical models can simplify the problems somewhat while still maintaining enough complexity to maintain a faithful representation of the biological reality. It is furthermore hoped that mathematical and statistical tools can play some role in highlighting some of the interactions and helping to describe the underlying biological realities.

Due to the complexity of the biological systems, they will not be fully described by basic mathematical models; however the approach of multiscale modeling and incorporating aspects of the underlying biological mechanisms should produce enhanced models capable of representing important aspects of the biological systems. For this reason it is important to study the role of cellular signaling, systems biology, and genetic programming in the functioning of stem cells and to find appropriate means for their inclusion in the relevant mathematical models.

Since we have just completed a few sections treating the role of gene expression and gene expression variables in the action of stem cells, we begin with a further treatment of related issues. In particular we would like to ask what additional information is available from the lists of upregulated and downregulated genes and how to extract information relating to the underlying biology. The article of Wong and Chang (2005)

mentions the goal of discovering influential genes that will allow for manipulation of the underlying biology, and they describe the use of gene modules and regulatory networks for further understanding the genetic basis of the gene expression patterns observed in microarrays. Numerous research has been directed toward the reconstruction of gene regulatory networks from experimentally determined gene expression profiles, and a survey of such methods can be found in the article of Cho et al. (2007). The article of Hickman and Hodgman (2009) gives a review of Boolean network methods used in modeling of genetic networks.

When considering the action of multiple genes and multiple genetic pathways and understanding how these complex interactions influence cellular behavior, we move into the realm of systems biology. This field also considers the wider influence of different components in the biological system and how their interactions determine cellular behavior. Understanding the action of gene regulatory networks leads us directly into this area of systems biology. A recent article, MacArthur et al. (2009), considers stem cell differentiation and fate determination from the perspective of systems biology, observing that control of stem cell fate has both deterministic and stochastic elements, and modeling of cellular reprogramming in the context of complex dynamics as navigation through a complex attractor landscape. The deterministic and stochastic models used in understanding systems behavior are also discussed in the article of Peltier and Schaffer (2010), and they further discuss the importance of statistical tools of Bayesian networks and PCA/PLS regression in working with large data sets to provide insight into action of regulatory networks.

Understanding the role of cellular signaling in the action of stem cells is a central concern, as signaling plays a central role in the interaction of the stem cell with the stem cell niche and in direction of the actions of the stem cells. Signal transduction is itself a large and important research area, interacting directly with both the areas of systems biology and gene expression. Understanding the complex processes of signal transduction requires aspects of systems biology and gene expression, together with a means of linking the signal to responses of the cell's genetic program. We mention the recent article of Peng et al. (2010) in which a new method has been developed for modeling in any signal transduction pathways, based on both forward and reverse engineering, which links signal transduction pathways with responses in relevant downstream genes. These three areas of signal transduction, systems biology, and gene expression are in some sense tied together within the action of biological systems, and each is a huge and complex field unto itself. However, as significant advances continue to be made in understanding of these complex fields, it becomes possible to apply aspects of this larger theory to the action of stem cells. In the newer generation of models describing stem cell behavior, inclusion of gene expression, systems biology, and signal transduction can be expected to play an important role in more fully describing stem cell behavior.

We conclude this section with the reminder that these issues of high complexity can be simplified by focusing on certain aspects of the larger system. We recognize the importance of bringing these areas of genetic expression, epigenetics, systems biology, and cellular signaling into our mathematical models. Although the ideas here are only preliminary, the development of models which address these issues is clearly an important direction. Even small advances in modeling which incorporate a small aspect of genetics or signaling help to clarify the larger picture. And the larger picture may well require many smaller models, each of which represents one specific aspect of the biological system. We do not mean to imply that the mathematical modeling has reached the level where we can effectively model these complex processes, but rather that we expect the exploration of these issues will yield more fruitful models in the future. We also mention we have not given a survey of these enormous fields but only hinted as to the potential that they hold. Quantitative biology is an important and expanding field, and we may expect that the future holds many important developments in these fields.

Conclusion

In conclusion we remark that mathematical models and tools form an important link for the ongoing development of this field of stem cell research. We have visited several representative uses of mathematical models in areas from proliferation and differentiation of stem cells to embryonic development and to the cancer stem cell hypothesis. In each case the quantitative tools available through mathematics can assist in understanding of the biological issues. In certain cases it is also possible to formulate and test hypotheses based on the mathematical models which may also carry over to formulation of hypotheses in the biological setting.

As this new and rapidly developing field continues to expand, we can expect the development of new mathematical tools and models to continue to play a role in the development of this research. While many researchers are excited by the potential for new breakthroughs and treatments through the use of stem cells, we are also excited by the increased understanding of the nature of stem cells, their role in biological processes, and the potential for their direction and control. We are also excited by the role mathematics can play in modeling of these processes and in providing new tools for exploration and discovery. Both in directions for mathematical modeling related to stem cells and in the potential for therapeutic uses of stem cells in medicine, it seems that the sky is the limit.

The nature of mathematical modeling includes application of developed tools and methods to an important applied problem, as well as development of new tools and methods. We believe there is much room for creative application of the wide range of known mathematics to these interesting and complex problems from biology. Although the original development of mathematics was generated by the physical sciences and underlying mathematical theories, the application to problems from biology is raising important issues and helping develop new and interesting directions for mathematics itself. What new mathematical creations will arise from areas of systems biology, genomics, and cellular signaling? Especially within this rapidly expanding field where the problems are complex and multi-faceted, we believe much can be gained from the development of a variety of mathematical tools and models that are suited to problems raised in this field. In this article we had the opportunity to review some existing tools and models and to suggest the development of certain other models representing specific aspects of stem cell biology. More generally we would like to suggest combinations of these various models in representing various aspects of stem cell research, together with explorations of what newer models are possible. We also suggest creativity in application of known models and in formulation of the underlying problems.

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Preoptic Regulatory Factor-2, a Rhogap Domain Protein that Modifies Cell Cycle Progression and Apoptosis in the CNS

19

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Contents

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Abstract

RhoGAPS are proteins that control Rho GTPases, small enzymes that regulate diverse cellular functions. Preoptic regulatory factor-2 (Porf-2) is a RhoGAP domain-containing protein that is both pro-apoptotic and anti-proliferative. It is widely distributed among vertebrate taxa and expressed in multiple organs and cell types. Porf-2 expression is regulated at the level of transcription by age, gender, location, gonadal hormones, and pituitary and growth regulating factors. It may help coordinate sexually dimorphic development and be a target for prevention or treatment of diseases that exhibit apoptotic or proliferative imbalance.

Introduction

The focus of this chapter is two-fold: (1) to summarize the work done to date to characterize preoptic regulatory factor-2 (Porf-2), a putative Rho GTPase modifying protein and (2) to discuss how these findings support the designation of Porf-2 as a functional RhoGAP (Rho GTPase activating protein).

The GTPases are low molecular weight enzymes that regulate a wide array of cellular processes, (Tcherkezian and Lamarche-Vane 2007) including cell proliferation and apoptosis, cell cycle progression, differentiation, polarity, synapse formation, gene transcription and organization of the actin cytoskeleton. The Rho

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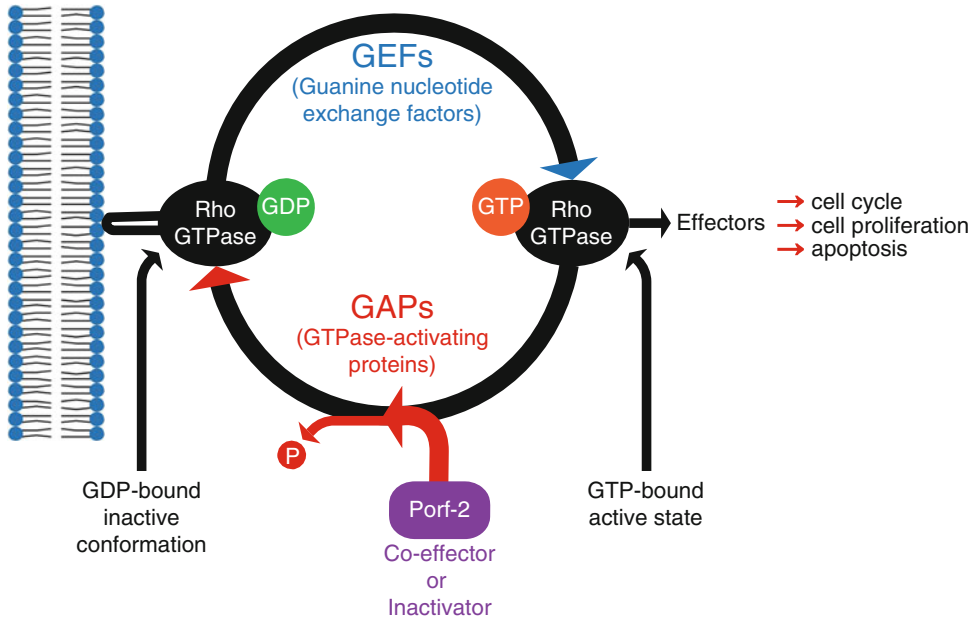

Fig. 19.1 The Rho GTPase cycle of activation and inactivation. Rho GTPases in the inactive GDP-bound conformation can be converted to the active state by the GEF family of proteins. Rho GTPases in the active GTP-bound conformation can be converted to the inactive state by the RhoGAP family of proteins. RhoGAPS can act as inactivators or repressors of Rho GTPase function or as co-effectors

GTPases, one family of these small enzymes that accelerate the hydrolysis of GTP to GDP, are organized into three subgroups: Ras, Rho and Cdc42. The GTPases are regulated by guanine nucleotide-exchange factors (GEFs), which activate Rho GTPases by promoting the exchange of GDP for GTP, and the GAPs (GTPase activating proteins) which enhance the intrinsic GTPase activity leading to the inactive state of the GTPase (Fig. 19.1). In addition, the GDIs (guanine nucleotide dissociation inhibitors) sequester Rho GTPases in the GDP-bound inactive state by regulating their intracellular location. Although in many cases, RhoGAP domain-containing proteins are Rho GTPase inactivators, RhoGAP domains in some proteins may serve as Rho GTPase interactive motifs and be co-effectors of Rho protein signaling (Moon and Zheng 2003).

While many of the RhoGAPS are localized to or highly expressed in the central nervous system (CNS) (Moon and Zheng 2003), others are expressed ubiquitously. A few are most highly expressed in other organs, such as testis, spleen or liver. Many RhoGAPS are highly expressed in both brain and testes (Moon and Zheng 2003). All RhoGAPs contain a highly conserved ~150 amino acid RhoGAP catalytic domain. Many RhoGAPs are multi-domain containing proteins (Tcherkezian and Lamarche-Vane 2007) with one RhoGAP domain and one or more others such as START, StAR (steroidogenic acute regulatory)-related lipid transfer; IQ, calmodulin-binding motif; Lim, a zinc-binding domain; ral BR, a Ral GTPase-binding region; or S/T Kinase, serine/threonine kinase domain. This may facilitate convergence of multiple regulatory pathways by interaction with these motifs, thus promoting rigorous regulation of the GAP activity. While there are approximately 75 RhoGAP domain proteins that regulate the 20 known Rho GTPases, only a few have been extensively characterized (Boettner and Van Aelst 2002).

Preoptic Regulatory Factor-2 (Porf-2)

Discovery and Early Characterization

Porf-2 was originally discovered as part of a cDNA library made from the hypothalamus of the castrated male rat (Nowak 1990). A peptide of 8–9 kd was translated *in vitro* using an RNA that was transcribed *in vitro* from a 375 base cDNA template (Nowak 2003). This peptide was isolated and purified by centrifugal fractionation followed by high pressure liquid chromatography and characterized by capillary electrophoresis and polyacrylamide gel electrophoresis as an 8.3 kd peptide with an isoelectric point of 8.0. A 10.5 kd peptide was also expressed in bacteria using a pFLAG construct. A 16 kd native peptide detected with an anti-Porf-2 antibody was also identified in the preoptic-anterior and medial basal hypothalamus, hippocampus, cerebral cortex, cerebellum and testes of 70 day old rat (Nowak 2003).

Distribution of Porf-2 mRNA and Gene

A subsequent series of investigations yielded substantial information about the expression and regulation of Porf-2 mRNAs. Multiple transcripts ranging in size from 300 to 1,100 bases were detected, many of which were tissue specific. Although expression was highest in the brain regions of the hypothalamus and hippocampus, Porf-2 mRNAs were also found to have a wide tissue distribution, including, in the rat, other brain regions such as cerebral cortex and cerebellum, adrenal gland, testes, liver, and kidney, as well as human hypothalamus, adrenal, prostate, placenta and testis and cultured cells including *cos-7* monkey kidney fibroblast-like cells, mouse cerebellar stem cells and Fischer rat thyroid-like (FRTL-5) cells. Table 19.1 summarizes the known sites of Porf-2 expression and the methods of detection used. Using gene dosage analysis, Porf-2 was shown in the rat to be a single copy gene (Nowak et al. 1997) Southern blot analysis indicates the presence of homologous DNA loci in rat, mouse, chicken, pig, sheep, cow, human and zebra fish (Schmerr et al. 2002). With the completion of the genome sequences for human and mouse, it was revealed that there are gene sequences in these two species that are highly homologous to rat Porf-2. These have been given various names, including KIAA_1688 in human and D15Wsu169e in the mouse (Peck et al. 2002). Table 19.2 lists the known phylogenetic distribution sites of Porf-2 and how these were determined. The retention of Porf-2 in diverse branches

Table 19.1 Tissue and cell type distribution of Porf-2

Tissue and cell type	Location and species	Method of determination
Brain:	Cerebral cortex (R)	N, NPA
	Hypothalamus (R, H)	N, NPA, ISH
	Hippocampus (R)	N, NPA
	Cerebellum (R)	N
	Amygdala (R)	ISH
Endocrine organs:	Testes (R, H)	N, ISH
	Testes (M)	EST[a]
	Anterior pituitary (R)	N
	Placenta (H, M)	N, EST[b]
	Adrenal (H)	N
Other organs:	Kidney (R)	PCR
	Prostate (low levels) (H)	N
	Liver (low levels) (R)	N
	Skin (M)	EST[a]
Cells	FRTL-5 cells (R thyroid origin)	PCR
	Pancreatic beta cells (H)	PCR[c]
	cos 7 cells (nhP kidney origin)	PAGE
	C17.2 cells (M cerebellar stem cells)	PCR, WB
	GT-1 cells (M hypothalamic neuronal origin)	N

Source: Data from Nowak lab except as follows; [a]Konno, H, NCBI, NLM (1999); [b]Ko, MSH, NCBI, NLM (1998); [c]Wang, J, personal communication (2012)
Species: *H* human, *M* mouse, *nhP* non-human primate, *R* rat
Method: *EST* expressed sequence tag, *ISH in situ* hybridization analysis, *N* Northern blot, *NPA* nuclease protection assay, *PAGE* polyacrylamide gel electrophoresis, *PCR* polymerase chain reaction, *WB* Western blot

Table 19.2 Phylogenetic distribution of Porf-2

Group	Animal	Method of determination
Primates	Human	Southern blot analysis
		Northern blot analysis
		Genome sequencing[a]
	Monkey	Protein electrophoresis
Ungulates	Pig, sheep, cow	Southern blot analysis
Rodents	Mouse	Southern blot analysis
		PCR
		Western blot analysis
		EST
		Genome sequencing[b]
	Rat	cDNA sequence analysis
		Southern blot analysis
		Northern blot analysis
		PCR
Birds	Chicken	Southern blot analysis
Fish	Zebrafish	Southern blot analysis

Source: Date from Nowak lab except as follows; [a]Collins et al. (2004); [b]Waterston et al. (2002)
Method: *cDNA* complementary DNA, *EST* expressed sequence tag, *PCR* polymerase chain reaction

of vertebrate species is suggestive of a selective advantage and therefore an essential function for this gene and its product(s) (Silander and Ackermann 2009).

Porf-2 Encodes a Putative RhoGAP Domain

There is a sequence-predicted Rho GAP domain at the carboxy-terminal end of the largest predicted Porf-2 open reading frame in mouse, rat and human. As mentioned earlier, highly homologous proteins may be translated from this open reading frame in human, mouse and rat. The mouse and human proteins also contain an actin binding domain. The human protein includes a RNA splicing regulator domain in the amino terminal portion. As noted earlier, many RhoGAPs are multi-domain containing proteins (Tcherkezian and Lamarche-Vane 2007) and at least one other RhoGAP, Myr5, has an actin filament binding site (Moon and Zheng 2003). As has been found for other RhoGAPs, the multiple protein-protein and protein-lipid interacting domains may determine the location of the Porf-2 RhoGAP within the cell. This also facilitates convergence of several regulatory pathways by interaction with multiple motifs, thus promoting rigorous regulation of the GAP activity.

Neural Expression of Porf-2 Is Modified by Pituitary and Gonadal Factors

Expression of Porf-2 in the hypothalamus is regulated by gonadal and pituitary factors. Both castration and hypophysectomy of 40–45 day old male rats increase levels of a brain-specific Porf-2 transcript in the preoptic area of the hypothalamus (POA), but not in the medial basal hypothalamus (MBH) or the cerebral cortex (Nowak 1997). This suggests that testicular secretagogues, either directly secreted by the testes or indirectly due to stimulation of the testes by pituitary factors, suppress Porf-2 in the POA in intact animals.

Porf-2 mRNA is also expressed in the female rat brain (Nowak et al. 1997). *In situ*

hybridization analysis of female rat hypothalamus shows distinct strand specific labeling with a Porf-2 antisense ^{3}H-RNA probe in discreet cells of the lateral POA (Nowak et al. 1997). In female rats ovariectomized at 44 days of age, treatment with 17β-estradiol (E_2) for 8 days results in an increase in Porf-2 mRNA in the POA and in the hippocampus, as measured by nuclease protection assay. Treatment with progesterone (P_4) increases Porf-2 mRNA in both the POA and the cerebral cortex. Combined treatment with E_2/P4 decreases Porf-2 mRNA in the POA and cerebral cortex, but increases the mRNA in the hippocampus. No difference is observed in intrinsic levels of Porf-2 mRNA in either the MBH or POA during the estrous cycle (estrous compared with proestrous), while the same animals do exhibit higher levels of gonadotropin releasing hormone (GnRH) in the MBH and lower levels of GnRH in the POA during proestrus compared with estrus.

Expression of Porf-2 in Rat Testes

In addition to those found only in the CNS, many RhoGAPS are highly expressed in both brain and testes (Moon and Zheng 2003). Tissue specific Porf-2 transcripts are expressed in the testis (Nowak 1990). Porf-2 mRNA is highest at 15 days of age, then declines substantially at 30 and 60 days, when the levels are approximately 40% of the 15 day value (Nowak et al. 1997). There is an additional 40–50% decline between 2 months and a plateau at 6 and 12 months. There is a further 60–75% decrease at 24 months. The mRNA has been localized by *in situ* hybridization analysis in the 60 day rat testes to the immature germ cells including spermatogonia and primary spermatocytes.

Hypophysectomy of 42 day old male Sprague-Dawley rats results in significantly increased testicular expression 12 days later when compared to intact 54 day males. This suggests that loss of the testiculotrophic pituitary hormones, luteinizing hormone and follicle stimulating hormone, results in increased levels of pro-apoptotic and anti-proliferative Porf-2. In a separate study that looked at the effect of hypophysectomy on specific Porf-2 transcripts (Nowak 1997), the relative levels of certain testes-specific transcripts were altered, but the functional significance of these changes awaits the detailed characterization of these transcripts. Another RhoGAP protein, tGAP1, is highly and uniformly expressed in testicular germ cells and may play a role in a basic cellular function such as nuclear import/export (Modarressi et al. 2004). However, Porf-2 is not uniformly expressed in germ cells and thus may play a role in testicular germ cell apoptotic deletion of damaged cells (Sinha Hikim et al. 2003).

Effects of Aging on Neural Expression of Porf-2 mRNA

Nuclease protection assays were done using a riboprobe for Porf-2 mRNA that corresponds to 375 bases in the 3' RhoGAP domain. In some regions of the adult male brain, levels of Porf-2 mRNA change with age (Hu and Nowak 1994), but not in others. Of the four brain regions examined, the MBH and POA, the hippocampus and the cerebral cortex, levels are highest in the hippocampus. In this region, Porf-2 mRNA levels are 40 μg/g total RNA at 2 months of age (young adult), increase significantly at 6 (mature adult) and 12 months (middle age) by over 100% to approximately 91 μg/g total RNA, then decline to levels seen in the young adult at 24 months (aged). In the POA, Porf-2 mRNA levels are highest at 2 months of age, at 19 μg/g total RNA, then decline steadily to approximately 6.5 μg/g total RNA in the aged 24 month animals. In the MBH and cerebral cortex there are no age-related changes observed. In the MBH, levels of Porf-2 mRNA are around 19 μg/g total RNA at 2 and 24 months of age, and slightly lower, averaging around 13.5 μg/g total RNA at 6 and 12 months but this difference is not statistically significant. Levels in the cortex are in the range of 20–23 μg/g total RNA at every age examined. The physiological significance of these changes in a pro-apoptotic factor in the brain with age remains to be established.

Sexually Dimorphic Expression of Porf-2 During Development

Expression of Porf-2 mRNA has also been examined during the perinatal and peri-pubertal stages of development. Porf-2 is expressed in the rat brain as early as embryonic day E18–19 (Nowak and Gore 1999). The levels of Porf-2 mRNA in the hypothalamus, relative to cyclophilin mRNA, are in the same range, (fg/pg), as those of two well-characterized neuromodulators, GnRH and neuropeptide Y (NPY). Both age and gender-related differences are observed in the levels of Porf-2 mRNA during the perinatal period. Porf-2 levels in the POA are highest at E18–19 in males (0.8 fg Porf-2 mRNA/pg cyclophilin mRNA), while the highest levels in females (0.65 fg Porf-2 mRNA/pg cyclophilin mRNA) are observed on the first day of life, P0. The gender differences on these 2 days are significant. Porf-2 mRNA levels are also higher in females (0.47 fg Porf-2 mRNA/pg cyclophilin mRNA) than in males (0.32 fg Porf-2 mRNA/pg cyclophilin mRNA) on postnatal day P15.

The levels of Porf-2 mRNA in the POA are also significantly higher on E18–19 than all other days examined, P0, P5, P10 and P15, while those on P0 are significantly higher than on P10 and P15. In the MBH, Porf-2 mRNA is most abundant on E18–19 and P0 (~6.5 fg Porf-2 mRNA/pg cyclophilin mRNA) and decreases significantly on P5, P10 and P15. By P15, Porf-2 mRNA measures 3.1 fg Porf-2 mRNA/pg cyclophilin mRNA, a greater than 50% decrease. In contrast, NPY mRNA is increasing during the perinatal period and GnRH mRNA shows no change. The observed gender and age differences in Porf-2 mRNA in both regions of the hypothalamus are statistically significant and may well be physiologically relevant, as the perinatal period is a critical time in the development and differentiation of the mammalian brain. Neuroendocrine sexual dimorphism begins during this period. There are differences in size and number of neurons. Neurogenesis, as well as apoptosis, contributes to the final outcome.

Several well-studied sex differences in the mammalian brain are the result of hormonal control of cell death. Deletion of Bax has been shown in the mouse forebrain to eliminate sex differences (Forger 2009) that are likely to be due to the perinatal surge of testosterone seen in mammals. Other sex differences in the hypothalamus have been linked to estradiol induction of caspase-dependent cell death or apoptosis. In the POA, estrogen up-regulates caspase-dependent death of dopaminergic neurons in the early postnatal period (Waters and Simerly 2009). Coupled with the finding that estradiol can increase Porf-2 mRNA in the hypothalamus (Nowak et al. 1999), the effects of Porf-2 on Bax-mediated and Bax-independent apoptosis (Fig. 19.2) (Ma and Nowak 2011) suggest that Porf-2 has a role in sexual differentiation of the brain.

Comparison of Porf-2 mRNA levels in male and female rats during the peri-pubertal period (Hu and Nowak 1995) at 15, 30 and 60 days of age shows no gender or age differences in the POA. However, in the MBH, Porf-2 mRNA decreases during the period of sexual maturation, and is higher at 15 days (pre-pubertal) than at 30 (peri-pubertal) and 60 (post-pubertal) days of age. Levels in males at 15 days of age are higher than in females, consistent with the proposed role of Porf-2 in gender-specific sexual maturation of the brain. Rho GAPs and Rho GTPases are known to regulate a variety of processes within neurons at different developmental stages and in response to the intracellular network of protein interactions as well as extracellular cues (Tolias et al. 2011).

There is also sexual dimorphism in developing hippocampus with higher levels of Porf-2 mRNA in the male compared to the female rat at 15 days of age (Hu and Nowak 1995). There is no age-related change in levels of expression in the male. In the female, Porf-2 mRNA levels are increased significantly at 60 days compared to day 15, and by 60 days of age, hippocampal expression is equal in males and females. Possibly the most interesting finding of this study is in the cerebral cortex, where at 15 days of age, Porf-2 mRNA levels are four times higher than those at 30 and 60 days. This may be related to the high degree of neuronal organization that takes place in the mammalian cerebral cortex in the early postnatal period.

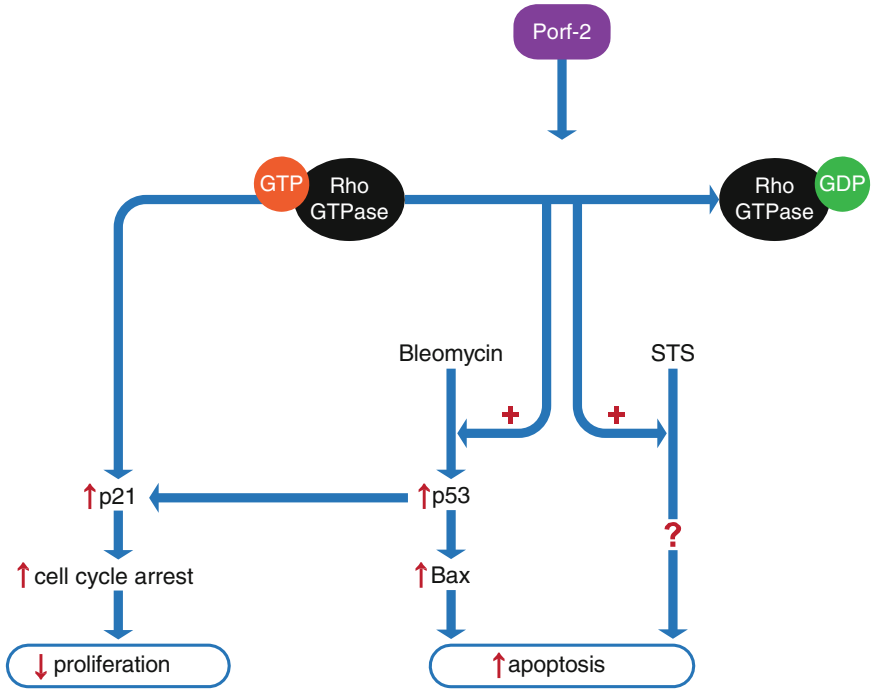

Fig. 19.2 Schematic illustration of the role of Porf-2 in apoptosis and proliferation of NSCs. Porf-2 inhibits cell proliferation by enhancing p21 protein levels and decreasing progression of cells from G1 to S phase of the cell cycle. Porf-2 enhances drug-induced apoptosis through both p53 transcription dependent and independent pathways. *STS* staurosporine

Porf-2 Has Pro-apoptotic and Anti-proliferative Activities

The first direct evidence that Porf-2 plays a role in cell survival was the demonstration that transfection of NIH-3T3 cells with a Porf-2 construct results in decreased incorporation of ^{3}H-thymidine (Nowak and Gilham 2004).

In order to investigate the effects of Porf-2 on cell survival and fate, a series of Porf-2 knockdown shRNAs was generated. One of these resulted in a 70% knockdown of Porf-2 mRNA in mouse C17.2 cells, and a corresponding 70% decrease in a 65 kd protein detected with an anti-Porf-2 polyclonal antibody (Ma and Nowak 2011). Comparison of these cells (Clone 184) with wild type cells, using the Alamar blue assay, shows that Porf-2 decreases cell proliferation. It is also shown by fluorescence activated cell sorting (FACS), based on identification of cell cycle populations by DNA content, that Porf-2 knockdown affects the distribution of cells in the G1 and S phases of the cell cycle, consistent with Porf-2 delaying the progression of cells from G1 to S. The Clone 184 cells also have decreased levels of p21, implying that Porf-2 increases p21 in intact cells. P21 is a cyclin-dependent kinase (CDK) inhibitor known to increase cell cycle arrest by inhibiting CDKs required for transition from G1 to S phase (Harper et al. 1993), resulting in decreased cell proliferation. P21 is required for neural stem cell renewal (Joseph and Hermanson 2010). There is substantial evidence that Rho GTPases affect the cell cycle (Boettner and van Aelst 2002). Rho activity suppresses the induction of p21. These actions would accelerate progression through the cell cycle. The effect of Porf-2 to decrease progress through the cell cycle could reflect inactivation of one or more Rho GTPases.

In addition to the effects on proliferation, Porf-2 also stimulates apoptosis through both p53-dependent and p53-independent pathways (Ma and Nowak 2011). The TUNEL, a nick end-labeling assay for DNA fragmentation, and the Annexin V assay for early loss of cell membrane integrity, were used. Populations of cells in which Porf-2 is knocked down display a decrease in both early and late stage drug-induced apoptosis, in cells treated with either staurosporine (STS) or bleomycin. Also, in the intact cells treated with bleomycin, but not in those treated with STS, both p53 and BAX are increased when compared to the Clone 184 cells. The effects of Porf-2 on cell survival are illustrated in Fig. 19.2.

Like many other Rho GAPs, Porf-2 may interact with more than one GTPase. A RhoGAP can act as a negative regulator that catalyzes the intrinsic GTPase activity of Rho proteins to return them to the inactive state. Thus Porf-2 could be anti-proliferative by inactivating a GTPase that stimulates proliferation, such as RhoA or RhoC (Ching et al. 2003; Kim et al. 2009). At the same time, Porf-2 could be pro-apoptotic by also acting as a co-effector for a pro-apoptotic GTPase, such as RhoB (Boettner and van Aelst 2002).

In contrast to the effects on proliferation and apoptosis, knockdown of Porf-2 in C17.2 cells induced to differentiate by serum starvation does not affect the phenotypic distribution of differentiated cells (Ma and Nowak 2011). Neurons were identified by antibody detection of expression of β tubulin III, type II astrocytes by detection of cell surface ganglioside A2B5, and glial precursors by detection of glial fibrillary acidic protein.

Insulin and Insulin-Like Growth Factor-1 (IGF-1) Modulate Expression of Porf-2

The first observation that Porf-2 expression could be modified by growth factors happened serendipitously when FRTL-5 cells were cultured in several different media, some with and some without insulin. Insulin at physiological levels decreased Porf-2 mRNA in these cells in a concentration and time dependent manner. It was considered that this effect might be mediated through insulin receptors (IR), IGF-1 receptors (IGF-1R), or both, including heterodimers of IR and IGF-1R. If so, which intracellular signaling pathways are responsible? It was also hypothesized that IGF-1 would affect Porf-2 mRNA in a fashion similar to insulin. IGF-1 does decrease Porf-2 mRNA in a dose and time dependent manner, similar to the response seen with insulin. When FRTL-5 cells are treated with specific inhibitors for phosphoinositol-3 (PI3) kinase, Akt (protein kinase B), rapidly accelerated fibrosarcoma (Raf) kinase or mitogen-activated protein kinase (MAPK), it is demonstrated that inhibition of PI3 kinase-Akt or Raf-MAPK (MEK) pathways results in dose-dependent partial abrogation of the effect on Porf-2. However, only wortmannin, which blocks both pathways at high concentrations, can cause a complete reversal (Wang 2011). It is hypothesized that the two pathways converge on a single effector molecule that causes the change in Porf-2 gene expression. Knockdown of IGF-1R in FRTL-5 cells results in partial loss of the effect of IGF-1 on Porf-2 (Wang 2011). The results of knockdown of IR and simultaneously of both IGF-1R and IR are forthcoming. A link has also been established between insulin and other RhoGAPs. The RhoGAP, DLC-1, which is a negative modifier of the RhoGTPases RhoA, RhoB, RhoC and Cdc42, is also negatively regulated by insulin through both PI3K and MEK pathways (Kim et al. 2009). It is hypothesized that suppression of Porf-2 is one of the ways that insulin and IGF-1 promote cell survival.

Summary: Modifiers of Porf-2 Expression

The degree of expression and also the distribution of alternative Porf-2 RNA transcript isoforms differs by tissue, cell type and region. Both age and gender regulate Porf-2 in specific areas of the brain and levels of Porf-2 RNA in brain change with developmental stage. Gonadal

hormones, including estradiol and progesterone, and pituitary factors up-regulate or down-regulate Porf-2 expression. Insulin and IGF-1 also down-regulate Porf-2 expression at the level of transcription.

Summary: Functions of Porf-2

Porf-2 decreases cell metabolism in neural stem cells. It also increases cell apoptosis in neural stem cells through p53-dependent and p53–independent pathways. Porf-2 increases both Bax and p53 when apoptosis is induced by bleomycin treatment in neural stem cells. Porf-2 increases p21 in proliferating neural stem cells and decreases cell cycle progression from G1 to S phase. It is possible that Porf-2 has similar functions in other cell types where it is found. However, it is also possible that Porf-2 function is context-dependent on intrinsic factors, such as the complement of Rho GTPases being expressed in the cell, as well as the presence or absence of extrinsic cues. In this case, the physiological effects of Porf-2 may diversify.

Potential Roles for Porf-2 in Normal Development

In many organs of the body, structural and functional homeostasis is maintained through a regulated balance of cell proliferation and cell death. This balance is also crucial during development, including the developing CNS. Astrocytes regulate neurogenesis in the CNS of fetal and young mammals. They are one of few endogenous cell types in the brain that have been shown to express IR. The regulation of Porf-2 expression by gender during development, as well as by sex steroids and insulin, supports the hypothesis that Porf-2 is one factor that regulates proliferation *vs.* apoptosis in the developing brain. Further, it may mediate maintenance of adult brain health by keeping cell proliferation in check. The expression of Porf-2 in a subset of primary germ cells in the testis suggests it also may also function in the elimination of cells that are genetically or metabolically compromised.

Many mammalian genes have been described that give rise to multiple functional transcripts and proteins (Sánchez-Pla et al. 2012; Montes et al. 2012). Additional Porf-2 proteins and peptides such as the previously described 8.3, 16 and 65 kd species (Nowak 2003; Ma and Nowak 2011), may be expressed from the Porf-2 genetic locus *in vivo*, including ones that are tissue and cell type specific. Multiple Porf-2 RNAs and proteins have been detected by Northern and Western blots, but the characteristics and functionality of many of them have not yet been explored.

Potential Roles for Porf-2 in Disease

Diabetic Nephropathy

The finding that insulin decreases the pro-apoptotic Rho GTPase modifier, Porf-2, raises the question of what happens to Porf-2 in an insulin-deprived or insulin-resistant state such as diabetes mellitus. Female Zucker rats, a model for Type 2 obesity related diabetes, show better preservation of renal architecture and function after 20 weeks on an anti-oxidant fortified diet than their counterparts on a control diet (Slyvka et al. 2009). Renal levels of Porf-2 mRNA are also lower in the antioxidant treated group, raising the possibility that promotion of apoptosis by Porf-2 contributes to deterioration in kidney function that is seen as a complication of diabetes.

Cognitive Dysfunction

The majority of excitatory synapses on principal neurons in the brain are located on the tips of dendritic spines. Rho A induces spine retraction and loss. Spine abnormalities are associated with neurodevelopmental, neuropsychiatric and neurodegenerative disorders. There appears to be a correlation between Rho GTPase signaling, spine abnormalities and mental retardation (Tolias et al. 2011); this implies that precise Rho GTPase

signaling is important for proper circuit development and cognitive function. Like other Rho GTPase modifying enzymes, Porf-2 may play a role in neural circuitry and cognitive function. However, proof of this concept awaits *in vivo* studies in which Porf-2 expression has been deleted or amplified, or the discovery of genetic or epigenetic modifications of Porf-2 in the setting of compromised CNS function.

Sexual Dimorphism

As discussed above, Porf-2 may also act as a mediator of sexually dimorphic brain development due to the time course and distribution pattern of its expression in the brain and its apparent influence by gonadal steroids. As such, discrepancies in Porf-2 expression or localization could lead to differences in sexually dimorphic attributes.

Neurodegenerative Disease

Accelerated apoptosis that is not matched by mitotic generation of new functionally integrated cells is the hallmark of neurodegenerative diseases including Alzheimer's (AD), Parkinson's, Huntington's, HIV-associated dementia, amyotrophic lateral sclerosis (ALS) and others (Nijhawan et al. 2000).

CNS development and its deconstruction, neurodegeneration, have been shown to involve several Rho GTPases and their regulatory RhoGAPs. Oligophrenin-1, a RhoGAP that suppresses Rho/ROCK, is an important regulator of synapse development and is encoded by a candidate gene for X-linked mental retardation. P190 RhoGAP is required for normal forebrain and neural tube structure, as well as structural remodeling of synapses involved in long term memory formation in the hippocampus and amygdala (Tolias et al. 2011). Alsin, a RhoGEF for Rac1, has been implicated in juvenile onset ALS. The Rho family GTPases may play key roles in the deleterious production and signaling of Abeta42. Rho/ROCK can promote increased levels of Abeta42, an amyloidogenic protein that underlies the pathogenesis of AD. In addition, Rac1 and Cdc42 are upregulated by Abeta42. This enhances F-actin polymerization and hippocampal neuronal damage (Linseman and Loucks 2008).

It is certainly feasible that acquired genetic or epigenetic defects in these and other additional RhoGAPs that are highly expressed in the CNS, such as Porf-2, play a role in neurodegeneration, either by direct stimulation of apoptosis or indirectly through disruption of cell-cell interactions leading to cell death. The novel role of Rho family GTPases in modulating neuronal survival and death is rapidly emerging as a fascinating area for exploration and development of innovative targeted intervention, treatment and prevention of CNS trauma and neurodegenerative diseases (Linseman and Loucks 2008).

Although spontaneous proliferation of neural stem and progenitor cells in adult mammals is largely limited to the subventricular (SVZ) and subgranular (SGZ) zones, quiescent neural stem cells appear to be much more widely distributed (Reynolds and Weiss 1992; Palmer et al. 1999; Arsenijevic et al. 2001). Functional neurogenesis from adult hippocampal stem cells is possible in co-culture with astrocytes from neonatal and adult hippocampus (Reynolds and Weiss 1992); the new neurons develop electrical activity and integrate into neuronal networks with functional synaptic transmission (Song et al. 2002) However, *in vivo* progenitor cells outside the SVZ and SGZ appear to be largely maintained in a non-proliferative state by inhibitory astrocytes that, at least in some settings, express high levels of ephrin-A2 and -A3 (Jiao et al. 2008). Activation of endogenous stem and progenitor cells through manipulation of anti-proliferation factors, such as ephrins, Porf-2 and others, may facilitate cell replacement therapies in CNS injury and disease.

Malignancy

Much recent evidence supports a role for the mutation or aberrant expression of RhoGAPs in many cancers (Kandpal 2006). Deletion of the RhoGAP, DLC-1, increases invasiveness and cell

motility in liver, breast, ovarian and lung cancer cell lines (Kim et al. 2009). The pro-apoptotic and anti-proliferative functions of Porf-2 make it a candidate for this role. Modifiers of RhoGAP tumor suppressor activity have been proposed as novel agents for anti-cancer therapy. The power of this approach is that the specific RhoGAP that is modified can be targeted, increasing specificity and decreasing the risk/benefit ratio.

Summary and Conclusions

Porf-2 encodes a RhoGAP domain protein that has anti-proliferative and pro-apoptotic functions. Its expression is highly regulated and varies with age, gender, exposure to sex steroids, insulin and IGF-1, and cell or tissue type. The gene has been conserved evolutionarily in vertebrates. Its roles in development, health and disease are in the early stages of discovery, but hold promise as one of the family of GTPase modifiers that control these powerful molecular switches.

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Molecular and Functional Characterization of Human Adipocytes

20

Antonella Poloni and Giulia Maurizi

Contents

Abstract

Adipose tissue, consisting mainly of adipocytes, functions as a critical organ for energy regulation, inflammation and immune response through intricate signals. Mature adipocytes were considered to be in the terminal stage of differentiation and stationary, having lost their proliferative ability. Recently, the capability of mature adipocytes to reprogram their gene expression profile and transform into different cytotypes has been demonstrated. Here, data of both mature and dedifferentiated adipocytes were collected and compared to underline structural and functional features of these cells. In particular, morphology, structure, molecular and immunophenotype markers, and dedifferentiation process of mature isolated adipocytes are analyzed. In addition, molecular and phenotype characterization of dedifferentiated fat cells is described, reporting important results on pluripotent differentiation ability, immunoregolatory and hematopoietic supporting functions of these cells. These findings highlight the concept that adipose lineage cells represent a suitable new cell source for clinical applications in such fields as cell therapy and regenerative medicine.

Introduction

Human adipose tissue is no longer considered simply as a storage depository of lipids, but as a critical organ involved in energy regulation,

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whole-body insulin action and inflammation, through intricate endocrine, paracrine and autocrine signals (Dandona et al. 2004). Adipose tissue contains adipocytes and non-adipose cells. Mature adipocytes are functionally the most important cell type in adipose tissue, while the stromal vascular fraction contains several cell types and represents a reservoir not only of specific adipocyte precursor cells but also of multipotent stem cells (Gimble and Guilak 2003).

The literature give higher attention to stromal stem cells than to mature adipocytes which demonstrated to have stem cells properties. The stemness features of fat and its in vitro dedifferentiation process is largely unknown, despite the potential clinical applications of both mature adipocytes and dedifferentiated adipocytes in such fields, as regenerative medicine.

White adipose tissue has attracted attention because of its great and reversible capacity for expansion, which appears to be permanent throughout adult life (Planat-Benard et al. 2004). The process of cellular differentiation in terminally differentiated mammalian cells is thought to be irreversible, but recent data suggest that the mature adipocytes, when under physiological stimuli, are able to reversibly change their phenotype and directly transform into cells with a different morphology and physiology (De Matteis et al. 2009; Poloni et al. 2012).

Mature adipocytes are functionally the most important cell type in adipose tissue. White adipocytes are spherical cells with a single large lipid droplet formed by triacylglycerols that accounts for >90% of the cell's volume (Cinti 2009). They have a variable size that depends mainly on the size of the lipid droplet stored in them. Even on electron microscopy there seems to be no distinct structure separating it from the thin rim of cytoplasm by a non-membranous electron-dense barrier containing functionally important proteins such as perilipin (Blanchette-Mackie et al. 1996). The thin cytoplasm contains the nucleus, characteristically squeezed by the large lipid vacuole, an usually under-developed Golgi apparatus, rough endoplasmic reticulum made up of short, isolated cisternae and rare lysosomes. Mitochondria are thin and elongated, with randomly oriented cristae, and variable in amount: in general, they are less numerous in the larger cells. Several caveolae are found on the outer surface. Numerous pinocytotic vesicles are found at the level of the outer cytoplasmic membrane, where a distinct basal membrane is present. Other organelles are usually poorly represented (Cinti 2009). Mature adipocytes should be studied using their buoyant property (Fig. 20.1).

To obtain isolated mature adipocytes free of stromal-vascular elements, after collagenase digestion, the disrupted tissues were filtered through a 200 μm nylon sieve. The filtered cells should be washed four–five times and eventually re-filtered if it is necessary. Only the floating top layer was collected after each washing step (Zhang et al. 2000).

The confocal microscopy analysis of adipogenic cellular fraction isolated is a good and easy way to exclude any contamination coming from other cell types contained in the whole tissue. These cells are large (50–70 μm), unilocular, perilipin-immunoreactive with a peripheral flattened nucleus.

The ceiling culture is the method that uses the buoyant property of adipocytes, allowing them to adhere to the top inner surface of a culture flask which is completely filled with medium (Sugihara et al. 1987). In this way, mature adipocytes adhere to the ceiling surface, then the flask should be reinverted to allow normal observation and the subsequent manipulation of the culture.

The aim of this chapter is to collect and compare the literature data about both mature and dedifferentiated adipocytes to better underline their stem cells properties. In addition to well characterize adipocytes morphologically and structurally, this chapter reports on the pluripotent differentiation ability, immunoregolatory and hematopoietic supporting functions of these cells. These findings open new perspectives on adipose tissue plasticity and highlight the way for cellular therapy and regenerative medicine based on the administration of adipose tissue stem cells.

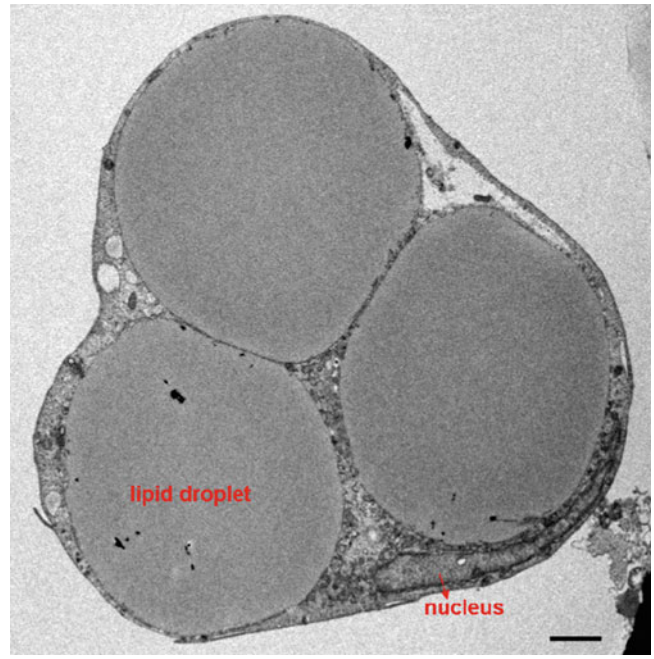

Fig. 20.1 Electron microscopy analysis. Mature adipocyte has thin cytoplasm and a nucleus characteristically squeezed by the large lipid vacuoles. The cytoplasmatic organelles are poorly represented. Scale bar = 4 μm

The Dedifferentiation Process of Mature Adipocytes

Mature adipocytes are generally considered terminally differentiated because they have lost their proliferative abilities, but recent data suggest that the process of cellular differentiation in terminally differentiated mammalian cells is not irreversible.

When maintained in culture, mature adipocytes undergo spontaneously a process of dedifferentiation (Matsumoto et al. 2008). Adipocytes lost their intracytoplasmatic lipid droplet, the nuclei became more centralized and the cells became elongated in shape, assuming a fibroblast-like morphology. At this step, the cells enter a proliferative log phase until cellular senescence (Fig. 20.2).

At molecular level, data revealed significant changes in gene expression during the dedifferentiation process (Ono et al. 2011; Poloni et al. 2012). Adipocytes downregulated many genes that play a role in lipid and fatty acid metabolism, while concomitantly upregulated genes involved in cell proliferation, altered cell morphology, movement and migration of cells, and regulation of differentiation.

As murine adipocytes (De Matteis et al. 2009), recent data demonstrated that also human mature adipocytes expressed genes required for the cell reprogramming process, which include Oct4, Klf4, c-myc and Sox2 (Poloni et al. 2012, 2013). Moreover, these cells expressed transcripts for embryonic stem cell genes that are required for self-renewal and pluripotency. Thus, recent data underline the potential role of mature adipocytes as stem cells (Poloni et al. 2012; Cinti 2009). The reprogramming of genes in isolated as well as cultivated adipocytes is in line with the plastic properties of these cells.

Immunophenotype Characterization and Pluripotential Properties of Mature and Dedifferentiated Adipocytes

While dedifferentiated fat cells are well characterized (Matsumoto et al. 2008), there are only few studies that provided the cell-surface antigen

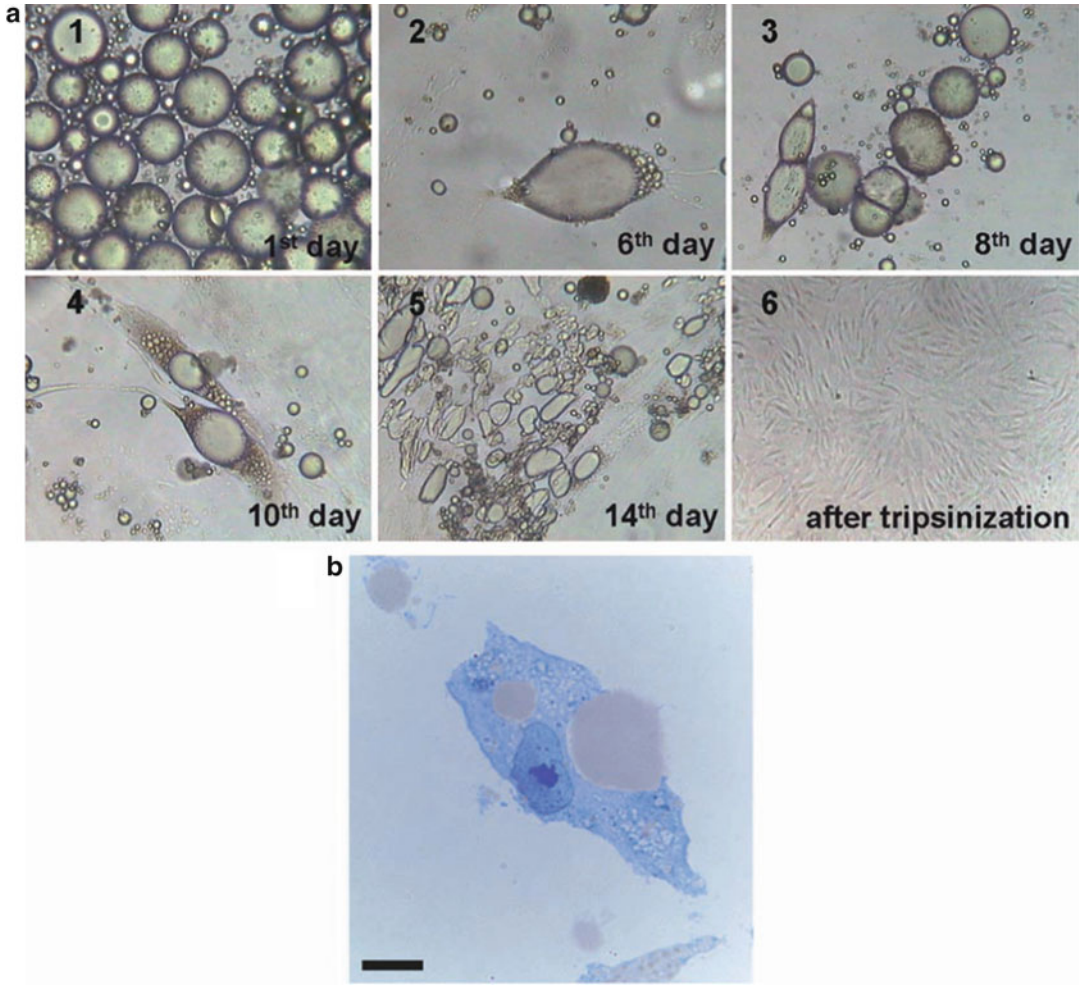

Fig. 20.2 Dedifferentiation process of mature adipocytes. During culturing, the cells attached to the upper surface of the flasks, followed by conversion to fibroblast-like dedifferentiated adipocytes, reached a morphology similar to BM-derived MSCs. (**a**) Morphological changes at different time points from mature adipocytes to dedifferentiated adipocytes. (**b**) Mature adipocytes lose their lipid droplets. Cells were stained by toluidine *blue*. Scale bar in B is equal to 4 μm, in A1 equal to 80 μm, in A2 equal to 40 μm, in A3 equal to 80 μm, in A4 equal to 80 μm, in A5 equal to 120 μm, and in A6 equal to 150 μm

profile of human mature adipocytes (Poloni et al. 2012). The stemness markers expressed by mature adipocytes at the molecular level are also present as surface antigens. These cells are positive stained by some stem cells markers, CD34 (hematopoietic progenitor cell antigen 1, HPCA 1), CD133 (prominin 1), CD90 (Thy-1), CD105 (endoglin), CD271 (NGFR) and CD117 (c-kit). During the dedifferentiation process, the typical mesenchymal stem cells markers are highly preserved at the molecular and antigenic levels, while CD34 and CD133 are lost as antigens.

After the dedifferentiation process, adipocytes are easy to isolate and cultivate, so there are many studies that described their immunophenotype. The cell-surface antigen profile of dedifferentiated cells was analyzed (Matsumoto et al. 2008) and this profile is consistent with previous findings for bone marrow MSCs (Pittenger et al. 1999) with uniformly positive expression of

CD13 (aminopeptidase N), CD29 (integrin β1), CD44 (hyaluronate receptor), CD49b (integrin α4-subunit), CD90, CD105, CD271, CD73 (ecto-5′-nuclotidase) and HLA-A, -B, -C, but negative for CD11b (integrin αM), CD31 (platelet endothelial cell adhesion molecule), CD34, CD133, CD45 (leukocyte common antigen), CD106 (vascular cell adhesion molecule-1) and HLA-DR.

These findings suggest that the dedifferentiated adipocytes are an homogeneous population expressing the same markers as bone marrow-derived MSCs and adipose tissue-derived MSCs. Data showed that dedifferentiated adipocytes are a highly homogeneous population of cells respect to adipose-derived stem cells, that contained a variety of cell types: high number of smooth muscle cells (19%), endothelial cells (3%) and blood cells (13%) (Yoshimura et al. 2006). These observations are convincing because dedifferentiated adipocytes originate from a fraction of highly pure mature adipocytes, whereas adipose-derived stem cells are an heterogeneous population.

Electron microscopy (EM) analysis of dedifferentiated adipocytes is shown in Fig. 20.3a, b. The images show most organelles described during the early stages of developing stromal vascular fraction-derived MSCs in primary cultures, i.e., well-developed Golgi complexes, short strands of rough endoplasmic reticulum, small lipid droplets, small mitochondria, lysosomes and small granules of glycogen. Nuclei were fusiform with smooth edges. Thus, the EM features of dedifferentiated adipocytes were very similar to developing stromal vascular fraction-derived MSCs (Fig. 20.3c). The spontaneous dedifferentiation process represents the manifestation of morphological, molecular and functional changes of mature adipocytes, that might be interpreted as a return back to a noncommitted status of the cells.

The methylation status of cells is the most common epigenetic modification of genome in mammalian cells (Bibikova and Fan 2010). Data showed that there are significant difference between the methylation statuses of mature adipocytes and dedifferentiated adipocytes, while there are no difference between dedifferentiated adipocytes and bone marrow-derived MSCs (Poloni et al. 2012). These findings suggested that during the dedifferentiation process a gene reprogramming event takes place, which leads to changes in cellular epigenetic status. Dedifferentiated adipocytes achieve the DNA methylation status of bone marrow-derived MSCs by this process.

Recent advances in regenerative medicine have created a broad spectrum of stem cell research. Among them, tissue stem cell regulations are important issues to clarify the molecular mechanism of differentiation. Tissue engineering and cell therapy techniques have been developed to reconstitute different tissues. Attention has been focused on cells that might be useful in regenerative medicine (Barrilleaux et al. 2006). However, because stem cells only represent a minor population of the cells in the body, invasive procedures are frequently needed to obtain the amount of stem cells required for cell therapy. Therefore, adult stem cells sources are needed to be easily isolated and expanded with high purity.

Several studies have demonstrated that mesenchymal progenitor cells from various tissues have the potential to differentiate into other cells, suggesting that pre-committed and committed cells have plasticity in cell fate determination (Sudo et al. 2007).

Mature adipocytes are the most abundant cell type in adipose tissue and they can be easily isolated without painful procedures or donor site injury. Recent data demonstrated that mature adipocytes are able to transdifferentiate into another cell type, suggesting a great plasticity of these cells. This process implies an in vivo phenomenon of physiological and reversible reprogramming of genes in mature cells (Cinti 2009). During this process a differentiated cell turns phenotypically and functionally into a differentiated cell of another type without undergoing dedifferentiation. Some other include the additional step of dedifferentiation (Tosh and Slack 2002).

Data showed two important examples of physiological and reversible transdifferentiation. In conditions of chronic cold exposure the amount brown adipose tissue (BAT) in the organ could

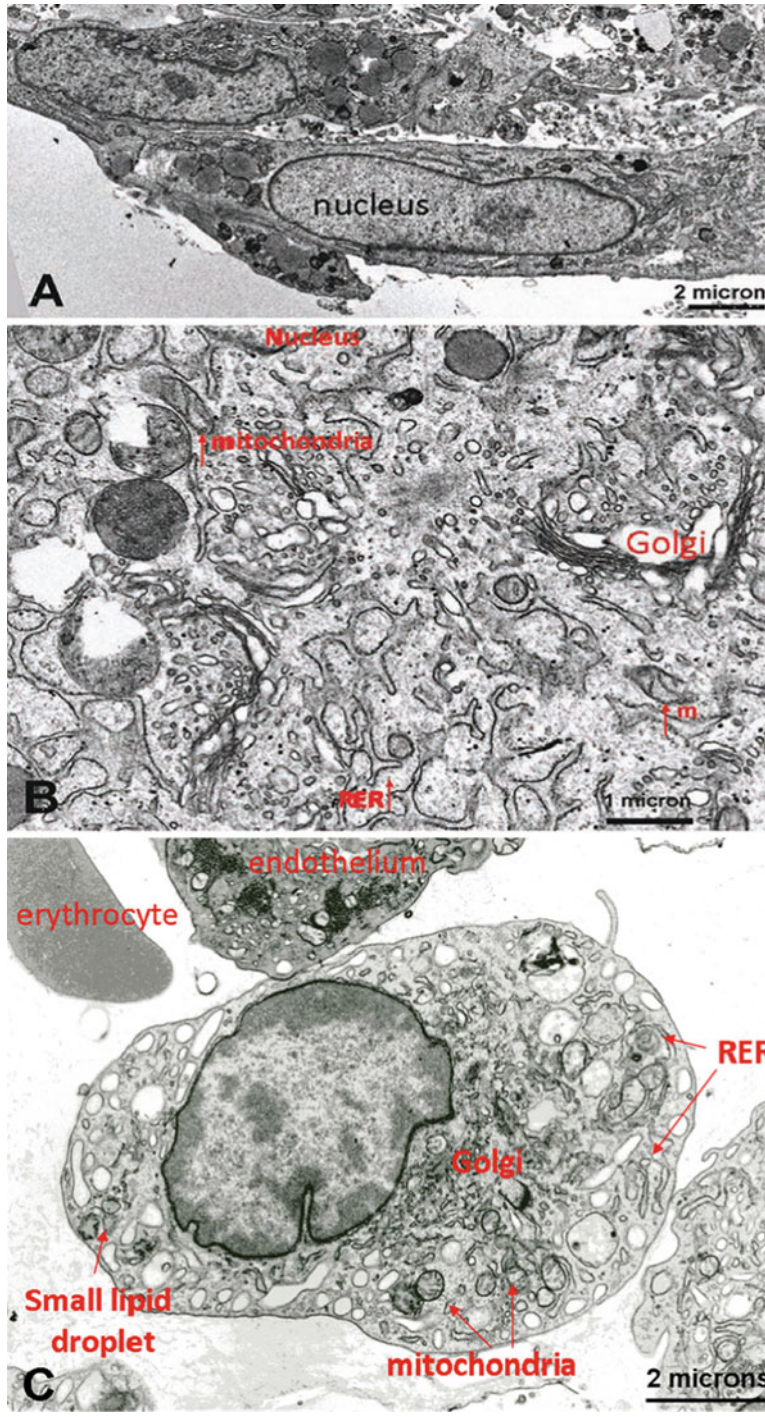

Fig. 20.3 **Electron microscopy analysis**. (**a**) Dedifferentiated adipocytes displayed structural organelle similarities (cytoplasm enlarged in **b**) to stromal vascular fraction-derived MSCs (**c**); *RER* rough endoplasmic reticulum, *m* mitochondria

increase via white-to-brown transdifferentiation and, vice versa, BAT could turn into WAT in case of exposure to an obesogenic diet, to enable greater energy accumulation (Cinti 2011). Moreover, murine mature adipocytes undergo a reversible process of adipocytic/epithelial differentiation during pregnancy and lactation. During pregnancy, adipocytes seem to transform progressively into epithelial cells, forming the lobolo-alveolar part of the mammary gland responsible for milk production. At the end of lactation the lobolo-alveolar component disappears and a new adipocyte population restores the prepregnancy anatomy (Cinti 2011).

While little is known about the potential differentiation of mature adipocytes, more informations are available regarding the dedifferentiated population. Because adipose tissue is abundant and easily accessible tissue at most ages, dedifferentiated cells may be an attractive source of mesenchymal lineages for tissue engineering and other cell-based therapy (Matsumoto et al. 2008). The plastic properties of mature adipocytes are present also in dedifferentiated adipocytes.

In line with these observations, results showed that dedifferentiated adipocytes can be converted into fully differentiated cells, like adipocytes both in vitro and in vivo (Fernyhough et al. 2008), chondrocytes and skeletal myocytes in vitro (Kazama et al. 2008) under the appropriate culture conditions. In particular, dedifferentiated cells may be applicable to bone tissue engineering startegies and cell-based therapies, because they could convert into differentiated osteoblasts in vitro only by transient all-trans retinoic acid stimulation. Thus, dedifferentiated cells can undergo terminal osteoblast differentiation and osteoblast matrix formation, following subcutaneous injection into the peritoneal cavity of mice (Oki et al. 2008).

Moreover, dedifferentiated adipocytes also have the potential to rapidly acquire the endothelial phenotype in vitro and to promote neovascularization in ischemic tissue and vessel-like structure formation, suggesting that cells of endothelial and adipocyte phenotype may have a common precursor (Planat-Benard et al. 2004). These results also highlight the concept that adipose lineage cells represent a suitable new source for therapeutic angiogenesis in ischemic disease.

Data of literature demonstrated that dedifferentiated fat cells can differentiate into smooth muscle cell lineages under specific culture conditions (Sakuma et al. 2009). Green fluorescence protein labelled dedifferentiated fat cells were injected into cryo-injured bladder walls in mice, examining the ability of the fat cells to regenerate smooth muscle tissue after 14 and 30 days after transplantation. Significantly a larger amount of cell expressing α-smooth muscle actin plus green fluorescence protein were observed at the bladder wall injection sites in transplanted mice than in saline injected control mice.

Furthermore, some studies have suggested a close relationship between mature white adipocytes and cardiovascular cells, providing evidence that adipocytes and endothelial cells have a common progenitor cells (Jumabay et al. 2010). Results showed that dedifferentiated adipocytes can act as sources of spontaneously contracting cardiomyocytes in vitro, in which cardiomyocyte phenotype was identified by morphological observations, expression of cardiomyocyte-specific markers and pacemaker activity revealed by electrophysiological studies. These results support a possible link between adipocyte and cardiomyocyte differentiation that might be of importance for pathology and cardiac regeneration.

Moreover, data showed that dedifferentiated adipocytes could differentiated even in cells different from mesenchymal lineages, like neurogenic differentiation. Dedifferentiated cells displayed the capability of forming neurosphere-like structures with significantly increased of nestin expression level respect to non treated control cells (Hermann et al. 2004).

Furthermore, results achieved megakaryocytes and platelets from adipocyte cells with a similar ultrastructures of cells obtained from bone marrow CD34-positive cell. In addition, adipocyte-derived platelets exhibited surface expression of P-selectin and bound fibrinogen upon stimulation with platelet agonists, suggesting that these platelets were functional (Matsubara et al. 2012).

Taken together, the results shown above indicate the capability of the dedifferentiated adipocytes to differentiate into multiple cell lineages. This results might be interpreted as a return back to a noncommitted status for dedifferentiated adipocytes, which was favoured by the culture conditions. These findings suggest that de-differentiated cells have the molecular signature of a reprogrammed cell with features similar to stem cells.

Mature and Dedifferentiated Adipocytes Maintain the Survival and Differentiation of Hematopoietic Stem Cells

Experiments performed in mice demonstrated the presence of hematopoietic progenitors in adipose tissue able to reconstitute hematopoiesis in lethally irradiated animals by systemic infusion of adipose-derived stem cells (Cousin et al. 2003). This beneficial effect could be assigned to the presence of hematopoietic stem cells in the graft, the differentiation of injected mesenchymal stem cells into hematopoietic repopulating cells or the ability of the donor adipose cells to promote the differentiation of residual endogenous hematopoietic progenitors in the host (Corre et al. 2006). In line with this latter hypothesis, results showed that dedifferentiated adipocytes support the complete in vitro differentiation of hematopoietic progenitors without a significant difference in the well-known hematopoietic-supporting capacity of adipose tissue-derived mesenchymal stem cells (Poloni et al. 2012).

Other data compared the hematopoietic supporting capacity of adipocytes differentiated in culture and adipose-derived MSCs (Ookura et al. 2007; Corre et al. 2006). Umbilical cord blood CD34+CD38- cells were co-cultured on MSCs or adipocytes and the results showed that the hematopoietic-supporting capacity of MSCs decreased with adipocyte differentiation. However, CD34+CD38- cells co-cultured with adipocytes preserved the ability to engraft in NOD/SCID mice, suggesting that adipocytes maintain the ability to support transplantable SCID-repopulating cells.

Moreover, a co-culture system of CD34+ cells seeded onto BM-derived adipocytes was studied to investigated their role in supporting hematopoiesis (Corre et al. 2004). These differentiated cells supported complete myeloid and lymphoid differentiation from hematopoietic stem cells, but they not supported the proliferation of immature progenitors. Moreover, the same authors showed that adipocytes differentiated from adipose-derived stem cells secreted cytokines promoting the differentiation of hematopoietic committed progenitors, like IL-6, G-CSF and GM-CSF, and cytokines inhibiting the proliferation of stem cells, like MIP1α (Corre et al. 2006).

Recently data demonstrated that isolated mature adipocytes co-cultured for long time with CD34+ cells has the ability to support the differentiation of hematopoietic stem cells as adipose tissue-derived MSCs (Poloni et al. 2012, 2013 submitted). Because it is abundant and accessible, adipose tissue could be a convenient source of cells for the short-term reconstitution of hematopoiesis.

For many years adipose tissue was regarded as just a heat insulator and store of excess free fatty acids that could be released when needed. Now, it is considered a critical organ involved in energy regulation, inflammation, immune response thought intricate signals and interrelationships with other cells (Kershaw and Flier 2004). The immunoregulatory capacity of mature adipocytes during the dedifferentiation process was analyzed, studying their behaviour in co-cultures with allogeneic lymphocytes. Results showed that the morphological changes of mature adipocytes during the culture time were associated with functional changes. Indeed, dedifferentiated adipocytes were able to inhibit the proliferation of stimulated lymphocytes in direct co-culture, while mature fat cells stimulated their growth. These features may be associated with the ability of adipose tissue to promote inflammation via cytokine production and with the immunoregolatory capacity of MSCs (Gregor and Hotamisligil 2011) derived from different sources such as adipose tissue (Kronsteiner et al. 2011) bone marrow, amniotic fluid and chorionic villi (Krampera et al. 2006; Poloni et al. 2011, 2013).

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Soluble CD40L in Stem Cell Products

21

Folker Wenzel

Contents

Abstract

Conditional on preparation techniques, stem cell products contain a considerable amount of platelets. But platelets are the major source of soluble CD40 ligand (sCD40L) in the blood. It has been demonstrated that CD40L is cleaved from the surface of activated platelets. So it can be expected that sCD40L accumulates in stem cell products reaching levels above the physiological range.

Therefore sCD40L concentrations were observed in peripheral blood samples and in the respective stem cell products of allogenic stem cell donors (peripheral stem cell apheresis or bone marrow) as well as of patients undergoing autologous stem cell apheresis. sCD40L concentrations were normally determined by different ELISA techniques.

In stem cell products, sCD40L concentrations were manifold elevated (range from 1,239 to 3,839 pg/mL) in comparison to concentrations of peripheral blood samples (range from 43 to 321 pg/mL). Experimentally, using MMP-9-, MMP-2- and MMP-2/9-inhibitors sCD40L release by platelets could be inhibited up to 90% of the control values. During autologous stem cell apheresis, the decrease in platelet count from 95,070/μL ± 58,234/μL at the beginning to 54,638/μL ± 26,944/μL at the end of the procedure was accompanied by a significant lowering of sCD40L concentrations in peripheral blood samples from 239 pg/mL ± 139 pg/mL to 126 pg/mL ± 71 pg/mL (dependent on platelet count, linearly correlated, r = 0.95).

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As known from platelet concentrates, an accumulation of sCD40L could also be observed in stem cell products pointing out the importance of sCD40L release by platelets. In addition, during stem cell apheresis sCD40L concentrations in peripheral blood were mainly influenced by alterations of platelet count.

Introduction

Soluble CD40L (sCD40L) is a member of the tumor necrosis family and well known to be a cytokine acting as a potent immune modulator. sCD40L can lead to B-cell immunoglobulin class switching under maturation of dendritic cells, by interaction with its receptor CD40 (Kaufman et al. 2007). Beside the well studied immunological phenomena, sCD40L seems to have effects on platelets and various non hematopoietic cells. So the expression of CD40 and CD40L is not restricted to cells of the immune system. It is known that smooth muscle cells, fibroblasts, keratinocytes and thrombocytes also using CD40/CD40L signalling (Kaufman et al. 2007; Mach et al. 1997). It is assumed that sCD40L influences the stability of arterial thrombi and the development of arterial occlusion (Andre, et al. 2002b). sCD40L acting as an inflammatory mediator plays a role in inflammatory diseases such as atherosclerosis, not only by binding to its classical receptor CD40, but also interacting with Mac-1, expressed abundantly on monocytes and macrophages (Zirlik et al. 2007). In addition sCD40L may support coagulation by inducing TF expression on monocytes (Andre et al. 2002b).

Cytokines released by platelets seem to play an important role in transfusion reactions, and especially platelets are assumed to be the major source for sCD40L in the peripheral blood (Andre et al. 2002a; Wenzel et al. 2008). CD40L is normally sequestered in resting platelets, but can be translocated to the membrane and released into the plasma after agonist activation. The release process is caused by a shedding of the platelet surface from CD40L and is mainly triggered by matrix metalloproteinase activity (Choi et al. 2010; Reinboldt et al. 2009). These effects cause the observed phenomenon of sCD40L accumulation in stored platelet concentrates (Blumberg et al. 2006; Kaufman et al. 2007; Wenzel et al. 2011a).

Although many cytokines are secreted by leukocytes during storage of blood products, platelet derived cytokines, like sCD40L, also contribute to transfusion reactions (Aye et al. 1995; Blumberg et al. 2006; Cognasse et al. 2008). Phipps et al. (2001) described a statistic correlation between the concentration of cytokines in platelet products and the incidence of febrile transfusion reactions. Especially, sCD40L should be involved by triggering adverse reactions in spite of leukocyte reduction. It was generally assumed that a reduction of sCD40L in platelet products should lead to a reduced incidence of adverse transfusion reactions. In addition, Khan et al. (2006) demonstrated that sCD40L may be a potential cofactor in the development of transfusion-related acute lung injury (TRALI).

Conditional on preparation techniques, stem cell products contain a considerable amount of platelets. Overall, considering these theoretical and clinical findings it can be expected that there is also a sCD40L accumulation in stem cell products.

Soluble CD40L Release

Platelet Count

It was shown that the sCD40L release of the peripheral blood is clearly dependent on platelet count (Wenzel et al. 2008). In plasma samples of healthy blood donors (n = 8), a lowering of the platelet count by autologous plasma dilution from 243 ± 35 platelets/nL to 39 ± 12 platelets/nL resulted in a significant reduction of sCD40L levels from 5,210 ± 1,645 pg/mL to 845 ± 634 pg/mL after platelet activation and clotting. Overall a linear correlation between platelet count and sCD40L release could be observed with a correlation coefficient r = 0.96.

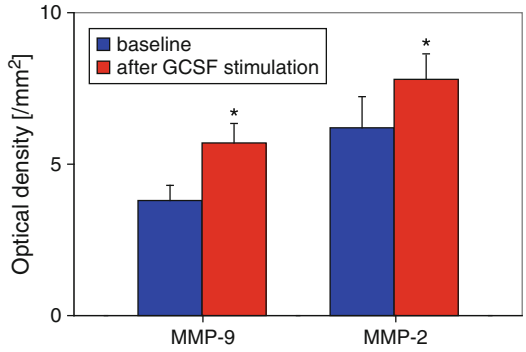

Fig. 21.1 Semiquantitative analysis of MMP-2- and MMP-9-concentrations by determining the optical densities of a respective zymogram using peripheral blood of allogenic stem cell donors (n = 6) before and after GM-GCSF-stimulation (5d, 10 µg/kg BW). Data are given as mean ± SD, *p < 0,05 compared to baseline, Mann-Whitney-Test

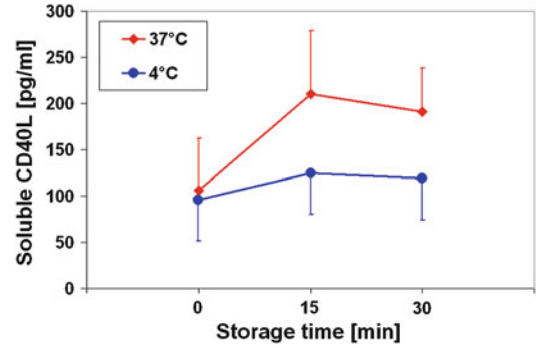

Fig. 21.2 sCD40L concentrations in platelet-rich-plasma samples of healthy blood donors (n = 8) under different storage temperatures. Data are given as mean ± SD

Matrix-Metallo-Proteinases (MMP)

While the inhibition of serine-, cysteine- and aspartate-proteases showed no considerable influence on sCD40L release, a significant reduction of sCD40L release was observed after MMP-inhibition. Especially, using MMP-9-, MMP-2- and MMP-2/9-inhibitors sCD40L release by activated platelets could be inhibited up to 90% of the control values (Menchen et al. 2009; Reinboldt et al. 2009). Conversely, GM-GCSF-stimulation in preparation for peripheral stem cell apheresis resulted in an increase in MMP-9- and MMP-2-concentrations of the peripheral blood (Fig. 21.1), and the sCD40L release kinetic in plasma samples is elevated from 1.4 ± 0.4 pg/mL/min at baseline to 2.9 ± 0.7 pg/mL/min after stimulation. A detailed description of the influence of GM-GCSF-stimulation on MMP concentrations is given in the literature (Domanovic, et al. 2005).

Temperature

As expected by an enzyme-triggered mechanism the CD40L release is temperature-dependent. As an example CD40L concentrations in plasma samples are shown during a storage period (Fig. 21.2). The CD40L release kinetic was lowered from 7.1 ± 2.9 pg/mL/min at 37 °C to 1.9 ± 1.2 pg/mL/min at 4 °C. So the CD40L accumulation should be decelerated in stem cell products during the storage at 4 °C after the respective preparation. These expectations could be confirmed by Woods et al. (2010) who observed in products obtained by peripheral stem cell apheresis of allogenic donors only a small increase in sCD40L after a 48 h storage period.

sCD40L Concentrations in Peripheral Blood and in Stem Cell Products

In the literature, for plasma sCD40L of the peripheral blood a range between 40–240 pg/mL (Chew et al. 2010; Wenzel et al. 2008) is described. These levels could also be found in autologous as well as allogenic stem cell donors. For example, a platelet loss (95/nL ± 57/nL at the beginning, to 75/nL ± 26/nL (during stem cell collection) and to 55/nL ± 27/nL at the end of the procedure ($p < 0.05$, Student's t-test)) during autologous stem cell apheresis (n = 6) was accompanied by a significant lowering of sCD40L concentrations in peripheral blood samples from 241 pg/mL ± 137 pg/mL at the beginning, to 192 pg/mL ± 91 pg/mL (during stem cell collection) and to 124 pg/mL ± 73 pg/mL at the end of the procedure ($p < 0.05$, Student's t-test, correlation coefficient r = 0.9 corresponding to the platelet count) (Fig. 21.3a). However, in the case of

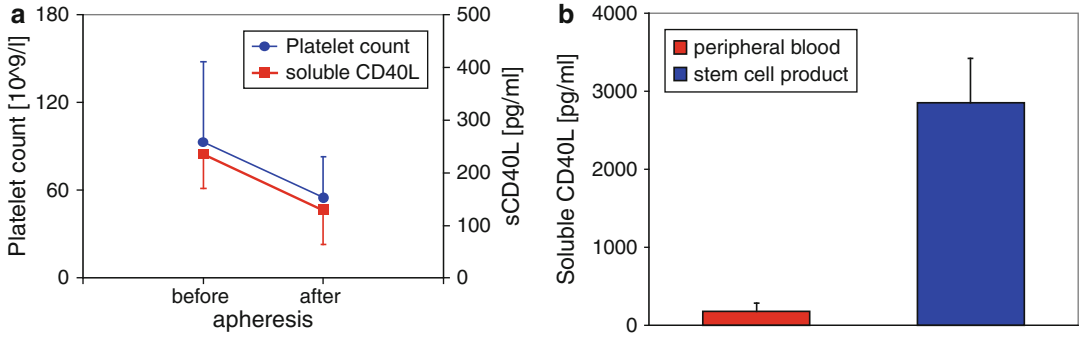

Fig. 21.3 (**a**) Platelet count and sCD40L concentrations in peripheral blood from autologous donors (n = 6) during stem cell apheresis. (**b**) Soluble CD40 Ligand (sCD40L) concentrations in plasma samples (peripheral blood) and in stem cell products from autologous donors (n = 5). Data are given as mean ± SD

platelet counts remaining in a physiological range instead of the apheresis procedure – especially in allogenic stem cell donors – sCD40L levels are also not altered.

In apheresis stem cell units, the platelet count showed normally pathophysiological levels and ranged from about 900/nL up to 3,000/nL. In parallel, sCD40L concentrations were elevated substantially above peripheral blood levels to a range from about 2,000 pg/mL up to 4,000 pg/mL. For example a comparison between sCD40L concentrations in the peripheral blood and in the respective stem cell product of patients (n = 6) undergoing peripheral stem cell apheresis is given (Fig. 21.3b). The sCD40L concentrations were elevated substantially above peripheral blood levels to 2,756 pg/mL ± 387 pg/mL (range: 2,189–3,641 pg/mL; $p < 0.05$ compared to peripheral blood) (Wenzel et al. 2011b).

In stem cell units collected from bone marrow, the sCD40L concentrations reached also a pathophysiological range from about 600 pg/mL up to 1,600 pg/mL in comparison to the peripheral blood, but remained in comparison to the apheresis products at lower levels. This may be due to the fact that the platelet count in the bone marrow showed also a reduced range from 80 to 160/nL.

In conclusion, with these facts the behaviour of soluble CD40L in stem cell concentrates could be interpreted: In line with the observations on sCD40L release in platelet concentrates, similarly a sCD40L accumulation in stem cell products could be observed, especially in the platelet-rich products prepared by apheresis. In support of the observations of Woods et al. (2010), our data showed a slow release of soluble CD40L at 4 °C. This may be attributable to reduced activity of the enzymes (MMP-2, MMP-9) responsible for sCD40L generation under these conditions. Thus the main amount of sCD40L that accumulates may be produced during the time interval of stem cell sampling and processing at room temperature before storing the stem cell unit at 4 °C.

At least Khan et al. (2006) demonstrated in an experimental work that sCD40L levels could be indirectly involved in the development of adverse transfusion reactions. But due to a lack of sufficient data regarding the pathophysiological sCD40L levels in the stem cell products under clinical routine use one may currently only speculate on effects of sCD40L influencing directly the haematopoietic stem cells in the product itself or triggering adverse transfusion reactions.

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