Stem Cells and Cancer Stem Cells 13
Therapeutic Applications in Disease and Injury

M.A. Hayat
Editor

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Volume 13
Therapeutic Applications in Disease and Injury

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Therapeutic Applications in Disease and Injury

Edited by

M.A. Hayat
Distinguished Professor
Department of Biological Sciences
Kean University, Union, NJ, USA

Springer

Editor
M.A. Hayat
Department of Biological Sciences
Kean University
Room 213, Library building
Morris Avenue 1000
Union, NJ 07083, USA

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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 nontherapeutical 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 13 of the multivolume 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 one 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 the normal lung and lung cancer is also explained.

A detailed overview of the progress from embryonic stem cells to transduced pluripotent stem cells is presented. The importance of cancer stem cells in clinical application and their dynamics and regulation are explained. Characteristics of glioblastoma multiforme stem cells are presented. The importance of stem cell markers in diagnosis is included. Also, included is the role of stem cells in angiogenesis.

The 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 the blood or bone marrow. 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. 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, as mentioned above, they can be produced in large quantities. The authors describe their proliferation and differentiation capacities in a variety of regenerative medicine, including neurodegenerative diseases. A method for isolating multipotent endothelial-like cells from human adipose tissue is presented.

The most serious late complication of allogeneic 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. However, with the possibility that transplantation of stem cells might cure HIV infection and multiple sclerosis, this technology is described in detail in this volume. The transplantation technology using oligodendrocytes and motoneuron progenitors from human embryonic stem cells to achieve locomotor recovery after spinal cord transaction is also explained here.

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. One 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 22 contributors representing 6 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 two subheadings: Treatments 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 disease 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 me 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
February, 2015

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Contributors

Nimer Adeeb California Neurosurgical Institute, Thousand Oaks, CA, USA

Ben Berkhout Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands

Aman Deep California Neurosurgical Institute, Thousand Oaks, CA, USA

Shentong Fang Translational Cancer Biology Research Program, University of Helsinki, Helsinki, Finland

Gero Hütter Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, Heidelberg University, Heidelberg, Germany

Kevin Kemp Multiple Sclerosis and Stem Cell Group, School of Clinical Sciences, University of Bristol, Bristol, UK

Mark J. Knights Leeds School of Medicine, University of Leeds, Leeds, UK

Cecilia Laterza Division of Neuroscience, Institute of Experimental Neurology-DIBIT 2, San Raffaele Scientific Institute, Milan, Italy

Elizabeth Mallam Multiple Sclerosis and Stem Cell Group, School of Clinical Sciences, University of Bristol, Bristol, UK

Gianvito Martino Division of Neuroscience, Institute of Experimental Neurology-DIBIT 2, San Raffaele Scientific Institute, Milan, Italy

Martin M. Mortazavi California Neurosurgical Institute, Thousand Oaks, CA, USA

Han S. Myat Division of Hematology-Oncology, University of California, Los Angeles, CA, USA

Juliana Redondo Multiple Sclerosis and Stem Cell Group, School of Clinical Sciences, University of Bristol, Bristol, UK

Upal Roy Department of Immunology, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

Petri Salven Department of Pathology, Haartman Institute, University of Helsinki, Helsinki, Finland

Neil Scolding Multiple Sclerosis and Stem Cell Group, School of Clinical Sciences, University of Bristol, Bristol, UK

Mary E. Sehl Department of Biomathematics, and Division of Hematology-Oncology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

J. Suaudeau Sezione Scientifica, Pontificia Accademia per la Vita, Rome, Italy

R. Shan Tubbs Pediatric Neurosurgery, Children's of Alabama, Birmingham, AL, USA

Alastair Wilkins Multiple Sclerosis and Stem Cell Group, School of Clinical Sciences, University of Bristol, Bristol, UK

Part I

Treatments

The Use of Mesenchymal Stem Cells for Treating Neurodegenerative Diseases

1

Kevin Kemp, Juliana Redondo, Elizabeth Mallam, Neil Scolding, and Alastair Wilkins

Contents

Abstract

Stem cell-based therapies are increasingly emerging as hopeful therapeutic options for treating currently incurable chronic degenerative and inflammatory diseases of the central nervous system (CNS). Among the variety of different stem cell types, mesenchymal stem cells (MSCs) possess a wide range of practical features, in addition to a plethora of neuroprotective properties, which make them an attractive candidate as a potential cell therapy for a wide range of neurodegenerative disorders. Here we will discuss the suitability of MSCs for clinical use. We will also summarize the underlying mechanisms that drive their multiplicity of neuroprotective effects alongside their use in the treatment of neurodegenerative disease from the bench side to clinical trials.

K. Kemp (✉) • J. Redondo • E. Mallam
N. Scolding • A. Wilkins
Multiple Sclerosis and Stem Cell Group,
School of Clinical Sciences, University of Bristol,
Bristol BS10 5NB, UK
e-mail: Kevin.Kemp@bristol.ac.uk

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Introduction

Since the early days of stem cell biology, their potential for treating neurological illnesses has been recognised. However, despite great strides in understanding methods of deriving stem cells, differentiation protocols and overall properties, there remains much to be understood before stem cells become a major therapy for neurodegenerative conditions. Not least to this is determining how precise mechanisms of neurodegeneration may be targeted.

In recent years a paradigm shift in considering how stem cells might be effective therapies has occurred. Initial considerations concerning the potential regenerative capacity of stem cells focused on their ability to differentiate into specific cell types and thus replace lost or injured cells. Considering CNS neurodegenerative conditions, particularly when trying to replace damaged neurons, the concept that a neuronally-differentiated stem cell could restore the multitude of synaptic connections and thus integrate in a functionally meaningful way is still far from reality, and so studies have focused on other mechanisms by which stem cells may aid repair. Stem cells possess a wide range of neuroprotective properties and, because of their multiple modes of neuroprotection, of stem cell types, MSCs offer one of the most enticing prospects of stem cell therapy for neurodegenerative disorders. These properties will be discussed in detail below.

Neurodegeneration

It might appear initially that discussing the whole range of neurodegenerative conditions as one entity is over-simplistic. However, neurodegenerative conditions share many common pathways and, although the aetiological factors may differ, pathways for neuronal injury and loss are often common amongst the diseases. In fact, determining whether stem cells are able to attenuate a number of disease processes may be key to their development as therapeutic agents. A list of some of the major cellular processes occurring in neurodegenerative disease is given in Table 1.1.

Table 1.1 Disturbances in common cellular pathways that may contribute to neuronal injury in neurodegenerative disease

Increased oxidative stress
Disturbed mitochondrial function and alterations in cellular energy requirements
Abnormal cellular transport mechanisms
Abnormal phosphorylation of key cellular molecules
Altered neuronal and axonal ion channel distributions
Abnormalities in ‘normal’ ageing processes

Oxidative Stress

Oxidative stress mechanisms are implicated to some extent in neuronal injury in most neurodegenerative conditions. The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) occurring in response to pathogens is a major cellular defence mechanism. However, generation of these species, either in response to altered immune responses or exposure of previously sequestered antigens to immunological responses, appears to occur in a variety of neurodegenerative diseases, both those with a primary immunological cause and those without. Of the immunological causes, multiple sclerosis (MS) is characterised by activation of ROS/RNS, most likely in response to aberrant activation of the immune system towards, as yet, unknown antigen(s). A major role for ROS in the pathophysiology of MS and CNS inflammatory disorders has been demonstrated in both pathological and experimental studies. Profound oxidative damage to oligodendrocytes and neurons has been demonstrated in MS lesions (Haider et al. 2011). The enzymes responsible for the generation of ROS have been detected in tissue derived from patients with MS and experimental models of CNS inflammation. NADPH oxidase is responsible for the generation of superoxide ions and is highly expressed in activated microglia and infiltrating macrophages within MS lesions (Fischer et al. 2012). In addition, expression of iNOS, which is responsible for the generation of nitric oxide, is up-regulated in microglia and macrophages in acute and chronic lesions (Hill et al. 2004). Oxidative damage to cell membranes leads to the formation of a number of toxic compounds

including 4-hydroxy-2-nonenal (4-HNE) and oxidised phospholipids which are abundantly present in active MS lesions (Haider et al. 2011).

As well as neurological conditions with a primary inflammatory cause, oxidative stress mechanisms operate in diseases which are not driven by inflammation *per se*. For instance, the genetic condition Friedreich's Ataxia (FRDA) is associated with oxidative injury. FRDA is caused by a trinucleotide repeat expansion which causes reductions in cellular frataxin protein expression. Frataxin has important roles in mitochondrial function, iron metabolism and the defence against oxidative stress. Frataxin deficient cells appear particularly sensitive to hydrogen peroxide-induced injury and have reductions in antioxidant detoxifying enzymes such as superoxide dismutase (SOD) (Dey et al. 2012).

Mitochondrial Dysfunction

Primary mitochondrial conditions, such as MELAS (myoclonic epilepsy with lactic acidosis and stroke-like episodes), commonly result in neurodegeneration. Neurons and axons, having extremely large energy demands, are particularly prone to defects in mitochondrial respiratory chain defects. Many neurodegenerative conditions also exhibit secondary mitochondrial failure. For example in MS, the increased energy demand for action potential propagation plus changes occurring to mitochondria as a result of the inflammatory environment may lead to mitochondrial deficits (Mahad et al. 2008). Failure of mitochondrial energy generation has been linked to disturbances of ion pumping by axons, leading to alterations in ion channel function and distribution which has been linked to axonal degeneration (Kapoor et al. 2003).

Abnormal Cellular Transport Mechanisms

The axon of a neuron is a unique cellular feature which can project for extremely long distances. This feature necessitates mechanisms to transport proteins and cellular organelles from the cell body to the distant nerve terminal. A well-defined series of molecular motors allows for this transport in both anterograde and retrograde fashions. However, this process is susceptible to pathological changes and axonal transport defects are commonly seen in neurological disorders. Genetic defects in motor protein genes may cause neurological disease. For instance, a form of hereditary spastic paraparesis (HSP) is caused by a missense mutation (N256S) in the KIF5A gene (spastic paraplegia type 10 (SPG10)) (Reid et al. 2002). This KIF5A mutation is implicated in disturbed intracellular axonal transport in HSP patients and pathologically, the disease is characterised by axonal loss in the corticospinal tract. Symptomatically, HSP patients present with weakness of the lower extremities and severe spasticity.

Secondary axonal transport abnormalities also appear commonly in a range of neurodegenerative conditions. Although these disorders are not associated with a primary defect in axonal transport, axonal transport defects may be seen as a consequence of pathological processes occurring throughout the neuron. This is relevant since reduced axonal transport leads to accumulation of substances along the course of the axon, axonal swelling and potentially this may lead to irreversible axonal transection. Thus, understanding mechanisms of preventing secondary axonal transport defects may be crucial in protecting axons from degeneration. In MS, axonal transport abnormalities are noted in the context of acute inflammatory lesions. Amyloid precursor protein (APP), which is normally rapidly transported along axons, accumulates in axons within inflammatory lesions and appears to be associated with axonal degeneration (Ferguson et al. 1997).

Abnormal Phosphorylation of Key Cellular Molecules

Abnormal or aberrant phosphorylation of key cellular proteins occurs in many neurodegenerative conditions and may be associated with

propagation of disease processes. Abnormal hyperphosphorylation of tau is associated with Alzheimer's disease and therapeutic strategies have been developed which aim to prevent this process (Wang et al. 2013). In addition, hyperphosphorylated alpha synuclein is found in Lewy bodies in the context of Parkinson's disease (PD), and hyperphosphorylated neurofilaments are found in a number of neurodegenerative disorders (Gray et al. 2013). Understanding mechanisms of abnormal phosphorylation and determining how specific therapies attenuate these processes are thought to be key to reversing neurodegeneration in these diseases.

Mesenchymal Stem Cells

Discovery and Terminology

During the 1960s and 1970s ground-breaking studies by Friedenstein et al. (1974) laid the foundations into what is today the vast area of MSC research and its many scientific applications. Maybe more specifically, for their pioneering work they are largely credited for observing that the bone marrow in adult life is a reservoir of stem cells for mesenchymal tissues, distinct from that of haematopoietic stem cells. His groups' initial work in rodents described cells harvested from the bone marrow, with a spindle-shaped fibroblast-like morphology, which could adhere to plastic and grow as highly proliferative monolayers of cells arising from single colonies. Taking all these physical characteristics into consideration they coined these newly discovered cells colony forming units-fibroblasts (CFU-F) and showed they could differentiate into haematopoietic supporting stroma when transplanted *in vivo* (Friedenstein et al. 1974).

Following on from this work, Owen et al. raised the notion of a stromal stem cell, with cells contained within CFU-F's having the capacity for self-renewal, multipotentiality and formation of ectopic bone marrow (Owen and Friedenstein 1988), features not observed with mature fibroblastic cells. They also described these cells being a heterogeneous population of stem and progenitor cells observed through differences in their potency at the colony level. In the 1990s Caplan popularised the term mesenchymal stem cell that is widely used today (Caplan 1991), acknowledging their role in both the formation of bone and cartilage. By the end of that decade, Pittenger reported the isolation and phenotypic profile of cells from human bone marrow that had all the characteristics of mesenchymal stem cells, these cells being multipotent having the tri-lineage capability of adipogenic, chondrogenic and osteogenic differentiation (Pittenger et al. 1999).

Finally in 2005/2006, the International Society for Cellular Therapy (ISCT) encouraged a minimum criterion within the scientific community for defining a mesenchymal stem cell. Given that only a relatively small proportion of marrow-derived plastic-adherent cells give rise to fibroblastic colonies *in vitro*, some have been reluctant to label these cells 'stem' cells per se. They initially concluded that the term 'multipotent mesenchymal stromal cells' should be used for fibroblast-like, plastic-adherent cells seen in culture, and the term 'mesenchymal stem cell' used only for cells that meet all specified stem cell criteria as a true 'mesenchymal stem cell' most certainly exists (Dominici et al. 2006).

Phenotype

Unlike the majority of alternative stem cell populations, MSCs express a number of non-specific markers, none of which individually or in combination yet yield to large and/or consistent numbers being isolated from tissues for enrichment. True MSCs reside in adult tissue in extremely low numbers; consequently with no single antigenic marker specific to the MSC population being found, there is a formidable obstacle in place hindering the study of MSCs *in vivo* and their use therapeutically. Without doubt the majority of data published to date regarding the phenotypic analysis of MSCs has been attained

through examination of MSCs expanded in *in vitro* cultures, while little is really known about the true phenotypic characteristics of the progenitor cells that reside *in vivo* responsible for the large turn-over of mesenchyme tissue. A major caveat to this predicament is that the *in vitro* antigen expression, proliferation and differentiation potential of MSCs all rely heavily on the culture conditions and experimental protocols used. Indeed, aspects such as passage number, cell density and culture media, as well as the species and tissue they were derived from all affect the phenotype of MSCs (Prockop 2009).

With many different methods of isolation, expansion and approaches to characterising MSCs being used worldwide, it was becoming increasingly difficult to compare and contrast study outcomes involving the use of MSCs. A collaborative meeting was therefore held that led the International Society for Cellular Therapy (ISCT) seeking to encourage a minimal criterion within the scientific community for defining MSCs for both laboratory investigation and preclinical studies (Dominici et al. 2006). They identified three key fundamental properties that MSCs should display:

1. Show plastic adherence under standard culture conditions
2. Express CD105, CD73, and CD90 and lack the expression of CD45, CD34, and markers for monocyte, macrophages and B-cells (CD14 or CD11b, CD79 or CD19 and HLA-DR)
3. Differentiate into adipocytes, osteoblasts, and chondrocytes under standard culture conditions and then be demonstrated by well excepted staining protocols

Isolation and Culture

Adult MSCs are thought to be mesodermal in origin and cells that display all characteristics of MSCs have been isolated from a vast number of tissues including the bone marrow, liver, lungs, cord blood, adipose tissue, brain, kidney, muscle among many others. It's likely that MSCs reside in virtually all post-natal organs and tissues however, within each of these tissues there are indeed variations in their yield, phenotype and differentiation potential. The most studied and accessible source of MSCs is the bone marrow where they comprise between 0.001 and 0.1 % of the total population of nucleated cells (Pittenger et al. 1999). In humans, bone marrow-derived MSCs are isolated from marrow harvests, obtained primarily from the posterior iliac crest of the pelvis, and also from the tibial and femoral marrow compartments. In rodents, bone marrow-MSCs are again typically obtained from the femur and/or tibia, however as the bones are relatively small, the bones are dissected out and completely flushed of all bone marrow so sufficient numbers of cells can be obtained for culture.

Once isolated, MSCs can be expanded extensively in *in-vitro* cultures without loss of function or phenotype. There are huge variations between laboratories in their enrichment and culture conditions. Antibody enrichment techniques for their positive selection, such as anti-CD271, are frequently utilized in isolation protocols. The addition of cytokines to growth media in conjunction with serum deprivation is also used to standardize culture conditions and to remove the need for animal products. However historically MSCs are selected from populations of mononuclear cells (MNCs) through their adherence to plastic in culture (allowing the non-adherent cell populations, such as hematopoietic cells, to be washed away) and expanded in medium consisting of Dulbecco's Modified Eagles Medium (DMEM) (low glucose), supplemented with foetal bovine serum and L-glutamine at 37 °C with 5 % CO_2. Cells with a spindle-shaped fibroblast-like morphology subsequently accumulate; with cultures containing both rapidly self-renewing cells that remain in high numbers for several passages if the cultures are maintained at low density, plus larger more mature cells that predominate in later passages (Pochampally et al. 2004).

Suitability for Clinical Use

Over recent years the wider therapeutic potential of MSCs has been realised, noticeably verified by the large numbers of trials investigating their clinical use. Taking into consideration the wealth of historical evidence relating to the administration of bone marrow-derived cells in humans, data almost entirely attained through their use over the last 40 years in haematological medicine, although not without caution, appears to validate their clinical safety. In response to this MSCs for therapeutic purposes have been designated as safe by the FDA, although expansion of MSCs is subject to FDA and EU regulation on advanced therapy medicinal products (ATMP) and must adhere to Good Manufacturing Practice for medicinal products. In corroboration, large numbers of reports have not found any significant adverse effects caused through the administration of MSCs when used as a potential treatment for a neurological disorder in humans. However, evidence obtained in both animal and *in vitro* studies indicate that there still remains the theoretical possibility of unwanted and potentially serious side effects to occur, including malignant transformation, the formation of fibrous masses and unwanted immunosuppressive effects. These are discussed below.

Maldifferentiation and Malignant Transformation

The differentiation of MSC, particularly down alternative lineages, is driven by environmental cues, and thus it would be hoped that MSC would only differentiate into cells appropriate for the tissues they find themselves in. However it has been reported that in mice with Experimental autoimmune encephalomyelitis (EAE) that intracerebroventricularly delivered MSC formed CNS parenchyma cellular masses characterised by focal inflammation, demyelination, axonal loss and increased collagen-fibronectin deposition (Grigoriadis et al. 2011). These studies highlight considerations that need to be made regarding the route of administration (none of these effects have been seen following intravenous or intraperitoneal administration) and also the number of cells used.

Another study found sarcomas in the lungs of mice that received intravenous MSC infusions – in this study, it was thought that transformation of the MSC had occurred during expansion *in vitro* (Tolar et al. 2007). Others however have demonstrated safe expansion of MSC after long-term culture, but it would seem prudent that MSC intended for human therapy should have regular phenotypic, functional and karyotyping tests during *in vitro* expansion to ensure their safety for transplant (Dominici et al. 2006).

Mesenchymal stem cells theoretically also have the potential to influence pre-existing malignancies: promoting growth through their trophic properties, suppressing the anti-tumour immune response, promoting angiogenesis within tumours, and promoting metastasis (Dai et al. 2011). Findings from *in vivo* studies however have so far been inconsistent, and thus this is a potential risk that investigators must consider and remain alert to.

Immunosuppression

One of the key features of MSC to be exploited are their immunomodulatory activities. Like MS, many of the diseases that MSC have been proposed as potential treatments have an immunological basis. Immunosuppression is the aim of those treatment regimes. There is of course a balance between immunosuppression that reigns in the target disease process, and that which allows other pathogenic processes to thrive. A literature search failed to provide any evidence of increased incidence of opportunistic infections after MSC treatment. Indeed, recent trials have demonstrated that the use of autologous MSC decreased the risk of opportunistic infection following solid organ transplantation (Tan et al. 2012).

The risk of over-immunosuppression and other unfavourable consequences of MSC trophic factor secretion could potentially be contained by the identification, isolation and pharmacological administration of predictable doses of the advan-

tageous factors secreted by MSC. This approach however disregards the other desirable properties of MSC, primarily their ability to migrate specifically to where they are required, act locally and respond to the changing pathophysiological environment.

Expansion and Biopreservation

A further consideration into the safe therapeutic use of MSCs is the minimal number of cells required to produce clinical benefit in patients. This is highly speculative and likely the answer differs depending on the disease being treated. Regardless, it is expected that prior to their use MSCs will need to be expanded in culture using cocktails of different cytokines, especially if a serum free culture is required. Protocols for the expansion of MSCs are well characterised and can indeed produce large numbers of highly pure cultures at levels accepted for clinical use. However conversely, culture techniques used for expanding MSCs, although advantageous, can lead to alterations in their reparative capacity (Crisostomo et al. 2006).

Long-term storage of MSCs also needs to be considered if 'banks' of MSCs are required. Given their immune evasive properties it is likely that banks of both autologous and allogeneic MSCs could be generated.

Evasion and Migration

Mesenchymal stem cells for therapeutic use can delivered both locally and/or systemically. If damage is localised, direct transplantation to the affected area may be beneficial. Alternatively, a distinct feature of many neurological (and non-neurological) disorders is that they present as highly dispersed focal areas of damage, thus direct transplantation strategies become impractical, favouring a systemic approach. An attractive therapeutic property of MSCs is they have the capacity to migrate to sites of inflammation and cellular damage after systematic administration and subsequently exert local effects in the resident nervous tissue. A significant body of literature exists relating to mechanisms of MSC migration towards areas of damage. The precise mechanisms by which MSCs are recruited are not yet fully understood, however it is thought that cell migratory processes involve events mediated through multiple adhesion/chemokine ligand interactions (Sohni and Verfaillie 2013). In healthy animals data suggests that genetically labelled MSCs distribute broadly following systemic infusion into a wide variety of tissues (Devine et al. 2003). More importantly, therapeutically there is indeed evidence that MSCs are attracted to chemokines produced in brain lesions (Rice and Scolding 2010) and able to preferentially migrate to areas of neurological damage (Kemp et al. 2011).

The migration and subsequent homing of MSCs to damaged tissue can be influenced by numerous factors including age, culture conditions, passage number and site of administration (Sohni and Verfaillie 2013). It is also clear that post intravenous infusion large quantities of MSCs are sequestered within the pulmonary circulation, thus not reaching their desired target tissue. However, it has been put forward that MSC trapped in the lung can alternatively secrete soluble factors into the blood stream to mediate a systemic effect (Lee et al. 2009).

Mesenchymal stem cells, at least in part, are able to evade the host immune system and avoid rejection, demonstrated in numerous studies utilizing either allogeneic or xenogeneic MSC transplantation *in vivo*. This survival technique is crucial if they are to have any significant therapeutic effect on CNS tissue. MSCs show extensive immunomodulatory capacities (as will be discussed below in more detail). However, exactly how they evade recognition by a foreign immune system in the allogeneic transplantation setting is largely unknown, but is likely to be mediated through both cell-cell interactions and indirectly via soluble factors. One explanation being MSCs express MHC class I molecules, very low levels of MHC class II, and no co-stimulatory molecules; a phenotype regarded as non-immunogenic leaving T cells anergic (Aggarwal and Pittenger 2005). Although the

complete immunoprivileged status of MSC cannot be solely attributed to this phenomenon.

Regardless of the precise mechanism, the immunosuppressive properties of MSCs make it possible that allogeneic as well as autologous cell therapy could be considered for therapeutic purposes. This also has bearing in diseases where there is the possibility that patient MSC function might be affected by the disease process itself (or its ongoing drug treatment), rendering them unsuitable for autologous therapy (Mallam et al. 2010).

Mechanisms of MSC Induced Neuroprotection

Mesenchymal stem cells have shown therapeutic effects in animal models of many neurologic disorders and are currently being tested in clinical trials, as a new therapeutic approach for tissue regeneration and repair. Understanding the underlying mechanisms that drive their multiplicity of neuroprotective and modulatory effects is important to optimize and enhance their function. Here we will discuss the main mechanisms that are currently believed to contribute to neuronal tissue repair and/or protection (also see Fig. 1.1).

Differentiation

The original rationale for stem cell-based therapy for neurodegenerative diseases was based on their presumed ability to replace damage cells by differentiating into cells of the neural lineage.

MSCs possess great plasticity and early reports suggested that these cells could overcome their germinal boundaries, differentiating not only into cells of the mesengenic lineage (as chondrocytes, adipocytes, osteocytes and muscle cells) but also into hepatocytes, endothelial cells and neurons. The presumed capacity for transdifferentiation received much attention as a potential mechanism of CNS repair and was thought to contribute to their efficacy in a wide variety of animal models of neurodegenerative disease. But differentiation of MSCs into functional neuronal lineages is still a matter of debate. There are a number of reports that have demonstrated that MSCs express neuronal proteins following *in-vitro* stimulation by growth factors, chemical compounds or neuro-sphere formation (Kim et al. 2002) and *in vivo* after transplantation into the brain (Zhao et al. 2002). But there is as yet no evidence that MSCs expressing neuronal proteins also possess the functional properties of neurons. *In vitro* neuron-like morphology was considered by some to be an artefact from culture, indeed Lu et al. (2004) showed that exposure to stressors such as detergents, chemicals, high pH and high molarity sodium chloride induces a neuron-like phenotype in MSCs, as well as fibroblasts and keratinocytes. Cytoskeletal alterations induced by these agents, rather than actual neurite outgrowth, accounted for the neuron-like morphology (Neuhuber et al. 2004).

Cell Fusion

Cell fusion relates to a process in which two or more cells fuse together and may represent a form of neuroprotection or rescue of mature cell types. Analysis of sex mismatched human bone marrow transplants, in which male donor cells are transplanted into female recipients, Purkinje cells containing the Y chromosome have been described with more than a diploid sex chromosome composition, suggesting that bone marrow-derived cells donate genetic material to Purkinje cells through fusion events (Weimann et al. 2003). In rodents, Purkinje cell fusion with bone marrow-derived cells has been shown, with its frequency increasing with age, toxic insult and inflammation exposure (Johansson et al. 2008). In humans, studies have also shown a disease-related increase in Purkinje cell fusion in patients with MS (Kemp et al. 2012).

Specifically, MSCs have shown the ability to fuse with Purkinje cells in both inflammatory and non-inflammatory rodent models of neurodegeneration. More importantly, these studies have also demonstrated that bone marrow-derived MSC and Purkinje cell fusion events develop into

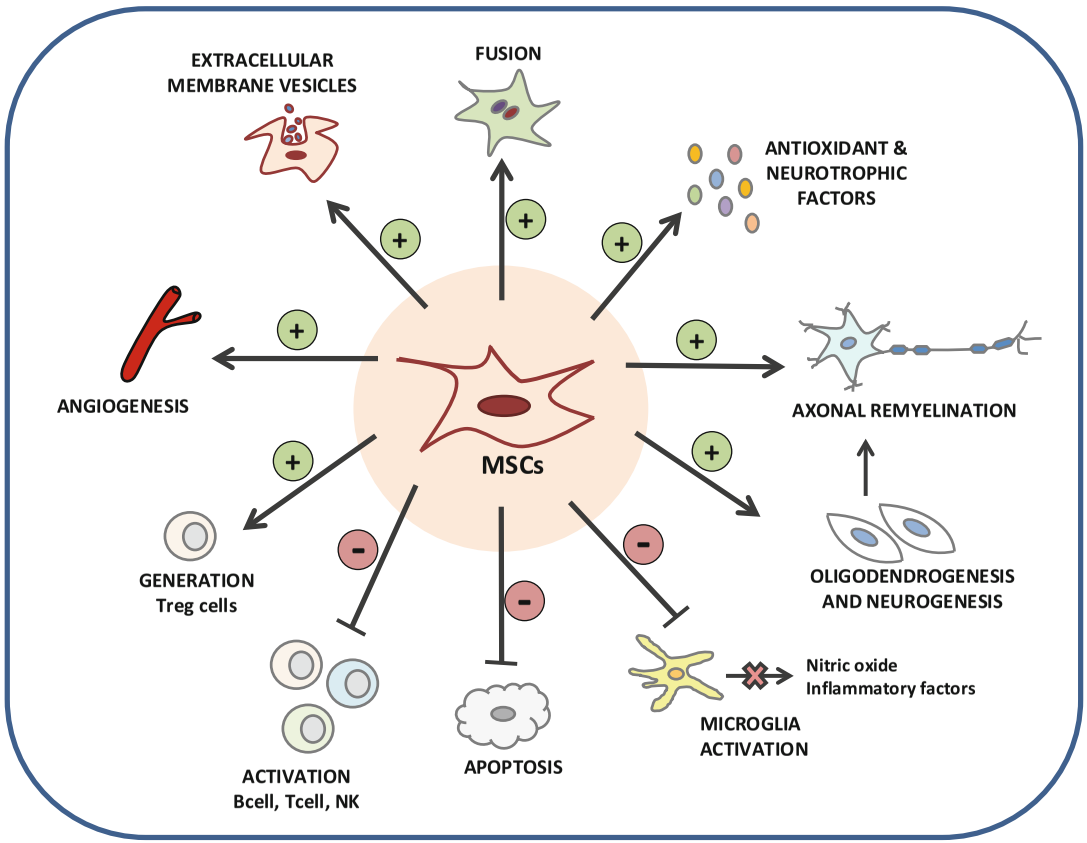

Fig. 1.1 A schematic representation of the mechanisms implicated in MSC induced neuroprotection

electrically active neurons with functional synaptic formation within a neurodegenerative cerebellar environment (Bae et al. 2007). This raises the possibility that MSCs are able to integrate into the CNS and aid the repair of highly differentiated cells types through mechanisms of cell fusion and subsequent donation of nuclear material.

Mesenchymal Stem Cells' Secretome

The complex set of proteins released by a cell/tissue that regulate a variety of physiological processes is defined as a "secretome". The initial rationale for cell replacement therapies is now being substituted with the new concept that the remarkable trophic, modulatory and reparative effects of MSCs are mediated by the paracrine secretion of bioactive molecules. Paracrine signalling is a means of cell-to-cell communication in which a donor cell secretes factors into the extracellular environment to induce changes in nearby target cells. Several studies have demonstrated that the therapeutic effect of MSCs requires minimal CNS integration and may not even be mediated primarily through direct cell to cell contact: despite sparse engraftment to the CNS, infusion of MSCs into animal models of neurological diseases is efficacious (Uccelli 2013). This apparent paradox is explained by findings that the beneficial actions of these cells can be mediated by their secretome: MSCs are able to detect and respond to cues released at the site of injury, producing cytokines, growth factors, anti-apoptotic factors and anti-oxidants to promote repair. Mounting evidence demonstrates that the beneficial effect of MSCs occurs even when they are distant to the target organs, thanks to the release of molecules that interact with the host environment activating endogenous repair processes. The repair mechanisms of MSCs can therefore be "direct", with the secretion of active molecules that can protect/rescue neurons from ongoing injury, or "indirect" through factors that can promote proliferation and differentiation of neural precursor cells that will enhance repair.

Soluble Growth Factors and Enhancement of Endogenous Repair

Mesenchymal stem cells can support tissue repair in the CNS both by protecting neural tissue and by promoting the induction of local neurogenesis. Several groups have demonstrated that MSCs can rescue neural cells from apoptosis, through their ability to secrete neurotrophic factors such as brain-derived neurotrophic factor (BDNF), several neurite-inducing factors, axon guidance and neural cell adhesion molecules (Crigler et al. 2006; Wilkins et al. 2009). Indeed, MSCs can modulate molecules involved in the cellular response to stress conditions: inhibiting death-inducing pathways whilst activating cell survival signalling, including the PI_3kinase/Akt and MAPkinase pathways (Wilkins et al. 2009). Thus the secretion of these neurotrophic molecules and cytokines by MSCs could explain the reduction of axonal injury/increased neuronal cell survival observed following the administration of MSCs in experimental models of several neurological diseases.

Several *in vivo* studies have suggested that MSCs can enhance endogenous repair within the CNS: the proliferation and differentiation of endogenous neural stem cells is stimulated when these cells are implanted into the brain after stroke in rat (Chen et al. 2003a). Recently Bai et al. (2012) also demonstrated that hepatocyte grow factor (HGF) secreted by MSCs can induce functional recovery in an animal model of multiple sclerosis (EAE), and promote the development of oligodendrocytes and remyelination (Bai et al. 2012).

Anti-oxidants

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced during normal cellular metabolism. However, disturbances in the normal redox state of cells, an overproduction of ROS and RNS and a failure of antioxidant mechanisms can have toxic effects through the production of free radicals that damage all components of the cell.

Mesenchymal stem cells can exert a protective effect against oxidative stress through the modulation of signaling pathways involved in cellular damage and through the production of antioxidant molecules. These cells are able to protect rodent cerebellar neurons from nitric oxide damage through the secretion of extracellular antioxidant superoxide dismutase 3 (SOD3) (Kemp et al. 2010b). SOD3 is a member of the SOD protein family and is the only enzyme that catalyses the conversion of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) in the extracellular compartment. H_2O_2 can then be detoxified to form water and oxygen by the enzymes catalase and glutathione. Thus SOD3 secretion limits the formation of strong neurotoxic oxidants thereby attenuating tissue damage and inflammation. It has been shown that SOD3 secretion by MSCs is increased by activated microglia and regulated synergistically in response to the inflammatory cytokines IFN-γ and TNF-α, elucidating a potential mechanism that can explain their anti-oxidative actions in the context of inflammation (Kemp et al. 2010a).

The anti-oxidant effect of MSCs has also been demonstrated *in vivo*, in a mouse model of EAE. Administration of MSCs reduced EAE-induced increases in the levels of tissue damage markers resulting from oxidative stress such as PARP1 (a nuclear enzyme functioning as a DNA-damage sensor, activated by DNA strand breaks) and the tumour suppressor protein P53 that triggers apoptosis. Furthermore, levels of antioxidant molecules SOD, catalase and metallothioneins (metal-binding proteins involved in scavenging of free radicals) were increased in those EAE mice treated with MSCs (Lanza et al. 2009).

Extracellular Membrane Vesicles

In addition to soluble factors, recent studies have shown that MSCs-derived membrane vesicles (MVs) may have an important role in the therapeutic effectiveness of these cells.

MVs are spherical structures that form intracellularly. They are released upon fusion with the plasma membrane and internalised in target cells through specific receptor-ligand interactions. MVs are therefore are an important mechanism helping to facilitate cell-to-cell communication.

Membrane vesicles can operate as paracrine or endocrine signalling vesicles, since they can travel to distant tissues to influence several cellular processes. They can be considered as complex vectors that have the potential to hold the wide of biological molecules present in the parental cells. A possible advantage of MVs as vehicles is that the molecular cargo is protected from enzymes present in plasma. In turn, this allows the safe delivery of biologically active molecules such as lipids, proteins, intracellular receptors and genetic material such as mRNA and microRNA (miRNA) (Biancone et al. 2012).

Kim et al. (2012) performed liquid chromatography and mass spectrometry analysis of the MSC-MVs proteome and by doing so identified 730 proteins (Kim et al. 2012). The proteome included surface receptors like platelet-derived growth factor receptor; signalling molecules controlling self-renewal and differentiation; cell adhesion mediators like fibronectin 1 and integrins; and MSC-associated antigens. These MV derived proteins all bear relation to the observed therapeutic effects of MSCs.

The physiological role of MSCs-derived vesicles is still not well understood. Beneficial effects of MSCs-MVs have been reported (Lai et al. 2010), but their use for brain repair is still not well characterized *in-vivo*. Nevertheless, MVs may represent a critical component of the cell-to-cell communication network involved in both physiological and pathological processes.

Angiogenesis

Angiogenesis is another mechanism by which MSCs may promote functional recovery of damaged brain. Angiogenesis is a physiological process by which new blood vessels form from pre-existing vessels. After stroke, enhancement of angiogenesis improves tissue perfusion and promotes neurological recovery. Intravenous

delivery of MSCs into rat models of stroke enhances angiogenesis by increasing endogenous levels of vascular endothelial growth factor (VEGF) and its receptor VEGFR2, essential for endothelial progenitor cell proliferation and differentiation (Chen et al. 2003b).

Immunomodulatory Properties

Mesenchymal stem cells have been shown to affect the behaviour of almost all immune cell populations including T cells, B cells, dendritic cells (DCs), macrophages and natural killer cells (NK) both *in-vitro* and *in vivo*. In-turn, it has been hypothesized that MSCs can ameliorate deficits in neurological disease through significant immunomodulatory effects, on both the innate and adaptive immunity.

Interestingly only small number of systemically infused MSCs infiltrate the brain, but despite little engraftment the infusion of MSCs in animal models of neurological diseases is efficacious. After intravenous injection many cells are trapped as emboli in the lungs, but recent findings suggest that these MSCs are activated in the lung to upregulate the expression of over 50 genes, including the anti-inflammatory factor TNF-α-induced protein 6 (Lee et al. 2009). Therefore, a combination of cell-to-cell contact and secretion of several cytokines and chemokines are likely to play a role in the MSCs immunomodulatory capacity.

Effect on T Cells

Mesenchymal stem cells suppress T-lymphocytes activities both *in-vitro* and *in-vivo*. MSCs have a marked effect on T cells inhibiting their proliferation by blocking the T cells in the G0/G1 phase of the cell cycle (Glennie et al. 2005). A number of mechanisms are implicated in the T-cell quiescence mediated by MSCs, but all appear dependent on cross-talk between the two cell populations. Several studies have proposed the role of various soluble factors and cell-to-cell contact in this immunosuppressive effect that is constitutive but induced by proinflammatory cytokines, such as interferon (IFNγ), tumour necrosis factor (TNFα) and interleukins (IL-1) (Prasanna et al. 2010).

Mesenchymal stem cell-induced T cell anergy has been proposed as a mechanism of immune suppression. MSCs have a non-immunogenic phenotype, with very low levels of MHC class II and no co-stimulatory molecules. After infusion, MSCs migrate to the lymph nodes and might be effective in inducing T cell tolerance due to their lack of co-stimulatory molecules. Inhibition of T cell proliferation by MSCs induces a shift in T cells from a pro-inflammatory state to an anti-inflammatory state, decreasing T-helper 1 secretion of IFN-γ and increasing the T-Helper 2 secretion of IL-4 (Aggarwal and Pittenger 2005).

Increased generation of regulatory T cells (T_{reg}) is another important immunosuppressive property of MSCs. T_{reg} cells are a specialized subpopulation of T cells that suppress activation of the immune system maintaining homeostasis and tolerance to self-antigens. MSCs have been found to induce the production of IL-10 by dendritic cells, which, in turn, triggered the generation of T_{reg} cells (Maccario et al. 2005).

Effect on B Cells

Mesenchymal stem cells can inhibit B cell proliferation *in-vitro* arresting B lymphocytes in the G0/G1 phase of the cell cycle (Corcione et al. 2006). The main function of B cells is the production of immunoglobulin, therefore inhibition of B cells proliferation impairs their activation and secretion of antibodies. As with T cells, cell contact and soluble factors, including IDO, mediate this effect of MSCs on B cells (Krampera et al. 2006).

Effect on Dendritic Cells, Macrophages and Natural Killer Cells

Dendritic cells (DCs) are the most potent antigen-presenting cells in the body, playing a major role in the uptake and presentation of antigens to naive T lymphocytes. During maturation, immature DCs upregulate their expression of MHC molecules and acquire the expression of co-stimulatory markers. MSCs have been shown to alter the cell-surface expression and the cytokine secretion profile of DCs cells, inducing a more anti-inflammatory

phenotype. In fact, in the presence of MSCs, DCs down-regulate both their expression of co-stimulatory molecules and the secretion of TNFα and IL-12, impairing both their antigen presenting function and the clonal expansion of T cells (Ramasamy et al. 2007). Conversely, MSCs increased the DC secretion of the anti-inflammatory interleukin 10 (Aggarwal and Pittenger 2005). IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, TNFα, IL-2 and IL-3 by macrophages, downregulating the expression of T-helper 1 cytokines, inhibiting the action of NK, blocking NF-kB activity (a major transcription factor for both innate and adaptive immune response) and also increasing the generation of T_{reg} cells. Studies have shown that MSCs also switched macrophages to a regulatory phenotype: macrophages co-cultured with MSCs expressed high levels of IL-10, low levels of TNFα and had increased phagocytic activity (Kim et al. 2009). Therefore MSC enhancement of IL-10 production is another important mechanism of immunomodulation.

Another immune cell type influenced by MSCs is the natural killer (NK) cell. NK cells are effectors of innate immunity, providing a rapid response to virally infected cells and tumour formation. NK cells are cytotoxic. Their release of enzymes in close proximity to target cells mediates apoptosis and cell lysis. MSCs can inhibit this cytotoxic activity of NK cells by down regulating the expression of receptors involved through cell-to-cell contact (Spaggiari et al. 2006). NK cells can also proliferate and acquire cytotoxic activity after activation by IL-2 or IL-15. However, MSCs have been found to suppress this NK-cell proliferation and IFN-γ production through the secretion of soluble factors (Krampera et al. 2006).

Clinical Application of Mesenchymal Stem Cell Transplantation

Encouraging therapeutic outcomes using MSC transplantation in animal models of neurodegeneration have now led to their use in the clinical setting for a number of neurological disorders. Initial trials have shown much promise and, in turn, increasing numbers of clinical trials are utilizing their efficacy. Many hurdles still need to be overcome before MSCs are likely to be considered for routine clinical use. However, as will be discussed below, they are indeed drawing attention as a viable therapeutic option for treating a wide range of neurological disorders. It should also be noted, based on the encouraging experimental data obtained in animal models, several clinical trials using MSCs as a treatment in humans (for example in patients with Alzheimer's disease and cerebellar ataxia) are currently registered to be undertaken (ClinicalTrials.gov).

Parkinson's Disease

Parkinson disease is a degenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra and by abnormal accumulation of α-synuclein protein (Lewy bodies) in the brain. To date, only a single clinical trial has reported the use of MSCs for PD. This trial by Venkataramana et al. (2010) published an open-label clinical pilot study of autologous bone marrow–derived MSCs to treat seven patients with advanced PD (Venkataramana et al. 2010). MSCs were transplanted into the sublateral ventricular zone by stereotaxic surgery and patients were followed up for a period that ranged from 10 to 36 months. Results indicated that three of the seven patients showed an improvement in their "off"/"on" Unified PD Rating Scale. Furthermore, authors reported subjective improvements in symptomology and quality of life after treatment with MSCs, in addition to two patients reducing their dosages of PD drugs. Unfortunately, despite the encouraging results found, the number of patients recruited and the open nature of the trial did not provide sufficient data to prove significant effectiveness of the treatment involved. Results did however indicate that transplantation of MSC (at least in the short term) seems to be safe in patients with PD, thus warranting similar (but larger and placebo-controlled) trials to be undertaken.

Multiple Sclerosis

Multiple sclerosis is an inflammatory demyelinating and neurodegenerative disease. The pathologic hallmarks of MS lesions include multifocal inflammation, demyelination, oligodendrocyte loss and axon degeneration. No curative therapies are available for patients, therefore innovative approaches are required and MSCs may provide a potential source of cells for reparative therapies.

In the treatment for MS, several phase I/II studies using MSC transplantation have been performed. The major aim of these studies has been to evaluate the feasibility, safety, and immunological effects of intravenous or intrathecal administration of autologous MSC. A pilot study reported by Mohyeddin Bonab et al. (2007) evaluated the potential therapeutic applications of intrathecally transplanted autologous MSC in ten patients with progressive disease that had not responded to disease modifying agents. During 13–26 months of follow-up, seven patients showed some form of improvement in either clinical score or their sensory, pyramidal, and cerebellar functions. MRI follow-up was also promising as only two patients showed increased disease activity (Mohyeddin Bonab et al. 2007). More recently, the same group increased their sample group to 25 patients. They again presented that MSC therapy can improve/stabilize the course of progressive MS in the first year after injection with no serious adverse effects (Bonab et al. 2012). In another trial, Yamout et al. (2010) again used intrathecally transplanted, culture expanded MSC in ten patients with advanced MS. After 3–6 months follow-up five out of seven patients showed improvements in clinical scores. However MRI revealed that disease activity continued. Authors also reported a major adverse event (transient encephalopathy) occurring few days after cell injection, however the patient did recover (Yamout et al. 2010). In the same year Karussis et al. reported the effects of intrathecal and intravenous administration of autologous MSC in 15 patients with active MS. No severe adverse events were reported. In addition, clinical follow-up showed improvements in expanded disability status scale scores, possible migration of MSCs to the meninges, subarachnoid space, and spinal cord; and also modulation of immune responses (Karussis et al. 2010). More recently Connick et al. (2012) have used intravenous infusion of autologous bone-marrow-derived MSCs in ten patients with secondary progressive MS. Improvements after treatment were observed, with the authors reporting increases in visual acuity, visual evoked response latency and an increase in optic nerve area. Again no severe adverse events were reported (Connick et al. 2012).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis is a neurodegenerative disease that selectively affects motor neurons in the brain, brain stem and spinal cord. The precise cause of ALS is still not known, but evidence suggests that failure of defences against oxidative stress (more than 100 different mutations in SOD1 have been linked with the disease) up-regulates apoptosis and motor neuron degeneration.

Several clinical trials involving either the intra-spinal, intrathecal or intravenous transplantation of MSC for ALS have been conducted. The major objectives of these trials were to assess the feasibility and toxicity of autologous MSC transplantation and to test the impact of a cell therapy on symptomatology. Two phase I trials by Mazzini et al., using autologous MSC delivered intra-spinally, in a total of 19 ALS patients, reported no significant acute or late side effects related to the procedure. Furthermore, at 4 years follow-up four patients showed a significant slowing down of the linear decline of the forced vital capacity and of the ALS-FRS score (Mazzini et al. 2008, 2010). These patients have now been followed-up for 9 years. Unfortunately, at this point, no clear clinical benefits have been detected, however the study did report that no adverse event had been observed (including tumour formation) using clinical, laboratory, and radiographic evaluations (Mazzini et al. 2012).

A separate trial by Karussis et al. (2010), to evaluate the safety and immunological effects of MSCs, when transplanted both intravenously and intrathecally in 19 patients with ALS, has been reported. In this trial no serious adverse effects related to the treatment were observed. Furthermore, clinical benefits have been suggested, with ALS functional rating scale scores remaining stable during 6 months of clinical follow up. Immediate *in vivo* immunomodulating effects of MSCs on both T cells and dentritic cells were also demonstrated (Karussis et al. 2010).

Stroke

Several studies now have investigated clinically the use of MSCs as a treatment for stroke. The first trial was undertaken by Bang et al. (2005) testing the efficacy of intravenously administered autologous MSCs in five patients 5–7 weeks post ischemic event. Neurological outcome measurements in MSC-treated patients at 1 year follow-up were consistent in displaying a trend toward improved scores in tests of functional recovery. Less prominent atrophy was also a consistent finding on serial MRI scans in patients treated with MSCs (Bang et al. 2005). Based on these encouraging results, the same group conducted a larger trail to evaluate the long-term safety and efficacy of intravenous MSCs transplantation in 85 patients with severe middle cerebral artery territory infarct. After a 5 year follow up, no significant side effects were reported and importantly a decreased mortality rate was observed in the MSC treated group (Lee et al. 2010). In another un-blinded study on 12 patients with ischaemic grey matter, white matter and mixed lesions, autologous culture expanded MSCs delivered intravenously 36–133 days post-stroke again proved safe. Some improvements in National Institutes of Health Stroke Scale scores were also seen alongside reductions in lesion volumes post infusion (Honmou et al. 2011). More recently, a succession of trials in chronic stroke patients (the largest recruiting 40 patients), Bhasin et al. (2013) showed the safety of intravenously infused, autologous MSCs (one trial using serum free culture conditions). Clinical outcomes after 6 months infusion in these trials indicated trends towards increased functional recovery using the parameters tested, including the modified Barthel Index (mBI) (Bhasin et al. 2013).

Future Strategies and Conclusion

Although still in its relative infancy, the transplantation of MSCs for the treatment of numerous neurological disorders shows much promise. MSCs appear to be a reliable and safe mode of cell therapy, utilizing a plethora of neuroprotective properties, which make them useful therapeutic tools against the dysregulation of numerous pathological systems. In recent times researchers have moved away from focussing on the MSCs ability to differentiate into neural tissue and focussed on their ability to modulate physiological systems through secretion of bioactive molecules. With this in mind, future strategies may take the extra step in engineering MSCs to express high levels of specific growth factors or anti-oxidant molecules with the engineered transgene product being tailored specifically for a given neurological disorder. In a similar fashion, it may be possible use MSCs to act as vehicles to donate nuclear material or organelles to injured and/or degenerating cells. We already know that bone marrow stem cells can fuse with neuronal cells and have the ability to protect tissues through transferring molecules or organelles to vulnerable cells. It is likely the transfer of such cellular components by MSCs could be exploited therapeutically and therefore key to cellular repair in a number of neurodegenerative conditions.

Regardless of the strategy used, it is expected over the next decade MSC-based therapies will continue to develop and flourish. With increasing experience in their clinical use and optimization in their isolation, culture and administration, it is clear that MSCs hold great promise as a cellular therapy for a number of neurological disorders.

References

Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105:1815–1822

Bae JS, Han HS, Youn DH, Carter JE, Modo M, Schuchman EH, Jin HK (2007) Bone marrow-derived mesenchymal stem cells promote neuronal networks with functional synaptic transmission after transplantation into mice with neurodegeneration. Stem Cells 25:1307–1316

Bai L, Lennon DP, Caplan AI, DeChant A, Hecker J, Kranso J, Zaremba A, Miller RH (2012) Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. Nat Neurosci 15:862–870

Bang OY, Lee JS, Lee PH, Lee G (2005) Autologous mesenchymal stem cell transplantation in stroke patients. Ann Neurol 57:874–882

Bhasin A, Srivastava MVP, Mohanty S, Bhatia R, Kumaran SS, Bose S (2013) Stem cell therapy: a clinical trial of stroke. Clin Neurol Neurosurg 115:1003–1008

Biancone L, Bruno S, Deregibus MC, Tetta C, Camussi G (2012) Therapeutic potential of mesenchymal stem cell-derived microvesicles. Nephrol Dial Transplant 27:3037–3042

Bonab MM, Sahraian MA, Aghsaie A, Karvigh SA, Hosseinian SM, Nikbin B, Lotfi J, Khorramnia S, Motamed MR, Togha M, Harirchian MH, Moghadam NB, Alikhani K, Yadegari S, Jafarian S, Gheini MR (2012) Autologous mesenchymal stem cell therapy in progressive multiple sclerosis: an open label study. Curr Stem Cell Res Ther 7:407–414

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650

Chen J, Li Y, Katakowski M, Chen X, Wang L, Lu D, Lu M, Gautam SC, Chopp M (2003a) Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. J Neurosci Res 73:778–786

Chen J, Zhang ZG, Li Y, Wang L, Xu YX, Gautam SC, Lu M, Zhu Z, Chopp M (2003b) Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. Circ Res 92:692–699

Connick P, Kolappan M, Crawley C, Webber DJ, Patani R, Michell AW, Du MQ, Luan SL, Altmann DR, Thompson AJ, Compston A, Scott MA, Miller DH, Chandran S (2012) Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of-concept study. Lancet Neurol 11:150–156

Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V, Uccelli A (2006) Human mesenchymal stem cells modulate B-cell functions. Blood 107:367–372

Crigler L, Robey RC, Asawachaicharn A, Gaupp D, Phinney DG (2006) Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. Exp Neurol 198:54–64

Crisostomo PR, Wang M, Wairiuko GM, Morrell ED, Terrell AM, Seshadri P, Nam UH, Meldrum DR (2006) High passage number of stem cells adversely affects stem cell activation and myocardial protection. Shock 26:575–580

Dai LJ, Moniri MR, Zeng ZR, Zhou JX, Rayat J, Warnock GL (2011) Potential implications of mesenchymal stem cells in cancer therapy. Cancer Lett 305:8–20

Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 101:2999–3001

Dey R, Kemp K, Gray E, Rice C, Scolding N, Wilkins A (2012) Human mesenchymal stem cells increase antioxidant defences in cells derived from patients with Friedreich's ataxia. Cerebellum 11:861–871

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317

Ferguson B, Matyszak MK, Esiri MM, Perry VH (1997) Axonal damage in acute multiple sclerosis lesions. Brain 120(Pt 3):393–399

Fischer MT, Sharma R, Lim JL, Haider L, Frischer JM, Drexhage J, Mahad D, Bradl M, van Horssen J, Lassmann H (2012) NADPH oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury. Brain 135:886–899

Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA (1974) Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 2:83–92

Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F (2005) Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 105:2821–2827

Gray E, Rice C, Nightingale H, Ginty M, Hares K, Kemp K, Cohen N, Love S, Scolding N, Wilkins A (2013) Accumulation of cortical hyperphosphorylated neurofilaments as a marker of neurodegeneration in multiple sclerosis. Mult Scler 19:153–161

Grigoriadis N, Lourbopoulos A, Lagoudaki R, Frischer JM, Polyzoidou E, Touloumi O, Simeonidou C, Deretzi G, Kountouras J, Spandou E, Kotta K, Karkavelas G, Tascos N, Lassmann H (2011) Variable behavior and complications of autologous bone marrow mesenchymal stem cells transplanted in experimental autoimmune encephalomyelitis. Exp Neurol 230(1):78–89

Haider L, Fischer MT, Frischer JM, Bauer J, Hoftberger R, Botond G, Esterbauer H, Binder CJ, Witztum JL, Lassmann H (2011) Oxidative damage in multiple sclerosis lesions. Brain 134:1914–1924

Hill KE, Zollinger LV, Watt HE, Carlson NG, Rose JW (2004) Inducible nitric oxide synthase in chronic active multiple sclerosis plaques: distribution, cellular expression and association with myelin damage. J Neuroimmunol 151:171–179

Honmou O, Houkin K, Matsunaga T, Niitsu Y, Ishiai S, Onodera R, Waxman SG, Kocsis JD (2011) Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. Brain 134:1790–1807

Johansson CB, Youssef S, Koleckar K, Holbrook C, Doyonnas R, Corbel SY, Steinman L, Rossi FM, Blau HM (2008) Extensive fusion of haematopoietic cells with Purkinje neurons in response to chronic inflammation. Nat Cell Biol 10:575–583

Kapoor R, Davies M, Blaker PA, Hall SM, Smith KJ (2003) Blockers of sodium and calcium entry protect axons from nitric oxide-mediated degeneration. Ann Neurol 53:174–180

Karussis D, Karageorgiou C, Vaknin-Dembinsky A, Gowda-Kurkalli B, Gomori JM, Kassis I, Bulte JW, Petrou P, Ben-Hur T, Abramsky O, Slavin S (2010) Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. Arch Neurol 67:1187–1194

Kemp K, Gray E, Mallam E, Scolding N, Wilkins A (2010a) Inflammatory cytokine induced regulation of superoxide dismutase 3 expression by human mesenchymal stem cells. Stem Cell Rev 6:548–559

Kemp K, Hares K, Mallam E, Heesom KJ, Scolding N, Wilkins A (2010b) Mesenchymal stem cell-secreted superoxide dismutase promotes cerebellar neuronal survival. J Neurochem 114:1569–1580

Kemp K, Gordon D, Wraith DC, Mallam E, Hartfield E, Uney J, Wilkins A, Scolding N (2011) Fusion between human mesenchymal stem cells and rodent cerebellar Purkinje cells. Neuropathol Appl Neurobiol 37:166–178

Kemp K, Gray E, Wilkins A, Scolding N (2012) Purkinje cell fusion and binucleate heterokaryon formation in multiple sclerosis cerebellum. Brain 135:2962–2972

Kim BJ, Seo JH, Bubien JK, Oh YS (2002) Differentiation of adult bone marrow stem cells into neuroprogenitor cells in vitro. Neuroreport 13:1185–1188

Kim YJ, Park HJ, Lee G, Bang OY, Ahn YH, Joe E, Kim HO, Lee PH (2009) Neuroprotective effects of human mesenchymal stem cells on dopaminergic neurons through anti-inflammatory action. Glia 57:13–23

Kim HS, Choi DY, Yun SJ, Choi SM, Kang JW, Jung JW, Hwang D, Kim KP, Kim DW (2012) Proteomic analysis of microvesicles derived from human mesenchymal stem cells. J Proteome Res 11:839–849

Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F (2006) Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 24:386–398

Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK (2010) Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 4:214–222

Lanza C, Morando S, Voci A, Canesi L, Principato MC, Serpero LD, Mancardi G, Uccelli A, Vergani L (2009) Neuroprotective mesenchymal stem cells are endowed with a potent antioxidant effect in vivo. J Neurochem 110:1674–1684

Lee RH, Pulin AA, Seo MJ, Kota DJ, Ylostalo J, Larson BL, Semprun-Prieto L, Delafontaine P, Prockop DJ (2009) Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell 5:54–63

Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY (2010) A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. Stem Cells 28:1099–1106

Lu P, Blesch A, Tuszynski MH (2004) Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J Neurosci Res 77:174–191

Maccario R, Podesta M, Moretta A, Cometa A, Comoli P, Montagna D, Daudt L, Ibatici A, Piaggio G, Pozzi S, Frassoni F, Locatelli F (2005) Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica 90:516–525

Mahad D, Ziabreva I, Lassmann H, Turnbull D (2008) Mitochondrial defects in acute multiple sclerosis lesions. Brain 131:1722–1735

Mallam E, Kemp K, Wilkins A, Rice C, Scolding N (2010) Characterization of in vitro expanded bone marrow-derived mesenchymal stem cells from patients with multiple sclerosis. Mult Scler 16:909–918

Mazzini L, Mareschi K, Ferrero I, Vassallo E, Oliveri G, Nasuelli N, Oggioni GD, Testa L, Fagioli F (2008) Stem cell treatment in amyotrophic lateral sclerosis. J Neurol Sci 265:78–83

Mazzini L, Ferrero I, Luparello V, Rustichelli D, Gunetti M, Mareschi K, Testa L, Stecco A, Tarletti R, Miglioretti M, Fava E, Nasuelli N, Cisari C, Massara M, Vercelli R, Oggioni GD, Carriero A, Cantello R, Monaco F, Fagioli F (2010) Mesenchymal stem cell transplantation in amyotrophic lateral sclerosis: a phase I clinical trial. Exp Neurol 223:229–237

Mazzini L, Mareschi K, Ferrero I, Miglioretti M, Stecco A, Servo S, Carriero A, Monaco F, Fagioli F (2012) Mesenchymal stromal cell transplantation in amyotrophic lateral sclerosis: a long-term safety study. Cytotherapy 14:56–60

Mohyeddin Bonab M, Yazdanbakhsh S, Lotfi J, Alimoghaddom K, Talebian F, Hooshmand F,

Ghavamzadeh A, Nikbin B (2007) Does mesenchymal stem cell therapy help multiple sclerosis patients? Report of a pilot study. Iran J Immunol 4:50–57

Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I (2004) Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J Neurosci Res 77:192–204

Owen M, Friedenstein AJ (1988) Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 136:42–60

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Pochampally RR, Smith JR, Ylostalo J, Prockop DJ (2004) Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. Blood 103:1647–1652

Prasanna SJ, Gopalakrishnan D, Shankar SR, Vasandan AB (2010) Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. PLoS One 5, e9016

Prockop DJ (2009) Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. Mol Ther 17:939–946

Ramasamy R, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F (2007) Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. Transplantation 83:71–76

Reid E, Kloos M, Ashley-Koch A, Hughes L, Bevan S, Svenson IK, Graham FL, Gaskell PC, Dearlove A, Pericak-Vance MA, Rubinsztein DC, Marchuk DA (2002) A kinesin heavy chain (KIF5A) mutation in hereditary spastic paraplegia (SPG10). Am J Hum Genet 71:1189–1194

Rice CM, Scolding NJ (2010) Adult human mesenchymal cells proliferate and migrate in response to chemokines expressed in demyelination. Cell Adh Migr 4:235–240

Sohni A, Verfaillie CM (2013) Mesenchymal stem cells migration homing and tracking. Stem Cells Int 2013:130763

Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L (2006) Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood 107:1484–1490

Tan J, Wu W, Xu X, Liao L, Zheng F, Messinger S, Sun X, Chen J, Yang S, Cai J, Gao X, Pileggi A, Ricordi C (2012) Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. JAMA 307:1169–1177

Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis Mortari A, McElmurry RT, Bell S, Xia L, Zhou N, Riddle M, Schroeder TM, Westendorf JJ, McIvor RS, Hogendoorn PC, Szuhai K, Oseth L, Hirsch B, Yant SR, Kay MA, Peister A, Prockop DJ, Fibbe WE, Blazar BR (2007) Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 25:371–379

Uccelli A (2013) Mesenchymal stem cells exert a remarkable regenerative effect requiring minimal CNS integration: commentary on: "Mesenchymal stem cells protect CNS neurons against glutamate excitotoxicity by inhibiting glutamate receptor expression and function" by Voulgari-Kokota et al. Exp Neurol 247:292–295

Venkataramana NK, Kumar SK, Balaraju S, Radhakrishnan RC, Bansal A, Dixit A, Rao DK, Das M, Jan M, Gupta PK, Totey SM (2010) Open-labeled study of unilateral autologous bone-marrow-derived mesenchymal stem cell transplantation in Parkinson's disease. Transl Res 155:62–70

Wang JZ, Xia YY, Grundke-Iqbal I, Iqbal K (2013) Abnormal hyperphosphorylation of tau: sites, regulation, and molecular mechanism of neurofibrillary degeneration. J Alzheimers Dis 33(Suppl 1):S123–S139

Weimann JM, Charlton CA, Brazelton TR, Hackman RC, Blau HM (2003) Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. Proc Natl Acad Sci U S A 100:2088–2093

Wilkins A, Kemp K, Ginty M, Hares K, Mallam E, Scolding N (2009) Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. Stem Cell Res 3:63–70

Yamout B, Hourani R, Salti H, Barada W, El-Hajj T, Al-Kutoubi A, Herlopian A, Baz EK, Mahfouz R, Khalil-Hamdan R, Kreidieh NMA, El-Sabban M, Bazarbachi A (2010) Bone marrow mesenchymal stem cell transplantation in patients with multiple sclerosis: a pilot study. J Neuroimmunol 227:185–189

Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC (2002) Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol 174:11–20

HIV Infection and Adipose Tissue Resident Stem Cells: Their Involvement in Pathology and Treatment

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Upal Roy and Ben Berkhout

Contents

U. Roy (✉)
Department of Immunology, Herbert Wertheim College of Medicine, Florida International University, 11200 SW 8th St. University Park, Miami, FL 33199, USA
e-mail: uroy@fiu.edu

B. Berkhout
Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam, Meibergdreef 15, K3-110, 1105 AZ, Amsterdam, The Netherlands
e-mail: b.berkhout@amc.uva.nl

Abstract

The HIV reservoir creates a true challenge for eradicating the virus from infected patients. Current Highly Active Antiretroviral Therapy (HAART) is very effective in controlling active viral replication in the periphery, although the drugs may not penetrate efficiently in all cellular and anatomical reservoirs. In these reservoirs, the already established HIV proviruses are stably integrated into the host cell genome and insensitive to antiviral therapy. The anatomical HIV reservoirs in the brain, lymph nodes and other compartments have been well described, but many questions remain on the actual cell types that constitute this reservoir. Recent advances in basic and clinical research have provided a better understanding of Adipose Tissue Resident Stem Cells (ASC) as possible HIV reservoir. Although ASC do not support active viral replication, the cells differentiating from ASC are susceptible to viral infection. A number of approaches have been proposed to characterize the virus from ASC and other cellular reservoirs. A detailed characterization of ASC and its association with HIV may elucidate new cellular targets for therapeutic intervention. Moreover, the current HAART treatment also affects ASC cell growth and division in adipose tissue. Laboratory and animal studies have shown a strong correlation between HAART and lypodystrophy in HIV infected patients treated with

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antiretroviral drugs. The current review describes disease progression during HIV infection and antiretroviral treatment, with a particular focus on the possible role of ASC as viral reservoir. These observations may suggest future treatment options to obtain better control of this chronic infectious disease.

Adipose Tissue and Immune System

Human Adipose Tissue Resident Stem Cell (ASC) Localization, Phenotype and Characterization

Adipose tissue is specialized connective tissue that functions as the major storage site for fat in the form of triglycerides. In addition to this role as major energy storage depot, adipose tissue is an endocrine organ that is involved in synthesizing and secreting cytokines, chemokines, and hormones such as adiponectin and resistin. These regulatory proteins and hormones are involved in numerous physiological functions including inflammation, immunity and metabolism. Adipose tissue is composed of many different cell types including adipocytes, pericytes, monocytes, macrophages, cells of endothelium (endothelial and vascular smooth muscle cells), and mesenchymal stem cells (MSC). Adipocytes are the most prominent cell types in this tissue and are differentiated from adipocyte progenitor cells (Maumus et al. 2011). MSC possess adipogenic, osteogenic, chondrogenic, myogenic and neurogenic potential. Adipose tissue represents an abundant and accessible source of adult stem cells that can differentiate along multiple lineages. Within the adipose tissue, the generation of adipocytes is through a sequential pathway of differentiation under the guidance of adipogenic micro-environmental factors, which include metabolites like glucose or lipids and other signaling molecules (Laharrague and Casteilla 2010). ASC are mainly $CD90^+$ and $CD105^+$ cells lacking the markers of hematopoietic (CD14, CD45) and endothelial (CD31) cells (Maumus et al. 2011).

Adipose Tissue and the Immune Response

Adipocytes, the dominant cell types within adipose tissue, participate in the regulation of proinflammatory cytokines and the generation of hematopoietic lineage specific cells. Recently, it has been shown that *in vitro* expanded ASC are capable of generating functional macrophages indicating the potential for hematopoietic lineage differentiation (Freisinger et al. 2010). Adipose tissue also contains cells referred to as the stromal vascular fraction (SVF). Several populations of cells within the SVF of adipose tissue contain hematopoietic markers. Considering the hematopoietic potential of ASC, it has been concluded that adipose tissue plays a role in the immune response. The mechanisms by which adipose tissue contributes to the immune response may be (**I**) through direct effects of resident immune cells within adipose tissues (**II**) through indirect effects whereby adipocytes modulate immune cell function in an endocrine or paracrine fashion or (**III**) through generation of hematopoietic cells from ASC. Adipose tissue most likely contributes to the immune response through all these mechanisms, but more studies are required to determine their relative importance. Cellular components of adipose tissue are functionally active and exert potent effects on adipocyte metabolism and endocrine function. Macrophages accumulate in adipose tissue during inflammation, which correlates with increased expression of cytokines and chemokines including tumor necrosis factor alpha (TNF-α), interleukine-1β (IL-1β), IL-6, IL-8, monocyte chemoattractant protein-1 (MCP1), and IL-18 (Weisberg et al. 2003; Whang et al. 1998; Whigham et al. 2007). The increased expression of cytokines has been correlated with enhanced hematopoietic differentiation of ASCs, decreased insulin sensitivity, increased lipolysis and increased leptin production (Trujillo et al. 2006). It has also been shown that adipocytes are highly responsive to endotoxins released from bacterial infections and that they produce high levels of proinflammatory cytokines (Lin et al. 2000).

Adipose Tissue and HIV Infection

HIV infection causes numerous metabolic abnormalities including dyslipidemia, insulin resistance, fat loss, lipodystrophy, lipoatrophy, and fat accumulation. The idea that adipocytes may play a role in HIV infection was suggested because of the significant changes in adipose tissue morphology and metabolism in HIV-infected individuals. The HIV infection lipodystrophy syndrome is particularly prevalent in patients on antiretroviral therapy and is also associated with other metabolic complications, including insulin resistance, dyslipidemia, cholesterol and fat redistribution. Within the adipose tissue all immune cells can serve as primary targets for HIV infection. Infection of lymphoid and myeloid lineages is mediated by recognition of CD4 and the chemokine co-receptor CXCR4 or CCR5 (Moore et al. 2004). These receptors promote viral attachment and fusion to cellular membranes, thus facilitating entry into the cell (Zaitseva et al. 2003). It has been shown that the receptors for HIV entry, CD4, CXCR4 and CCR5, are expressed on preadipocytes and adipocytes (Hazan et al. 2002). However, *in vitro* infection of adipose tissue with the virus was not successful as these receptors on ASCs did not support cellular entry of the virus (Munier et al. 2003). HIV exposure to hematopoietic cells may cause changes in the tissue microenvironment, which may alter the differentiation process of ASC. As mentioned earlier, macrophages are one of the main targets for HIV infection. Macrophages also play an important role in viral latency and the recurrence of infection upon stopping of therapy. Furthermore, progenitor cells differentiating towards macrophages have been documented to be susceptible to HIV infection (Duncan and Sattentau 2011).

Numerous research efforts have focused on whether ASC serve as HIV reservoir. Nazari-Shafti et al. 2011 measured significant expression of certain markers in hematopoietic differentiated (HD) cells derived from ASC. In the initial assessment, it was observed that HD cells express the HIV receptors CD4, CXCR4 and CCR5, unlike undifferentiated ASC (Fig. 2.1a). HD cells also express certain genes that have been implicated in regulating HIV infection, which includes IL-8, SERPINA1, CCL8, CD69 and the interleukins 2, 10 and 16 (Fig. 2.1b). However, this study did not address whether ASC could harbor latent HIV-1 proviruses and serve as reservoir. Munier et al. (2003) investigated the biopsies from patients for the level of expression of the HIV entry receptors (CD4, CXCR4 and CCR5) on ASC. Expression of CD4 and CCR5 was not detected, and CXCR4 expression was variable on those biopsy samples. On the other hand, early research indicated that bone marrow derived CD34+ progenitor cells from HIV infected patients are infected (Folks et al. 1988). More recently, HIV infection and killing of hematopoietic progenitor cells (HPC) has been demonstrated both *in vitro* and *in vivo* (Carter et al. 2010). The possible reason could be that HIV can affect HPC and induce cell death by affecting their hematopoietic potential (Iglesias-Ussel and Romerio 2011). Overall, several investigations suggested that HPC and ASC could contribute to the HIV reservoir (Lafeuillade and Stevenson 2011). It would be interesting to study whether CD34 cells produce any viral proteins in case of HAART treated HIV patients with viral levels staying below the detection limit. *In vivo* studies have thus far not provided evidence of ASC as viral reservoir, but further characterization of ASC in HIV infected patients may help to elucidate the contribution of these cells to the total viral reservoir.

Effect of HIV on ASC

HIV-1 predominantly infects hematopoietic cell types such as helper T lymphocytes, monocytes and macrophages. Infection of lymphoid and myeloid cells is mediated by the receptor CD4 (Nazari-Shafti et al. 2011). Although adipocytes also express CD4 that may facilitate viral entry, no evidence of viral replication in human adipocytes has been reported *in vitro* (Sankale et al. 2006). Hazan et al. (2002) demonstrated the

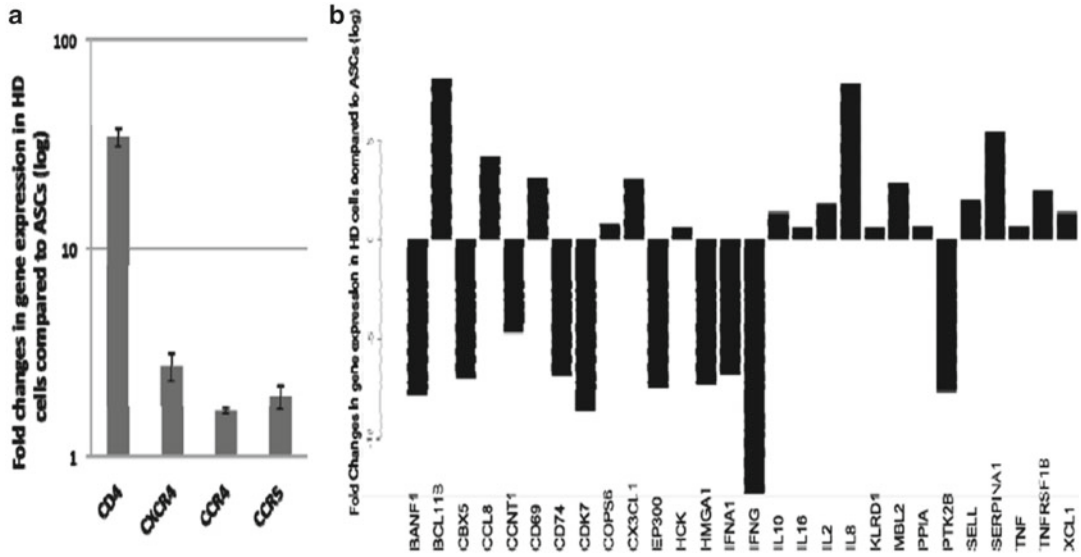

Fig. 2.1 Gene expression analysis of HD cells following hematopoietic differentiation. (**a**) Fold change expression of HIV receptor gene HD cells following differentiation compared to ASCs. (**b**) Expression of several genes involved in innate and adaptive immune reaction. Relative expression of cell cycle regulator genes in HD cells compare to ASCs. The fold change was used to select genes ($p<0.05$). All values are normalized to ASCs (Adapted from Nazari-Shafti et al. (2011) and permission obtained from Biomed Central)

presence of the CXCR4 and CCR5 co-receptors on human adipocytes, supporting the possibility of viral entry into these cells and further evidence for this was obtained by PCR methods. Along the same line, Nazari-Shafti et al. (2011) observed that HIV exposure can significantly alter the expression of cell cycle and apoptosis regulatory genes in HD cells. Immunocytochemical analysis indicated profound expression of CCR4, CCR5, NOS2 and CXCR4 protein on HIV-exposed HD cells, although CD4 expression was undetectable (Fig. 2.2). Additionally, HIV may facilitate the macrophage type commitment of ASC, which may also support productive viral infection in differentiated cells. During differentiation of ASC into certain stromal cell lineages, the cells get more susceptible to viral infection (Nazari-Shafti et al. 2011). The bone marrow (BM) stroma is a major component of the microenvironment that regulates the hematopoietic activity. Stroma is a heterogeneous mixture of cells including fibroblast, macrophages, endothelial cells, adipocytes and other cell types. HIV infection of some of these cell types may thus directly influence the hematopoietic cell microenvironment. Primary human stroma appears to be susceptible to *in vitro* infection with the HIV-1_{ADA} strain (Cheng et al. 2013). The causal relationship between infected stroma cells and the loss of hematopoietic cells is still unresolved. There are at least two possible causes of the reduction of stem cell numbers in HIV infected patients. Either there is inhibition of a cellular factor that stimulates hematopoiesis or there is induction of cytokines that inhibit the hematopoiesis process (Bahner et al. 1997).

It is noteworthy that HIV-1 is able to induce transforming growth factor beta (TGF-β) expression in other cell types like macrophages and hematopoietic stem cells (HSC). TGF-β is a pleiotropic cytokine that negatively regulates hematopoiesis and induces apoptosis. A recently characterized member of the TNF family, known as proliferation-inducing ligand (APRIL), positively regulates the proliferation of megakaryocytes (MK) during differentiation. In fact, TGF-β and APRIL work together in regulating hematopoiesis and MK cell replication. In an

HIV infected environment, the HIV gp120 Envelope protein interacts with the CD4 receptor to down regulate APRIL and TGF-β. In this regard, the HIV gp120 protein potently down regulates the differentiation process towards MK cells (Gibellini et al. 2007). Fc epsilon Receptor 1 (Fcr R1) that is present on hematopoietic cells induces the synthesis and release of IL4 in HIV infected tissue. The virus sheds HIV gp120 molecules that bind to IgE/FceR1 complexes on hematopoietic cells to induce IL-4 secretion. This event may contribute to the initiation of the gradual immune deficiency in patients (Becker 2004). The HIV-1 regulatory protein Tat has been suggested to play a role in AIDS pathogenesis by interaction with CD34 progenitor cells (Gibellini et al. 2003). Tat is an early transcriptional trans-activator protein that is released from HIV infected cells and readily taken up by uninfected cells. Tat has the potential to induce a large number of host cellular genes and to initiate various signal transduction pathways. CXCR4 is a member of the transmembrane G protein family that is present on many cells including CD34 haematopoietic cells and CXCR4 has a high affinity for the chemokine stromal cell derived factor-1 alpha (SDF-1α). A possible scenario for cell apoptosis is thus Tat-induced CXCR4 expression and subsequent induction of SDF-1α that may contribute to the gradual loss of stem cells in HIV infected patients. It was confirmed that $CD34^+$ cells from patients with HIV infection are committed to apoptosis (Gibellini et al. 2003). In general, HAART treatment improves the CD34 cell viability and function in HIV infected patients, although the underlying mechanism is not yet clear. The HIV-1 Protease Inhibitors (PIs) atazanavir (ATV) and lopinavir (LPV), frequently used in HAART regimens, can reduce the resistance of $CD34^+$ cells to an apoptotic stimulus even in healthy adults. RTV has no effect on $CD34^+$ cell apoptosis when used in combination with ATV or LPV. The combined data suggested that certain PI drugs and the HIV gp120 protein may increase the apoptotic susceptibility of $CD34^+$ hematopoietic progenitor cells (MacEneaney et al. 2011).

Effect of Antiretroviral Therapy on ASC

HIV infected patients, particularly those on HAART, are frequently characterized by adipose dysregulation, dyslipidemia and insulin resistance, which are the hallmarks of HIV related lipodystrophy. Lipodystropy is often regarded as toxicity attributed to various antiretroviral drugs used in HAART therapy. The specific mechanisms are not yet known, but it was observed that viral exposure dramatically increases the secretion of adiponectin from human adipocytes, even without an active infection of these cell types (Sevastianova et al. 2008). HIV significantly affects adiponectin endocrine regulation that cannot be physiologically sustained even though the viral loads are down due to HAART (Sankale et al. 2006). It has also been demonstrated that HIV infected lipodystrophy patients show 40 % reduction in plasma adiponectin levels compare to patients without lipodystrophy (Vernochet et al. 2005). The symptoms develop with the increased use of antiviral PI drugs. Nonetheless, recent clinical trials indicated that lipidostrophy is observed in PI-naïve patients and patients treated with nucleoside reverse transcriptase inhibitors (NRTIs). Currently, five PIs are approved for AIDS therapy: amprenavir (APV), indinavir (IDV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV). These antiviral drugs significantly reduce the viral load, but also interfere with adipocyte and/or fat cell differentiation. That in turn affects the adipose tissue and its body distribution, resulting in changes in lipid metabolism or adipogenesis.

Adipogenesis is mainly controlled by two receptors, peroxisome proliferator activated receptor gamma (PPAR-ɣ) and retinoid X receptor alpha (RXR-α), which form a heterodimer and affect cellular gene expression. It has been observed that SQV, NFV and RTV alter the fat metabolism in murine mesenchymal stem cells (C3H10T1/2). These PIs inhibit the conversion of stem cells to adipocytes. Interestingly, other than SQV, none of the PIs bound to PPAR-ɣ. On the other hand, APV and IDV have very little effect on adipogenesis. Recent data have shown

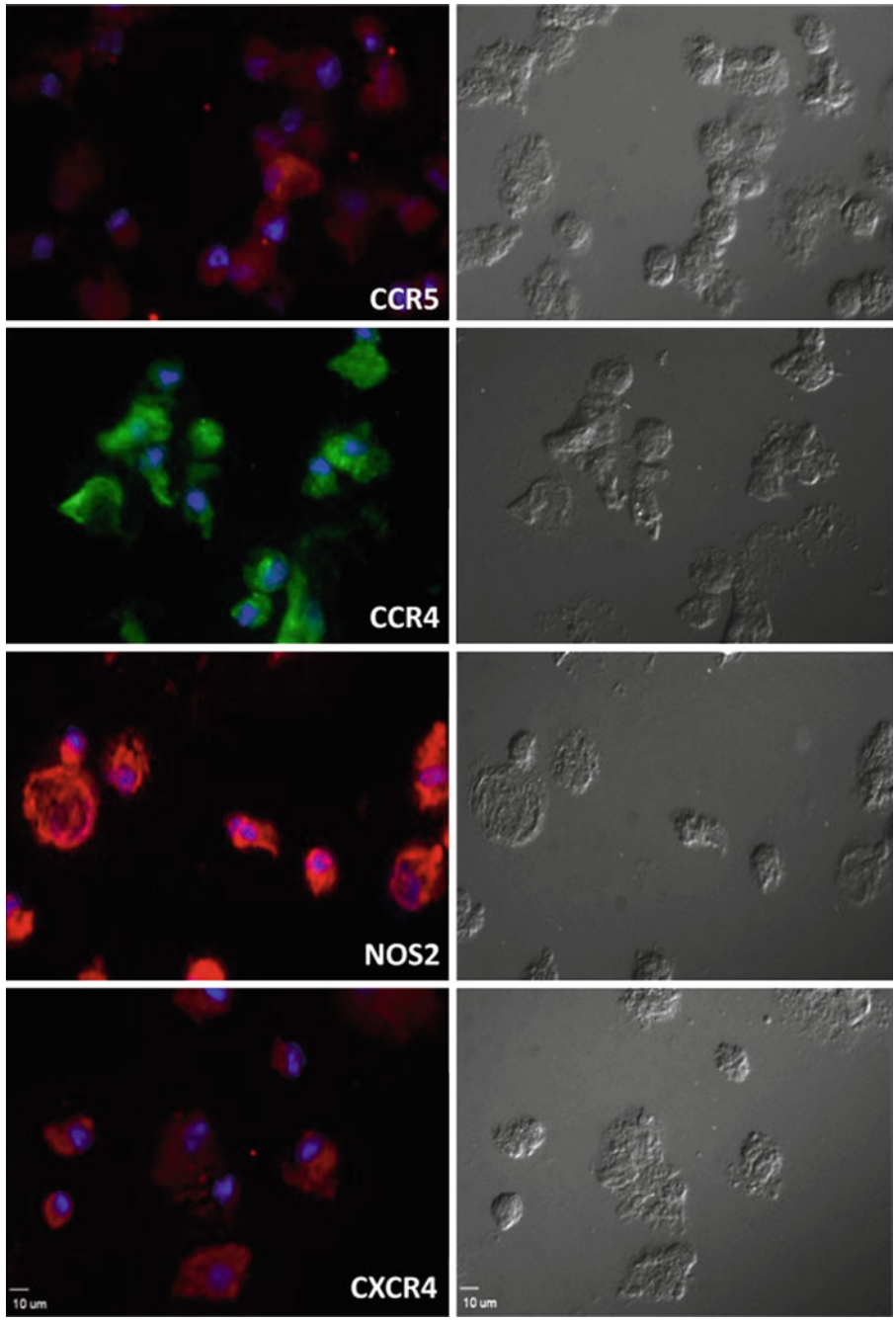

Fig. 2.2 Expression of hematopoietic markers in HD cells following HIV infection. *Left panel*: immunohistochemistry of HIV-exposed HD cells indicating the expression of CCR5, CCR4, NOS2 and CXCR4. *Right panel*: Differential Interference Contrast (DIC) images of the identical fields. Images were obtained with the Leica TCS SP-2 confocal microscope. Scale bar 10 μm (Adapted from Nazari-Shafti et al. (2011) and permission obtained from Biomed Central)

that 50 μM IDV and 10 μM SQV inhibit the differentiation of primary human adipocytes (Lenhard et al. 2000). IDV also impairs differentiation at an early stage of adipose conversion, probably involving the process that controls the intracellular localization of sterol regulatory element binding protein-1 (SREBP-1). SREBP-1 is a well-known regulator of multiple genes that are involved in the metabolism of cholesterol, triglycerides and fatty acids, particularly in the liver. SREBP-1 also controls the expression of PPAR-ɣ, which is an important adipocyte differentiation factor. In fact, SREBP-1 mediates the effect of insulin by increasing the expression of insulin target genes such as PPAR-ɣ. The matrix metalloproteinase protein (MMP) family plays a major role in the adipocyte differentiation process. Bouloumié et al. (2001) have shown that human adipocytes and preadipocytes produce and release two members of the MMP family, MMP-2 and −9. The use of MMP inhibitors can decrease the rate of adipocyte differentiation in rats, suggesting that MMP activities are required for adipogenesis in rodents. Furthermore, the broad-spectrum MMP inhibitor Batimastat strongly inhibits human adipocyte differentiation. PIs like IDV, RTV, SQV and NFV also lead to a strong reduction of the human adipocytes differentiation process by a mechanism that involves MMP-9 inhibition. The decrease in MMP-9 secretion might be linked to the reduced MMP-9 gene expression observed in pre-adipocytes following a PI-containing treatment regime. Interestingly, the effect of most of the PIs (IDV, NFV, SQV, and RTV) on the differentiation process was similar. The mechanism by which the PIs affect MMP-9 expression in human preadipocytes may involve the degradation of I-kappaB (IKb) molecule that sequesters the NFkB transcription factor. While reducing MMP secretion, PIs may also alter the proteolytic cleavage of several circulating, cell surface and pericellular proteins, which regulate cell behaviors in numerous way. The extracellular level and activity of adipocytokines (IL-8, IL-1b, IL-6) and transforming growth factor-β (TGF-β) may be regulated by MMPs, which can affect adipocyte differentiation (Bourlier et al. 2005).

In clinical practice, it was observed that long-term PI usage may cause a loss of fat from the face and limbs, but an increase of fat at the abdomen and the back of the neck (Caron et al. 2001). The molecular mechanism behind this process may include the inhibition of one of the main receptors, PPAR-ɣ or RXRalpha (Zhang et al. 1999). PI and non-nucleoside reverse transcriptase inhibitors (NNRTI) were detected in the adipose tissue of patients, but a direct effect of these drugs on the metabolism of adipocytes has not been established. Adipose tissue was also described to play a critical role in insulin resistance through the expression of tumor necrosis factor-α (TNF-α), IL-6 and adiponectin (Vernochet et al. 2005). Increased accumulation of macrophages in subcutaneous adipose tissue was observed by CD68 cell staining. These tissue macrophages are involved in several immune functions, including phagocytosis of cellular debris and triggering of immune response via cytokine release.

Increased expression of macrophage markers and inflammatory cytokines in the liver of HIV infected lipodystrophic patients is well documented (Sevastianova et al. 2008). In adipocytes, RTV was able to up-regulate TNF-alpha, IL-6 and adiponectin. As a result of up-regulation of IL6 and TNF-α, insulin resistance occurs in lipodystrophic patients on HAART (Vernochet et al. 2005). Some HIV drugs could inhibit the differentiation of precursor cells isolated from human adipose tissue. Concerning the lipodystrophy associated with the use of PIs, alternative therapeutic regimens based on NNRTIs efavirenz (EFV) or nevirapine (NFV) have been proposed. However, recent studies indicated that EFV can also accumulate in adipose tissue (Dupin et al. 2002), although the intracellular accumulation is low compared to PIs. Of note, NFV does not interfere with lipid accumulation during adipocyte development in human ASCs (Vernochet et al. 2005).

Present Clinical Scenarios

The introduction of antiretroviral therapy (ART) has changed the morbidity and mortality of HIV disease. In the era of successful HAART, HIV-infected individuals have a near normal life expectancy, with relatively minor medical complications. However, the effective drug treatment does not eradicate the virus from HIV reservoir organs in the body like the central nervous system (CNS), lymph nodes, testis etc. (Pippi 2008). This is due to integration of the HIV proviral genome into the host cell DNA genome in long-lived cellular reservoir like resting T cells.

The concept of HSC based gene therapy for HIV is gaining popularity in pre-clinical cure research because of its potential to address the difficult issue of viral reservoirs and possible virus eradication (Deeks and McCune 2010). Initial attempts of engineering HIV-resistant haematopoietic progenitor cells (HPS) faced many hurdles because of general toxicity of the gene therapy *in vivo*, which includes immune suppression and induction of leukemia (Tamhane and Akkina 2008). Over the past two decades, investigators have focused mostly on bone marrow and adipose derived MSC to optimize the efficacy and safety of stem cell based gene therapy. This includes challenges of development of *in vitro* protocols for clinical grade cell preparation and *in vivo* studies to probe the long-term adverse effects. These multiple lines of research have generated a wealth of basic and clinical research data documenting the potential of MSC therapy, bringing the gap from bench to bed side (Gimble et al. 2010). Two recent developments in this field are worth mentioning. The major cell surface receptor (CCR5) for HIV seems an ideal target for drug treatment or gene therapy because it has no obvious role in human physiology (Contento et al. 2008; Deeks and McCune 2010). In 2008, an HIV infected patient who was treated in a Berlin clinic was declared virus-free some 20 months after a bone marrow transplant with cells from a CCR5-negative donor and upon discontinuation of HAART (Hutter et al. 2009).

Chemokine Receptors

Chemokine receptors are known for their role in cell migration and the significant contribution to host defence in case of inflammation and infection. There are two primary receptors involved in HIV infection besides CD4: CCR5 and CXCR4, which the virus exploits to enter host cells (O'Hayre et al. 2010). The majority of transmitted HIV variants use CCR5 (R5 variants). As disease progresses, the virus mutates and starts recognizing the CXCR4 receptor in some patients (Contento et al. 2008). The development of the CCR5 antagonist Maraviroc and the fusion inhibitor T20 demonstrated the value of blocking cell entry for the treatment of HIV infection (O'Hayre et al. 2010). In case of the Berlin patient, the donor of the bone marrow transplant carried a 32-base pair deletion in the CCR5 gene that leads to a protein production defect. The deletion of base pair 32 within the coding region of CCR5 gene results in a frameshift during translation and the synthesis of a non-functional receptor that does not support HIV infection (Samson et al. 1996). This rare kind of genetic mutation is found in only 1–3 % of the population of northern European ancestry, but is absent in the population from western and central Africa and Japan (Hutter and Thiel 2011; Samson et al. 1996). The homozygous delta-32 population is generally protected against HIV infection, whereas heterozygous persons exhibit a slightly slower disease progression. These important observations triggered experimentalists to design a therapy based on the genetic engineering of cells that would make the patient at least partially resistant to HIV. At the same time, pharmaceutical companies had invested their resources to discover antibody or small molecule CCR5 inhibitors. In both cases, a considerable amount of success has been achieved over the years (Deeks and McCune 2010).

The Berlin Patient

A 40-year-old man was presented with acute myeloid leukemia (AML) at Berlin's Charite

University Medical Center in February 2007. He had been diagnosed with HIV infection for some 10 years and had been on HAART for four years prior to AML diagnosis. Seven months later, when his leukemia relapsed, he underwent an allogeneic hematopoietic stem cell transplantation using progenitor cells from a donor with the homozygous CCR5-delta32 deletion. Most importantly, after this treatment the patient did not show any sign of viral replication in blood and organs even after discontinuation of HAART. Moreover, his CD4 counts increased more than 800 cells/ul and his entire hematopoietic stem cell compartment consisted of CCR5 negative cells (Hutter et al. 2009; Hutter and Thiel 2011).

Presently, researchers are trying to create the same resistance to HIV by alternative means. This includes the silencing of CCR5 expression by the RNA interference (RNAi) mechanism or even the complete removal of the CCR5 gene from the host cell genome by sequence-specific endonucleases. In such an *ex vivo* gene therapy scenario one could target the mature T cells or the hematopoietic precursor cells. It should be mentioned that the Berlin patient represents only a single case and this approach should be repeated in a larger cohort, combined with long term monitoring. Very recently, two more transplantation successes were reported, but surprisingly with cells that encode a functional CCR5 receptor (Hutter and Thiel 2011). In contrast, allogeneic stem cell transplantation with a wild-type CCR5 gene was reported not to be successful (Hutter et al. 2009). These conflicting studies indicate the need to develop standardized protocols and to organize larger clinical trials. Anyhow, it is likely that the approach will not help patients that already developed an X4-using virus variant.

A recent report provided an update on the status of the Berlin patient, indicating the complete systemic recovery of CD4 $^{+}$ T-cells. After stem cell therapy the expansion of activated CD4^{+} T-cells usually enriches the pool of target cells of HIV infection. In this patient, an approximately normal number of CD4^{+} T-cells were recovered, but HIV remained undetectable. Although this patient still remains susceptible to X4-using HIV strains, the results indicate that these strains did not evolve (Benito 2011). This result revived hope that one could achieve the same effect with a gene therapy, which would also open new avenues for a complete cure of HIV infection. This remains an important future goal as current HAART therapy helps to control the infection by keeping the viral load low, but it is not able to achieve complete viral eradication from the reservoirs.

Gene Therapy

Gene therapy includes the introduction of a functional gene (transgene) in certain cells of the body to combat a persisting virus infection. The transgene is expressed in the target cells and as a consequence it will rescue a genetic defect or provide the cell with a new property, e.g. durable resistance against viral infection. Ideal target cells for such a gene delivery will be the stem cells. These cells have self-renewal and differentiation capacity that will allow the expression of the antiviral transgene in all progeny cells. Human mesenchymal stem cells (MSC) have been recognized as attractive targets for gene therapy because of their multilineage differentiation potential and ex vivo expansion capacity.

Retroviral and lentiviral vector systems are commonly used for gene therapy applications because these vectors integrate into the host cell genome, thus achieving permanent gene transduction. For instance, a lentiviral vector can be used to express certain transgene proteins from a housekeeping gene promoter. It was shown that mesenchymal progenitor cells from adipose tissue can maintain transgene expression during lineage-specific differentiation, which seems essential for a durable therapeutic effect (Morizono et al. 2003). Alternative viral vector systems have been developed (e.g. based on Adenovirus and Adeno-associated virus) and transposon-mediated gene therapy has been proposed in combination with a HIV-resistance gene. The Sleeping Beauty (SB) transposon system offers a non-viral vector for gene transfer

that bypasses the risk of vector-induced oncogenesis.

The SB system has been used to evaluate the stable gene transfer of CCR5 and CXCR4 *in vitro*. This system consists of a synthetic transposon and an associated transposase. Gene transposition is initiated by recognition of a short direct repeat sequence and excision by the transposon. Subsequently, the transposon gets attached to the target DNA at sites with the TA-dinucleotide sequence. In the GHOST-R3/X4/R5 cell culture model that expresses both the CXCR4 and CCR5 receptors, 94 % down-regulation of both receptors after SB mediated small interfering RNA (siRNA) gene transfer was observed. The SB system needs to be evaluated further in CD34 progenitor cells *in vitro* to compare the efficacy with vector-mediated gene transfer (Tamhane and Akkina 2008). Other antiviral approaches that have been proposed include diverse RNA interference (RNAi) based antivirals (Liu et al. 2009; ter Brake et al. 2009), the use of the human TRIM (tripartite motif)5α gene that encodes a potent HIV restriction factor and the *Herpesvirus saimiri* subgroup C transformation associated protein (StpC) that modulates HIV replication (Pham et al. 2010; Turner et al. 2006).

As a first step towards such a stem cell based gene therapy protocol, patients will be administered the *ex vivo* modified hematopoietic progenitor cells expressing multiple RNA-based anti-HIV moieties (e.g. short hairpin RNA (shRNA), TAR decoy, CCR5 ribozyme) or inhibitory proteins such as restriction factors. It is important to attack the virus with a combinatorial approach to avoid the evolution of escape variants (ter Brake et al. 2006; von Eije et al. 2008). In the first clinical trial, the lentiviral vector-modified cells were transplanted in autologous HIV-positive non-Hodgkin lymphoma patients and these cells showed sustained expression of the shRNA and ribozyme inhibitors for up to 24 months (DiGiusto et al. 2010). This clinical trial presents a milestone for cell based gene therapy for HIV infection.

Another retrovirus vector-based clinical trial demonstrated safety, although no therapeutic effect was scored. An anti-HIV ribozyme gene made up the antiviral payload, but it was observed that hematopoietic stem cells produce six times less ribozyme over a period of 6 months than expected (Mitsuyasu et al. 2009). The goal of ongoing research is to introduce an effective anti-HIV gene into progenitor stem cells or mature T cells. The progenitor cells will continuously produce HIV-resistant T cells, macrophages and dendritic cells to provide a long-term immune reconstitution. Because HIV-infection will trigger the removal of non-modified cells, the expectation is that the genetically modified cells will preferentially survive because they resist HIV infection, leading to a (slow and partial) reconstitution of the immune system (Bandi and Akkina 2008).

New Treatment Strategies

In recent years anti-HIV drug discovery efforts have included the chemokine receptors. Most approaches focus on small molecule inhibitors, but monoclonal antibodies and peptide analogs are in different stages of development. The first FDA-approved CCR5-specific antagonist for HIV infection that arrived in 2007 is Selzentry (Maraviroc, Pfizer), which can block R5-tropic HIV variants (O'Hayre et al. 2010). Based on a similar concept, the second approved drug was Mozobil (AMD3100, Genzyme) in 2008, which targets CXCR4 and also mobilizes hematopoietic stem cells in infected patients. The clinical evaluation of AMD3100 confirmed that the drug does mobilize CD34 cells. In a murine model, a dose of 5 mg/kg AMD3001 mobilized HPC within 1 h after injection (Broxmeyer et al. 2005). A level of complexity that may hinder the path of drug discovery is the existence of homo-, hetero-dimeric and higher order oligomeric receptor complexes. Recent findings indicated that CCR2, CCR5 and CXCR4 form functional homo-dimers and hetero-dimers on T cells (Contento et al. 2008). Evidence was also presented for the formation of CCR2, CCR5 and CXCR4 hetero-oligomeric complexes when recombinantly expressed on HEK293 cells. These complexes were also

reported to exist when endogenously expressed on primary leukocytes (Sohy et al. 2009).

As CCR5 is the most important co-receptor for HIV infection, blocking CCR5 on human CD34 stem cells would give rise to a polyclonal multi-lineage progeny cells in which CCR5 will be permanently disrupted. Engineered zinc finger nuclease (ZFN), which comprise of series of linked zinc fingers domains especially designed to recognize specific DNA sequences, can be designed to delete the CCR5 gene. CD4 T cells modified to express CCR5-targeting ZFNs are currently under investigation in clinical trials (Holt et al. 2010). Deletion of the CCR5 gene in stem cells may provide the most durable antiviral effect that is transferred to CCR5-negative lymphoid and myeloid cells, but the virus may still escape through CXCR4-usage. The safety of genome manipulation by CCR5-specific ZFNs in modified T lymphocytes is currently under investigation. Such gene therapeutic approaches can form a back-up plan for HAART therapy, especially for patients that develop resistance against most antiviral drugs. That use of CCR5-specific ZFNs may help to repopulate the CD4 cell compartment that was seriously affected by HIV infection (Holt et al. 2010; Lafeuillade and Stevenson 2011).

These new strategies are now being investigated along with current HAART regimens to achieve viral eradication from its tissue reservoir. The current approaches are to exhaust, shock-and-kill or to permanently silence the latent HIV reservoir (Frater 2011). While HAART keeps the viral load below the detection level, Immune Activation Therapy (IAT) could be used to stimulate the latent HIV-1 reservoir. As virus resides in resting memory CD4 T cells, the strategy is focused on activation of these cells to produce more virus. Virus-producing cells will be recognized and killed by cytotoxic T lymphocytes. Antiviral agents that produced promising *in vitro* results include histone deacetylase inhibitors, methylation inhibitors and NFkB activators. These agents are very effective in triggering the production of virus from a latent reservoir (Frater 2011). In an animal model, it was observed that IAT combined with HAART treatment triggered the complete removal of virus (Pippi 2008). The recent success with HIV-based vectors for a gene therapy against leukemia caught the attention of many researchers and may also stimulate investigations on the path towards a molecular gene therapy of HIV. In this particular case, a HIV-based lentiviral vector was used to infect T cells of Chronic Lymphoid Leukemia patients (Berkhout 2013; Porter et al. 2011). The complete recovery of these patients nicely adds to the control of both HIV and leukemia in the "Berlin patient". As mentioned earlier, transplantation with CCR5delta 32/delta32 stem cells demonstrated a successful reconstitution of CD4 T cells at the systemic level (Hutter et al. 2009). As immune reconstitution is a major hurdle in stem cell transplantation, the systemic recovery of CD4 cells after CCR5 delta32/delta32 stem cell transplantation is a major success (Allers et al. 2011). It should be noticed that the delta32 deletion is not common in HIV infected patients across the world, and the proposed stem cell gene therapy will not form a universal solution. However, these examples provide hope that HIV will eventually become a curable virus infection.

Conclusions

This review describes the complex HIV-host interaction as it occurs in *vivo*, which remains partially understood. Although the currently available drug cocktails have dramatically improved the life expectancy of HIV-infected individuals, a complete cure is not within easy reach. We described several novel treatment approaches that have provided the proof of concept of durable HIV-1 inhibition in the laboratory, but these results need to be verified in *in vivo* model systems and subsequently translated towards clinical application. In this regard, stem cell based gene therapy recently received much attention due to its success in some patients (Aiuti et al. 2013; Biffi et al. 2013). However, it is noteworthy that stem cell therapy for HIV infection may not be an immediate solution because of its side effects, which include teratoma formation, disease progression and genomic stability

(Benderitter et al. 2014). It is likely that routine drug therapy should be combined with novel therapeutic strategies to purge HIV-1 from reservoirs and to reach a functional cure, which means that the patient can discontinue regular drug treatment. It is important to realize that such patients will still harbor integrated copies of the HIV provirus in some of their cells. A functional cure without complete virus eradication seems the next goal in clinical HIV research.

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References

Aiuti A, Biasco L, Scaramuzza S, Ferrua F et al (2013) Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341:1233151-1-9

Allers K, Hutter G, Hofmann J, Loddenkemper C et al (2011) Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. Blood 117:2791–2799

Bahner I, Kearns K, Coutinho S, Leonard EH et al (1997) Infection of human marrow stroma by human immunodeficiency virus-1 (HIV-1) is both required and sufficient for HIV-1-induced hematopoietic suppression in vitro: demonstration by gene modification of primary human stroma. Blood 90:1787–1798

Bandi S, Akkina R (2008) Human embryonic stem cell (hES) derived dendritic cells are functionally normal and are susceptible to HIV-1 infection. AIDS Res Ther 5:1–9

Becker Y (2004) HIV-1 gp120 binding to dendritic cell receptors mobilize the virus to the lymph nodes, but the induced IL-4 synthesis by FcepsilonRI+ hematopoietic cells damages the adaptive immunity–a review, hypothesis, and implications. Virus Genes 29:147–165

Benderitter M, Caviggioli F, Chapel A, Coppes R et al (2014) Stem cell therapies for the treatment of radiation-induced normal tissue side effects. Antioxid Redox Signal 21(2):338–355

Benito JM (2011) HIV cure following CCR5 delta 32 stem cell transplantation-an update. AIDS Rev 13:58

Berkhout B (2013) HIV, leukemia, and new horizons in molecular therapy. J Formos Med Assoc 112:441–444

Biffi A, Montini E, Lorioli L, Cesani M et al (2013) Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341:1233158

Bouloumié A, Sengenès C, Portolan G, Galitzky J, Lafontan M (2001) Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. Diabetes 50(9):2080–2086

Bourlier V, Zakaroff-Girard A, De Barros S, Pizzacalla C, de Saint Front VD, Lafontan M, Bouloumie A, Galitzky J (2005) Protease inhibitor treatments reveal specific involvement of matrix metalloproteinase-9 in human adipocyte differentiation. J Pharmacol Exp Ther 312:1272–1279

Broxmeyer HE, Orschell CM, Clapp DW, Hangoc G et al (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J Exp Med 201:1307–1318

Caron M, Auclair M, Vigouroux C, Glorian M, Forest C, Capeau J (2001) The HIV protease inhibitor indinavir impairs sterol regulatory element-binding protein-1 intranuclear localization, inhibits preadipocyte differentiation, and induces insulin resistance. Diabetes 50:1378–1388

Carter CC, Onafuwa-Nuga A, McNamara LA, Riddell JT et al (2010) HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs. Nat Med 16:446–451

Cheng K, Rai P, Lan X, Plagov A et al (2013) Bone-derived mesenchymal stromal cells from HIV transgenic mice exhibit altered proliferation, differentiation capacity and paracrine functions along with impaired therapeutic potential in kidney injury. Exp Cell Res 319:2266–2274

Contento RL, Molon B, Boularan C, Pozzan T et al (2008) CXCR4-CCR5: a couple modulating T cell functions. Proc Natl Acad Sci U S A 105:10101–10106

Deeks SG, McCune JM (2010) Can HIV be cured with stem cell therapy? Nat Biotechnol 28:807–810

DiGiusto DL, Krishnan A, Li L, Li H et al (2010) RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. Sci Transl Med 2:36.1–12

Duncan CJ, Sattentau QJ (2011) Viral determinants of HIV-1 macrophage tropism. Viruses 3:2255–2279

Dupin N, Buffet M, Marcelin AG, Lamotte C, Gorin I et al (2002) HIV and antiretroviral drug distribution in plasma and fat tissue of HIV-infected patients with lipodystrophy. AIDS 16:2419–2424

Folks TM, Kessler SW, Orenstein JM, Justement JS, Jaffe ES, Fauci AS (1988) Infection and replication of HIV-1 in purified progenitor cells of normal human bone marrow. Science 242:919–922

Frater J (2011) New approaches in HIV eradication research. Curr Opin Infect Dis 24:593–598

Freisinger E, Cramer C, Xia X, Murthy SN et al (2010) Characterization of hematopoietic potential of mesenchymal stem cells. J Cell Physiol 225:888–897

Gibellini D, Re MC, Vitone F, Rizzo N et al (2003) Selective up-regulation of functional CXCR4 expression in erythroid cells by HIV-1 Tat protein. Clin Exp Immunol 131:428–435

Gibellini D, Vitone F, Buzzi M, Schiavone P et al (2007) HIV-1 negatively affects the survival/maturation of cord blood CD34(+) hematopoietic progenitor cells differentiated towards megakaryocytic lineage by HIV-1 gp120/CD4 membrane interaction. J Cell Physiol 210:315–324

Gimble JM, Guilak F, Bunnell BA (2010) Clinical and preclinical translation of cell-based therapies using adipose tissue-derived cells. Stem Cell Res Ther 1:19

Hazan U, Romero IA, Cancello R, Valente S et al (2002) Human adipose cells express CD4, CXCR4, and CCR5 [corrected] receptors: a new target cell type for the immunodeficiency virus-1? FASEB J 16:1254–1256

Holt N, Wang J, Kim K, Friedman G et al (2010) Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Nat Biotechnol 28:839–847

Hutter G, Thiel E (2011) Allogeneic transplantation of CCR5-deficient progenitor cells in a patient with HIV infection: an update after 3 years and the search for patient no. 2. AIDS 25:273–274

Hutter G, Nowak D, Mossner M, Ganepola S et al (2009) Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N Engl J Med 360:692–698

Iglesias-Ussel MD, Romerio F (2011) HIV reservoirs: the new frontier. AIDS Rev 13:13–29

Lafeuillade A, Stevenson M (2011) The search for a cure for persistent HIV reservoirs. AIDS Rev 13:63–66

Laharrague P, Casteilla L (2010) The emergence of adipocytes. Endocr Dev 19:21–30

Lenhard JM, Furfine ES, Jain RG, Ittoop O et al (2000) HIV protease inhibitors block adipogenesis and increase lipolysis in vitro. Antiviral Res 47:121–129

Lin Y, Lee H, Berg AH, Lisanti MP et al (2000) The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. J Biol Chem 275:24255–24263

Liu YP, von Eije KJ, Schopman NC, Westerink JT et al (2009) Combinatorial RNAi against HIV-1 using extended short hairpin RNAs. Mol Ther 17:1712–1723

MacEneaney OJ, Connick E, DeSouza CA (2011) Effects of HIV-1 gp120 and protease inhibitors on apoptotic susceptibility of CD34+ hematopoietic progenitor cells. J Acquir Immune Defic Syndr 56:e49–e50

Maumus M, Peyrafitte JA, D'Angelo R, Fournier-Wirth C et al (2011) Native human adipose stromal cells: localization, morphology and phenotype. Int J Obes (Lond) 35(9):1141–1153

Mitsuyasu RT, Merigan TC, Carr A, Zack JA et al (2009) Phase 2 gene therapy trial of an anti-HIV ribozyme in autologous CD34+ cells. Nat Med 15:285–292

Moore JP, Kitchen SG, Pugach P, Zack JA (2004) The CCR5 and CXCR4 coreceptors – central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 20:111–126

Morizono K, De Ugarte DA, Zhu M, Zuk P et al (2003) Multilineage cells from adipose tissue as gene delivery vehicles. Hum Gene Ther 14:59–66

Munier S, Borjabad A, Lemaire M, Mariot V, Hazan U (2003) In vitro infection of human primary adipose cells with HIV-1: a reassessment. AIDS 17:2537–2539

Nazari-Shafti TZ, Freisinger E, Roy U, Bulot CT et al (2011) Mesenchymal stem cell derived hematopoietic cells are permissive to HIV-1 infection. Retrovirology 8:3.1–12

O'Hayre M, Salanga CL, Handel TM, Hamel DJ (2010) Emerging concepts and approaches for chemokine-receptor drug discovery. Expert Opin Drug Discov 5:1109–1122

Pham QT, Bouchard A, Grutter MG, Berthoux L (2010) Generation of human TRIM5alpha mutants with high HIV-1 restriction activity. Gene Ther 17:859–871

Pippi F (2008) A novel approach to HIV therapy: highly active antiretroviral therapy and autologous hematopoietic cell transplantation. Med Hypotheses 70:291–293

Porter DL, Levine BL, Kalos M, Bagg A, June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365:725–733

Samson M, Libert F, Doranz BJ, Rucker J et al (1996) Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 382:722–725

Sankale JL, Tong Q, Hadigan CM, Tan G et al (2006) Regulation of adiponectin in adipocytes upon exposure to HIV-1. HIV Med 7:268–274

Sevastianova K, Sutinen J, Kannisto K, Hamsten A et al (2008) Adipose tissue inflammation and liver fat in patients with highly active antiretroviral therapy-associated lipodystrophy. Am J Physiol Endocrinol Metab 295:E85–E91

Sohy D, Yano H, de Nadai P, Urizar E et al (2009) Hetero-oligomerization of CCR2, CCR5, and CXCR4 and the protean effects of "selective" antagonists. J Biol Chem 284:31270–31279

Tamhane M, Akkina R (2008) Stable gene transfer of CCR5 and CXCR4 siRNAs by sleeping beauty transposon system to confer HIV-1 resistance. AIDS Res Ther 5:16.1–9

ter Brake O, Konstantinova P, Ceylan M, Berkhout B (2006) Silencing of HIV-1 with RNA interference: a multiple shRNA approach. Mol Ther 14:883–892

ter Brake O, Legrand N, von Eije KJ, Centlivre M et al (2009) Evaluation of safety and efficacy of RNAi against HIV-1 in the human immune system (Rag-2(−/−)gammac(−/−)) mouse model. Gene Ther 16:148–153

Trujillo ME, Lee MJ, Sullivan S, Feng J et al (2006) Tumor necrosis factor alpha and glucocorticoid synergistically increase leptin production in human adipose tissue: role for p38 mitogen-activated protein kinase. J Clin Endocrinol Metab 91:1484–1490

Turner LS, Tsygankov AY, Henderson EE (2006) StpC-based gene therapy targeting latent reservoirs of HIV-1. Antiviral Res 72:233–241

Vernochet C, Azoulay S, Duval D, Guedj R et al (2005) Human immunodeficiency virus protease inhibitors accumulate into cultured human adipocytes and alter expression of adipocytokines. J Biol Chem 280:2238–2243

von Eije KJ, ter Brake O, Berkhout B (2008) Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. J Virol 82:2895–2903

Weisberg SP, McCann D, Desai M, Rosenbaum M et al (2003) Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796–1808

Whang KT, Steinwald PM, White JC, Nylen ES et al (1998) Serum calcitonin precursors in sepsis and systemic inflammation. J Clin Endocrinol Metab 83:3296–3301

Whigham LD, Dhurandhar NV, Rahko PS, Atkinson RL (2007) Comparison of combinations of drugs for treatment of obesity: body weight and echocardiographic status. Int J Obes (Lond) 31:850–857

Zaitseva M, Peden K, Golding H (2003) HIV coreceptors: role of structure, posttranslational modifications, and internalization in viral-cell fusion and as targets for entry inhibitors. Biochim Biophys Acta 1614:51–61

Zhang B, MacNaul K, Szalkowski D, Li Z et al (1999) Inhibition of adipocyte differentiation by HIV protease inhibitors. J Clin Endocrinol Metab 84:4274–4277

Transplantation of CCR5-Δ32/Δ32 Stem Cells May Cure HIV Infection

3

Gero Hütter

Contents

Abstract

HIV-1 can persist in a latent form in resting memory CD4+ cells and macrophages carry an integrated copy of the HIV genome. Because of the presence of these stable reservoir cells, eradication by antiretroviral therapy is unlikely, and in order to achieve eradication, alternative treatment options are required. Recently, we have described a successful hematopoietic stem cell transplantation in an HIV-1 infected patient by transferring donor derived cells with a natural resistance against HIV infection (CCR5-delta 32 deletion). At present the patient is more than 6 years after allogeneic transplantation without requirement of any antiretroviral treatment. Here we report the potential and limitations concerning this stem cell based approach.

Introduction

Antiretroviral therapy (HAART) was the major breakthrough in HIV-1 treatment leading to a significant benefit in survival of HIV-patients. Nevertheless, HAART is not sufficient to remove the virus from the body and viral rebound is commonly observed when HAART medication is discontinued. The need of a life-long treatment is associated with serious medical side effects. The increasing economical burden for all aspects of

G. Hütter (✉)
Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, Heidelberg University, Heidelberg, Germany
e-mail: Gero.Huetter@medma.uni-heidelberg.de

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the health care system and the alarming number of infected people worldwide has promoted the search for an HIV cure.

The hope that a sustained suppression of viral replication can eliminate the virus over a couple of years were in vain because the half-time of these latent infected cells (which are causative for rebounds after discontinuing HAART) is much longer than expected (Siliciano and Siliciano 2004). Theoretically, it would take the whole life-time for eradication could be achieved which is somehow ineligible for practice.

Therefore, alternatives or more efficient strategies are required to clear the body form the virus. One strategy is the use of hematopoietic stem cells for autologous or allogeneic transplantation. These techniques already have shown efficiency in cancer treatment (Hayat 2012). The principle of eradication of a malignant clone in patients with leukemia has been well established, and, has become a standard treatment procedure in oncology.

CCR5 Deficieny and HIV Infection

The entry of human immunodeficiency virus type 1 (HIV-1) into target cells requires both CD4 and a coreceptor, predominantly the chemokine receptor CCR5. A 32 base pair deletion in the CCR5 gene results in a truncated protein that is insufficient for HIV entry reported from Dragic et al. (1996). Consequently, CCR5-delta32 homozygosity provides natural protection against HIV infection.

Allogeneic Stem Cell Transplantation and HIV

The first experimental attempt to affect the HIV-infection by stem cell transplantation (SCT) was made in the early 1980s by Hassett et al. (1983) by transfusion of large amounts of allogeneic T cells and CD34+ stem cells. Clinically, the patients did not substantially improve from this procedure and the immunological condition remained stable or worsened. Retrospective analysis of reported cases indicate that the outcome of allografted HIV positive patients is only negligible in comparison to HIV negative patients as analyzed by Hütter and Zaia (2011).

However, allogeneic SCT alone is not sufficient to improve the course of HIV-1 infection because of the reinfection of the newly arising immune system (due to the latently infected host cells) commonly survive the transplantation procedure. Surprisingly, the introduction of HAART during allogeneic transplantation did not change the situation. There were seven reports of patients which discontinued HAART after allogeneic SCT, and all of them rebounded after a couple of days (Table 3.1).

In this context the report by Henrich et al. (2013b) of two patients from the Brigham and Woman's Hospital, Boston, USA which were called "Boston patients" was surprising. Both received allogeneic stem cells with no special donor selection expect the necessary 10/10 HLA match. Both had episodes of graft versus host disease (GVHD). During the follow up of 4.5 and 2.5 years, respectively, Henrich et al. (2013a, b) reported that residual virus was all negative even using techniques with the highest sensitivity. In one patient there were additional gut biopsies available which were found also negative for HIV. Both patients had a declining anti-HIV antibody titer and the chimerism was less than 0.001 % of circulation host cells, indicating a complete exchange of the hematopoitic cell system. Therefore, the Boston researches decided to stop HAART medication and after a short period of enthusiastic reports, the virus rebounded in both patients after a couple of weeks (Lazar 2013). In summary, allogeneic SCT alone is not sufficient to eradicate HIV-1.

The "Berlin Patient" as a Proof of Principle

In 2009, Hütter et al. and later Allers et al. (2011) described successful hematopoietic stem cell transplantation (SCT) in an HIV-1 infected patient by transferring CCR5-delta32 donor derived cells that harbor a natural resistance

Table 3.1 Summary of reported cases oh patients with HIV-1 infection and antiretroviral treatment interruption

Graft	HAART stopped (days after transplantation)	Viral outcome	Reference
MRD	5–14	Rebound day 42	Sora et al. (2002)
double UCB	0–28	Rebound day 33	Tomich et al. (2005)
MUD	0–34	Rebound	Wolf et al. (2007)
MUD	114–134	Rebound	Avettand-Fenoel et al. (2007)
MUD	0–365	Rebound day 30	Polizzotto et al. (2007)
MUD	0–34	Rebound	Kamp et al. (2010)
MUD (2 patients)	From day 900 and 1980, respectively	Both rebounded after 2 and 5 months, respectively	Henrich et al. (2013a), Lazar (2013)

All patients displayed a viral rebound after discontinuation. The data are suggestive that a longer interval from transplantation to treatment interruption is associated with a later rebound
UCB umbilical cord blood, *MUD* matched unrelated

against HIV infection. These hematopoietic stem cells engrafted, proliferated and differentiated into mature myeloid and lymphoid cells. At present the patient is more than 5 years post-allogeneic transplantation without the requirement of any antiretroviral treatment. Analyzing peripheral blood cells and different tissue samples, including gut, liver, and brain, no viral RNA load or proviral DNA could be detected.

Benefits and Risks of Transplanting CCR5 Deficient Stem Cells

Recipients of organ allografts homozygous for CCR5-delta32 show longer survival of transplant function than those with other genotypes. This has been shown by Fischereder et al. (2001) and Heidenhain et al. (2009) for renal and liver transplants, suggesting that patients with CCR5-delta32 might be candidates for a reduced immunosuppressive therapy. Consequently, interaction and blockade of the CCR5 receptor may also reduce alloantigen-specific T lymphocyte proliferation, and may be effective in preventing acute and chronic rejection of the allograft as reported by Schnickel et al. (2008). Furthermore, Bogunia-Kubik et al. (2006) reported the presence of the CCR5-delta32 allele represents a protective factor in terms of the risk of developing GVHD after allogeneic SCT. Taken together, the expression of the CCR5-delta32 allele in recipients of allografts constitutes an independent and protective factor associated with a decreased risk of GVHD and graft rejection. However, the mechanism of this beneficial effect of the deletion regarding GVHD is not known. In the past there was much speculation regarding the association of the CCR5-delta32 genotype and other diseases with chronic autoimmune inflammation. The results of these retrospective studies are controversial. No association or beneficial effect was observed in diabetes mellitus type 1, asthma, rheumatoid arthritis, and Behcet's disease summarized by Burke et al. (2013). Glass et al. (2006) have reported individuals with the CCR5-delta32 homozygous genotype being associated with an increased risk of symptomatic West Nile virus infection. Thus, there is no evidence that the CCR5-delta32 genotype is associated with significant negative comorbidities or risks in terms of transplantation, and the presence of the CCR5-delta32 genotype should not have negative implications for the recipient of a CCR5 targeted therapy.

Challenges in Repeating the "Berlin Patient"

HLA Matched Related or Unrelated Donors

More than 16,000,000 people are already registered worldwide as stem cell donors. Based on a 10/10 allele HLA-match, the probability in

finding a matching donor is more than 80 %, and there is commonly more than 1 donor and sometimes more than 100 donors available for each patient. According to the frequency of 1 % homozygous CCR5-delta32 Caucasians, there is a small but reasonable chance to find both an HLA-identical donor without CCR5 surface expression.

The major limitations include that donors are not generally tested for CCR5 genotype, and there is commonly not enough time to complete the screening process. Therefore, Hütter's group organized a workshop in 2009 bringing together leading European stem cell registries for a discussion of the possibilities and limitations of a CCR5-based donor screening. The meeting reached the agreement to support further attempts to use CCR5-delta32 deleted stem cells in appropriate candidates. However, none of these registries initiated a program of preemptive CCR5 genotyping.

With a grant from the Bill & Melinda Gates foundation, Hütter's group was able to carry on 20 additional donor searches requesting units for transplantation. In four cases (20 %!) it was possible to find an HLA matching CCR5-delta32 homozygote donor. Unfortunately, one patient died prior to initiation of the transplantation procedure, one received transplant (not published yet) and in two cases the decision for transplantation is pending.

Furthermore, Hütter's group initiated a CCR5 genotype prescreening program for the "German Red Cross Donor Registry" of Mannheim, Germany, which currently has tested 8,000 of 40,000 adult unrelated stem cell donors. Together with the cord blood bank of Stemcyte, Covina, CA, USA (>25,000 units) and the M.D. Anderson CB Bank, Houston, Texas, USA (>10,000 units), these repositories represent the largest potential source of CCR5 negative stem cells for transplantation.

Cord Blood: An Alternative Stem Cell Source

As an alternative to HLA matched related or unrelated donors, cord blood units are an interesting stem cell source. Since 2001 R. Chow (founder of StemCyte Inc.) and L. Petz built up a database with more than 25,000 cord blood units genotyped for the CCR5-delta32 deletion, and over 300 of these UCB units have already been identified as being CCR5 negative. This would be sufficient to provide an 4/6 HLA matched unit with a 73.6 % probability to Caucasian paediatric patients, and 27.9 % for Caucasian adults in the case that a minimal cell dose of $\geq 2.5 \times 10^7$ total nucleated cell count (TNC)/kg is accepted according to Petz et al. (2012).

However, since StemCyte started the CCR5 testing in 2001, there were no CCR5-delta 32 homozygous UCB units administered up to 2011. The reason is probably due to the ready access of perfectly matched stem cells from adult donors. Some countries, such as Germany, do not use UCB in significant amounts. Some transplant centers are concerned that a delayed engraftment of UCB and the consequent higher risk of opportunistic infections during aplasia would narrow the success of the transplant procedure.

To overcome these limitations, strategies to increase the probability of administration of UCB are required. One option could be the so called "dual transplant" method consisting of the coinfusion of one UCB unit with a relatively low number of highly T-depleted mobilized $CD34^+$ cells from an adult third party donor, after myeloablative conditioning regimen shown by Kwon et al. (2012).

This approach has several advantages:

1. High accessibility of a haploidentical donor that bridges the time until the CCR5-negative UCB engrafts.
2. Increased probability of finding matching UCB due to the reduction of the required $CD34^+$ cell dose. Lowering the required cell dose from 2.5 to 1.0×10^7TNC/kg will increase the probability from 27.9 to 82.1 %.
3. Reduced time of aplasia due to the haploidentical proportion of the transplant.
4. Finally, after engraftment, the UCB unit will take over the myeloid function while the haploidentical part disappears after a period of time resulting in a 100 % chimerism of CCR5 depleted and HIV resistant peripheral blood cells.

Competition between grafts of various sources is commonly observed after double UCB transplantation and a similar competition phenomenon occurs after combined)haploidentical/UCB transplantation (Fig. 3.1). Immunological mechanisms likely play a role in the UCB cord blood dominance, and in this regard the T-cell depletion of the haplograft may be important but probably not essential. This approach has already been performed in some selected patients (publication of first results is in progress). Unfortunately, one of these patients died due to relapse of the malignancy 2½ months after the transplantation procedure (personal communication from Prof. Jose L. Díez Martín, Chairman of Hematology Department, HGU Gregorio Marañón, Madrid, Spain). Due to the difficulties in finding a second candidate for SCT from an adult unrelated donor, the meeting suggested the initiation of a program to promote the idea of combined haploidentical/CCR5-negative UCB based on the experience at the University of Utrecht, Netherlands (Table 3.2).

Limitations of the CCR5-Delta32 Approach

During transmission of HIV, CCR5 is the preferred coreceptor for cell entry. However, during the time course of HIV infection, the virus is able to change its tropism to other chemokine coreceptors, such as CXCR4, and the role of CCR5 in maintaining HIV infection is still unclear. The "Berlin patient" harbored a CXCR4 tropic variant before transplantation that did not emerge after discontinuation of antiretroviral therapy, a phenomenon that is discussed by Symons et al. (2012) and cannot yet be explained.

Secondly, there is a broad discussion about sterilizing cure in the "Berlin patient". However, current investigations and several attempts with different techniques from tissues, cerebral fluid, and blood have led to contradicting results.

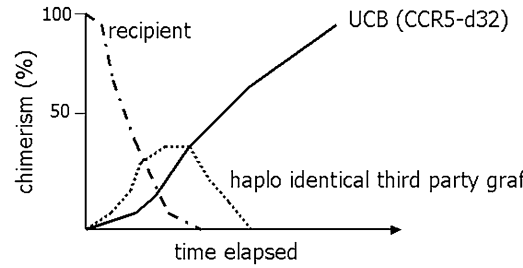

Fig. 3.1 Course of double transplant of CCR5-delta32 homozygous umbilical cord blood (UCB) and a bridging third party donor, in this case from a haploidentical family donor. The haploidentical part engrafts first bridging the time of cytopenia. After up to 6 months after transplantation the UCB part has replaced the haploidentical part as well as the remaining cells from the recipient

Table 3.2 Patients who received allogeneic stem cell transplantation from an CCR5-delta32 homozygous donor after the "Berlin patient"

Donor/graft	Patient	Follow up
Combined CCR5-d32 homozygous UCB combined with haplo identical third part donor	MDS	HAART continued after SCT, died 2 months after SCT due to MDS relapse
Single CCR5d32 homozygous UCB	Leukaemia	No rebound during 3 months of HAART discontinuation, patient died from leukaemia
MUD CCR5-d32 homozygous	Lymphoma	HAART discontinued after SCT, X4 rebound within 4 weeks
MMUD CCR5-d32 homozygous	Lymphoma	Data pending
MRD CCR5-d32 homozygous	Lymphoma	Data pending

UCB umbilical cord blood, *SCT* stem cell transplantation, *MDS* myeloid dysplastic syndrome, *MUD* matched unrelated, *MMUD* mismatch unrelated, *MRD* matched related

However, the patient is well, more than 6 years off antiretroviral medication, without HIV related symptoms, without viral replication showing a perfect clinical remission of the infection as extensively investigated by Yukl et al. (2013).

Summary and Outlook

Currently, the "Berlin patient" is the only reported case of an successful allogeneic stem cell transplantation with CCR5-deficient cells. However, there have been several attempts to repeat this approach using different sources of stem cells. Taken into account that allogeneic SCT is associated with a direct and indirect mortality rate of up 30 % it will be difficult to estimate the value of this approach as long as there were such a small number of patients evaluable. There is some hope that improved technique in safe and easy gene therapy methods may have the same efficiency as using natural CCR5 depleted cell sources to improve the cell based HIV-1 therapy.

References

Allers K, Hütter G, Hofmann J, Loddenkemper C, Rieger K, Thiel E, Schneider T (2011) Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. Blood 117:2791–2799

Avettand-Fenoel V, Mahlaoui N, Chaix ML, Milliancourt C, Burgard M, Cavazzana-Calvo M, Rouzioux C, Blanche S (2007) Failure of bone marrow transplantation to eradicate HIV reservoir despite efficient HAART. AIDS 21:776–777

Bogunia-Kubik K, Duda D, Suchnicki K, Lange A (2006) CCR5 deletion mutation and its association with the risk of developing acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Haematologica 91:1628–1634

Burke BP, Boyd MP, Impey H, Breton LR, Bartlett JS, Symonds GP, Hütter G (2013) CCR5 as a natural and modulated target for inhibition of HIV. Viruses 6:54–68

Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381:667–673

Fischereder M, Luckow B, Hocher B, Wuthrich RP, Rothenpieler U, Schneeberger H, Panzer U, Stahl RA, Hauser IA, Budde K, Neumayer H, Kramer BK, Land W, Schlondorff D (2001) CC chemokine receptor 5 and renal-transplant survival. Lancet 357:1758–1761

Glass WG, McDermott DH, Lim JK, Lekhong S, Yu SF, Frank WA, Pape J, Cheshier RC, Murphy PM (2006) CCR5 deficiency increases risk of symptomatic West Nile virus infection. J Exp Med 203:35–40

Hassett JM, Zaroulis CG, Greenberg ML, Siegal FP (1983) Bone marrow transplantation in AIDS. N Engl J Med 309:665

Hayat MA (ed) (2012–2014) Stem cells and cancer stem cells. Therapeutic applications in disease and injury, vol 1–12. Springer, Heidelberg/New York

Heidenhain C, Puhl G, Moench C, Lautem A, Neuhaus P (2009) Chemokine receptor 5Delta32 mutation reduces the risk of acute rejection in liver transplantation. Ann Transpl 14:36–44

Henrich T, Hanhauser E, Sirignano M, Davis B, Lee TH, Keating S, Busch M, Marty F, LaCasce A, Armand P, Soiffer R, Altfeld M, Kuritzkes D (2013a) In depth investigation of peripheral and gut HIV-1 reservoirs, HIV-specific cellular immunity, and host microchimerism following allogeneic hematopoetic stem cell transplantation. In: 7th international AIDS society conference on HIV pathogenesis, treatment and prevention, Kuala Lumpur, p. WELBA05

Henrich TJ, Hu Z, Li JZ, Sciaranghella G, Busch MP, Keating SM, Gallien S, Lin NH, Giguel FF, Lavoie L, Ho VT, Armand P, Soiffer RJ, Sagar M, Lacasce AS, Kuritzkes DR (2013b) Long-term reduction in peripheral blood HIV type 1 reservoirs following reduced-intensity conditioning allogeneic stem cell transplantation. J Infect Dis 207:1694–1702

Hütter G, Zaia JA (2011) Allogeneic haematopoietic stem cell transplantation in patients with human immunodeficiency virus: the experiences of more than 25 years. Clin Exp Immunol 163:284–295

Hütter G, Nowak D, Mossner M, Ganepola S, Mussig A, Allers K, Schneider T, Hofmann J, Kucherer C, Blau O, Blau IW, Hofmann WK, Thiel E (2009) Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N Engl J Med 360:692–698

Kamp C, Wolf T, Bravo IG, Kraus B, Krause B, Neumann B, Winskowsky G, Thielen A, Werner A, Schnierle BS (2010) Decreased HIV diversity after allogeneic stem cell transplantation of an HIV-1 infected patient: a case report. Virol J 7:55

Kwon M, Balsalobre P, Serrano D, Perez CA, Buno I, Anguita J, Gayoso J, Diez-Martin JL (2012) Single cord blood combined with HLA-mismatched third party donor cells: comparable results to matched unrelated donor transplantation in high-risk patients with hematologic disorders. Biol Blood Marrow Transpl 19:143–149

Lazar K (2013) HIV virus returns after cure hope rose. In: Boston Globe, 6 Dec 2013 https://www.bostonglobe.com/lifestyle/health-wellness/2013/12/05/hiv-virus-returns-after-curehope-rose/kSUyH1YkgJ27AP4a-ZwpUXJ/story.html

Petz LD, Redei I, Bryson Y, Regan D, Kurtzberg J, Shpall E, Gutman J, Querol S, Clark P, Tonai R, Santos S,

Bravo A, Spellman S, Gragert L, Rossi J, Li S, Li H, Senitzer D, Zaia J, Rosenthal J, Forman S, Chow R (2012) Hematopoietic cell transplantation with cord blood for cure of HIV infections. Biol Blood Marrow Transpl 19:393–397

Polizzotto MN, Shortt J, Cole-Sinclair MF, Opat SS, Spencer A, Avery S (2007) Allogeneic peripheral blood stem cell transplantation for hematological malignancies in patients with HIV. ASH Annu Meet Abstr 110:4941

Schnickel GT, Bastani S, Hsieh GR, Shefizadeh A, Bhatia R, Fishbein MC, Belperio J, Ardehali A (2008) Combined CXCR3/CCR5 blockade attenuates acute and chronic rejection. J Immunol 180:4714–4721

Siliciano JD, Siliciano RF (2004) A long-term latent reservoir for HIV-1: discovery and clinical implications. J Antimicrob Chemother 54:6–9

Sora F, Antinori A, Piccirillo N, De Luca A, Chiusolo P, Cingolani A, Laurenti L, Rutella S, Ortona L, Leone G, Sica S (2002) Highly active antiretroviral therapy and allogeneic CD34(+) peripheral blood progenitor cells transplantation in an HIV/HCV coinfected patient with acute myeloid leukemia. Exp Hematol 30:279–284

Symons J, Deeks S, Hütter G, Wensing A, van Ham P, Vandekerckhove L, Nijhuis M (2012) CCR5-dependence prevents X4-tropic HIV-rebound post CCR5-delta32-SCT in the 'Berlin. JIAS 15:16–17

Tomich CH, da Silva P, Carvalho I, Taft CA (2005) Homology modeling and molecular interaction field studies of alpha-glucosidases as a guide to structure-based design of novel proposed anti-HIV inhibitors. J Comput Aided Mol Des 19:83–92

Wolf T, Rickerts V, Staszewski S, Kriener S, Wassmann B, Bug G, Bickel M, Gute P, Brodt HR, Martin H (2007) First case of successful allogeneic stem cell transplantation in an HIV-patient who acquired severe aplastic anemia. Haematologica 92:e56–e58

Yukl SA, Boritz E, Busch M, Bentsen C, Chun TW, Douek D, Eisele E, Haase A, Ho YC, Hütter G, Justement JS, Keating S, Lee TH, Li P, Murray D, Palmer S, Pilcher C, Pillai S, Price RW, Rothenberger M, Schacker T, Siliciano J, Siliciano R, Sinclair E, Strain M, Wong J, Richman D, Deeks SG (2013) Challenges in detecting HIV persistence during potentially curative interventions: a study of the Berlin patient. PLoS Pathog 9, e1003347

Transplantation of Stem Cells to Treat Patients with Multiple Sclerosis

4

Cecilia Laterza and Gianvito Martino

Contents

Abstract

Multiple sclerosis (MS) is an acquired inflammatory and neurodegenerative immune-mediated disorder of the central nervous system (CNS), characterized by inflammation, demyelination and axonal degeneration. In the most recent years, pre-clinical data have been accumulated in experimental models of demyelination – mimicking the pathogenic processes occurring in human MS – suggesting that therapies based on the transplantation of stem/precursor cells from various sources (i.e. neural stem/precursor cells, oligodendrocyte precursor cells, mesenchymal stem cells) can contribute to prevent or repair the CNS damage via different mechanisms. However, there are still unsolved issues concerning how to repair chronic MS lesions and whether transplanted cells can overcome the inhibitory microenvironment that restrain the endogenous repair. In order to translate preclinical research from bench-to-bedside, it is still mandatory to assess the optimal cell source, the timing and route of cell delivery, the therapeutic windows and the best cohort of patient potentially responsive for stem cell-based treatments.

C. Laterza • G. Martino (✉)
Division of Neuroscience, Institute of Experimental Neurology-DIBIT 2, San Raffaele Scientific Institute, Milan, Italy
e-mail: Martino.gianvito@hsr.it

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Introduction

Multiple sclerosis (MS) is an immune-mediated inflammatory demyelinating disease of the central nervous system (CNS) whose aetiology is still indeterminate (Noseworthy et al. 2000). It is a complex multifactorial disorder in which environmental factors might contribute to the occurrence of the disease in genetically susceptible individuals (Compston and Coles 2008). The unknown aetiology and the complexity (heterogeneity) of the immune-mediated pathological process have, at least in part, restrained the development of efficacious treatments for MS. Available therapies do reduce the disease activity but do not prevent the disease evolution into the progressive phase that is always accompanied by the development of permanent disability.

There are two generally accepted strategies for treating MS: (i) preventing CNS damage indirectly through immunomodulation, and (ii) repairing CNS damage by promoting remyelination and axonal protection. Both approaches also address the unmet need of providing neuroprotection, either by reducing inflammation that causes axon damage or by restoring the normal cytoarchitecture of myelinated tissue on which axonal health depends (remyelination) (Martino et al. 2010).

While the approved as well as the next generation of disease-modifying therapies are likely to have enhanced efficacy, particularly in the early phases of the disease, these therapies will not benefit the large numbers of MS patients in the progressive phase with advanced disability who represent the major social burden. Indeed, current treatments prevent demyelination and neuronal damage by regulating or suppressing the immune response but do not address axon pathology directly. Thus, the treatment of the neurodegenerative component of MS, including remyelination failure and axonal/neuronal loss, is still far from being established.

Several pre-clinical and clinical evidence supports the notion that neurodegeneration is already present at the onset of MS imposes a change of perspective in MS treatment. MS therapy should not only focus on controlling neuroinflammation but also on protecting and repairing damaged axons *ab initio* (Centonze et al. 2009). Therefore, *neuroprotective* strategies focused on dampening futile energy dispersion and optimizing cellular metabolism in order to limit axonal degeneration and myelin loss have been developed. Several 'neuroprotective' drugs – e.g. sodium channel-blocking compounds, potassium channel-blocking compounds and glutamate receptor antagonists such as phenytoin, lamotrigine, dalfampridine, topiramate or amiloride – have been already tested in pre-clinical models of MS or in patients with MS but the results so far obtained are still inconclusive. On the other hand, promotion of endogenous remyelination has been the main objective of a new anti-LINGO1 antibody based treatment that targets a negative regulator of myelination expressed on neurons; an approach able to preserve axonal integrity and ameliorate clinical outcome in the animal model of MS, namely experimental autoimmune encephalomyelitis (EAE) (Mi et al. 2013).

The Role of Stem Cells in MS Therapy

Since late '70, cell-based therapies for MS have been initially developed in order to promote remyelination and tissue repair via replacing damaged myelin forming cells. Although successful in focal myelin disorders, most of these therapeutic approaches have failed to foster repair in multifocal myelin disorders, such as MS, where the anatomical and functional inflammation-induced cell damage is widespread.

More recently, stem cells have been proposed as an alternative therapeutic strategy to promote tissue repair in MS but the results, so far obtained, are still preliminary to draw any conclusion about the efficacy of this approach. This is owing to the fact that there are some preliminary questions that need to be solved before envisaging any potential human applications of such therapies: (i) the ideal cell candidate; (ii) the cell source for transplantation; (ii) the route of cell administration.

Furthermore, the functional and long-lasting integration of transplanted cells into the host tissue have to be achieved without side/toxic effects.

Different Sources of Stem Cells in MS Therapy

The "gold standard" cell for cellular therapies has to be plastic. Stem cells can meet this criterion since they are intrinsically able to adapt their specification fate to different environmental needs. For some disease, the differentiation potential of stem cells does not always equate to their therapeutic potential. The therapeutic plasticity of stem cells and progenitors form different sources is a key element for their clinical use in neurological and non neurological disease. However it is really important to consider which is the candidate disease to be addressed by cell therapy and which is the therapeutic function (or functions) of stem cells that is relevant in this disease when choosing the type of stem cells to be used.

Several stem cell based platforms have been proposed over the years for the treatment of MS (Fig. 4.1). Apart from haematopoietic stem cells that have been already used in hundreds of MS patients as immunosuppressive therapy (Mancardi and Saccardi 2008), neural and oligoglial precursor cells (adult, embryonic or derived from induced pluripotent stem cells (iPSCs), non-CNS myelinating cells (Schwan cells, olfactory bulb cells, boundary cap cells), non-CNS stem cells (MSCs form bone marrow, skin, fat, placenta and umbilical cord) have been variably shown to be potentially useful in such a disease. In this chapter we will focus on stem cell types that have been variably proven in preclinical models of MS to be potentially useful as a pro-regenerative treatment.

NeuralStem/Precursor Cells (NPCs)

NPCs are multipotent cells that can be isolated from embryonic, foetal, neonatal or adult CNS tissues and can be expanded and maintained safely in a chemically defined serum-free medium. In cultures with epidermal growth factor (EGF) and fibroblast growth factor (FGF2), NPCs proliferate almost indefinitely as multicellular free-floating spheres (neurospheres) or as an adherent monolayer, and spontaneously differentiate into post-mitotic CNS daughter cells (such as neurons, astrocytes or oligodendrocytes) after growth factor withdrawal.

In vivo experiments, aimed at repairing injured CNS by transplanting NPCs, have shown that these cells might survive to transplantation procedures within the host CNS, maintaining their multipotency and migrating from the site of grafting (Pluchino et al. 2004). At the beginning, NPC transplantation was aimed at *replacing* damaged cells, but the results obtained so far, indicate that this approach represent a rational and realistic therapeutic strategy only for restricted category of neurological disorders and is very much depending on the cell type to be substituted and the intrinsic characteristics of the damaged CNS region (De Feo et al. 2012). As a matter of fact, cell replacement is obtainable via NPC transplantation only in disorders in which degeneration is caused either by intrinsic cellular defects or by extrinsic factors that are no longer active in a specific cell population residing within a discrete CNS area (e.g. Parkinson's disease, genetic demyelination). Contrarily, cell replacement has been only partially successful when NPC transplantation has been performed in a persistently unfavourable environment, i.e. inflammation, where different cell subpopulations in different CNS areas are affected, such as MS.

Irrespective from the characteristics of the experimental disease (e.g. acute vs. chronic, focal vs. multifocal), functional recovery obtained by NPC transplantation scarcely correlates with absolute numbers of terminally differentiated neuronal cells generated by transplanted cells (Kokaia et al. 2012). This suggests that transplanted NPCs might be therapeutic efficacious via a *bystander mechanism(s)* alternative to cell replacement capable of promoting tissue repair by preventing tissue damage, interfering with the pathogenic process, rescuing endogenous neural

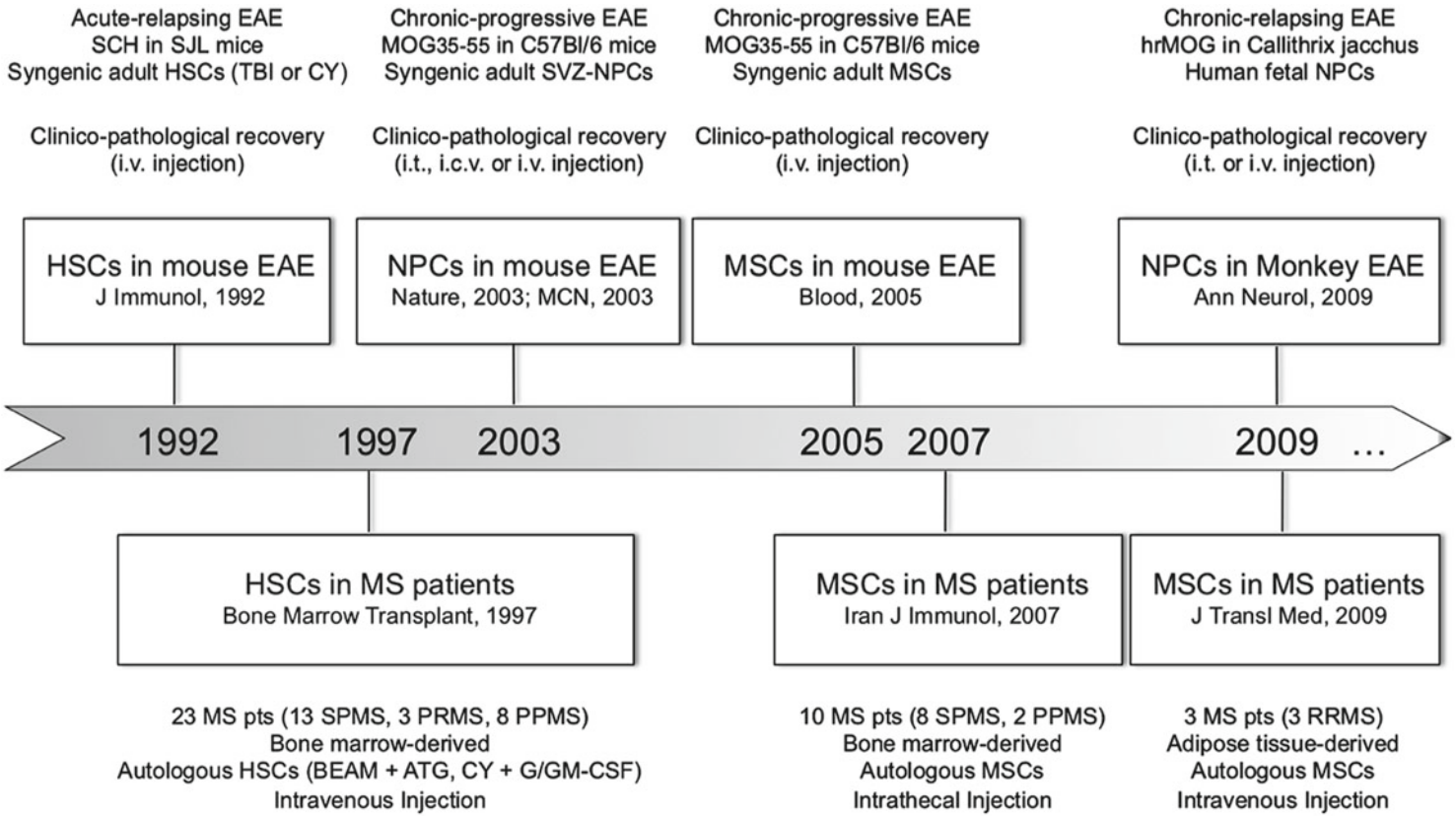

Fig. 4.1 The main steps that paved the way to the clinical application of NPC and MSC based treatments for MS. The first experimental evidence that stem cells could be a useful therapeutic approach in MS comes from transplantation studies in which bone marrow derived stem cells were used as immunosuppressive therapy in EAE mice (Karussis et al. 1992). Soon after, the first trial has been undertaken encompassing the transplantation of bone marrow derived haematopoietic stem cells into patients with MS (Fassas et al. 1997). Nowadays, such immunosuppressive therapy is still considered an experimental therapy but useful in malignant cases of MS. Few years later, in 2003, stem cell based regenerative therapies have been initiated in pre clinical models of MS. The first stem cells to be used were NPCs (Pluchino et al. 2003). Such cells, transplanted intrathecally and intravenously in mice with EAE after disease onset, promoted both clinical and pathological disease amelioration. Positive results obtained in mice have been later on confirmed in non human primates: human foetal brain-derived NPCs have been successfully injected, both intrathecally and intravenously, in a non-human primate model of MS (Pluchino et al. 2009a). Mesmerised by the positive results obtained using NPCs, in 2005 MSCs have been intravenously inject into EAE mice (Zappia et al. 2005). Results have been extremely positive and this was considered sufficient to start safety trials in MS patients using autologous bone marrow derived mesenchymal stem cells (Fassas et al. 1997; Mohyeddin Bonab et al. 2007; Riordan et al. 2009)

cells and orchestrating CNS-resident cells reactive to the pathological injury (mainly astrocytes and microglia) and inflammatory blood-borne cells. This bystander mechanism of action of NPCs can be obtained by systemic (e.g. intravenous, intrathecal) transplantation that allows cells to reach all site of lesion in multifocal disease as MS. Transplanted NPCs can be therapeutically efficacious owing to the ability to follow, via the blood stream or cerebrospinal fluid circulation, a gradient of chemoattractants (e.g. cytokines, chemokines) occurring at the site of inflammatory lesions (Kokaia et al. 2012; Martino and Pluchino 2006). While promoting interaction between transplanted NPCs and activated endothelial and ependymal cells surrounding inflamed CNS tissues, this chemoattractive gradient leads to selective and specific homing of transplanted cells in multifocal inflammatory CNS areas. The exact molecular mechanism sustaining this phenomenon has been detailed in EAE: NPC tethering, rolling, and firm adhesion to inflamed endothelial cells and extravasation into inflamed CNS areas are sequentially mediated by the constitutive expression of functional cell adhesion molecules (e.g. CD44), integrins (e.g. α4, β1) and chemokine receptors (e.g. CCR1, CCR2, CCR5, CXCR3, CXCR4) expressed on NPCs surface (Martino et al. 2011). Upon migration into inflamed CNS areas, transplanted NPCs survive in close proximity to blood vessels where they interact with CNS-infiltrating blood-borne inflammatory cells, endothelial cells and CNS-resident astrocytes and microglia. Within perivascular areas, these latter cell populations secrete a series of molecules (i.e. BMP4, noggin, Notch, Jagged and Sonic Hedgehog) that are capable, on one hand, to recapitulate the microenvironment of the prototypical SVZ germinal niche and, on the other hand, to inhibit the NPC cell cycle. As a consequence of these cellular interactions, transplanted NPCs retain an undifferentiated phenotype forming an atypical ectopic perivascular niche. There, undifferentiated NPCs continue to express and produce a wide array of constitutive transmembrane and secreted molecules that, in turn, promote tissue repair through stimulation of endogenous repair mechanisms (e.g. trophic support, cellular plasticity) and immunomodulation (Martino and Pluchino 2006).

On one hand, NPCs are able to exert a *neuroprotective* effect preventing neuronal programmed cell death and glial scar formation mainly via the paracrine secretion of neurotrophins (e.g. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and glial-derived neurotrophic factor (GDNF), and enhancing the viability of OPCs by a parallel decrease of proastrogliotic factors TGFβ and FGF2 (Pluchino et al. 2003). As a partial confirmation of this mechanism, in toxic-induced demyelination, NPC transplantation induces proliferation and differentiation of OPCs via the secretion of PDGF, FGF-2 (Einstein et al. 2009) and leukaemia inhibitory factor (LIF) (which promote mature oligodendrocyte survival) (Marriott et al. 2008).

On the other hand, undifferentiated transplanted NPCs may promote bystander *immunomodulation* since they can release soluble molecules (e.g. cytokines and chemokines), and express immune-relevant receptors (e.g. chemokine receptors, CAMs), capable of profoundly altering inflammatory environment(s). In particular, systemic transplantation of NPCs inhibit T-cell proliferation and promotes the apoptosis of encephalitogenic CNS-infiltrating T cells either via the expression of death receptor ligands (i.e. FasL, Trail and Apo3L) or the production of soluble mediators (i.e. nitric oxide synthase (iNOS), IFNγ, GDNF, LIF) involved in mitochondrial-mediated apoptosis (Pluchino et al. 2005). Similarly to other stem cell types (i.e. MSCs), NPCs can exert a bystander immunomodulatory effects also outside the CNS upon systemic transplantation. In EAE mice, intravascularly injected NPCs may inhibit the initiation and maintenance of the inflammatory events occurring within the secondary lymphoid organs (Einstein et al. 2007; Pluchino et al. 2009b). In peripheral lymphoid organs from EAE mice, dendritic cell antigen presentation (Pluchino et al. 2009b) and antigen-specific T-cell proliferation (Einstein et al. 2007) are impaired upon intravascular or subcutaneous NPC transplantation. Recently, the peripheral immunomodulatory

effect of NPCs has been further confirmed showing that NPCs inhibit the differentiation of pathogenic Th17 cells through LIF secretion. LIF, on turn, acts through the extracellular signal-regulated MAP kinase–suppressor of cytokine signalling 3 (SOCS3) inhibitory signalling cascade that antagonizes IL-6-mediated phosphorylation of signal transducer and activator of transcription 3 (STAT3), both required for Th17 cell differentiation in peripheral lymphoid organs (Cao et al. 2011).

Whatever is the precise mechanism of action of transplanted NPCs, the evidence accumulated over the years has been considered sufficient to start the first safety human trials with using foetal brain-derived NPCs. So far, such cells have been safely transplanted in patients with Batten Disease, Pelizeus-Merzbacher disease, amyotrophic lateral sclerosis, PD, Huntington disease, spinal cord injury and glioma (See Trounson BMC medicine 2012) (Gupta et al. 2012).

Oligodendrocyte Precursor Cells (OPCs)

Although NPCs remyelinate efficiently when injected directly into areas of demyelination, recent challenging data do indicate that these cells, when delivered by the clinically more attractive intravascular or intrathecal routes into EAE mice, very little differentiate into oligodendrocytes directly engaged in remyelination (Pluchino et al. 2003). Thus, a more efficient 'migratory' cell source of myelinating cells is necessary.

The best-characterised neural progenitor cell displaying remyelinating potential is the OPCs, sometimes also called the $NG2^{+}$ cells. This is a population of stem/precursors cells widely distributed throughout the CNS parenchyma that displays self-renewing and multipotent characteristics so to be regarded also as a type of adult NPCs (Tripathi et al. 2010). Indeed, OPCs are the major source of remyelinating oligodendrocytes in MS although it appears that such cells are not able to contribute efficiently to axon remyelination (Franklin 2002).

Since the first demonstration of successful myelination by grafted CNS tissue containing OPCs (Lachapelle et al. 1983), there has been a substantial literature providing pre-clinical proof-of-principle that OPCs transplanted directly into areas of CNS demyelination are capable of remyelinating demyelinated axons. This has been shown using both non-human and human cells in both focal lesions and in genetic disorders with widespread myelination deficits throughout the neuraxis (Groves et al. 1993; Windrem et al. 2008). Moreover, remyelination associated with OPC transplantation is associated with functional recovery at both electrophysiological and locomotor level. As such, human glial restricted progenitors, isolated on the basis of their expression of the ganglioside A2B5, give rise to extended areas of myelination when grafted within the shiverer mouse brain (Windrem et al. 2004). Moreover, multiple injections of these glial restricted progenitors in the same model, lead to massive colonization of the host brain, increased survival of some of the grafted animals and reduced clinical signs, opening new perspectives of cell therapy for patients affected with leucodystrophies (Windrem et al. 2008).

The translation of these studies in clinical practice has, however, not progressed at the rate many would have predicted a decade ago for several reasons. First, while OPCs migrate and proliferate within injured tissue they are unable to survive and therefore migrate through normal intact adult CNS (Franklin 2002). This means that individual lesions would need to be directly targeted for transplantation, restricting the approach to a handful of the most clinically informative lesions. Second, obtaining large numbers of autologous human OPCs has proven to be less than straightforward. Deriving OPCs from syngeneic human embryonic-like cells remains a possible solution (Wang et al. 2013). Third, there is clearly little benefit to be gained by transplanting OPCs into lesions (such as those that occurs in MS) that already contain abundant cells with the ability to generate new oligodendrocytes. Here, the environment is inhibiting (or fail to promote) differentiation and regeneration, and this should also limit the effect of exogenous

cells. The environmental inhibition could be overcome by modifying the cells before transplantation – for example, to inhibit the activity of any deleterious component of the diseased environment – but in this case it would be more logical to try to manipulate the resident precursor cells (Franklin and Ffrench-Constant 2008).

It should be noted that much of this data is derived from toxin-induced models of demyelination or in animals with genetic abnormalities of myelination, neither of which will accurately reproduce the environments of MS lesions. While these studies have provided a wealth of information of the myelinogenic properties of various cell populations and the factors that govern the efficiency of remyelination, they do not necessarily predict the behaviours of transplanted cells in MS. Moreover an increasing consensus in MS indicates that the mere cell replacement might be not useful in this disease, and the manipulation of the microenvironment to foster endogenous myelin repair could represent a more efficacious approach(Franklin and Ffrench-Constant 2008).

Mesenchymal Stem Cells (MSCs)

MSCs are a heterogeneous mixture of progenitor cells at different stages of differentiation along the mesodermal lineage that can be isolated from almost any connective tissue including bone marrow (first tissue in which they have been identified), adipose tissue, umbilical cord blood and perivascular tissues (Prockop and Oh 2012). Within the bone marrow, MSCs provide the appropriate microenvironment for maturation, differentiation and survival of blood-born cells (Keating 2006). MSC can differentiate into cells of the mesenchymal lineage, such as bone, cartilage and fat but, under certain circumstances, have been reported to acquire the phenotype of cells of the endodermal and neuroectodermal lineage, suggesting some potential for 'transdifferentiation'. However this transdifferentiation potential is still a matter of debate and at present the consensus is that MSCs cannot make cells belonging to lineages other than mesodermal (Rice et al. 2013).

MSCs derived from the bone marrow, after expansion in culture are able to modulate innate and adaptive immunity in a variety of animal models (Prockop and Oh 2012). In particular, MSCs have been shown to modulate many T cell, B cell, NK cell and dendritic cell functions. Inhibition of T cell proliferation by MSCs appears to be subsequent both to cell-to-cell interaction and to release of soluble factors (Uccelli et al. 2008). The intravenous injection of MSCs in diabetic NOD/scid mice resulted in an increased number of pancreatic islets and insulin producing beta cells (Lee et al. 2006) and prevented tissue destruction by dampening T cell attack to the cartilage in an experimental model of rheumatoid arthritis. Recently, intravenous infusion of MSCs following the onset of disease have been shown to ameliorate EAE through the induction of T cell tolerance and the suppression of pathogenic B cell responses in vivo (Zappia et al. 2005). Interestingly, labelled MSCs were detected inside the damaged CNS without any evidence of trans-differentiation into neural cells (Constantin et al. 2009; Zappia et al. 2005). Moreover, a protective effect on damaged tissue was also ascribed to MSCs once transplanted in EAE mice, leading to a decreased neuronal loss: this neural protective effect seems to stem also from the infusion of MSCs in other experimental neurological diseases including spinal cord injury (Akiyama et al. 2002) and stroke (Li et al. 2002). Rather than a true trans-differentiation effect, MSC-supported neuroprotection might possibly occur through the recruitment of local endogenous NPCs and their subsequent induction to differentiate into neural cells (Constantin et al. 2009). Among those indirect mechanisms, it can be postulated that MSCs might affect NPC behaviour via a direct anti-oxidant effect on neural cells (Lanza et al. 2009) and/or via the release of anti-apoptotic molecules (Ohtaki et al. 2008) and neurotrophins (Wilkins et al. 2009). Apart from the above mentioned paracrine effects, it is also clear that MSCs might attenuate the inflammation and demyelination in chronic EAE without significant engraftment in the inflamed CNS, thus suggesting that their beneficial effect is largely

related to their ability to suppress the ongoing autoimmune response (Zappia et al. 2005).

The capacity of MSCs to migrate to sites of inflammation following the gradient of cytokines and to protect damaged tissues, including the CNS, supported their use as an immunosuppressive strategy for immune-mediated diseases such as MS. Thus, MSCs derived from various tissues have been already used in pilot studies for MS treatment (Mohyeddin Bonab et al. 2007) (Fig. 4.1).

Timing and Route of Stem Cell Administration

Despite pre-clinical and clinical data support the use of certain types of stem cells in MS, we still need to understand how to optimize the treatment in order to avoid unwanted side-effects. This is particularly true in diseases such as MS, in which repeated inflammatory insults determine the coexistence of both neurodegenerative and regenerative processes. As recently suggested, treatment optimization might be achieved by tightly controlling the timing and route of cell administration.

An early time-window for NPC and MSC transplantation seems to be the most appropriate approach, in order to target pathogenic inflammation as well as to exploit the expression of genes coding for molecules supporting tissue growth soon after CNS damage (Pluchino et al. 2005; Uccelli and Mancardi 2010). Moreover, an early transplantation setting should be preferred given the recent evidences that immunomodulatory and neurotrophic properties of transplanted NPCs and MSCs are limited in time and tend to be lost with the persistence of the cells in the tissue (Fainstein et al. 2013).

The route of cell administration represents another key issue for stem cell transplantation procedures for CNS diseases. On one hand, the anatomic-pathological features of focal CNS disorders, would suggest that direct intralesional cell transplantation might facilitate tissue regeneration (e.g. neurons of the *substantia nigra* or naked axons within the spinal cord). On the other hand, the multifocality of certain CNS disorders such as MS or Alzheimer's disease, limits per se stem focal cell-based therapies. However, concerning MS, the emergence of leading concepts of therapeutic plasticity of stem cells have shift research from the original cell-replacement-oriented/intralesional approach to alternative transplantation approaches. Some recent experiments have shown that in multifocal inflammatory brain disorders the systemic injection of stem cells into the blood stream or intratechally into the cerebrospinal fluid could overcome these limitations. The specific homing and engraftment after the injection through both routes of administration has been already demonstrated for NPCs (Pluchino et al. 2003) and MSCs (Payne et al. 2013).

Another fundamental issue that has to be taken into consideration regarding the administration route is the safety. Indeed, homotopic transplantation should be preferred to heterotopic ones as recent data indicate that stem cells might form tumours, in response to micro environmental mediated signals. The transplantation of autologous hematopoietic $CD34^+$ stem cells into the renal parenchyma of a patient with lupus nephritis resulted into an angiomyeloproliferative lesion (Thirabanjasak et al. 2010). Intracerebellar and intrathecal injection of poorly characterized human foetal NPCs into a patient with ataxia telangiectasia evolved into a multifocal brain tumour derived from donor cells (Amariglio et al. 2009). Co-transplantation of NPC and pancreatic islet cells under the kidney capsule of diabetic mice induced the generation, at the site of cell transplantation, of insulin-induced neuroblastoma-like neoplasms (Melzi et al. 2010). Moreover, transplantation of genetically engineered MSCs overproducing stem cell factor into mice heart immediately after myocardial infarction promoted the formation of a chest wall-invading soft tissue sarcoma (Fazel et al. 2008).

Selection of Patients for Stem Cell Therapy

Given the variety of stem cell sources, routes of administration and multiple therapeutic effects of stem cells it is important to understand who are the ideal candidate MS patient for cell therapy

and which type of MS could be better addressed by this therapeutic approach. Concerning RR-MS patients, there are several limitations against the application of this therapeutic tool; first, it is unfeasible translating the experimental transplantation setting (at the inflammatory peak of the disease) to the clinical practice since it is impossible to foresee where and when an autoimmune attach would occur into the CNS. Second, it does not make much sense transplanting the cells during the remission phase since cells would not sense the inflammatory stimuli necessary for the homing into the inflamed CNS and would not migrate towards the lesions. Third, several efficient therapeutic options are already available and stem cells transplantation seems to be not advantageous both in terms of costs and time-limited function of cells after the transplantation.

On the contrary, concerning progressive MS patients, cell therapy emerges as a highly attractive therapeutic option. Available therapeutic agents, while acting on the immune component of the MS might have only a marginal effect on progressive MS forms without relapses and no approved efficient treatment is available for these patients. Moreover, they present a uniform progression of disability with a chronic active disease course that may positively respond to the various therapeutic mechanism of action of transplanted stem cells. Between progressive patients, patients with significant disability but sufficient residual function that might be preserved by cell therapy may be the good candidates. In addition to these ethical considerations, this cohort of patients has also the big advantage to be less heterogeneous, making it easier to evaluate the efficacy of treatment.

Nevertheless, without first conducting clinical trials it is difficult to predict which type of MS will benefit from which type of cells.

Future Advances in Stem Cell Transplantation Field

Both NPCs (including OPCs) and MSCs might represent an alternative therapeutic approach in demyelinating disorders of immune mediated origin, as they are intrinsically able to adapt their specification fate to different environmental needs (Kokaia et al. 2012).

Concerning MSC, bone marrow derived stem cells have proven efficacy in animal model and are used in human, in these days, for safety/efficacy trials in humans: the possibility of using autologous cells for treating patients is certainly major benefit. Among NPCs, adult NPCs have limited proliferation capacity during serial passages in vitro and only foetal tissue-derived NPCs might represent, so far, a ready-to-use cell source for cell-based therapies (Galli et al. 2003). While used successfully in pre clinical models of human diseases, there are so far limited amount of data on the use of such cells in humans. However, given the HLA-mismatched origin of these cells, the major hurdle for using these cells in human trials is the necessity to concomitantly adopt immunosuppressive treatments. As a matter fact, the same order of problems would also occur using embryonic stem cell derived NPCs, for which ethical and safety concerns would further complicate their clinical application. The recent discovery of induced pluripotent stem cells (iPSCs) – a new source of pluripotent stem cells recently obtained by genetic reprogramming of somatic cells (e.g., fibroblasts) (Takahashi and Yamanaka 2006) – might overcome this impediment. Indeed, 'autologous' somatic cells of different origin can be reprogrammed into iPSCs by the expression of a combined set of four transcription factors (i.e. Oct4, Sox2, Klf4, and c-Myc) and then differentiated *in vitro* into large quantities of autologous NPCs and/or OPCs. However, few data are so far available on the use of iPSC-derived NPCs in MS. On one hand, only one report has been published so far (Song et al. 2011), showing the generation of iPSCs from one RR-MS patient. These cells were successfully differentiated into mature astrocytes, oligodendrocytes and neurons. On the other hand, while a highly enriched population of human iPSC-derived OPCs was able to robustly myelinate the brain of myelin-deficient shiverer mice (Wang et al. 2013), still few data are available on the therapeutic potential of such cells in immune mediated demyelinating disease

such as MS. Nevertheless, encouraging results have been recently reported showing that mouse iPSC-NPCs, transplanted into the mouse model of MS, might ameliorate the disease course through a bystander effect rather than a cell replacement. Such cells were capable of fostering the endogenous mechanism of repair and promote remyelination via the *in situ* production of LIF (Laterza et al. 2013).

While the expectations from the scientific community are reasonably high owing to the fact that iPSCs represent at the moment the only potential source of large quantities of cells of any lineage, still a lot of work should be done to find the best cell reprogramming method and to assess the safety and therapeutic potentialities of iPSCs before envisaging bench-to-bedside translation (Yamanaka 2009).

In conclusion, given the therapeutic plasticity of stem/precursors cells, alternative stem cell-based approaches for neurological disorders should be seriously considered. The different therapeutic properties of stem cells – the so called 'therapeutic plasticity' (Martino and Pluchino 2006) – could be the major advantage of such approach to foster tissue repair in a very complex and pathologically heterogeneous disease such as MS. However, we still need to confront with unsolved questions regarding the best way to control the *in vivo* fate of transplanted cells and how to enhance one specific mechanism of action compared to the others. The proper choice of candidate patient, the type of stem/precursor cells, the route and timing of cell delivery will be certainly crucial for the clinical success of cell therapy.

References

Akiyama Y, Radtke C, Honmou O, Kocsis JD (2002) Remyelination of the spinal cord following intravenous delivery of bone marrow cells. Glia 39:229–236

Amariglio N, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, Paz N, Koren-Michowitz M, Waldman D et al (2009) Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med 6, e1000029

Cao W, Yang Y, Wang Z, Liu A, Fang L, Wu F, Hong J, Shi Y, Leung S et al (2011) Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural progenitor cell therapy in autoimmune disease. Immunity 35:273–284

Centonze D, Muzio L, Rossi S, Cavasinni F, De Chiara V, Bergami A, Musella A, D'Amelio M, Cavallucci V et al (2009) Inflammation triggers synaptic alteration and degeneration in experimental autoimmune encephalomyelitis. J Neurosci 29:3442–3452

Compston A, Coles A (2008) Multiple sclerosis. Lancet 372:1502–1517

Constantin G, Marconi S, Rossi B, Angiari S, Calderan L, Anghileri E, Gini B, Bach SD, Martinello M et al (2009) Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. Stem Cells 27:2624–2635

De Feo D, Merlini A, Laterza C, Martino G (2012) Neural stem cell transplantation in central nervous system disorders: from cell replacement to neuroprotection. Curr Opin Neurol 25:322–333

Einstein O, Fainstein N, Vaknin I, Mizrachi-Kol R, Reihartz E, Grigoriadis N, Lavon I, Baniyash M, Lassmann H et al (2007) Neural precursors attenuate autoimmune encephalomyelitis by peripheral immunosuppression. Ann Neurol 61:209–218

Einstein O, Friedman-Levi Y, Grigoriadis N, Ben-Hur T (2009) Transplanted neural precursors enhance host brain-derived myelin regeneration. J Neurosci 29:15694–15702

Fainstein N, Einstein O, Cohen ME, Brill L, Lavon I, Ben-Hur T (2013) Time limited immunomodulatory functions of transplanted neural precursor cells. Glia 61:140–149

Fassas A, Anagnostopoulos A, Kazis A, Kapinas K, Sakellari I, Kimiskidis V, Tsompanakou A (1997) Peripheral blood stem cell transplantation in the treatment of progressive multiple sclerosis: first results of a pilot study. Bone Marrow Transplant 20:631–638

Fazel SS, Angoulvant D, Butany J, Weisel RD, Li RK (2008) Mesenchymal stem cells engineered to overexpress stem cell factor improve cardiac function but have malignant potential. J Thorac Cardiovasc Surg 136:1388–1389

Franklin RJ (2002) Why does remyelination fail in multiple sclerosis? Nat Rev Neurosci 3:705–714

Franklin RJ, Ffrench-Constant C (2008) Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci 9:839–855

Galli R, Gritti A, Bonfanti L, Vescovi AL (2003) Neural stem cells: an overview. Circ Res 92:598–608

Groves AK, Barnett SC, Franklin RJ, Crang AJ, Mayer M, Blakemore WF, Noble M (1993) Repair of demyelinated lesions by transplantation of purified O-2A progenitor cells. Nature 362:453–455

Gupta N, Henry RG, Strober J, Kang SM, Lim DA, Bucci M, Caverzasi E, Gaetano L, Mandelli ML et al (2012) Neural stem cell engraftment and myelination in the human brain. Sci Transl Med 4:155ra137

Karussis DM, Slavin S, Lehmann D, Mizrachi-Koll R, Abramsky O, Ben-Nun A (1992) Prevention of experimental autoimmune encephalomyelitis and induction of tolerance with acute immunosuppression followed by syngeneic bone marrow transplantation. J Immunol 148:1693–1698

Keating A (2006) Mesenchymal stromal cells. Curr Opin Hematol 13:419–425

Kokaia Z, Martino G, Schwartz M, Lindvall O (2012) Cross-talk between neural stem cells and immune cells: the key to better brain repair? Nat Neurosci 15:1078–1087

Lachapelle F, Gumpel M, Baulac M, Jacque C, Duc P, Baumann N (1983) Transplantation of CNS fragments into the brain of shiverer mutant mice: extensive myelination by implanted oligodendrocytes. I. Immunohistochemical studies. Dev Neurosci 6:325–334

Lanza C, Morando S, Voci A, Canesi L, Principato MC, Serpero LD, Mancardi G, Uccelli A, Vergani L (2009) Neuroprotective mesenchymal stem cells are endowed with a potent antioxidant effect in vivo. J Neurochem 110:1674–1684

Laterza C, Merlini A, De Feo D, Ruffini F, Menon R, Onorati M, Fredrickx E, Muzio L, Lombardo A et al (2013) iPSC-derived neural precursors exert a neuroprotective role in immune-mediated demyelination via the secretion of LIF. Nat Commun 4:2597

Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, Prockop DJ (2006) Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proc Natl Acad Sci U S A 103:17438–17443

Li Y, Chen J, Chen XG, Wang L, Gautam SC, Xu YX, Katakowski M, Zhang LJ, Lu M et al (2002) Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. Neurology 59:514–523

Mancardi G, Saccardi R (2008) Autologous haematopoietic stem-cell transplantation in multiple sclerosis. Lancet Neurol 7:626–636

Marriott MP, Emery B, Cate HS, Binder MD, Kemper D, Wu Q, Kolbe S, Gordon IR, Wang H et al (2008) Leukemia inhibitory factor signaling modulates both central nervous system demyelination and myelin repair. Glia 56:686–698

Martino G, Pluchino S (2006) The therapeutic potential of neural stem cells. Nature reviews. Neuroscience 7:395–406

Martino G, Franklin RJ, Van Evercooren AB, Kerr DA (2010) Stem cell transplantation in multiple sclerosis: current status and future prospects. Nat Rev Neurol 6:247–255

Martino G, Pluchino S, Bonfanti L, Schwartz M (2011) Brain regeneration in physiology and pathology: the immune signature driving therapeutic plasticity of neural stem cells. Physiol Rev 91:1281–1304

Melzi R, Antonioli B, Mercalli A, Battaglia M, Valle A, Pluchino S, Galli R, Sordi V, Bosi E et al (2010) Co-graft of allogeneic immune regulatory neural stem cells (NPC) and pancreatic islets mediates tolerance, while inducing NPC-derived tumors in mice. PLoS One 5, e10357

Mi S, Pepinsky RB, Cadavid D (2013) Blocking LINGO-1 as a therapy to promote CNS repair: from concept to the clinic. CNS Drugs 27:493–503

Mohyeddin Bonab M, Yazdanbakhsh S, Lotfi J, Alimoghaddom K, Talebian F, Hooshmand F, Ghavamzadeh A, Nikbin B (2007) Does mesenchymal stem cell therapy help multiple sclerosis patients? Report of a pilot study. Iran J Immunol 4:50–57

Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG (2000) Multiple sclerosis. N Engl J Med 343:938–952

Ohtaki H, Ylostalo JH, Foraker JE, Robinson AP, Reger RL, Shioda S, Prockop DJ (2008) Stem/progenitor cells from bone marrow decrease neuronal death in global ischemia by modulation of inflammatory/immune responses. Proc Natl Acad Sci U S A 105:14638–14643

Payne NL, Sun G, McDonald C, Layton D, Moussa L, Emerson-Webber A, Veron N, Siatskas C, Herszfeld D et al (2013) Distinct immunomodulatory and migratory mechanisms underpin the therapeutic potential of human mesenchymal stem cells in autoimmune demyelination. Cell Transplant 22:1409–1425

Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G, Galli R, Del Carro U, Amadio S et al (2003) Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 422:688–694

Pluchino S, Furlan R, Martino G (2004) Cell-based remyelinating therapies in multiple sclerosis: evidence from experimental studies. Curr Opin Neurol 17:247–255

Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A et al (2005) Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature 436:266–271

Pluchino S, Gritti A, Blezer E, Amadio S, Brambilla E, Borsellino G, Cossetti C, Del Carro U, Comi G et al (2009a) Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates. Ann Neurol 66:343–354

Pluchino S, Zanotti L, Brambilla E, Rovere-Querini P, Capobianco A, Alfaro-Cervello C, Salani G, Cossetti C, Borsellino G et al (2009b) Immune regulatory neural stem/precursor cells protect from central nervous system autoimmunity by restraining dendritic cell function. PLoS One 4, e5959

Prockop DJ, Oh JY (2012) Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol Ther 20:14–20

Rice CM, Kemp K, Wilkins A, Scolding NJ (2013) Cell therapy for multiple sclerosis: an evolving concept with implications for other neurodegenerative diseases. Lancet 382:1204–1213

Riordan NH, Ichim TE, Min WP, Wang H, Solano F, Lara F, Alfaro M, Rodriguez JP, Harman RJ et al (2009)

Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. J Transl Med 7:29

Song B, Sun G, Herszfeld D, Sylvain A, Campanale NV, Hirst CE, Caine S, Parkington HC, Tonta MA et al (2011) Neural differentiation of patient specific iPS cells as a novel approach to study the pathophysiology of multiple sclerosis. Stem Cell Res 8:259–273

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Thirabanjasak D, Tantiwongse K, Thorner PS (2010) Angiomyeloproliferative lesions following autologous stem cell therapy. J Am Soc Nephrol 21:1218–1222

Tripathi RB, Rivers LE, Young KM, Jamen F, Richardson WD (2010) NG2 glia generate new oligodendrocytes but few astrocytes in a murine experimental autoimmune encephalomyelitis model of demyelinating disease. J Neurosci 30:16383–16390

Uccelli A, Mancardi G (2010) Stem cell transplantation in multiple sclerosis. Curr Opin Neurol 23:218–225

Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726–736

Wang S, Bates J, Li X, Schanz S, Chandler-Militello D, Levine C, Maherali N, Studer L, Hochedlinger K et al (2013) Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. Cell Stem Cell 12:252–264

Wilkins A, Kemp K, Ginty M, Hares K, Mallam E, Scolding N (2009) Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. Stem Cell Res 3:63–70

Windrem MS, Nunes MC, Rashbaum WK, Schwartz TH, Goodman RA, McKhann G 2nd, Roy NS, Goldman SA (2004) Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. Nat Med 10:93–97

Windrem MS, Schanz SJ, Guo M, Tian GF, Washco V, Stanwood N, Rasband M, Roy NS, Nedergaard M et al (2008) Neonatal chimerization with human glial progenitor cells can both remyelinate and rescue the otherwise lethally hypomyelinated shiverer mouse. Cell Stem Cell 2:553–565

Yamanaka S (2009) A fresh look at iPS cells. Cell 137:13–17

Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F et al (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 106:1755–1761

5 Locomotor Recovery After Spinal Cord Transection: Transplantation of Oligodendrocytes and Motoneuron Progenitors from Human Embryonic Stem Cells

Nimer Adeeb, R. Shan Tubbs, Aman Deep, and Martin M. Mortazavi

Contents

Abstract

During the last few years, human embryonic stem cells have begun to take a place in the stem cell therapy panorama, especially in respect to the nervous system. The extensive experimental research efforts have focused on translating in vitro cellular regeneration to in vivo transplantation and survival of the transplants, in order to improve clinical outcomes. For spinal cord injury recovery, two major types of cells are in focus: the oligodendrocytes and motor neurons. In this chapter, we will discuss the progressive development of the cellular generation protocols and the locomotor outcome of their transplantation at sites on spinal cord injury. The newly advanced method of motor neurons and oligodendrocytes generation form human induced pluripotent stem cells will be also discussed.

Introduction

In 1908, the Russian histologist Alexander Maksimov proposed that all the blood cells and the process of hematopoiesis are derived from hematopoietic stem cells. Fifty-five years later, in 1963, the Canadian scientists James E. Till and Ernest A. McCulloch demonstrated the presence

N. Adeeb • A. Deep • M.M. Mortazavi
California Neurosurgical Institute,
Thousand Oaks, CA 91361, USA

R.S. Tubbs (✉)
Pediatric Neurosurgery, Children's of Alabama,
Birmingham, AL 35233, USA
e-mail: Shane.tubbs@chsys.org

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of these stem cells for the first time in mice bone marrow. Since then, research on the characteristics and therapeutic applications of these cells has initiated a new era of medicine.

Stem Cells

The presence of more than 200 types of cells in the human body gives a clue of the high differentiation potential of the stem cells. Stem cells are non-differentiated cells that have the capability of proliferation, self-renewal, formation of large numbers of different types of cells, and regeneration of the already differentiated tissues. The potency of the stem cells can be determined depending on how many types of differentiated cells, and of what germ layers, the stem cells are capable to produce. This is defined as the 'differentiation potential'. The totipotential stem cells constitute the first line of cells during fetal development following fertilization, and may give rise to extraembryonic and embryonic cells. Following the separation of inner and outer cell masses, the cells of the inner cell mass (embryonic cells) are defined as pluripotential stem cells, and may give rise to any type of cells from the yet-to-be formed three germ layers: ectoderm, mesoderm, and endoderm. When these layers are separated, cells of each layer are classified as multipotent, oligopotent, and unipotent, depending on their differentiation potentials (Cherian et al. 2011).

Of the above mentioned types of the stem cells, two have received most attention in regard to stem cells therapy: embryonic stem cells (ESC) and adult stem cells (ASC). The ESC are pluripotent cells that present during early stages of human development, and have a high proliferative capacity, and may differentiate into any type of cells. After the formation of the tissues and organs, most of them retain a population of stem cells during childhood and adulthood. These cells are defined as the ASC, which, under normal conditions, give rise to that specific line of cells of the retaining organ. However, other tissues, for unknown reason, including brain, spinal cord, heart, and kidneys, with minor exceptions, do not maintain their stem cells, which limit the use of ASC as a therapeutic choice in these tissues (Kiessling 2003). Recently, novel studies were capable of reprogramming these somatic cells into pluripotent stem cells, known as induced pluripotent stem cells (iPSC), as is discussed later in this chapter.

More than two decades of intense research on mouse ESC has provided insight into human ESC (hESC) research despite the differences between the two types of cells (Kang et al. 2007). They have also provided the proper methods of differentiating mouse ESC into several clinically relevant neural and non-neural cell types (Keirstead et al. 2005). In 1998, Thomson et al. were the first to isolate the hESC, using 14 inner cell masses of in vitro fertilization (IVF)-produced embryos as a source. Since then, the blastocysts of IVF- embryos constituted the major source of hESC. Other sources of ESC include nuclear transfer and therapeutic cloning. The former is achieved by transferring the nucleus of an adult differentiated egg into an enucleated egg, which is then stimulated to form blastocysts in a backward fashion. Therapeutic cloning is so called due to use of cloning to create ESC for therapeutic purposes (Cherian et al. 2011). Nevertheless, the directed generation and isolation to purity of specific clinically important neuronal phenotypes from human ES cells has yet to be accomplished.

A comparison between experimental generation of oligodendrocytes and motor neurons is summarized in Table 5.1.

hESC as a Source of Oligodendrocytes

Oligodendrocytes (OL) are one type of glial cells that provide support to the central nervous system (CNS), mainly by the formation of the myelin sheath. They extend into high numbers of branches and sub-branches expanding into sheets of myelin membranes that wrap around multiple neural axons. This myelin sheath facilitates the rapid salutatory conduction and insulation of the nerve cells (Hatch et al. 2007; Izrael et al. 2007).

Table 5.1 Characteristics and comparison between oligodendrocytes and motor neurons linages

	Oligodendrocytes linage	Motor neurons linage
First use	Nistor et al. (2005)	Li et al. (2005)
Inducers	RA, Noggin, SHH, AA, EGF, PDGF, FGF, CNTF, IGF, HGF, T3, puromorphamine	RA, Noggin, SHH, puromorphamine, SAGA, dorsomorphin, BDNF, CNTF, GDNF, FGF, IGF1, AA, cAMP, NT-3, ROCK
Inhibitors	BMP	BMP
Expressed genetic markers	Sox8, Sox9, Sox10, Olig1, Olig2, Nkx2.2, Nkx6.2, A2B5, NG2, PDGF-R, PLP, Ngn3, Gli1, Gli2, OMG, MBP, GalC, RIP, O4, O1	Pax6, Sox1, Sox2, Sox3, Nestin, Otx2, NG2, HOXB1, HOXB4, HOXB6, HOXC5, HOXC8, HOXC10, HLBX9, Olig2, Nkx2.2, Nkx6.1, Irx3, GFP, HB9, Islet1, islet2, ChAT, MAP2, β III-tubulin, Musashi1, PTCH, Tuj1
Secreted factors	TGF-β1, TGF-β2, activin A, VEGF, BDNF, midkine, SCF	–
Integration and maturation after transplantation	Yes, at early and late stages	Yes
Locomotor improvement	Yes, only at early stage	Yes
Studies on humans	No	No

However, this is not the limit of their function, which far exceeds their insulating role and extends to promoting neuronal and axonal survival by secreting different types of neurotrophic factors. Loss of the OL function and consequent demyelination and axonal degeneration can be seen in a number of neurological pathologies, including multiple sclerosis, Pelizaeus-Merzbacher, Alzheimer's disease, stroke, progressive multifocal leukoencephalopathy, multisystem degeneration, oligodendrogliomas, and spinal cord injury (Keirstead et al. 2005; Nistor et al. 2005).

Oligodendrocytes in Spinal Cord Injury

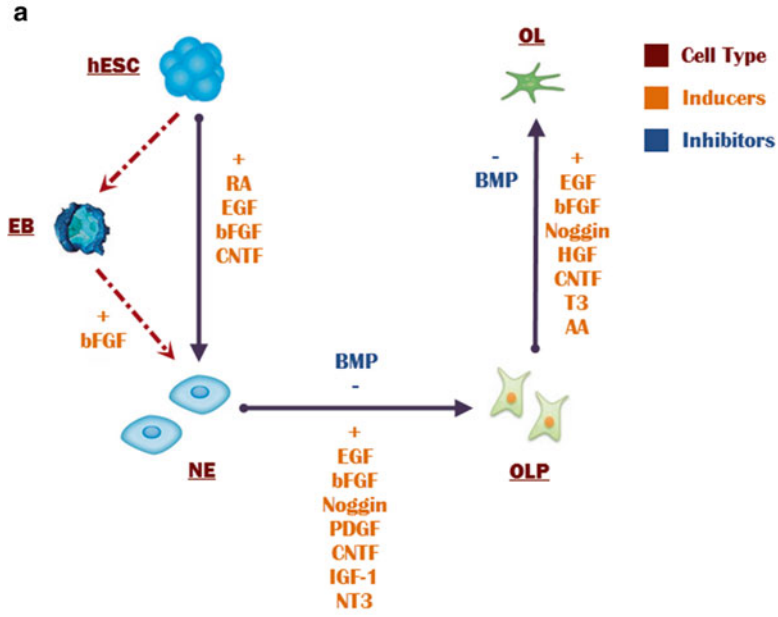
Following spinal cord injury (SCI), loss of OL and oligodendrocytes progenitor cells (OPC) occur via two main processes that are timed during the initial insult and the secondary degenerative changes: acute necrosis and subacute apoptosis. Autophagy may also occur for at least 21 days following injury. The acute necrotic phase starts as early as minutes to few hours after injury, and the subacute apoptotic phase extends up to weeks. During the early phase of injury, vascular rupture leads to release of toxic digestive proteolytic enzymes. Blood components may also induce apoptosis and necrosis of OPC, and inhibit their proliferation and migration. Other factors contributing to the acute injury include the free radical (reactive oxygen and nitrogen species) formation following ischemia and reperfusion, and excitotoxicity. The latter is mainly attributed to the release of glutamate and adenosine triphosphate (ATP) at the site of injury, which in turn activate the glutamate and P2X7 receptors, respectively. These receptors attract the OL and OPC to the site of injury and cause further cellular loss. They also lead to intracellular calcium release, which can trigger cellular apoptosis. Infiltration of the injury site by the early and late phase immune cells, including neutrophils, monocytes and their derived macrophages and microglia, and lymphocutes, and their various types of secreted inflammatory mediators lead to direct lyses and apoptosis of the OL and OPC, and inhibit their growth and proliferation. On the long term, Wallerian degeneration of the nerve axons induces sustained apoptosis of the OL, which is supported by the trophic factor released from these axons. Few weeks after injury, the above mentioned mechanisms lead to detected wide-spread demyelination of the nerve axons, and may continue to progress for 1–22 years following the injury. However, wide-range demyelination is prevented by concomitant remyelination, which may also start few weeks after the

insult. Although remyelination is not perfect, it is sufficient to preserve function of spared intact axons, and maintain their integrity. This acute phase remyelination is mediated by the proliferating myelogenic progenitor cells that present at the margins of injury, and identified by the expression of nerve/glial antigen 2 (NG2) or platelet derived growth factor receptor (PDGFR). Mature form of the OPC has less capacity to remyelinate, and needs prolonged exposure to growth factors to convert them into proliferating cells. The specification of OL from the progenitor cells is induced by the sonic hedgehog (SHH) and opposed by the bone morphogenetic factor (BMP). Both SHH and BMP are up-regulated at the site of injury. The presence of the astrocytes, often produced by the proliferating OPC, is essential. They play a role in maintaining the survival, proliferation and differentiation of the OPC and OL by secreting different typed of growth factors. These factors include PDGF, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and ciliary neurotrophic factor (CNTF). Other less important factors include neurotrophin (NT-3), brain-derived neurotrophic factor (BDNF), chemokines CXCL1, CXCL12, and CXCR4, and cytokines IL-1β, Leukemia inhibitory factor (LIF), transforming growth factor (TGF) β1. The increased expression of thyroid hormone receptor, the nuclear receptors peroxisome proliferator activated receptor (PPAR)-δ, and retinoic acid receptor also play a significant role in the OL differentiation. Most of these factors are secreted during the early phase of spinal cord injury. Thus, over long time, the capability of the OL and their progenitors decreases progressively in terms of maintaining the axonal function. Moreover, despite the essential early role of the astrocytes, at the 2nd to 4 weeks of injury, they begin to form a dense astrocytic scar surrounding the demyelinating axons, which prevent the OL and OPC from reaching the site of injury. They may also express other molecules (e.g. Jagged1) that inhibit maturation and differentiation of the OL and OPC. For these reasons, in addition to younger age, early treatment of spinal cord injury is significant in enhancing the locomotor function. This period is called the 'therapeutic window', and extends from the acute inflammation to before the scar formation (Faulkner and Keirstead 2005; Sharp and Keirstead 2007; Almad et al. 2011). Thus, one of the promising treatment options in the SCI is the OL and OPC transplantation, as replacement of the non-functioning cells may provide an ideal solution to preserve the axonal function and suppress their progressive loss.

Experimental Generation of Oligodendrocytes from hESC

The first step in OL transplantation is to isolate a pure, functional lineage of these cells. Sources of human OL may include aborted fetuses, olfactory biopsies of the neuroepithelium, and differentiated hESC. The latter may provide the ideal source due to its high proliferation and differentiation potentials (Sundberg et al. 2010). The major challenge in this situation is the ability to produce high purity differentiated OL and OPC from directed differentiation of hESC, and reduced the of teratoma formation. The process of selective proliferation and differentiation of the hESC into fully mature OL passes through neural embryoid body (EB) cells, neural progenitor (NP) or neuroectodermal (NE) cells, glial-restricted precursor (GP) cells, and OPC formation (Fig. 5.1a).

In 2005, Nistor et al., Keirstead et al., and Faulkener et al. were the pioneers of this field, and were able to direct the hESC differentiation into high purity and functioning OL and OPC through yellow neurosphere or NE formation. They used a modified retinoic acid (RA) caudalizing protocol to induce restricted differentiation to a multipotent neural lineage, which could generate neurons, OL, and astrocytes by employing RA exposure, along with preferential selection of OL lineage cells by media components. The used glial restriction media (GRM) contained insulin and IGF, differentiation factor triiodothyroidin hormone, FGF, and epidermal growth factor (EGF), and it played important role in the proliferation and survival of the OL (Nistor et al. 2005). Their method generated about 80 %



b **OL Linage Markers**

hESC	NE	OLP	OL
SSEA4	Pax6	Olig1	PLP
OCT4	Nestin	Olig2	O1
TRA-1-60	Mash1	SOX8	O4
TRA-1-81	OPC	SOX9	MBP
		SOX10	GalC
		A2B5	RIP
		NG2	
		OMG	
		PDGF-R	
		Nkx2.2	
		Nkx6.2	
		Gli1	
		Gli2	

Fig. 5.1 (**a**) Generation of oligodendrocytes from human embryonic stem cells. *hESC* human embryonic stem cells, *EB* embryoid bodies, *NE* neuroepithelial cells, *OPC* oligodendrocytes progenitors, *OL* oligodendrocytes. (**b**) Cellular markers of the oligodendrocytes linage. *hESC* human embryonic stem cells, *NE* neuroepithelial cells, *OPC* oligodendrocytes progenitors, *OL* oligodendrocytes

population of OL capable of myelin formation in vivo, but no highly branched, ramified and mature OL were reported in vitro. Markers of the OL differentiation included Olig1, SOX10, A2B5, NG2, and PDGFR.

In 2007, Izrael et al. added Noggin, antagonist of BMP, following treatment with RA. The addition of Noggin turned out to be the key for the development of highly branched and mature OL in vitro, which also significantly enhanced their capacity to myelinate after transplantation. The RA functions through stimulation of the Nkx2.2 gene that is required for terminal differentiation of OL. However, for proper function, the Nkx2.2 needs the Sox10 gene activation, which is also an OL-specific gene, and is induced by the addition of Noggin at specific stages of development (Izrael et al. 2007). Olig2 transcription factor gene induction is also an important step in the differentiation of human OL and their

progenitors. Evidences of such role of Olig2 were provided following its induction using sonic hedgehog (SHH) (Hu et al. 2009a) and inhibition using BMP (Izrael et al. 2007). In 2007, Kang et al. used the growth factors EGF and PDGF to induce the formation of 81–91 % OPC from neural precursors after their isolation and expansion from the hESC using specific media. These progenitors were then treated with the removal of the growth factors and the addition of the thyroid hormone T3 to generate mature OL. The formed mature OL represented 81 % of the total cells number.

Hu et al. (2009b) described a simpler method that included the removal the G5 supplement media (containing insulin, transferrin, selenite, biotin, hydrocortisone, FGF, and EGF), which was applied for a certain period of time, and the addition of the hepatocyte growth factor (HGF) to enhances the proliferation of neural progenitors derived from hESC and promote the generation and maturation of OL. This method yielded OL with high purity (about 80 %).

In 2010, Sundberg et al. introduced a novel method for the generation of the OL from hESC using human recombinant growth factors and extracellular matrix (ECM) proteins. This was in contrast to the previous protocols that employed animal-derived (Matrigel) media, which is less suitable for clinical applications in humans. Their ECM media contained laminin, collagen IV, and nidogen-1 that together facilitate the OPC survival, maturation, and myelination. The growth factors used included; FGF, EGF, and CNTF for the initial neural differentiation; and PDGF-AA, EGF, bFGF, CNTF, and IGF-1 to enhance the survival, proliferation, and differentiation of the OL and their precursors. At the last stage of the cellular maturation, the CNTF, ascorbic acid (AA), and T3 were added. All these growth factors were associated, in variant degrees, with the expression of large number of genes, including PDGF-R, NG2, Nkx2.2, Sox10, Olig1/2, myelin basic protein (MBP), proteolipid protein (PLP), Ngn3, Sox9, Sox8, Sox10, Gli1, Gli2, Nkx6.2, oligodendrocyte-myelin glycoprotein (OMG), O4, and GalC. The expression of these genes is dependent on the stage of development and cellular differentiation from hESC to fully mature OL (Sundberg et al. 2010) (Fig. 5.1b).

Important regulators of the genes expression during these stages are the MicroRNAs. These molecules are known as the "micromanagers" of gene expression, and they function by binding to the mRNA of protein coding genes. Full profile of the MicroRNA of human OL formation has been described for the first time by Letzen et al. in 2010. Identification of these molecules may provide key markers of OL maturation (Letzen et al. 2010).

In 2006, Zhang et al. studied the ability of the OPC derived from hESC to secret neurotrophic factors. Of all the genes tested, 49 growth factors were expressed by OPC at highly significant levels. Of these factors, TGF-β1, TGF-β2, activin A, vascular endothelial (VEGF), BDNF, midkine, and stem cell factor (SCF) proteins were of particular interests. These factors were found to play a remarkable role in neural regeneration and function restoration (Zhang et al. 2006).

hESC as a Source of Motor Neurons

Motor neurons (MN) are large nerve cells with extensive dendritic extension that are located in specific areas in the nervous system, including the brain cortex (upper motor neuron), brainstem, and spinal cord (lower motor neuron). By their specific location, the MN form connecting links between the CNS and skeletal muscles, and facilitate movement and breathing (Nizzardo et al. 2010; Takazawa et al. 2012).

Motor neuron diseases (MND) are group of neurodegenerative diseases that selectively affect the MN with consequent loss of function and motor paralysis. The MND can be divided into three categories: the first form involves both upper and lower MN, such as amyotrophic lateral sclerosis, which is the most common MND. The second form involves the lower MN, and includes spinal muscular atrophy and spinobulbar muscular atrophy or Kennedy's disease; hereditary motor neuropathies and progressive spinal muscular atrophy. The third form involves the upper MN and includes primary lateral sclerosis and

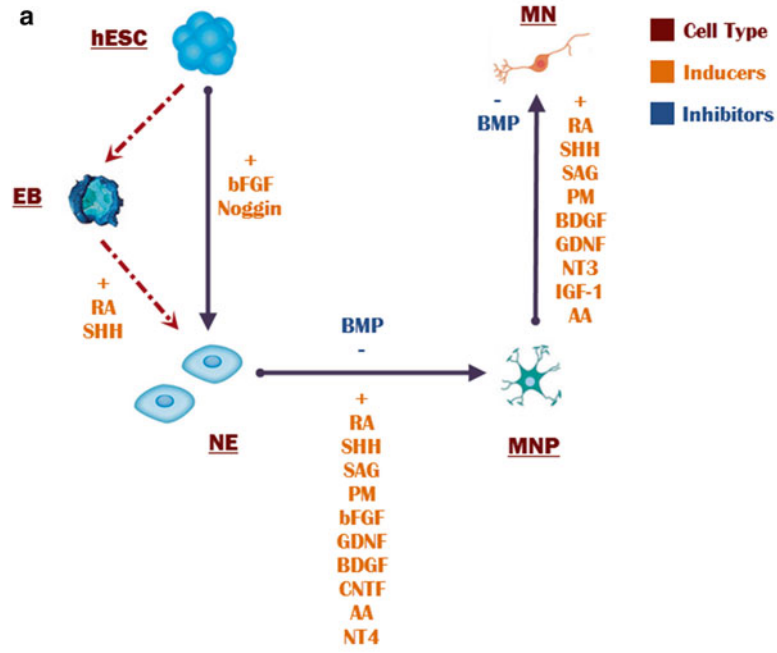
hereditary spastic paraplegia (Nizzardo et al. 2010). Beside these degenerative disorders, trauma (e.g. SCI) with its associated direct and delayed axonal loss remains one of the most common causes of permanent disability (Lopez-Gonzalez and Velasco 2012). Up to date, no effective therapy has been found to treat these conditions. However, because the degeneration of the MN is the main pathology in these conditions, their replacement has been proposed as a therapeutic potential. One major source for these cells is the hESC.

Experimental Generation of Motor Neurons from hESC

The process of MN derivation from the hESC can be achieved using different protocols. As in the case with the OL, the first step is the proper obtainment of the NE cell form the pluripotent hESC. This can be achieved either directly or with and intermediate process of EB formation. Then, these NE are used as a source of the motor neurons progenitors (MNP) and MN (Fig. 5.2a).

The first experiential generation of MN from hESC was done Li et al. (2005). After culturing the hESC in a feeder layer, differentiation into NE cells was induced using neural induction medium consisting of F12/DMEM, N2 supplement and heparin with or without bFGF. Two stages of NE cells could be identified: early and late stages. The early stage could be identified with the expression of Pax6 transcription factor gene but not Sox1. Thus, Pax6 is the earliest NE marker expressed during neural plate and tube formation. Both stages expressed Nestin. Even in the late stage, the NE cells were also positive for Otx2, a homeodomain protein expressed by forebrain and midbrain cells, but negative for HoxC8, a homeodomain protein produced by cells in the spinal cord, and engrailed 1 (En1), which is produced by midbrain. Thus, more selective differentiation was needed to produce caudal cells. Treating the NE cells, at early stage, with RA or bFGF was associated with caudalization of the NE cells. BFGF also induced rostral cells formation. The caudalization of the cells was marked by the expression of HOXB1, HOXB6, HOXC5, HOXC8, and HOXC10. All of these markers were induced by bFGF, whereas, HOXC10 was not induced by RA. BFGF, in contrast to RA, did not eliminate the expression of Otx2. Following caudalization, inducing ventral neural cell types was required for MNP formation. This was achieved by treating the culture with SHH, and was evident by the balanced expression of Class II (Olig2, Nkx2.2, Nkx6.1) and Class I (Irx3, Pax6) homeodomains. Continued exposure to bFGF suppressed the expression of SHH, class I and class II genes, and subsequently inhibited MN differentiation. The use of neuronal differentiation medium was needed at the differentiation stage. It consisted of the neurobasal medium, N2 supplement, and cAMP in the presence of RA and SHH for 1 week. Following the appearance of Olig2-MNP, BDNF, glial-derived neurotrophic factor (GDNF), IGF1 and a low concentration of SHH were added to the medium. The differentiated MN were characterized by the expression of HB9, Islet1/2, choline acetyltransferase (ChAT), MAP2, and β III-tubulin (Fig. 5.2b). The authors concluded that the cells with combined expression of Islet1/2 and HB9 are likely MN, whereas the cells with negative HB9 and positive Islet1/2 are likely interneurons. The differentiated cells represented almost 20 % of the culture, and the identity of the remaining 80 % of the cells remained unknown. The duration of the process ranged from 28 to 35 days.

In 2005, Shin et al. used the mouse feeder layer for hESC culture, followed by the DMEM/F12 medium with N2, L-glutamine, penicillin, streptomycin, and bFGF. Following the removal of the feeder layer, the formed NE were suspended in neurobasal medium supplemented with L-glutamine, penicillin, streptomycin, B27, bFGF, and LIF. Differentiated NE cells were characterized by the expression of Nestin, Musashi1, or SOX 1, 2, and 3. Later on, exposure of the culture to SHH and RA, along with bFGF favored the differentiation into MN. The effect of SHH on the NE could be predicted by the expression of PTCH, the SHH receptor. However, it does not necessitate differentiation into MN. The effect of the bFGF was found to increase the MNP Olig2 gene expression,



b

MN Linage Markers

hESC	NE	MNP	MN
SSEA4	Pax6	β III-tubulin	β III-tubulin
OCT4	Sox1	MAP2	ChAT
TRA-1-60	Nestin	Islet1	HB9
TRA-1-81	Otx2	Tuj1	GFP
	BF1	HB9	Islet1
	PITCH	Lim3	Tuj1
		HOXB1	
		HOXB4	
		HOXB6	
		HOXC5	
		HOXC6	
		HOXC8	
		HLBX9	
		Olig2	
		Nkx2.2	
		Nkx6.1	

Fig. 5.2 (**a**) Generation of motor neurons from human embryonic stem cells. *hESC* human embryonic stem cells, *EB* embryoid bodies, *NE* neuroepithelial cells, *MNP* motor neurons progenitors, *MN* motor neurons. (**b**) Cellular markers of the motor neurons linage. *hESC* human embryonic stem cells, *NE* neuroepithelial cells, *MNP* motor neurons progenitors, *MN* motor neurons

which is further increased by combination of the three factors. Their combination also significantly increased the expression of HLBX9 gene. However, some of the cultured NE gave rise to MN even without SHH and RA exposure. This might be due to continuous exposure to low level of bFGF, but the exact mechanism was not defined. To detect the presence of MN within the culture, their phenotype markers were examined. These markers included Islet1, Tuj1, and ChAT, and based on this criterion, 20–30 % of the cultures were MN. The duration of the process was around 53 days.

Few months later, Singh Roy et al. (2005) induced differentiation of the hESC to MN through EB formation. The EB formation was induced using the mouse embryonic feeder cells and treated with collagenase type IV. It was also fed with the KO-medium (KO-DMEM supplemented with 20 % KO-Serum replacement) and bFGF. The EB were then treated with RA and SHH until the expression of neuronal marker β III-Tubulin. Then, the MN differentiation was induced by the use of DMEM/F12 media supplemented with N2, GDNF, BDNF or NT4, CNTF, B27, and fetal bovine serum (FBS), and with continuous application of RA and SHH. The differentiated MN were identified by the expression of Islet1, HB9, and ChAT genes. In their experiment, 37 % of β III-Tubulin cells were HB9 positive. The co-expression of the Green Florescent Protein (GFP) and the Hb9 gene in the differentiating MN allowed the use of fluorescence-activated cell sorting (FACS) for isolation of the MN from the culture to purity of more than 99 %. Their method, although faster to conduct and is associated with higher purity final selection, it resulted in less initial proportions of induced MN, and losing 76 % of all potential MN in the target population.

In 2007, Lee et al. used the F12/DMEM, N2 supplement medium to induce the neural differentiation of the hESC co-cultured with MS5 stromal cells, and with the addition of Noggin. The formation of the neural rosette was evident by the expression of Pax6 and Sox1, and, later, BF1 and Otx2, which are associated with anterior neural identity. Gbx2 and HoxB4, which marks the posterior neural identity, were not expressed during this default conditions. Isolated neural rosettes were then re-plated on polyornithine/laminin coated culture dishes and N2 medium supplemented with Noggin, AA, and BDNF in the presence of RA and SHH. The addition of RA and SHH was associated with induced caudalization and ventralization of the cells, respectively. This was marked by BF1 suppression and HOXB4 and HOXC8 (caudal identity markers), and Nkx6.1 and Nkx2.2 (ventral identity genes characteristic of the caudal CNS) up-regulation. With additional culture under the presence of RA and SSH, genetic expression indicated the formation of MNP (Nkx6.1, Olig2), early postmitotic motor neurons (NG2, Isl1), and more mature MN (ChAT and vesicular acetylcholine transporter). Many of the cells also expressed somatic MN marker HB9. In the third step, further differentiation was induced in the same medium with the absence of RA and SHH, but in the presence of GDNF, BDNF, and AA. This lead to expression of more mature MN markers, including ChAT, the gene required for Ach synthesis. Co-expression of ChAT and HB9 confirmed MN identity of the hESC progeny, and the additional expression of Lhx3 suggested MN of the medial motor column. According to the authors, a single hESC plated at day 0 on MS5 for neural induction yielded approximately 100 HB9 MN at day 50 with almost 20 % efficiency.

In 2008, Li et al. induced the NE cells differentiation using the DMEM/F12 medium supplemented with N2, Heparin, and cAMP. RA and SHH were then added to the culture. Purmorphamine was occasionally used to replace the variable activity and high cost of SHH, with the same level of action. In contrast to the first protocol, exposure to SHH was maintained during the MNP Olig2-cells phase. This exposure increased proliferation of these cells, but if sustained until the differentiation phase it will suppress MN formation and induce OL formation. Thus, after maximal proliferation of the Olig2-cells, the SHH may be reduced. BDNF, GDNF, and IGF1 were also added to the culture. The differentiated MN produced this way represented more than 96 % of the total hESC-differentiated progenies. Which is the largest percentage produced so far.

In 2009, Wada et al. used a 1:1 mix of DMEM/F12 supplemented with N2 and Neurobasal medium supplemented with B27, with addition of Noggin or Dorsomorphin, to induce hESC differentiation. Following the neural rosette formation, differentiation into MNP and MN was induced by the addition of RA and either SHH or SAGA. Higher concentration of RA had no effect on raising the MN cell numbers. The addition of RA and SHH was also associated with caudalization of the cells, marked by increased expression

of HOXB4 and the suppression of the BF1. However, no significant difference in the expression level of β III-tubulin was noticed, suggesting no difference in neurogenesis itself. Similar results were obtained using SAGA (SHH agonist) as a substitute to SHH. For further maturation, the culture was supplemented with BDNF, GDNF, and NT-3. These mature MN expressed both HB9 and ChAT gene markers. The authors proclaimed that the use of the FGF neurospheres following the first stage of NE cells formation and before the second stage (neural rosette) may expand the numbers of the NE cells up to more than 30-fold while preserving the potency of motor neuron differentiation. They also added that this effect was more significant than using a EGF/FGF neurosphere. That is due to the EGF effect on suppressing the caudalization of the cells. Their last step included purification of the MN from the culture by gradient centrifugation, which raised the purity of the isolated MN from 30 to 80 %.

In 2012, Takazawa et al. induced the EB differentiation from hESC using the DMEM:F12 medium with 20 % Knockout Serum Replacer (KSR), betamercaptoethanol (BME), L-Glutamine and non essential Amino Acids (NEAA). Rho-associated kinase (ROCK) inhibitor, Noggin, and bFGF were added to the culture. The formed EB were then re-suspended on DMEM F:12 medium with N2 supplement, NEAA, L-Glutamine, Heparin, bFGF, Noggin, and ROCK inhibitor. The ROCK inhibitor, and Noggin and bFGF were discontinued at days 5 and 10, respectively, and a diluted Wnt3a-L-cell conditioned medium, RA, AA, db-cAMP, and SHH protein were added regularly until day 18. Then Wnt3a-conditioned medium was discontinued, SHH was increased, and the BDNF was added. At day 25, the medium was switched to the neural differentiation medium (Neurobasal medium with N2 and B27, L-Glutamine, NEAA, AA, db-cAMP), with BDNF, GDNF, IGF-1, CNF, SHH, and RA. The GFP and Hb9 expression were the marker for differentiated MN appeared around the day 31. Morphological changes, including soma size, branching and neurite outgrowth, were also monitored from day 31 to 40.

Locomotor Outcomes

Oligodendrocytes Transplantation

In almost all the experimental studies, induced SCI in animal models, including mice and rats, was the target of the hESC-derived OL and OPC transplantation. Shiverer mice are genetically modified with an autosomal trait mutation in the MBP-gene located on chromosome 18. The absence of this gene and its coded MBP protein causes severe myelin deficiency throughout the CNS. These mice have an average life span of 8 week, which give a relatively short but sufficient window for follow-up. Transplantation of the OL-linage cells, generated in the experiment of Nistor et al. (2005), into these mice was associated with integration and differentiation into functional OL over a 6 weeks follow-up period. This was evident by the formation of compacted, multilayered myelin sheath under microscope, and the expression of MBP, with exclusive distribution within the white mater of the spinal cord surrounding the site of injection. No data on the locomotor improvement were included and only early transplantation was examined. Keirstead et al. (2005), and Faulkner and Keirstead, in the same year, transplanted the OPC into adult rats with induced SCI at early and late phases, 1 and 10 weeks post-injury, respectively. Eight weeks following the injection at the early phase, at least 55 % of the axons around the site of injury were remyelinated. This is almost 136 % more than the endogenous remyelination in control group. To assess the functional improvements, the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) and the four-parameter kinematic analyses were used. The later measures the rear paw stride length, rear paw stride width, rear paw toe spread, and rear paw rotation. On both scales significant gradual improvement was noticed with the early phase transplantation compared to the control group, and persisted for almost 1 month following injection. On the other hand, none of these microscopic and functional improvements were noticed in the late phase transplantation. In addition to the previously mentioned limitation of later transplantation, the formation of OPC-directed

immune response has been suggested. These findings strongly support the therapeutic window theory. In their experiments also, the number of transplanted OL showed no difference in the ultimate outcome.

Izrael et al. (2007) transplanted the Noggin-treated OL into the brain of Shiverer mice. These were associated with significant local and distant integration and myelination within the brain tissue. In another study by Sharp et al. (2010), they proclaimed that, beside the final result, the progressive improvement in the symptoms was faster as compared to control group. Their results were recorded using Forelimb-movement scores, which measures the forelimb stride length, proximal forelimb step range, and passed-perpendicular step frequency. These could be detected as early as 1 week after transplantation. In the same year, Cao et al. described the use of neurotrpohic factors, i.e. CNTF with the OL transplantation to improve their survival, integration and differentiation (Cao et al. 2010). Karimi-Abdolrezaee et al. also proved that the combined use of chondroitinase ABC (ChABC) and growth factors EGF, bFGF, and PDGF-AA with the transplant significantly improve the outcome of OL differentiation, myelination, and functional outcome of chronic spinal cord injury. The ChABC antagonizes the chondroitin sulfate proteoglycans (CSPG) of the glial scar, which negatively influence the long-term survival, migration and differentiation of the transplanted cells (Karimi-Abdolrezaee et al. 2010) (Table 5.2).

Motor Neurons Transplantation

Following the *in vitro* culture, differentiation, and isolation of the MN, functional maturation, including the electrical properties, actions potential generation and conduction, and the receptive and terminal synaptic function, is analyzed. Measurement of the intrinsic membrane properties of the MN shows more hyperpolarized resting membrane potential and decreased input resistance with maturation. The generation and repetitive firing of action potential was also proved using the whole-cell patch clamp and/or the voltage-clamp configuration, which measure the function of the ion-channels within the cells, especially the voltage-gated Na and K-channels. Other electrical characteristics including spike frequency adaptation and rebound action potential firing are also measured, and are more consistent with spinal MN (Takazawa et al. 2012). The neurotransmitter sensitivity of the MN can be also assessed by applying the desired neurotransmitter, e.g. GABA, glutamate, dopamine and acetylcholine to the MN culture, and measuring the resultant current formation (Erceg et al. 2008). The synaptic function are being also studied with neighboring neurons (Li et al. 2005) and skeletal muscle cells (Wada et al. 2009). This detects the maturation of the synaptic vesicles at the axonal terminal, and the consequent up-regulation of the neurotransmitter, i.e. acetylcholine, receptor at the post-synaptic membrane. Co-culture of the MN with astrocytes derived from the respective hESC line was reported to be essential for electrophysiological maturation (Lee et al. 2007).

Following the MN differentiation and isolation, Lee et al. tested the survival capability of the cells via transplanting them into the spinal cord of a chick embryo. They found that these MN were able to survive and extend axonal fibers outside the CNS. The next step was to test their fate in adult CNS. Thus, they injected the HB9-cells into the ventral spinal cords of 3-month-old Sprague-Dawley rats. Observations at 1 day, 2 weeks, and 6 weeks after transplantation revealed progressive loss of the HB9 expression and increase in ChAT expression, which corresponds to physiological MN maturation. There were also evident of extensive fiber outgrowth and cell migration toward the ventral surface of the spinal cord. However, over the 6 weeks period, no signs of axonal outgrowth outside the CNS were addressed (Lee et al. 2007).

In the study of Erceg et al. (2010), the MNP transplantation into rats with transected spinal cord showed significant locomotor improvement in a 4 months follow-up period. The locomotor improvement was assessed clinically, were the samples showed partial recovery of the hindlimb movements, and on the BBB score with 6 out of 21. This is noticeably higher compared to the

Table 5.2 Experimental generation of oligodendrocytes from hESC and their transplantation

Study	Year	Media	Factors	Duration (days)	Purity (%)	Recipient	Integration and differentiation	Locomotor Improvement
Nistor et al.	2005	DMEM/F12, B27	RA, bFGF, EGF, IGF-1, T3	42	>95	Shiverer mice	Yes	–
Keirstead et al.	2005	DMEM/F12, B27	RA, bFGF, EGF, IGF-1, T3	42	>95	Rats	Yes	Significant, only at early stage transplantation
Faulkner and Keirstead	2005	DMEM/F12, B27	RA, bFGF, EGF, IGF-1, T3	42	>95	Shiverer mice	Yes	Significant, only at early stage transplantation
Izrael et al.	2007	ITTSPP/B27	RA, Noggin, SHH, bFGF, EGF, T3	>70	94	Shiverer mice	Yes	–
Kang et al.	2007	DMEM/F12, N2	bFGF, EGF, PDGF, T3	<50	81	–	–	–
Hu et al.	2009	DMEM/F12, N2	RA, SHH, purmorphamine, Noggin, bFGF, EGF, IGF-1, PDGF	>50	84	–	–	–
Hu et al.	2009	DMEM/F12	HGF	>50	>80	–	–	–
Sundberg et al.	2010	DMEM/F12, N2	EGF, bFGF, IGF-1, PDGF, CNTF, AA, T3	>80	>90	–	–	–
Sharp et al.	2010	DMEM/F12, B27	RA, EGF, FGF	42	<98	Rats	Yes	Significant

control group. On the electrophysiological study 4 months after the transplantation, partial conduction at the site of injury was present, compared to blind conduction in the controls. Immunohistological examination at the injection site showed clear evidence that these progenitors have the capacity to differentiate into mature OL and neurons in the lesion site. Nevertheless, no evidence of anatomically, physiologically, and functionally active motor units was seen (Erceg et al. 2010) (Table 5.3).

In the above experiment, the first application of combined cellular transplantation was documented. Erceg et al. (2010) used both the OL and MNP in rats with transected spinal cord. Over a 4 months follow-up, the functional locomotor recovery showed a better hindlimb recovery, and significantly higher BBB score and electrophysiological function compared to single-cell transplantation.

Lastly, transplantation of these cells proved that the generation of differentiated and highly pure cell line from the hESC is associated with decreased risk of tumor formation, i.e. teratoma. It also decreases the risk of undesired differentiation, including astorocyte and scar formation, graft-induced sprouting, and allodynia (Sharp and Keirstead 2007).

Motor Neurons and Oligodendrocytes Derived Human Induced Pluripotent Stem Cells

During fetal development, the pluripotent stem cells start to acquire a restricted and specific potency that gradually becomes directed towards one cell line generation. This process was always considered unidirectional. In 2006, Takahashi and Yamanaka were able to go back in cellular time by reprogramming adult somatic cells into pluripotent cells similar to the ESC using defined pluripotency-related transcription factors (i.e. Oct3/4, Sox2, c-Myc and Klf4) (Fig. 5.3). Yamanaka was honored with the Nobel Prize for this remarkable discovery. These novel types of stem cells were named "Induced pluripotent stem cells" or iPSC. Since this first description, various studies have been conducted using different protocols and cells of origin from different species. However, the main cellular origin stills the skin fibroblast. These protocols also tried to decrease the risks and health concerns and obstacles associated with the use of iPSC which include the integration of transcription factors with oncogenic properties, mutagenesis from insertion of the genes, the use of viral vectors and, lastly, the slow and relatively inefficient reprogramming process that may creates a situation that may favor incomplete reprogramming vectors. Detailed review of these methods is beyond the scope of this chapter.

The use of these cells is still considered an extensive, rapid, and important advancement that may solve the ethical issue related to the use of ESC. They may also be used for cell replacement therapy without requiring immunosuppressive therapy. Moreover, the fact that these cells are derived from individual patients makes it possible to develop customized stem cell therapies, generate disease-specific stem cell lines, and perform genetic corrections.

Genetic Variations

Despite the reported close genetic similarities between ESC and iPSC, genetic variations have been found. The use of single-nucleotide polymorphism (SNP) analysis on the pluripotent (i.e. ESC and iPSC) and non-pluripotent cells by Laurent et al. (2011) showed that the former have an increased overall frequency of sub-chromosomal copy number variations (CNV), with variations enriched in specific genomic regions. Comparing the ESC and iPSC showed that the ESC exhibit large number of duplications, compared to moderate amount of deletions found throughout the iPSC lines. These deletions were mainly present during reprogramming at the sites of tumor suppressor genes. Moreover, the oncogenic genes were duplicated with extended culture of the iPSC. Similar results were obtained by other authors (Gore et al. 2011; Hussein et al. 2011; Lister et al. 2011) who found

Table 5.3 Experimental generation of motor neurons from hESC and their transplantation

Study	Year	Media	Factors	Duration (days)	Purity (%)	Recipient	Integration and differentiation	Locomotor Improvement
Li et al.	2005	DMEM/F12, N2	RA, SHH, GDNF, BDNF, IGF1, cAMP	28–35	20	–	–	–
Shin et al.	2005	DMEM/F12, N2	RA, SHH, bFGF	53	20–30	–	–	–
Singh Roy et al.	2005	DMEM/F12, B27	RA, SHH	>40	20	–	–	–
Lee et al.	2007	DMEM/F12, N2	RA, Noggin, SHH, BDNF, GDNF, AA	50	20	Chick embryo Rats	Yes	–
Li et al.	2008	DMEM/F12, N2	Heparin, SHH, puromorphamine, RA, cAMP, GDNF, BDNF, IGF1	<30	50	–	–	–
Wada et al.	2009	DMEM/F12, N2, B27	SHH, SAG, RA, GDNF, BDNF	38	30	–	–	–
Erceg et al.	2010	DMEM/F12, N2	RA, SHH, GDNF, BDNF, CNTF	38	20	Rats	Yes	Significant
Takazawa et al.	2012	DMEM/F12N2	RA, SHH, AA, cAMP, BDNF, GDNF	>30	>30	–	–	–

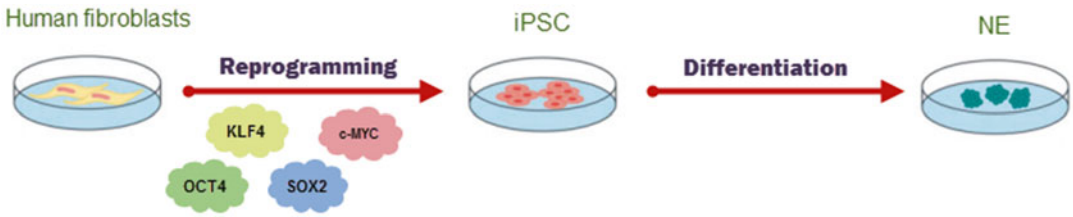


Fig. 5.3 Reprogramming of the human induced pluripotent stem cells from adult fibroblasts using pluripotency-related transcription factors (c-MYC, OCT4, KLF4, SOX2). *iPSC* induced pluripotent stem cells, *NE* neuroepithelial cells

that half of these mutations arise during the reprogramming process, despite the method used, and the other half is present in the original cells with lower expression. An average of five protein-coding point mutations per region was found by Gore et al. (2011) independent of the used method. Although these genes are cancer-related genes, gene ontology analysis did not reveal any pathway enrichments, which suggest a random rather than selective process (Gore et al. 2011; Panopoulos et al. 2011). In addition to these genetic mutations, epigenetic mutations were also reported by Lister et al. (2011) who detected more than a thousand differentially methylated regions or hotspots between iPSC and ESC lines.

All of these findings and others (Ben-David and Benvenisty 2011) suggest that the iPSC are more carcinogenic than the ESC, with chromosomal aberration acquired during three stages: from the original cells, during reprogramming, and during culture. However, despite these findings, the functional, morphological, and oncogenic manifestations and their in vivo variations and impact are still unclear (Panopoulos et al. 2011).

Neuronal Regeneration

As in the case of hESC, human iPSC (hiPSC) are capable of differentiating toward all neural cell types, including neurons, glia, NE, and MN, which in turn are used to treat various kinds of neurological pathologies. Moreover, due to their beneficial characteristics and despite their potential risks, the hiPSC is becoming an appealing alternative source for neuronal generation.

Experimental studies on MN (Ebert et al. 2009; Karumbayaram et al. 2009) and OL (Ogawa et al. 2011) generation from fibroblast-derived human iPSC were capable of inducing functional cells through and intermediate EB, in a process exactly similar to the hESC. However, none of these cells were tested in vivo. Other studies that involved direct transplantation of the human iPSC –derived neural spheres will be discussed in the next chapter.

References

Almad A, Sahinkaya FR, McTigue DM (2011) Oligodendrocyte fate after spinal cord injury. Neurotherapeutics 8(2):262–273

Ben-David U, Benvenisty N (2011) The tumorigenicity of human embryonic and induced pluripotent stem cells. Nat Rev Cancer 11(4):268–277

Cao Q, He Q, Wang Y, Cheng X, Howard RM, Zhang Y, DeVries WH, Shields CB, Magnuson DS, Xu XM, Kim DH, Whittemore SR (2010) Transplantation of ciliary neurotrophic factor-expressing adult oligodendrocyte precursor cells promotes remyelination and functional recovery after spinal cord injury. J Neurosci 30(8):2989–3001

Cherian E, Nandhini G, Kurian A (2011) Stem cells, book stem cells. 1. Jaypee Brothers Medical Pub, New Delhi, p 116

Ebert AD, Yu J, Rose FF Jr, Mattis VB, Lorson CL, Thomson JA, Svendsen CN (2009) Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 457(7227):277–280

Erceg S, Lainez S, Ronaghi M, Stojkovic P, Perez-Arago MA, Moreno-Manzano V, Moreno-Palanques R, Planells-Cases R, Stojkovic M (2008) Differentiation of human embryonic stem cells to regional specific neural precursors in chemically defined medium conditions. PLoS One 3(5), e2122

Erceg S, Ronaghi M, Oria M, Rosello MG, Arago MA, Lopez MG, Radojevic I, Moreno-Manzano V, Rodriguez-Jimenez FJ, Bhattacharya SS, Cordoba J, Stojkovic M (2010) Transplanted oligodendrocytes and

motoneuron progenitors generated from human embryonic stem cells promote locomotor recovery after spinal cord transection. Stem Cells 28(9):1541–1549

Faulkner J, Keirstead HS (2005) Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl Immunol 15(2):131–142

Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N, Panopoulos AD, Ruiz S, Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471(7336):63–67

Hatch MN, Nistor G, Keirstead HS (2007) Oiigodendrocyte differentiation from human embryonic stem cells. In: Peterson S, Loring JF, Wesselschmidt RL, Schwartz PH (eds) Human stem cell manual: a laboratory guide, 1st edn. Academic Press, Elsevier, London

Hu BY, Du ZW, Li XJ, Ayala M, Zhang SC (2009a) Human oligodendrocytes from embryonic stem cells: conserved SHH signaling networks and divergent FGF effects. Development 136(9):1443–1452

Hu Z, Li T, Zhang X, Chen Y (2009b) Hepatocyte growth factor enhances the generation of high-purity oligodendrocytes from human embryonic stem cells. Differentiation 78(2-3):177–184

Hussein SM, Batada NN, Vuoristo S, Ching RW, Autio R, Narva E, Ng S, Sourour M, Hamalainen R, Olsson C, Lundin K, Mikkola M, Trokovic R, Peitz M, Brustle O, Bazett-Jones DP, Alitalo K, Lahesmaa R, Nagy A, Otonkoski T (2011) Copy number variation and selection during reprogramming to pluripotency. Nature 471(7336):58–62

Izrael M, Zhang P, Kaufman R, Shinder V, Ella R, Amit M, Itskovitz-Eldor J, Chebath J, Revel M (2007) Human oligodendrocytes derived from embryonic stem cells: effect of noggin on phenotypic differentiation in vitro and on myelination in vivo. Mol Cell Neurosci 34(3):310–323

Kang SM, Cho MS, Seo H, Yoon CJ, Oh SK, Choi YM, Kim DW (2007) Efficient induction of oligodendrocytes from human embryonic stem cells. Stem Cells 25(2):419–424

Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Schut D, Fehlings MG (2010) Synergistic effects of transplanted adult neural stem/progenitor cells, chondroitinase, and growth factors promote functional repair and plasticity of the chronically injured spinal cord. J Neurosci 30(5):1657–1676

Karumbayaram S, Novitch BG, Patterson M, Umbach JA, Richter L, Lindgren A, Conway AE, Clark AT, Goldman SA, Plath K, Wiedau-Pazos M, Kornblum HI, Lowry WE (2009) Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. Stem Cells 27(4):806–811

Keirstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, Steward O (2005) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci 25(19):4694–4705

Kiessling AA (2003) Human embryonic stem cells: an introduction to the science and therapeutic potential. Jones and Bartlett Publishers, Inc., Sudbury, p 240

Laurent LC, Ulitsky I, Slavin I, Tran H, Schork A, Morey R, Lynch C, Harness JV, Lee S, Barrero MJ, Ku S, Martynova M, Semechkin R, Galat V, Gottesfeld J, Izpisua Belmonte JC, Murry C, Keirstead HS, Park HS, Schmidt U, Laslett AL, Muller FJ, Nievergelt CM, Shamir R, Loring JF (2011) Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. Cell Stem Cell 8(1):106–118

Lee H, Shamy GA, Elkabetz Y, Schofield CM, Harrsion NL, Panagiotakos G, Socci ND, Tabar V, Studer L (2007) Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. Stem Cells 25(8):1931–1939

Letzen BS, Liu C, Thakor NV, Gearhart JD, All AH, Kerr CL (2010) MicroRNA expression profiling of oligodendrocyte differentiation from human embryonic stem cells. PLoS One 5(5), e10480

Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC (2005) Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 23(2):215–221

Li XJ, Hu BY, Jones SA, Zhang YS, Lavaute T, Du ZW, Zhang SC (2008) Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. Stem Cells 26(4):886–893

Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S, Downes M, Yu R, Stewart R, Ren B, Thomson JA, Evans RM, Ecker JR (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471(7336):68–73

Lopez-Gonzalez R, Velasco I (2012) Therapeutic potential of motor neurons differentiated from embryonic stem cells and induced pluripotent stem cells. Arch Med Res 43(1):1–10

Nistor GI, Totoiu MO, Haque N, Carpenter MK, Keirstead HS (2005) Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49(3):385–396

Nizzardo M, Simone C, Falcone M, Locatelli F, Riboldi G, Comi GP, Corti S (2010) Human motor neuron generation from embryonic stem cells and induced pluripotent stem cells. Cell Mol Life Sci 67(22):3837–3847

Ogawa S, Tokumoto Y, Miyake J, Nagamune T (2011) Immunopanning selection of A2B5-positive cells increased the differentiation efficiency of induced pluripotent stem cells into oligodendrocytes. Neurosci Lett 489(2):79–83

Panopoulos AD, Ruiz S, Izpisua Belmonte JC (2011) iPSCs: induced back to controversy. Cell Stem Cell 8(4):347–348

Sharp J, Keirstead HS (2007) Therapeutic applications of oligodendrocyte precursors derived from human embryonic stem cells. Curr Opin Biotechnol 18(5):434–440

Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS (2010) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells 28(1):152–163

Shin S, Dalton S, Stice SL (2005) Human motor neuron differentiation from human embryonic stem cells. Stem Cells Dev 14(3):266–269

Singh Roy N, Nakano T, Xuing L, Kang J, Nedergaard M, Goldman SA (2005) Enhancer-specified GFP-based FACS purification of human spinal motor neurons from embryonic stem cells. Exp Neurol 196(2):224–234

Sundberg M, Skottman H, Suuronen R, Narkilahti S (2010) Production and isolation of NG2+ oligodendrocyte precursors from human embryonic stem cells in defined serum-free medium. Stem Cell Res 5(2):91–103

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676

Takazawa T, Croft GF, Amoroso MW, Studer L, Wichterle H, Macdermott AB (2012) Maturation of spinal motor neurons derived from human embryonic stem cells. PLoS One 7(7), e40154

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147

Wada T, Honda M, Minami I, Tooi N, Amagai Y, Nakatsuji N, Aiba K (2009) Highly efficient differentiation and enrichment of spinal motor neurons derived from human and monkey embryonic stem cells. PLoS One 4(8), e6722

Zhang YW, Denham J, Thies RS (2006) Oligodendrocyte progenitor cells derived from human embryonic stem cells express neurotrophic factors. Stem Cells Dev 15(6):943–952

Part II

General Applications

Progress from Embryonic Stem Cells to Transduced Pluripotent Stem Cells. An Overview

6

J. Suaudeau

Contents

Abstract

The report of Thomson and collaborators on the first successful derivation and subsequent *in vitro* culture of human embryonic stem cells (hESCs), in 1998, opened up great expectative for a future regenerative medicine, but also sparkled a sharp ethical debate in favor or against the use of these cells. Moreover, hESCs have also serious biological limitations due to their immune rejection by the organism in which they are grafted and because of the formation of tumors when they are injected in organisms. This prompted some scientists to look for alternatives to the hESCS. They wished to obtain pluripotent stem cells, offering the same qualities as hESCS in terms of proliferation, self-renewal and differentiation capacities *in vitro*, but that could be obtained without having to sacrifice human embryos. Were particularly investigated the cells

J. Suaudeau (✉)
Sezione Scientifica, Pontificia Accademia per la Vita, 1 via della Conciliazione, Rome 00193, Italy
e-mail: jacques@suaudeau.fr

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reprogrammed through nuclear transfer, the hESCs obtained from nonviable human embryos, or through parthenogenesis, or derived from blastomeres through embryo biopsy, and the cells reprogrammed through cellular fusion with ESCs. None of these solutions proved to be clearly adequate or superior to the other solutions. Finally, the proposal of inducing pluripotency by introducing in the somatic cell a set of specific transcription factors, presented by Yamanaka in 2006, has offered a surprisingly simple, valid and reliable alternative to hESCs.

Introduction

The field of stem cells research is one of the most busy and creative in biology today, and also one of the most promising in translational medicine, given the hope of reaching that way an actual regenerative medicine. While the field of stem cell research had opened up with the discovery of adult stem cells (Till and McCulloch 1961), followed by that of umbilical cord blood stem cells (Knudtzon 1974), subsequent progresses have come from developments with embryonic stem (ES) cells. Evans and Kaufman (1981) and Martin (1981), independently, isolated ES cells from the inner cell mass of mouse blastocysts and have them grown into culture. But the true revolution in that field came in 1998, with the announcement by Thomson and colleagues that they had managed to isolate and put into culture human embryonic stem cells (hESCs). This technological breakthrough immediately triggered a burst of research and a whirlwind of ethical debate. It is this achievement that has given its impetus to the whole research on stem cells.

Embryonic Stem Cells

Embryonic stem cells obtained from the inner cell mass (ICM) of blastocysts are pluripotent cells that can undergo unlimited self-renewal divisions while retaining the capacity for multilineage specification and differentiation.

ESCs Are Pluripotent Stem Cells

ESCs are pluripotent cells, with the capacity to engender all cell lineages and germ cells. ESCs represent immortalization of the naive epiblast. Under appropriate conditions (co-culture with mouse embryo fibroblasts that produce cytokine leukemia inhibitory factor, Lif) they exhibit unlimited self-renewal capacity while retaining the attributes of preimplantation epiblast identity and potency. Mouse ESCs depend on LIF/STAT3 signalling for maintenance of their pluripotency. The use of a combination of two inhibitors (2i), inhibitor of Erk pathway and inhibition of glycogen synthase kinase-3 (GSK3) is sufficient to stabilize and sustain mouse ESCs with full pluripotency (Ying et al. 2008). Mouse ESCs may represent as such a "*naïve ground state*" of pluripotency, without any prespecification of lineage choice. Human ESCs differ significantly from mouse ESCs in their culture requirements, and are more similar to postimplantation mouse epiblasts cells, EpiSCS (Rossant 2008). Alike EpisSCS their self-maintenance in culture is assured by fibroblast growth factor (FGFD) and activin, but not by 2i and LIF. They may represent a "*primed*" *state* of pluripotency, in favor of particular fates. Like EpiSCs they are heterogeneous between and within cell lines and can show marked biases in differentiation.

Properties of hESCS

hESCs are endowed with unique properties, coming from their nature of pluripotent stem cells: – Prolonged undifferentiated proliferation in culture when maintained in a suitable medium which secure their illimited self-renewal; – Stable developmental potential to form derivatives of all three embryonic germ layer, even after prolonged culture; – Remarkably stable karyotypes and expression of high levels of telomerase, which is correlated with immortality in human cell lines,

and this even after more than 300 population doublings and passage for more than 1 year in culture (Amit et al. 2000). All these properties confer to ESCs a unique capacity to secure the repair of damaged tissues and organs, superior to that of any other type of stem cell.

Limits and Problems with hESCS

However, hESCs have proved problematic, since the beginning of their individuation, from a double point of view, practical and ethical. From a practical point of view, hES cannot be used as such for regenerative medicine, and this is for two main reasons: The first is that these cells are known to be tumorigenic, growing into teratomas or teratocarcinomas when injected anywhere in the adult organism (Fujikawa et al. 2005). This property is linked to pluripotency. Only their derivatives, at the level of progenitors, could be safe in patients, great care been taken to eliminate all pluripotent remaining cells from the administered cell suspension. The second reason which makes difficult to use hESCs in patients is that they are rejected by the immune defenses of the patient's organism (Drukker and Benvenisty 2004). This second obstacle did not appear to be absolute to the first experimenters because it has been observed that ESCs expressed reduced levels of major histocompatibility (MHC) class I genes, and did not expressed MHC class II and HLA-G genes on their surface (Drukker et al. 2002). This suggested that pluripotent stem cells could be "immune-privilege" and were unable to induce immune reactions. However, recent studies have shown that some hESCs are recognized by immune cells and induce an immunological reaction, without been explained the differences between ESCs at this point of view. In order to apply hESCs or their derivatives to patients for regenerative medicine purpose, it will therefore be necessary to associate to their administration an immunodepressive treatment, which will make more burdensome the regenerative treatment. From the point of view of ethics, the obstacle is clear and, for some, absolute: to obtain the hESCs which one's need, human embryos have to be sacrificed. Even if one does not actually destroy human embryos for preparing hES, and use only readily commercially available hESCs lines, one still share in the moral responsibility of the destruction of these human embryos which have served to derive the hESCs lines that one is using.

In order to overpass these difficulties, scientist have proposed various alternative methods for obtaining pluripotent stem cells of the same functional value as hESCS, but free of the biological and ethical roadblocks proper to these cells. They looked therefore for pluripotent stem cells which would not trigger immunological reaction from recipient organisms, and would not require the sacrifice of human embryos for their collection. These various proposals include :– using embryonic germ cells (EGS) instead of ES; – avoiding some of the biological obstacles found with hES, while still using human embryos as a source of these pluripotent stem cells; – avoiding the ethical obstacle found with hES, but not the biological, with the use of defective preimplantation human embryos. – Avoiding both the biological obstacle and the ethical obstacle, using biopsied blastomeres as a source of hES, or reprogramming somatic cells to the pluripotent state.

Embryonic Germ Cells

Pluripotent embryonic stem cells, called "embryonic germ cells" (EG) have been derived from foetal primordial germ cells (PGCs) in culture. This was first achieved on mouse cells in 1992 by Matsui and colleagues (Vanderbilt University, Tennessee), and by Resnick and colleagues 2003 (Frederick, Maryland) working independently. These authors found out that a modification of PGCs culture for promoting their proliferation and survival changed in fact the nature of these cells. In adequate conditions, with bFGF in presence of LIF, they became indeed "reprogrammed" to a pluripotential state in which they behave very much alike ESCs derived from blastocyst. Then, in November 1998, Gearhardt and colleagues announced that they had derived human embryonic germ cells (hEGCs) from foetal primordial

germ cells (Schamblott et al. 1998). EGCs are equivalent of ESCs in terms of capacity for *in vitro* proliferation and broad cell differentiation. EGCs from mice and rats can be established with high efficiency using 2i in combination with the cytokine leukemia inhibitory factor LIF. They exhibit therefore properties consistent with the generic pluripotent ground state found in mouse ESCs. However, hEGCS are more difficult to obtain than hESCS, and tend to differentiate spontaneously in culture (Turnpenny et al. 2003). Moreover, they do not resolve the biological difficulties encountered with hESCs. The potential use of hEG cells in cell therapy applications will depend on a more thorough understanding of how to derive and maintain these cells *in vitro*. From the ethical point of view, such a practical application of EGSCs would involve harvesting germ cells from aborted human fetuses (at 59 weeks after fertilisation). Thus the use of hEGCs instead of hESCs while permitting to avoid the ethical problem of preimplantation embryo destruction, will bring nonetheless one another ethical problem of possible moral cooperation with provoked abortion.

Alternative Proposals Using Human Embryos as a Source of Pluripotent Stem Cells

The development of somatic cell nuclear transfer into enucleated oocyte (SCNT) (*somatic cell nuclear transfer*) (Wilmut et al. 1997) has opened to stem cell researchers a new avenue of research. Two different proposals have been put forward, using SCNT:– "Therapeutic cloning"; – Creation of human cytoplasmic hybrid embryos ("cybrids").

"Therapeutic Cloning" to Produce Immuno-Compatible Human ES Cells

"Therapeutic cloning", better called "research cloning" (Lanza et al. 1999), would create a human embryo by nuclear transfer of one of the patient's somatic cells into an enucleated human oocyte. hESCs extracted from that embryo when arrived at the blastocyst stage, would therefore be immunologically compatible with the organism of the patient. Administered to the patient for regenerative purpose, they would not be rejected. However, this proposal immediately came up against serious biological and ethical difficulties. From the biological standpoint, the nuclear transfer technique, even limited simply to the production of blastocysts from which to obtain stem cells, has produced positive, consistent results in only some species (mouse, bovine, rabbit). In primates, the Oregon National Primate Research Center team, after years unfruitful attempts, managed in 2007 to obtain two ES cell lines from 35 *macacus rhesus* blastocysts created by SCNT (out of 213 prepared embryos), then, more recently, two other ES cell lines from six blastocysts created by SCNT (out of 71 prepared embryos) (Sparman et al. 2009). In humans, therapeutic cloning, despite repeated efforts has not produced any consistent results. In May 2005, Stojkovic and Murdoch (Newcastle, UK) did indeed announce that they had created the first cloned human embryo, but they had not extracted stem cells from it. More recently (January 2008), a group of researchers at *Stemagen Corporation* (French et al. 2008) reported the creation by cloning of 14 human embryos, derived from 29 oocytes. Five of these embryos were said to have been able to develop to the blastocyst stage, but the authors had not tried to extract stem cells from them. Li et coworkers (Shandong, China) (Li et al. 2009) also reported in 2009 the creation of human blastocysts by SCNT (5 out of 26 prepared embryos, produced from 135 oocytes from 12 donors), but again did not seek to derive hES cells from them. "Therapeutic cloning" in humans is therefore still a costly undertaking, severely limited by the number of human oocytes it requires, and which has given no positive results. From an ethical standpoint, "therapeutic cloning" requires the deliberate creation of an early-stage human embryo, followed by its disaggregation. Its ethical acceptability thus depends on the degree of "moral value" the preimplantation embryo is recognized to possess.

Human Cytoplasmic Hybrid Embryo ("Cybrid") Proposal

Because of the difficulty of collecting a sufficient number of human oocytes in order to be able to create human embryos by cloning and extract the stem cells from them, some have suggested using the interspecies somatic cell nuclear transfer (iSNCT) technique that seeks to create cloned embryos by the nuclear transfer of a human somatic cell into an enucleated animal oocyte. These would be "cytoplasmic hybrid" embryos ("cybrids") with a human nuclear DNA and an animal cytoplasm containing the animal mitochondrial DNA. This technique has been used primarily to ensure the survival of certain endangered species, but it has had very little success. Despite these mediocre results, iSCNT has been seen as a possible solution to the problem posed by the difficulty of recruiting human oocytes for therapeutic cloning. The idea would be to use enucleated bovine oocytes as host and activator of human somatic cell nuclei. Human-animal cytoplasmic hybrid embryos would thus be created (99.9 % human due to the nuclear DNA, 0.1 % animal due to the mitochondrial DNA), from which human-like embryonic stem cells could be extracted once these embryos have developed to the blastocyst stage. These ES cells would be genetically human, and could therefore be used therapeutically, without the risk of rejection. The *Advanced Cell Technologies* (Massachusetts) team was the first to study this possibility. In 1999, these authors reported that they had created embryos by nuclear transfer of human somatic cells into enucleated cow oocytes (Lanza et al. 1999). Twenty-six percent of these embryos (n = 6) was said to have been able to develop to the 4–16 cell stage and only one of them to have reached the 400-cell stage. The success of this operation was thus more than limited. Chang et al. (2003) reported the creation, using the same technique, of human/cow hybrid embryos, with modest results: out of 286 embryos prepared in this way, only four embryos were able to develop to the morula or blastocyst stage, and there was no mention of ES cell extraction from these embryos. The same authors published a follow-up to this study in October 2004. Out of 194 human/cow hybrid embryos created by SCNT, only two embryos reached the blastocyst stage. Illmensee et al. (2006) reported the creation of 37 human/cow hybrid embryos, seven of which were said to have reached the blastocyst stage, but no ES cells could be extracted from these blastocysts. Despite this repeated lack of success, the British HFEA (*Human Fertilisation and Embryology Authority*) declared on 5 September 2007 that it was in favour of the creation of such "cybrids", declaring them "necessary and desirable in both scientific and ethical terms". The creation of "cybrids" is a serious matter from an ethical point of view. Even if the individual resulting from this transfer into a bovine oocyte is genetically 99 % human, the process itself does not respect the humankind of which the cybrid is a part.

Alternative Proposals to Obtain hES-Like Cells from Human Embryos with No Development Potential

In the second line of response to problems encountered with hES cells, what is primarily sought is the development of ethically acceptable methods that would enable hES cells to be obtained without viable human embryos being disaggregated. Three proposals have thus been put forward: – using poor-quality embryos rejected by the IVF centers; – using parthenogenetically created embryos; – using human embryos planned to be defective and unable to implant.

Use of Poor-Quality Embryos

A fifth of all embryos generated for *in vitro* fertilization, classed as "nonviable" for transfer because they fail to develop properly or because of their poor morphology, could be in fact

considered as dead, taking as criteria the hypocellularity and the lack of compaction on embryonic day 5. Such embryos, which are discarded, still contain live cells that are a potential source of hES cells. Landry and Zucker (2004) have, therefore, proposed to derive hESCs from such "nonviable embryos". Mitalipova et al. (2003) reported that they had been able to derive hES cells from surplus human embryos offered to research because of their lack of development and morphological anomalies, and that they had been able to obtain four hES cell lines from such embryos. Pickering et al. (2003) reported obtaining three hES cell lines from 58 human embryos created by IVF, subjected to preimplantation diagnosis, and judged to be defective and unfit for implantation. Lerou et al. (2008) have shown that there was a possibility, albeit very low (0.6 %), of deriving hES cells from 3-day embryos considered to be of poor quality, and with low probability of uterine implantation. hES cell derivation efficiency was clearly better (4.1 %) when these poor-quality embryos had reached 5 days of development, and it was 8.5 % when these embryos had become blastocysts. From a biological standpoint, this proposal of using non-viable embryos for the derivation of hESCS does not offer a real solution to the problem of the limited number of human embryos available for obtaining hES cells, because to be more effective it requires "cavitated embryos", that is blastocysts, and most arrested embryos or those presenting morphological changes do not reach this stage of development. Nor does it solve the problem of the immunological rejection of hES cells, if these cells were to be used clinically. However, despite the poor success rate of the process, this proposal should not be underestimated because it does take advantage of the actual availability of these arrested nonviable embryos from IVF centers. Moreover, the idea of using embryos facing certain death as a source of hES cells might be defended from an ethical standpoint, based on the analogy of taking organs from brain-dead patients. However, at present, there are no reliable, early criteria for declaring an embryo "dead". One can only speak about "arrested non viable embryos".

Parthenogenesis

Parthenogenesis is the process by which a new individual develops from a non-fertilised oocyte. It can be induced in mammals by artificial, chemical or electrical stimulation of an oocyte, which then develops to form an embryo that only has the genetic programme of the mother and is called a "parthenote". In primates, embryos created in this way cannot develop correctly and are generally lost before they can implant, probably due to a lack of expression of the paternal imprinted genes. Recourse to parthenogenesis was proposed by Cibelli et al. (2002) as a way of obtaining immunocompatible hES cells from a female donor. This approach would moreover be ethically acceptable, because it does not lead to destroying normal human embryos. These authors had derived embryonic-like parthenogenetic stem cells from four monkey (*Macaca fascicularis*) embryos at the blastocyst stage, obtained through the development of metaphase II oocytes that were not fertilized but activated by a calcium wave. These parthenogenetic embryonic stem cells (PESCs) were capable of multiplying *in vitro* for more than 10 months. PESCs harvested from "parthenote" embryos have characteristics similar to those of hES cells collected from viable embryos created by fertilisation. They express the pluripotency key ESC-specific markers (OCT4, Nanog, Sox-2, Foxd3, Rex-1, Stat-3, cMYC). They form embryoid bodies structure. They spontaneously differentiate into derivatives of the three embryonic germ layers as confirmed by ectodermal, mesodermal and endodermal markers. Their differentiation can be successfully oriented toward the neuronal lineage. hPESCs can be differentiated into multipotent human mesenchymal stem cells (hMSC), and thus be a valuable source for cell-based therapies. They do have robust germline competence enabling the production of transgenic mouse strains from genetically modified haploid parthenogenetic ESCs. Differentiation of haploid parthenogenetic PESCs in the embryo correlates with the gain of a diploid Karyotype by endoreduplication. Because these PESCs are histocompatible with the oocyte donor, they could represent a solution to the immunocompatibility

problem of ESCs, at least for female patients. They could that way provide a valuable source of progenitors for regenerative medicine. Parthenogenetic embryonic stem cells have already been used as equivalent to ES cells, in preclinical studies in the animal. They ameliorate cardiac dysfunction and remodelling after myocardial infarction in mice. They can be differentiated toward retinal pigment epithelium. Parthenogenetic dopamine neurones derived from parthenogenetic primate hES cell lines and transplanted into the right striatum of rats made hemiparkinsonian by treating them with 6-hydroxy-dopamine restored the motor function of these animals. However the genetic implications of the parthenogenetic origin of these ES cells do cast doubts on their possible future use. First of all, the high level of homozygosis and the lack of paternal genome might pose a safety challenge for their therapeutic use. We can fear in particular in these parthenogenetic ES cells an abnormal expression of the imprinted genes. Genome-wide expression study by microarray analysis shows that, although overall gene expression of parthenogenetic iPSCs (*induced pluripotent stem cells*) resembles that of biparental ES cells, significantly broader changes (accumulation of instability) occurs in this expression, compared with biparental controls, when these parthenogenetic iPS are further differentiated into parthenogenetic mesenchymal stem cells. This highlights the effect of the interplay of epigenetic reprogramming on a monoparental background, as well as the importance of heterozygosis and biparental imprinting for stable epigenetic reprogramming. mRNA expression analysis show Insulin-Like Growth Factor I and Uteroglobin to be overexpressed in parthenote embryos, compared to non parthenote embryos. The parthenogenetic embryonic ES cell has an excessive number of centrioles, while lacking the centriole which is inherited through the sperm of mammalian species. They demonstrate centrosome amplification, over expression of PLK2 and down regulation of CCNF respectively involved in the stimulation and inhibition of centrosome duplication, and a high incidence of aneuploidy. But it appears that these anomalies could be corrected to a certain extent in the pluripotent cells derived from such parthenogenetic embryos. Therefore, with cells derived from partheogenetically created embryos, we would have pluripotent cells of a quality equal to that of ES cells, which would moreover be histocompatible with the recipient when the parthenogenetically activated oocyte came from that same person. The authors of these studies considered that the derivation of these embryonic-like pluripotent cells from non-fertilised human oocytes activated to parthenotes would be ethically acceptable, because the product of this activation, i.e. the parthenogenetically created embryo should not be regarded and treated as a real embryo. In their opinion this "parthenote", incapable of developing beyond the blastocyst stage, with no future potential, should be considered as potentially dead, and treated as such. This opinion seems however questionable, because these activated human oocytes behave exactly like normal embryos until their epigenetic imbalance curbs their development and stops them implanting *in utero*.

Altered Nuclear Transfer

The proposal presented by Hurlbut (2005) entitled "*Altered Nuclear Transfer*" (ANT) aimed to create by cloning (SCNT), an "altered" human embryo, that is to say, incapable of implanting or developing after implantation, which could become a morally licit source of hES cells. Meissner and Jaenisch have showed in the mouse that it is possible to neutralise the *cdx2* gene in mouse fibroblasts and use these fibroblasts to create through SCNT a *cdx2*-deficient embryo, unable to implant. From this defective embryo it is possible to derive ES cells, to which a normal *cdx2* function could be restored afterward by transferring the gene. Hurlbut's plan is to create by RNA interference embryos deficient in the *cdx2*, from which embryonic stem cells would be derived. Once these cells had been obtained, they would have their *cdx2* gene re-expressed, turning that way into normal pluripotent hES cells. According to Hurlbut, the biological result of "*altered nuclear transfer*" with *cdx2* deficiency would not be an embryo, since it would not have

the ability to develop, but a "group of cloned cells", comparable to a teratoma. Hurlbut thinks that it would be morally licit to destroy such "altered embryos" in order to collect their inner cell mass. This proposition of Hurlbut is not without weak points, scientifically and ethically. (1) From a scientific standpoint, it is not known what might happen to human embryos if they were made *cdx2*-deficient, and allowed to develop, given that this gene is a "homeobox" gene, of the "Hox" essential gene family, which definitely plays a number of important roles in embryo development. (2) From an ethical standpoint, the comparison between a defective embryo that does not manage to implant and a teratoma is not correct. The *cdx2*–deficient embryo behaves like an organism with a development plan, up to the blastocyst stage. The fact that it is unable to implant takes nothing away from its quality as a biological individual. (3) The proposal to manipulate the genome of human embryos created by cloning to make them incapable of implantation is perplexing. The fact that many embryos produced through sexual relations have chromosomal or genetic anomalies that prevent them from developing properly or implanting does not justify the deliberate creation of such anomalies.

Alternative Proposals That Do Not Involve the Destruction of Human Embryos

Other proposals have been made to obtain hES cells or pluripotent stem cells hES-like without having to destroy human embryos. These are: – (1) Using blastomeres harvested by embryo biopsy. – (2) Reprogramming somatic cells to embryonic-like cells.

hES Cells Derived from a Single Blastomere Collected by Embryo Biopsy

One proposal to obtain hES-like cells without having to sacrify a human embryo is to remove by biopsy one blastomere from an embryo in the segmentation phase. ES cell lines have been that way derived from the blastomeres of early-stage mouse embryos, in the very first stages of cleavage, the two-cell embryo blastomeres having the highest success rates (Wayayama et al. 2007). This idea was taken up by Chung et al. (2006) as a method for obtaining hES cells without having to destroy human embryos. However, the procedure proved to be very inefficient, as only 2 % of the blastomeres collected in this way had given rise to an hES cell line. In the best conditions, three blastomeres out of 15 had generated stable hES cell lines. These studies also reported frequent chromosomal and genetic anomalies affecting the hES cells in these lines. Different derivations of hESCS lines from biopsied blastomeres have been recently reported. One study (Ilic et al. 2009) has reported an increase of efficacy from 12.5 % (one hESC line out of 13 biopsies) to 50 % (3 lines out of 6 biopsies). In that study all derived lines maintained normal karyotype and expressed totipotent phenotype including the ability to differentiate into trophectoderm and all three germ layers. However, all these derivations were realized using the whole available material of the human embryos, without trying to save them. Only one study (Taei et al. 2010) has been performed within the conditions of a biopsy for preimplantation diagnosis, but again without saving the embryo. Derivation of hESC lines from blastomeres of biopsied embryo would be a way of taking advantage of these human embryos which are eliminated in the IVF clinics through preimplantation diagnosis. From an ethical point of view, however, it does not bring a valuable alternative to hESCs harvesting from cryopreserved, left over blastocysts, nor to the extraction of hESCs from poor-quality embryos discerded from IVF clinics.

Somatic Cell Reprogramming

The last proposal for obtaining embryonic-like, pluripotent stem cells without having to sacrifice human embryos is "nuclear reprogramming", that is, the change that occurs in the

nucleus of a somatic cell when it is induced to revert from its differentiated state and assume a pluripotent state. In order to demonstrate that a nucleus from a differentiated cell still possessed the same genetic material than the nucleus of an embryonic undifferentiated cell, Spemann proposed in 1938 to transfer the nucleus of a differentiated, adult cell, into an egg whose own nucleus had been removed. But the experiment so designed had to wait 14 years – till the end of the Second World War – to be carried out. It was finally accomplished by Briggs and King (1952) who injected nuclei of blastomeres from *Rana pipiens* into previously enucleated frog eggs and obtained, that way, early cleavage embryos. It is that technique of nuclear transfer which enabled Gurdon (1960), studying on *Xenopus*, to demonstrate the reality of reprogramming. Gurdon showed that the nucleus of a differentiated cell was able to recover the capacities of the nucleus of a zygote when it was transferred into the enucleated ovocyte of *Xenopus*. From this finding, Gurdon drew two teachings: on one side he was able to demonstrate that the dogma of the irreversibility of the differentiated state of adult cells was wrong, on the other side he could tell that there were factors within the cytoplasm of eggs that were able to achieve this nuclear reprogramming. The announcement, on February 27, 1997, of the birth of the healthy lamb created by Wilmut and. Campbell, using the technique of nuclear transfer, has illustrated in a eloquent way the rightness of these views.

Lessons from Transdifferentiation

Henry Harris and colleagues in 1969 provided important background to cellular reprogramming by showing that malignant cancer cells fused to fibroblasts were no longer malignant due to gene repression. Then, key finding had been in 1979 the observation by Taylor and Jones that the treatment of fibroblast cell lines (10T1/2, 3T3 cells) with the DNA analogue 5-azacytidine induced their conversion into adipocytes, chondrocytes and myogenic cells. The recognition that 5-azacytidine blocks DNA methyltransferase and is thus a DNA demethylating agent suggested the importance of epigenetic mechanisms in regulating cell fate.

Lessons from Heterokaryons

In 1983–1984 Blau showed that fusion of one differentiated cell (a muscle cell) with a cell in which muscle genes are not normally expressed (a human amniocyte as example) leads to the activation of muscle genes in the amniocyte. Blau obtained such a transdifferentiation through interspecies cell fusion to form mixed species cell hybrids, called "hétérokaryons". This indicated the existence of transacting factors capable of inducing transdifferentiation.

Reprogramming Using Cell-Free Extracts

From these findings on transdifferentiation came the idea that nuclear reprogramming with the activation of silent genes could be accomplished without nuclear transfer in ovocytes, using only somatic cell-free extracts of active cells. This was shown in 2002 by Collas and colleagues (Håkelien et al. 2002) on quiescent human T cells and on fibroblasts, by incubation in an extract of T-cell cytoplasm. These authors proposed their method as an alternative way to somatic cell nuclear transfer for bringing somatic cells to the pluripotent state (Collas et al. 2006).

Epigenetic Reprogramming of Somatic Cells by Hybridisation (Fusion with ES Cells)

One another way of inducing the activation of silenced genes in somatic cells, without passing through somatic cell nuclear transfer, and without cell extracts treatment is fusion of the somatic cell with a pluripotent cell (Tada et al. 1997). This demonstrated that the same intrinsic transacting factors which in the oocytes allows the reprogramming of the transfered somatic cell nucleus, are also acting in the cytoplasm of the pluripotent cell, allowing DNA demethylation, histone acetylation and modifications of the chromatine structure involved in the establishment of the pluripotent state. *Epigenetic nuclear reprogramming of somatic cells by in vitro hybridisation with ES cells* (Tada et al. 2001) has therefore

been proposed (Cowan et al. 2005) as an alternative method to hESCs extraction from human blastocysts for obtaining pluripotent stem cells. The result of this "reprogramming" are tetraploid cells with capabilities comparable to those of ES. This method for reprogramming somatic cells to the pluripotent state can be considered as a valid alternative to hESCs: the pluripotent stem cells can be derived from somatic cells of the patient, eliminating that way the immunological problem of HeLa incompatibility; they can be produced in an abundant, unrestricted way, because the somatic cells to be derived are obtained from a skin biopsy and not from an embryo. Finally, this method does not require any human embryo, and this removes the ethical roadblock of hESCs. However, this method do rise its own problems. It would be indeed questionable to apply the product of this hybridization, i.e. tetraploid cells, on patients, given the uncertainties of such cells when administered in an organism. For that reason, some have proposed to proceed to targeted elimination of the ESC-derived chromosomes following cell-fusion, using a universal chromosome elimination cassette (CEC) (Matsumura and Tada 2008). From an ethical standpoint, this method presupposes the use of an ES cell line to reprogram the somatic cells. But the use a hESC line means cooperating with the process that has permitted to obtain that line; that is the removal of the ICM from a human blastocyst, destroying it. It will still be not approved by some on ethical grounds.

Inducing Pluripotency Through the Intervention of a Set of Specific Transcription Factors: The Induced Pluripotent Stem Cells (iPSC)

Yamanaka (University of Kyoto, Japan) took up the challenge of individuating these transcription factors which were needed for converting a somatic cell into a pluripotent, undifferentiated cell ESC-like. In a relatively short time, he was able to reach the groudbreaking achievement of inducing pluripotency in somatic cells by introducing a specific set of such transcription factors.

The Announcement and Its Verification

In July 2006. Yamanaka and colleagues at the Congress of the *International Society for Stem Cell Research* (ISSCR) in Toronto reported that, using a technique of transferring some of the factors responsible for pluripotency in preimplantation embryos blastomeres and hESCs (*Oct4*, *Sox2*, *c-Myc* and *Klf4)*, they had been able to change ordinary, "somatic", mouse skin differentiated cells (fibroblasts) in undifferentiated "*ES-like*" cells, which they named "*induced pluripotent stem cells*" (iPS) (Takahashi and Yamanaka 2006). A year later, these authors published identical cell reprogramming results (Takahashi et al. 2007), but this time using human fibroblasts. The scientific world realised the importance of this work, and its value, when the results of Yamanaka and colleagues were confirmed in 2007 by three different teams of researchers, one in Japan and two in the United States. What was astonishing in the method developed by Yamanaka et al. was the reliability of the approach and the fact that only four factors, Oct4, Sox2, Klf4 and c-Myc were needed to induce pluripotency in somatic cells.

iPSCs Are Comparable to ES Cells

Human iPS cells (hiPS), as produced by Yamanaka, Takahashi and colleagues, present all the characteristics of embryonic stem cells regarding morphology, self-renewal, and proliferation capability. They provide the expression of pluripotency related transcription factors (presence of 27 "specific epigenetic marker" genes of ES cells, and signature of the ES state, especially *Nanog*, *Oct3/4*, *Sox2*, *Cripto*, and *GDF3*). ES-specific surface antigens are found on the iPS. By restoring pluripotency in the fibroblasts, the reprogramming resets the biological age of the cell to its starting point, which can be seen in

an activation of the telomerase, causing a lengthening of the telomeres. hiPSCs are capable of producing *in vitro* derived cells representing the three embryonic germ layers, and can subsequently produce all the cell types of the organism, including germ cells. Like ES cells, iPS cells form embryoid bodies *in vitro* and develop into teratomas when they are injected subcutaneously into laboratory mice. When iPS cells are introduced into embryos at the blastocyst stage, they take part, as do ES cells, in the development of the three primitive embryonic germ layers, producing a "chimerical" embryo in which the descendants of the injected iPS cells are of all cell types, including gametes. Zhao et al. (Beijing, China) in 2009 have shown that iPSCs are able to generate a whole animal when aggregated into tetraploid embryos. Human iPSCs share with hES the same characteristic of been more similar to mouse EpiSCs than to mouse ESCs. They do not respond functionally to LIF, and they self-renew in response to FGF and activin. Like EpiSCs they are heterogenous between and within cell lines and can show marked biases in differentiation. hPSCS present a reprogramming-specific epigenetic signature comprised of nine aberrantly methylated genes that enable to segregate hESC and hiPSC lines regardless of the somatic cell source or differentiation state (Ruiz et al. 2012). The therapeutic significance of these nine genes awaits further research.

An Effective Alternative to hESCs, in Biology and Ethics

Human iPS cells offer distinct biological and ethical advantages over hESCs. First, a major advantage of using reprogrammed somatic cells is that they provide the possibility of autologous transplant in regenerative therapy, eliminating the need for immunosuppressive therapy. Second, iPSCs possess an unlimited resource capacity because they require only a tissue biopsy (or some blood collection) for derivation, whereas hESCs have severely limited resource capacity. Finally, and perhaps more important, human embryos need never to be destroyed to obtain iPS cells. Thus iPSCs avoid all the ethical problems and disagreements surrounding embryo destruction for hESCs collection.

The Weak Points of iPSCs

One of the main criticisms addressed to. Yamanaka's nuclear reprogramming technique concerns the inefficiency of the process. Indeed, fewer than 0.1 % of the skin fibroblasts thus treated demonstrate effective reprogramming. Various strategies have since been developed to increase the efficiency of cell reprogramming, such as the addition of SV40LT and hTERT, the inhibition of DNA methyltransferase (DNMT), the silencing of p53 and the introduction of UTF1 into the human fibroblasts to be reprogrammed, or the use of the GLI transcription factor. Another way to improve reprogramming efficiency would be to resort to embryonic stem cell-specific microRNAs (*mir*-291, 294, 295) which increase reprogramming efficiency and can replace *c-myc* in this reprogramming. A second criticism of Yamanaka's technique regards the use of the *c-myc* transgene for the cell reprogramming, given that this oncogene causes cancers in the host. However it is possible to substitute in the Yamanaka protocol the *n-myc* gene for the *c-myc* oncogene, without adversely affecting reprogramming efficiency. A third criticism of the method of reprogramming used by Yamanaka and colleagues concerns the use of multiple retroviral vectors – one for every gene transferred. These retroviral vectors were indeed responsible for insertional mutagenesis leukaemias in some of the children treated with gene therapy for severe immunodeficiency (SCID-X) at the Necker Hospital in Paris. More recent studies have shown that other vectors, less risky, can be used for reprogramming through bioengineering. Induced reprogramming has been obtained for example by using adenoviral vectors, a single lentiviral, polycistronic vector (carrying four reprogramming genes), a non-viral polycistronic vector (plasmid), a transposon, a vector carrying transgenes that was excised once the reprogramming had been done or a non-integrating episomal

vector. However the efficiency of iPS cell generation when an adenoviral vector or a plasmid is used is very low. Yamanaka, Okita and colleagues have devised a simple and more efficient method to generate integration-free human iPS cells: they use episomal plasmid vectors combined with p53 suppression and nontransforming L-Myc. The efficiency of iPSC induction using a plasmid combination can also be increased adding EBNA1, a factor for episomal amplification. iPS can also be generated using small molecules that promote or facilitate cell reprogramming, and can replace one or two reprogramming factors during iPS cell generation. Finally, the reprogramming proteins can be directly delivered to the cells by combining them with peptides or with recombinant proteins (Ding at the Scripps Research Institute).

Applications of iPS

Future Clinical Use

With their capacity to undergo unlimited self-renewal and to differentiate into all cell types in the body, iPSCs reprogrammed from somatic cells of human patients do hold promise for future clinical use, especially in regenerative medicine. Moreover, they can provide a renewable source of autologous cells for cell therapy which eliminate the concern for immune rejection.

Given the time needed to get a sufficient number of iPSCs from a skin biopsy, autologous iPSCS derived from the future recipient would not be very useful for pathologies that necessitate a rapid intervention – such as spinal cord injury – This is why Yamanaka is developing at the present an iPSCs bank with various HLA haplotypes which would cover the needs of Japan in homozygous iPSCs donors. iPSCS will not be applied directly to patients because of the risk of teratomas carried by pluripotent stem cells, but instead tumor-free progenitor stem cells derived from these iPSCs. The use of iPSCS in the clinical field has been already explored for many different pathologies: in hematology, for the treatment of Diabetes 1, in neurological diseases, in neurodegenerative diseases, for the derivation of dopaminergic neurones for Parkinson's disease, for the study of Parkinson's disease, for Huntington's disease, cardiac regeneration, muscular dystrophy, spinal cord injuries, immune modeling, the procurement of hematopoietic cells, and the production of red blood cells, megacaryocytes and platelets. Based on the good results obtained with iPSCs in the induction of retinal pigment in macular degeneration, Takahashi from RIKEN (Japan), is hoping to launch a clinical trial on age-related macular degeneration (AMD) treatment derived from hiPSCs (prepared without any viral vector). iPSCs can be combined with gene therapy for the treatment of numerous pathologies of genetic origin. Gene modified iPSCs have been experimented with success in animal models for sickle cell disease, Fanconi anaemia, laminopathy-associated *LMNA* mutations, α1-antitrypsine deficiency, osteogenesis imperfecta, thalassemia, congenital erythropoietic porphyria, Huntington's disease. Russell and colleagues from the University of Washington, Seattle, using a gene targeting method, have been able to correct the trisomy in Down syndrome in iPSCs derived from trisomic individuals (2012).

iPSC as cellular models, patient specific, of human diseases hiPS enable pathology modelling ("disease in a dish") with distinct advantages over hES (Ellis and Bhatia 2011). Many different cell models of various pathologies have been produced that way. Most recent models include human cell model of spinocerebellar ataxia type 2, spinal muscular atrophy, Huntington's disease, cystic fibrosis, Down syndrome, amyotrophic lateral sclerosis (ALS). iPSCs can constitute excellent cell models of pathologies which can be exploited to illuminate disease physiopathology, especially in the field of genetic disorders. One of the benefits from these cell models of genetic diseases is that they can reveal disease related phenotypes, and that these phenotypes can be reversed *in vitro* using appropriate drugs. hiPSCs cell models of pathologies offer pharmaceutical research a material of choice for the screening of molecules that could potentially be used therapeutically against these diseases, and for identify

novel drug targets (Inoue and Yamanaka 2011). hiPSCs will permit a patient-specific drug screening which is the prime condition for the advent of personalized medicine (as example for amyotrophic lateral sclerosis) (Egawa et al. 2012).

Limitations of iPS

Despite these advantages, hiPS have their limitations. The first is that there is often heterogeneity in the degree of effective reprogramming reached by the cells that are qualified as iPS. Only some of these cells have properties comparable to those of ES, which explains the rather variable results found by different authors in their evaluation of iPS properties (Soldner and Jaenisch 2012). One particularity of iPSCs which has cast some doubt about their functional capacities is that these cells seem to keep an epigenetic "memory" of their cellular type of origin, which explains why the transcriptional profiles of these cells are not identical whether they are derived from skin fibroblasts, hematopoietic cells, or myogenic cells. Three studies, published in the March 2011 issue of "Nature" showed that some genetic and epigenetic anomalies appear during the process of reprogrammation for obtaining iPSCs or in the following cell culture. Such anomalies are not seen in somatic cells reprogrammed in hESCs through ovocyte nuclear transfer. The iPSCs which bear genomic abnormalities are not fully pluripotent, as evaluated by the tetraploid complementation assay. One another limitation of iPSCs concerns their efficiency at forming the various cell types by differentiation. Hu and colleagues from the University of Wisconsin have found that iPSCS generated neuroepithelia and various types of functional neurones with less efficiency and greater variability than hESCs. Feng and Lanza from the *Stem cell and Regenerative Medicine International* (Worcester, MA) have reported that hES cells generated a thousand times more of the desired cells than iPS. These results must, of course, be interpreted in the light of more specific studies because they may be simply the result of incomplete reprogramming. The most important question concerns the risk of causing cancer associated with a future use of hiPSCs in patients, either because of the inclusion of oncogenic transgenes, or because of the persistence of undifferentiated cells in the differentiated batch that will be administered to the patient, or because of still unknown factors connected with the reprogramming. Experimental studies on animals have been reassuring in that regard up to now.

Ethical Perspective on iPS

From the ethical standpoint, the development of iPS technology allows to obtain in abondance pluripotent stem cells of a quality comparable to that of ESCs, without necessitating the sacrifice of human embryos. One cannot underestimate this achievement. On the other hand, the development of iPSCs raises new questions. One particular issue concerns the possible generation of germ cells from iPS. A number of laboratories are aiming at deriving gametes from human iPSCS. It has been shown recently (Easley et al. 2012) that hESCs ad hIPSCs can be induced to differentiate into advanced male germ cell lineages, including postmeiotic, spermatid-like cells. These studies may lead to the procurement of male gametes derived from somatic cells of an individual of either sex. This paves the way to personal procreative choices which may conflict with ethics and local laws.

Possibilities are opening up for the translation of the iPS research into clinical applications on patients. But we are not yet sure of the safety of iPS cells. The question of genomic stability and of quality of reprogrammation has to be answered. There are therefore still many "roadblocks" on the way to clinical application of iPSCS. It would be tempting to apply already the iPS technology to some patients, in a compassionate perspective, for desperate issues such as spinal cord injuries. But wisdom and precaution principle require more studies on animals. Because of these uncertainties of iPSCs, some think that more "natural" processes for reprogramming, such as those that are acting in salamander limb regeneration, should be considered rather than to use

bioengineered reprogrammed cells (Zhang et al. 2012). One another way to avoid these uncertainties of iPSCs in regenerative medicine could be to have recourse instead to direct conversion ("lineage reprogramming") (Asuelime and Shi 2012), a field in full development today.

Conclusion

The development of induced pluripotent cells, obtained by somatic cell dedifferentiation, constitutes a prime order progress, not only for stem cell studies, but for cell biology in general. It shows in effect that it is possible to "reprogramme" differentiated cells epigenetically, making these cells go back to their developmental starting point by erasing their epigenetic adult cell "memory" and re-activating the expression of pluripotency genes in their genome. iPS cells offer a clear, simple and effective alternative solution to embryonic stem cells. But there remain a great deal to learn about iPSC safety, the underlying mechanisms of reprogramming, and ways to direct their differentiation into specific functional cell types.

References

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknis MA, Itskovitz-Eldor J, Thomson JA (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227:271–278

Asuelime GE, Shi Y (2012) A case of cellular alchemy: lineage reprogramming and its potential in regenerative medicine. J Mol Cell Biol 4:190–196

Blau HM, Chiu CP, Webster C (1983) Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell 32:1171–1180

Briggs R, King TJ (1952) Transplantion of living nuclei from blastula cells into enucleated frog's eggs. Proc Natl Acad Sci U S A 38:455–463

Chang KH, Lim JM, Kang SK, Lee BC, Moon SY, Hwang WS (2003) Blastocyst formation, karyotype, and mitochondrial DNA of interspecies embryos derived from nuclear transfer of human cord fibroblasts into enucleated bovine embryos. Fertil Steril 80:1380–1387

Chung Y, Klimanskaya I, Becker S, Marh J, Lu S-J, Johnson J, Meisner L, Lanza R (2006) Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. Nature 439:216–219

Cibelli JB, Grant KA, Chapman KB, Cunniff K, Worst T, Green HL, Walker SJ, Gutin PH, Vilner L, Tabar V, Dominko T, Kane J, Wettstein PJ, Lanza RP, Studer L, Vrana KE, West MD (2002) Parthenogenetic stem cells in nonhuman primates. Science 295:819

Collas P, Taranger CK, Boquest AC, Noer A, Dahl JA (2006) On the way to reprogramming cells to pluripotency using cell-free extracts. Reprod Biomed Online 12:762–770

Cowan CA, Atienza J, Melton SD, Eggan K (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309:1369–1373

Drukker M, Benvenisty N (2004) The immunogenicity of human embryonic stem-derived cells. Trends Biotechnol 22:136–141

Drukker M, Katz G, Urbach A, Schuldiner M, Markel G, Itskovitz-Eldor J, Reubinoff B, Mandelboim O, Benvenisty N (2002) Characterization of the expression of MHC proteins in human embryonic stem cells. Proc Natl Acad Sci U S A 99:9864–9869

Easley CA 4th, Phillips BT, McGuire MM, Barringer JM, Valli H, Hermann BP, Simerly CR, Rajkovic A, Miki T, Orwig KE, Schatten GP (2012) Direct differentiation of human pluripotent stem cells into haploid spermatogenic cells. Cell Rep 2:440–446

Egawa N, Kitaoka S, Tsukita K, Naitoh M, Takahashi K, Yamamoto T, Adachi F, Kondo T, Okita K, Asaka I, Aoi T, Watanabe A, Yamada Y, Morizane A, Takahashi J, Ayaki T, Ito H, Yoshikawa K, Yamawaki S, Suzuki S, Watanabe D, Hioki H, Kaneko T, Makioka K, Okamoto K, Takuma H, Tamaoka A, Hasegawa K, Nonaka T, Hasegawa M, Kawata A, Yoshida M, Nakahata T, Takahashi R, Marchetto MC, Gage FH, Yamanaka S, Inoue H (2012) Drug screening for ALS using patient-specific induced pluripotent stem cells. Sci Transl Med 4:145ra104. doi:10.1126/scitranslmed.3004052

Ellis J, Bhatia M (2011) iPSC technology: platform for drug discovery. Clin Pharmacol Ther 89:639–641

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156

French AJ, Adams CA, Anderson LS, Kirchen JR, Hughes MR, Wood SH (2008) Development of human cloned blastocysts following somatic cell nuclear transfer (SCNT) with adult fibroblasts. Stem Cells 26:85–493

Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE (2005) Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol 166:1781–1791

Gurdon JB (1960) The developmental capacity of nuclei taken from differentiating endoderm cells of Xenopus laevis. J Embryol Exp Morphol 8:505–526

Håkelien A-M, Landsverk HB, Robl JM, Skålhegg BS, Collas P (2002) Reprogramming fibroblasts to express T-cell functions using cell extracts. Nat Biotechnol 20:460–466

Harris H, Miller OJ, Klein G, Worst P, Tachibana T (1969) Suppression of malignancy by cell fusion. Nature 223:363–368

Hurlbut WB (2005) Altered nuclear transfer as a morally acceptable means for the procurement of human embryonic stem cells. Natl Cathol Bioeth Q 5:145–151

Ilic D, Giritharan G, Zdravkovic T, Caceres E, Genbacev O, Fisher SJ, Krtolica A (2009) Derivation of human embryonic stem cell lines from biopsied blastomeres on human feeders with minimal exposure to xenomaterials. Stem Cells Dev 18:1343–1350

Illmensee K, Levanduski M, Zavos PM (2006) Evaluation of the embryonic preimplantation potential of human adult somatic cells via an embryo interspecies bioassay using bovine oocytes. Fertil Steril 85: 1248–1261

Inoue H, Yamanaka S (2011) The use of induced pluripotent stem cells in drug development. Clin Pharmacol Ther 89:655–661

Knudtzon S (1974) In vitro growth of granulocyte colonies from circulating cells in human cord blood. Blood 43:357–361

Landry DW, Zucker HA (2004) Embryonic death and the creation of human embryonic stem cells. J Clin Invest 114:1184–1186

Lanza RP, Cibelli JB, West MD (1999) Human therapeutic cloning. Nat Med 5:975–977

Lerou PH, Yabuuchi A, Huo H, Takeuchi A, Shea J, Cimini T, Ince TA, Ginsburg E, Racowsky C, Daley GQ (2008) Human embryonic stem cell derivation from poor-embryos. Nat Biotechnol 26:212–214

Li J, Liu X, Wang H, Zhang S, Liu F, Wang X, Wang Y (2009) Human embryos derived by somatic cell nuclear transfer using an alternative enucleation approach. Cloning Stem Cells 11:39–50

Martin GR (1981) Isolation of a pluripotential line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634–7638

Matsui Y, Zsebo K, Hogan BL (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70:841–847

Matsumura H, Tada T (2008) Cell fusion-mediated nuclear reprogramming of somatic cells. Reprod Biomed Online 16:51–56

Mitalipova M, Cahoun J, Shin S, Wininger D, Schultz T, Noggle S, Venable A, Lyons I, Robins A, Stice S (2003) Human embryonic stem cell lines derived from discarded embryos. Stem Cells 21:521–526

Pickering SJ, Braude PR, Patel M, Burns CJ, Trussler J, Bolton V, Minger S (2003) Preimplantation genetic diagnosis as a novel source of embryos for stem cell research. Reprod Biomed Online 7:353–364

Resnick JL, Bixler LS, Cheng L, Donovan PJ (2003) Long-term proliferation of mouse primordial germ cells in culture. Nature 359:482–483

Rossant J (2008) Stem cells and early lineage development. Cell 132:527–531

Ruiz S, Diep D, Gore A, Panopoulos AD, Montserrat N, Plongthongkum N, Kumar S, Fung HL, Giogetti A, Bilic J, Batchelder EM, Zaehres H, Kan NG, Schöler HR, Mercola M, Zhang K, Izpisua Belmonte JC (2012) Identification of a specific reprogramming-associated epigenetic signature in human induced pluripotent stem cells. Proc Natl Acad Sci U S A 109:16196–16201

Schamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhardt JD (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A 95:13726–13731

Soldner F, Jaenisch R (2012) iPSC disease modeling. Science 338:1155–1156

Sparman M, Dighe V, Sritanaudomchai H, Ma H, Ramsey C, Pedersen D, Clepper L, Nighot P, Wold D, Hennebold J, Mitalipov S (2009) Epigenetic reprogramming by somatic cell nuclear transfer in primates. Stem Cells 27:1255–1264

Spemann H (1938) Embryonic development and induction. Yale University Press, New Haven, Haffner Publishing, pp 210–211, 371–372

Stojkovic M, Stojkovic P, Leary C, Hall VJ, Armstrong L, Herbert M, Nesbitt M, Lako M, Murdoch A (2005) Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes. RBM Online 11:226–231

Tada M, Tada T, Lefebvre L, Barton SC, Surani MA (1997) Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J 216:6510–6520

Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T (2001) Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 11:1553–1558

Taei A, Gourabi H, Seifinejad A, Totonchi M, Shahbazi E, Valojerdi MR, Eftekhari P, Karimian L, Baharvand H (2010) Derivation of new human embryonic stem cell lines from preimplantation genetic screening and diagnosis-analyzed embryos. In Vitro Cell Dev Biol 46:395–402

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblasts cultures by defined factors. Cell 126:663–676

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Taylor SM, Jones PA (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocytes. Science 282:1145–1147

Till JE, McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222

Turnpenny L, Brickwood S, Spalluto CM, Piper K, Cameron IT, Wilson DI, Hanley NA (2003) Derivation of human embryonic germ cells: an alternative source of pluripotent cells. Stem Cells 21:598–609

Wayayama S, Hikichi T, Suetsugu R, Sakaide Y, Bui HT, Mizutani E, Wakayama T (2007) Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. Stem Cells 25:986–993

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813

Ying QL, Wary J, Nichols J, Battle-Morera L, Doble B, Woodgett J, Cohen P, Smith A (2008) The ground state of embryonic cell self-renewal. Nature 453: 519–523

Zhang Y, Yao L, Yu X, Ou J, Hui N, Liu S (2012) A poor imitation of a natural process: a call to reconsider the iPSC engineering technique. Cell Cycle 11:4536–4544

Zhao XY, Li W, Lv Z, Liu L, Tong M, Hai T, Hao J, Guo C-I, Ma Q-W, Wang L, Zeng F, Zhou Q (2009) iPS cells produce viable mice through tetraploid complementation. Nature. doi:10.1038/nature8267 (advanced online publication)

Cancer Stem Cell Dynamics and Regulation

7

Han S. Myat and Mary E. Sehl

Contents

Abstract

Mathematical models provide many insights into both the biology of cancer and the optimization of its treatment. Cancer stem cells represent a novel target of cancer therapeutics. While cancer stem cells can represent a relatively small proportion of the total cells within a tumor, they are responsible for driving the dynamics of tumor growth and invasiveness. Modeling in combination with experimental validation has advanced quantitative understanding of cancer stem cells and their interaction with their microenvironment. We present here the salient features of a set of examples of mathematical models that have contributed to our current understanding of how cancer stem cells are regulated and describe further opportunities for modeling.

Introduction

Mathematical models have proven tremendously useful for several decades in cancer research. Models range from molecular and cellular level models inspired by results from basic science experiments to population level models based on epidemiological observations, and address key questions that have important implications in the care of the cancer patient. Multiscale models bridge these components, using models and experimental

H.S. Myat
Division of Hematology-Oncology, University of California, Suite 2333, PVUB, 957059, Los Angeles, CA 90095, USA

M.E. Sehl (✉)
Department of Biomathematics, and Division of Hematology-Oncology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA
e-mail: MSehl@mednet.ucla.edu

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data at each level to make predictions about the dynamics of emergent properties of these complex systems. Figure 7.1 shows the varying levels of detail in cancer models, providing examples of modeling questions that have advanced cancer research at each level.

The cancer stem cell and its microenvironment represent a complex dynamic system that presents exciting opportunities to address compelling modeling questions (Ashkenazi et al. 2007; Sehl et al. 2009). While stem cells can comprise only a small proportion of the total tumor population, they drive the growth and invasive nature of the tumor. Stem cells possess two important abilities: long term asymmetric self-renewal, and potency to generate all of the cell types that comprise a tissue. Eradication of cancer stem cells offers the hope for cure even in advanced settings of malignancy. Figure 7.2 reveals the expected change in population size with expected with gradual reduction in self-renewal capacity with aging, and abrupt increase in self-renewal in a proliferative disorder. A quantitative understanding of cancer stem cells would greatly enhance understanding of the underlying biology of carcinogenesis and the ability to optimally devise targets that would eliminate cancer stem cell populations. Elements of the stem cell niche govern the frequency with which stem cells self-renew, differentiate, and undergo programmed cell death. Recent research suggests that aberrancies in the regulatory behavior of the niche may contribute to carcinogenesis. Mathematical models can shed light on the dynamics of cancer stem cells and stochastic simulation can help to untangle the complex overlapping regulatory feedback networks that influence the behavior of stem cells. While diverse models have been explored in cancer stem cell research and have advanced our understanding of stem cell kinetics in compelling ways, we review here the salient features of a few examples.

Extrapolation of Stem Cell Kinetics from Animal Models

When constructing mathematical models of cancer stem cells, critical questions arise related to the total number stem cells in the human body, and what are the rates of self-renewal and death of these cells. Mathematical models using data from animal models to extrapolate these

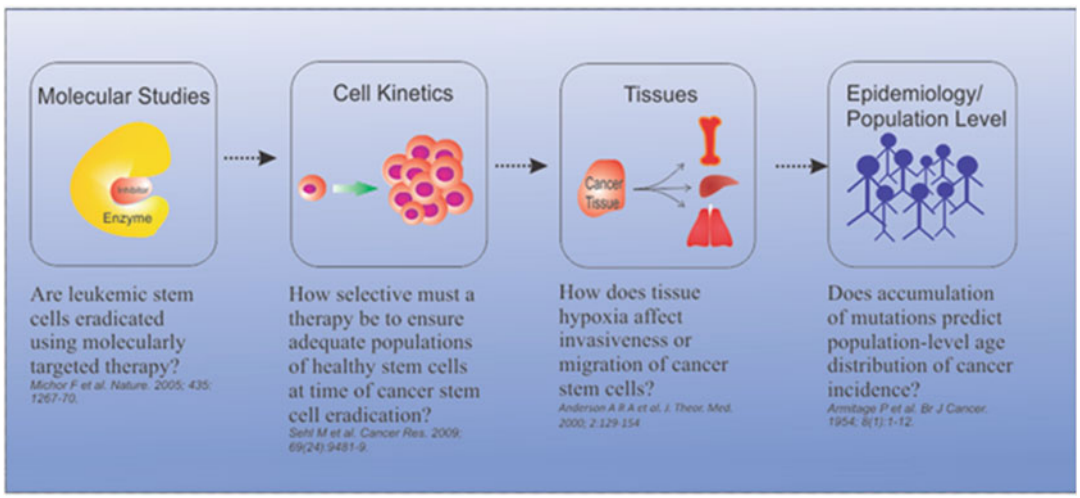

Fig. 7.1 Examples of compelling oncology questions addressed with mathematical modeling. Mathematical models have been used to address cancer biology questions at varying levels from molecular to population studies. Multiscale models can be used to integrate information from each level and address potential interactions and emergent properties about cancer stem cell dynamics

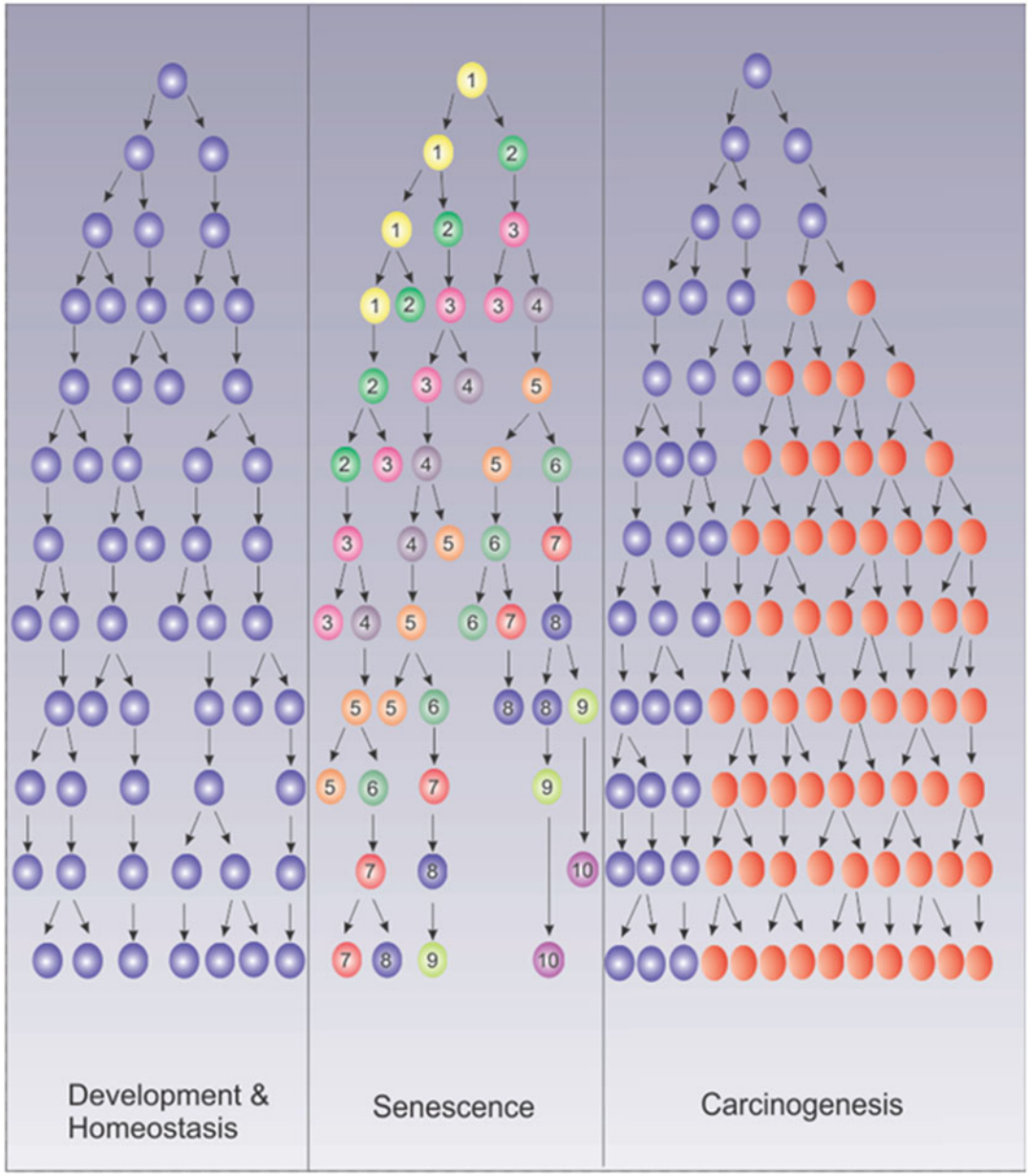

Fig. 7.2 Stem cell population dynamics. In normal tissue homeostasis (*left panel*), a mixture of asymmetric and symmetric self-renewal is required to maintain the stem cell population size relatively constant over time. As stem cells undergo a series of genetic and epigenetic changes with age, the replicative capacity of a stem cell diminishes, gradually leading to a reduction in the size of the stem cell compartment. A mutation in a stem cell or an aberrancy in microenvironmental signaling that leads to an increase in the frequency of symmetric stem cell self-renewal causes a rapid increase in the stem cell compartment size and in their offspring

quantities provide the best estimates of these parameters. Classic results from Till et al. (1964) and Abkowtiz et al. (2002) using murine, feline, and primate data have facilitated the development of informed mathematical models comparing normal stem cell and cancer stem cell dynamics. Here we briefly describe the impact of these results.

Stem cells have ability to proliferate extensively, differentiate, and self-renew over long periods of time. Since spleen colony-forming cells have these properties, Till and McCulloch deduced that they must be stem cells (1964). In mouse hematopoietic tissue, macroscopic colonies can be counted to determine the number of stem cells, because each colony is derived from a single stem cell. Hematopoiesis is under precise control where the stem cells are the sites of regulation. While the numbers of differentiated cells in the blood remain essentially constant, rapid changes in cell number are seen under conditions of stress or increased demand.

Distributions of spleen colony-forming cells in the colonies can be explained by a random "birth-and-death" process, where birth occurs when colony-forming cells self-renew, and death occurs when colony-forming cells differentiate. A three-compartment mathematical model is developed assuming that the process is random and expected to agree with experimental data. The first compartment consists of stem cells with extensive proliferative capacity that give rise to new stem cells and differentiated cells. The second compartment contains early differentiated cells with limited proliferative capacity which can give rise to differentiated cells and the third compartment contains mature cells only. Each colony-forming cell in the model may divide and produce two new colony-forming cells or may differentiate and lose the ability to form colonies but can still divide to produce fully differentiated cells. Results from the model matched the observed distribution, suggesting that individual cells within the population are not closely regulated but the population size is regulated by control mechanisms varying the birth and death rates.

Abkowtiz et al. (1996)) also showed that patterns of clonal contributions to hematopoiesis seen in experimental animals can be explained by stochastic differentiation. A stem cell's behavior is governed by its unique microenvironment and integration of input from other cells. Since it is difficult to isolate the stem cells, they determined the contribution of stem cells to progenitors by comparing simulation to data from autologous marrow transplantation studies of G6PD heterozygous female Safari cats. The estimated frequency of quiescent hematopoietic stem cells in cats is 6 per 10^7 nucleated marrow cells whereas that in mice is 50–300 per 10^7 nucleated marrow cells, suggesting that hematopoietic stem cell concentrations decrease with increasing animal size or lifespan. A replication rate of once every 10 weeks resulted in the distributions that reveal good agreement between predicted outcome and experiment. Thus, clonal dominance does not require specialized phenotype or genetic advantage.

In a later study, Abkowitz et al. used the murine data to study the kinetics of murine hematopoietic stem cells (Abkowtiz et al. 2000). The experimental murine data (density of percent donor cell) was obtained by limiting-dilution, competitive-repopulation studies. Different sets of parameter values were explored in simulations and compared with experiment. In murine experiments, the average replication rate is once every 2.5 weeks and the frequency of hematopoietic stem cells is 8 per 10^5 nucleated marrow cells. In feline, the average replication rate is once every 8.3–10 weeks and the frequency of hematopoietic stem cells is 6 per 10^7 nucleated marrow cells. This implies that as animals increase in size, the number of hematopoietic stem cells decreases and their replication rates decrease.

Although larger mammals have longer lifespan and need more blood cells per life time than smaller ones, the total number of hematopoietic stem cells per animal is conserved among mammals (Abkowtiz et al. 2002). The total number of HSCs per animal is the product of total number of nucleated marrow cells, determined by ^{59}Fe distribution analysis, and the frequency of HSCs in the marrow, determined by limiting-dilution, competitive repopulation assays. Since the total number of HSCs is the same in both cats and mice and cats need larger numbers of mature blood cells, each feline HSC must produce more progenitors than each murine HSC does. Based on these, the frequency of HSCs in human is estimated to be 0.7 to 1.5 per 10^8 nucleated marrow cells. In addition, this suggests that the frequency of HSCs in infants and young children is expected to be higher than that in adults.

In another experiment, Shepherd et al. estimated the HSC replication rate of nonhuman primates and used the values to extrapolate HSC replication rates for the humans (Shepherd et al. 2007). Experiments, including retroviral-mediated gene transfer and quantitation of the average telomere length of granulocytes, were combined with stochastic simulation were used to estimate the parameters. These studies revealed that the rate of self-renewal per animal is conserved, and provided a firm basis for many modeling studies that have utilized these estimates in modeling studies of both normal HSCs and leukemic stem cells.

Predicting Susceptibility to Molecularly Targeted Therapies

Chronic myelogenous leukemia (CML) is the first malignancy recognized as a stem cell disorder, marked by the chromosomal translocation t(9;22) and resultant expression of the fused proto-oncogene BCR-ABL in all cells of the myeloid lineage. It is also among the first malignancies for which a molecularly targeted therapy caused dramatic responses and improvements in survival. Imatinib targets the constitutively active ABL tyrosine kinase. In 2005, Michor et al. used a four compartment model including stem cells, progenitors, differentiated cells and fully differentiated cells to investigate the behavior of CML (Michor et al. 2005). In this model, both normal and leukemic stem cells have the same hierarchy. Levels of BCR-ABL transcripts exhibit a biphasic exponential decline with the first slope of 0.05 ± 0.02 per day between 0 and 3 months and the second slope of 0.008 ± 0.004 per day between 6 and 12 months. Mathematical modeling showed that the first slope corresponds to the turnover rate of differentiated leukemic cells, with an average lifespan of 20 days, and the second slope corresponds to the turnover rate of leukemic progenitors, with an average lifespan of 125 days. In patients who discontinued imatinib therapy because of side effects, transcripts returned quickly to pre-treatment level even if they had been treated with imatinib for up to 3 years. The average rate of the exponential increase after discontinuation of imatinib was used to estimate the rate at which leukemic stem cells result in terminally differentiated leukemic cells. The authors concluded that long-term therapy does not deplete leukemic stem cells. The model was extended to describe the development of resistance in CML. Even prior to treatment, patients may have leukemic cells with "advantageous mutations" that promote growth of leukemic cells. There are several suggested cellular mechanisms for drug resistance of leukemic stem cells, including imatinib being a substrate for a multidrug resistance protein and less dependence of leukemic stem cells on BCR-ABL. Thus, at that point, there were two problems with therapy: unresponsiveness of leukemic stem cells and development of drug resistance.

In 2009, therapy that combined imatinib with Granulocyte-Colony Stimulating Factor (G-CSF) was studied in order address the question of whether quiescence of leukemic stem cells is a possible mechanism for drug resistance (Foo et al. 2009). The G-CSF was used to awaken leukemic stem cells and pulsed imatinib with G-CSF is used to reduce the proliferative effect of G-CSF. Pulsed imatinib with G-CSF had shown a decrease in leukemic stem cells in vitro. Changes to parameters were made to test the sensitivity of the model that affects the quiescent stem cells. Varying the equilibrium frequency of cycling stem cells is very sensitive. The therapy is effective only if the initial LSC population is mostly made up of quiescent leukemic stem cells. In vivo studies along with the mathematical model showed that the therapy does not actually eradicate LSC since the initial LSC composition was rarely quiescent. In addition, the model suggests that the therapy increases the probability of developing resistance.

Mathematical modeling combined with observations from 10 years of imatinib treatment later revealed a triphasic exponential decline in BCR-ABL transcript levels (Tang et al. 2011). A similar approach as in the 2005 study was used to conclude that the third slope corresponds to the turnover rate of leukemic stem cells and appears after 34 months of treatment. This study rejects the previous idea that leukemic stem cells are

insensitive to targeted treatment and confirms that given enough time the imatinib therapy can actually eliminate the leukemic stem cells.

However, the problem with treatment resistance still remains. Recent mathematical modeling suggests that combination therapy might be effective against resistance, in the absence of resistance mutations to both drugs, and drugs should be administered simultaneously rather than sequentially (Bozic et al. 2013). In the future, more drugs targeting different pathways should be combined to overcome resistance.

Aging, Cancer, and Stem Cells

Cancer and aging are known as rival demons because tumor suppressor mechanisms that are in place to protect against accumulation of oncogenic mutations with age also lead to cell senescence (Campisi 2003). Mathematical models have been used to explore the link between aging and cancer by relating rising incidence of cancer with advancing age to mutation accumulation (Armitage and Doll 1954). Recently, genetic and epigenetic changes in stem cells have been implicated in both normal aging processes and cancer risk (Campisi 2003; Sharpless and DePinho 2004; Teschendorff et al. 2010; Bocklandt et al. 2011). These include telomere shortening, increased expression of the cell cycle regulatory gene $p16^{INK4A}$, and altered methylation patterns among polycomb group target genes. Models of telomere length dynamics have been used to study stem cell turnover in vivo, during normal hematopoiesis, after stem cell transplantation, and in disease states such as acquired bone marrow failure syndromes and myeloproliferative disorders (Brümmendorf and Balabanov 2006).

Stress, inflammation, and hormesis are also important mediators of the link between cancer and aging. Normal aging is associated with immunosenescence of lymphocytes leading to increased secretion of cytokines such as Il-6 and TNF-α (Michaud et al. 2013). This state of chronic immune activation has been associated with DNA-modifying events that lead to an increased risk of malignancy (Breen et al. 2011). Inflammatory cytokines are also important regulators of stem cell states, increasing the transition of stem cells from a proliferative, epithelial like state to a quiescent, invasive state (Korkaya et al. 2011). The association between stress, inflammation, and carcinogenesis is an important area of study. Mathematical models have proven helpful in the study the effects of stress and hormesis on lifespan and the relationship between accelerated aging and carcinogenesis (Butov et al. 2001, 2002, 2003). Induced expression of heat shock proteins is required for all organisms to survive exposures to acute stress. Stochastic models have been employed to study the balance between damage and repair in stressed worms. Predictions of lifespan using this model match experimental observations well (Butov et al. 2002). Further stochastic models have been used to examine levels of free radicals and cumulative damage to DNA, lipid structures, and proteins, leading to genetic instability and malignant transformation (Butov et al. 2001). Predictions from these models match both experimental data of survival and fertility curves in Mediterranean fruit flies, and cancer incidence in rats exposed to bromodeoxyuridine (Butov et al. 2002, 2003). Future models should endeavor to examine the effects of immunosenescence, chronic inflammatory networks, and DNA-modifying events on stem cell behavior.

Mathematical Models of Stem Cell Regulatory Networks

Microenvironmental signals that normally regulate stem cell behavior, including self-renewal and differentiation, may go awry during carcinogenesis (Li and Neaves 2006). Figure 7.3 reveals the interactions between stem cells and the components of their microenvironment, including the effects of intracellular signaling, cytokines, vascular effects, and changes associated with aging and chronic immune activation. Mathematical models have been used to study the role of feedback in cancer stem cell dynamics (Sun and Komarova 2012). Using a Moran process model involving negative control loops on proliferation

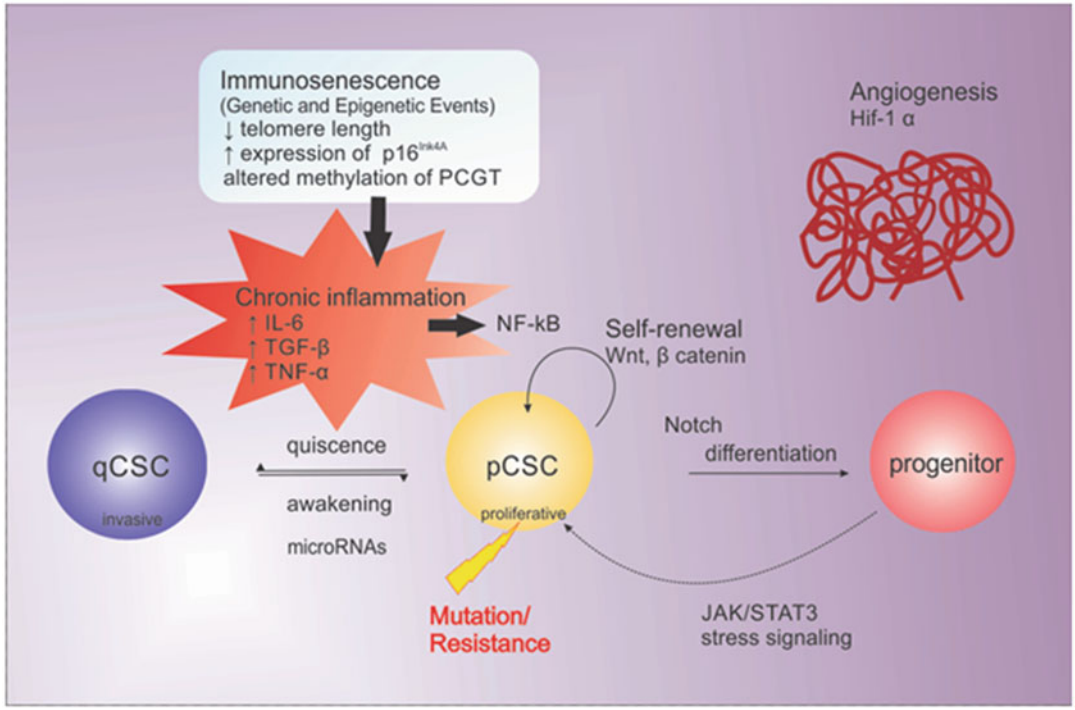

Fig. 7.3 Regulatory dynamics of the tumor microenvironment. The ability of the stem cell to undergo quiescence, differentiation, and self-renewal is regulated by elements of the stem cell niche. Inflammatory cytokines govern the transition from a proliferative (pCSC) to a more invasive, quiescent state (qCSC). Wnt, Notch, and β-catenin signaling pathways as well as Jak/Stat feedback stress signaling from differentiated cells regulate self-renewal and differentiation. Immunosenecence leads to a chronically activated immune state with increased levels of cytokines. Hypoxia leads to activated Wnt signaling and increased numbers of cancer stem cells. Compelling modeling questions highlighted in this review address varying components of these pathways

and differentiation, the authors showed that nonlinear regulatory control loops have evolved to maintain a homeostatic level of stem cells and reduce fluctuations in normal tissues. These models reinforce the need to include feedback effects when studying cancer stem cell dynamics in response to therapy.

Modeling changes associated with vasculature has become a priority in cancer research. According to Judah Folkman's model of tumor angiogenesis, expanding tumor cells can sense that they are growing away from the blood supply and secrete angiogenic factors (Folkman 1971). The growth of tumor cells in a hypoxic environment has been thought to induce angiogenesis. Once the angiogenic switch is turned on, angiogenic activators are promoted and angiogenic inhibitors are repressed so that the rates of tumor cell proliferation increase (Liao and Johnson 2007). The tumor cells from later stages of tumor progression can induce a potent angiogenic response while the tumor cells from early stages cannot. In tumors, stromal cells such as macrophages and neutrophils can also induce angiogenic response. Tumors of less than 1 mm in diameter do not need an angiogenic switch since they are already well vascularized. Factors involved in hypoxia-induced angiogenesis include hypoxia-inducible factors (HIF), vascular endothelial growth factor (VEGF), nitric oxide synthetase (NOS), and other growth factors. Mathematical models are helpful in describing the effects of various angiogenic and anti-angiogenic factors on the tumor microenvironment (Mantzaris et al. 2004). HIF-1α is activated by hypoxia or by mutations in oncogenes and tumor suppressor genes and

shown to promote tumor growth by regulating endothelial cell activity (Liao and Johnson 2007).

Hypoxia poses several challenges. Hypoxia can induce drug-resistance because some drugs require oxygen to exert its cytotoxic effect and also promote genetic instability of tumor cells (Teicher 1994). Furthermore, new blood vessels formed via hypoxia-induced angiogenesis is chaotic and inefficient which makes the delivery of drugs to the tumor difficult. The vasculature is leaky causing the interstitial fluid pressure to increase (Jain et al. 2007), and fluid convection at the tumor margin causes the growth factors and cells to go out of tumor leading to angiogenesis and metastasis (Jain et al. 2007). Mathematical modeling of tumor invasion and migration at both macro- and micro-levels matches the invasion patterns observed in clinics and allows us to see how tumor's interaction with extracellular matrix leads to metastasis (Anderson et al. 2000). Parameter estimation in the model can predict the size of tumor mass necessary for adequate removal of solid tumor (Anderson et al. 2000). In addition to allowing the tumor to grow larger, angiogenesis promotes tumor metastasis to other tissues and allows the tumor to thrive at the secondary sites. A mathematical model by Scott et al. reveals that the tumor proliferates through a process called self-seeding in which the secondary seeding is the key player (Scott et al. 2013). Self-seeding is an event where the circulating tumor cell migrates back to the original tumor through the vasculature and repopulate. The secondary seeding occurs when the tumor growing at a secondary revisit the primary tumor.

Treatment of cancer with angiogenisis inhibitors, including bevacizumab and sorafenib, targets genetically stable endothelial cells, and has been shown to delay time to progression. However, the development of resistance to anti-angiongenic drugs remains an important concern. Despite the delay in tumor progression, cancers that recur after anti-angiogenesis therapy are often more aggressive. Combination therapy with chemotherapy and anti-angiogenic therapy is associated with improved outcomes, including delayed time to progression. This results from normalization of blood vessels and improved delivery of drugs before they are destroyed (Jain 2001). Mathematical modeling demonstrates that vascular normalization can indeed improve the delivery of drugs to tumor and lower the chance of metastasis (Jain et al. 2007). These models study interaction of the tumor with the vasculature to test the efficacy of combination therapy and confirm the beneficiary effects observed in experimental and clinical studies (Kohandel et al. 2007). Modeling can also be useful in predicting the optimized drug dose and schedule (Benzekry et al. 2011).

Importantly, a recent discovery using human breast cancer xenograft experiments shows that anti-angiogenesis therapies, including sunitinib and bevacizumab cause an increase in cancer stem cells (Conley et al. 2012). Hypoxia leads to activation of Wnt/Akt/β-catenin signaling, driving cancer stem cell self-renewal. These findings suggest an underlying mechanism for the aggressive nature of the tumor recurrence following anti-angiogenic therapies and suggest the promise of combining stem cell targeted therapies with anti-angiogenesis therapies.

Conclusions and Opportunities for Future Modeling

Recent advances in technology allow for isolation and analysis of genetic expression within single cells. Systems biology approaches and biomathematical modeling will allow for an integrated analysis of large amounts of data generated from these genetic and epigenetic experiments. Combining mathematical modeling and analytic tools will advance our understanding of normal healthy stem cell behavior as well as aberrancies that arise in the stem cell and its microenvironmental regulation during carcinogenesis. Mathematical models of stem cell behavior can be used in combination with experiment to optimize therapeutic approaches in stem cell regenerative medicine and in targeting cancer stem cells. Future challenges and opportunities in modeling include screening for combinations of agents that target the cancer stem cell and its niche, incorporating spatial effects, senescence,

and the effects of the immune system. We expect that mathematical models will prove especially useful in guiding treatment strategies that combine conventional chemotherapies with molecularly targeted therapies, and novel approaches that utilize hematopoietic stem cells genetically engineered to attack tumor antigens.

References

Abkowtiz JL, Catlin SN, Guttorp P (1996) Evidence that hematopoiesis may be a stochastic process in vivo. Nat Med 2(2):190–197

Abkowtiz JL, Golinelli D, Harrison DE, Guttorp P (2000) In vivo kinetics of murine hematopoietic stem cell. Blood 96(10):3399–3405

Abkowtiz JL, Catlin SN, McCallie MT, Guttorp P (2002) Evidence that the number of hematopoietic stem cells per animal is conserved in mammals. Blood 100(7):2665–2667

Anderson ARA, Chaplain MAJ, Newman EL, Steele RJC, Thompson AM (2000) Mathematical modelling of tumour invasion and metastasis. J Theor Med 2:129–154

Armitage P, Doll R (1954) The age distribution of cancer and a multi-stage theory of carcinogenesis. Br J Cancer 8:1–12

Ashkenazi R, Jackson TL, Dontu G, Wicha MS (2007) Breast cancer stem cells-research opportunities utilizing mathematical modeling. Stem Cell Rev 3:176–182

Benzekry S, Chapuisat G, Ciccolini J, Erlinger A, Hubert F (2011) A new mathematical model for optimizing the combination between antiangiogenic and cytotoxic drugs in oncology. C R Acad Sci Paris Ser I 350:23–28

Bocklandt S, Lin W, Sehl ME, Sánchez FJ, Sinsheimer JS, Horvath S, Vilain E (2011) Epigenetic predictor of age. PLoS One 6, e14821

Bozic I, Reiter JG, Allen B, Antal T, Chatterjee K, Shah P, Moon YS, Yaqubie A, Kelly N, Le DT, Lipson EJ, Chapman PB, Diaz LA Jr, Vogelstein B, Nowak MA (2013) Evolutionary dynamics of cancer in response to targeted combination therapy. eLife 2, e00747

Breen EC, Hussain SK, MagpanRabkin CS, Kaslow RA, Variakojis D, Bream JH, Rinaldo CR, Ambinder RF, Martinez-Maza O (2011) B-cell stimulatory cytokines and markers of immune activation are elevated several years prior to the cytokines and markers of immune activation are elevated several years prior to the diagnosis of systemic AIDS-associated non-Hodgkin B-cell lymphoma. Cancer Epidemiol Biomarkers Prev 20:1303–1314

Brümmendorf TH, Balabanov S (2006) Telomere length dynamics in normal hematopoiesis and in disease states characterized by increased stem cell turnover. Leukemia 20:1706–1716

Butov A, Johnson T, Cypser J, Sannikov I, Volkov M, Sehl M, Yashin A (2001) Hormesis and debilitation effects in stress experiments using the nematode worm Caenorhabditis elegans: the model of balance between cell damage and HSP levels. Exp Gerontol 7:57–66

Butov AA, Volkov MA, Anisimov VN, Sehl ME, Yashin AI (2002) A model of accelerated aging induced by 5-bromodeoxyuridine. Biogerontology 3(3):175–182

Butov AA, Carey JR, Volkov MA, Sehl ME, Yashin AI (2003) Reproduction and survival in Mediterranean fruit flies: a "protein and energy" free radical model of aging. Biogerontology 4:387–395

Campisi J (2003) Cancer and ageing: rival demons? Nat Rev Cancer 3:339–349

Conley SJ, Gheordunescu E, Kakarala P, Newman B, Korkaya H, Heath AN, Clouthier SG, Wicha MS (2012) Antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia. Proc Natl Acad Sci U S A 109:2784–2789

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285(21):1182–1186

Foo J, Drummond MW, Clarkson B, Holyoake T, Michor F (2009) Eradication of chronic myeloid leukemia stem cells: a novel mathematical model predicts no therapeutic benefit of adding G-CSF to imatinib. PLoS Comput Biol 5(9), e1000503. doi:10.1371/journal.pcbi.1000503

Jain RK (2001) Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nat Med 7(9):987–989

Jain RK, Tong RT, Munn LL (2007) Effect of vascular normalization by antiangiogenic therapy on interstitial hypertension, peritumor edema, and lymphatic metastasis: insights from a mathematical Model. Cancer Res 67(6):2729–2735

Kohandel M, Kardar M, Milosevic M, Sivaloganathan S (2007) Dynamics of tumor growth and combination of anti-angiogenic and cytotoxic therapies. Phys Med Biol 52:3665–3677

Korkaya H, Liu S, Wicha MS (2011) Breast cancer stem cells, cytokine networks, and the tumor microenvironment. J Clin Invest 121:3804–3809

Li L, Neaves WB (2006) Normal stem cells and cancer stem cells: the niche matters. Cancer Res 66:4553–4557

Liao D, Johnson RS (2007) Hypoxia: a key regulator of angiogenesis in cancer. Cancer Metastasis Rev 26(2):281–290

Mantzaris NV, Webb S, Othmer HG (2004) Mathematical modeling of tumor-induced angiogenesis. J Math Biol 49:111–187

Michaud M, Balardy L, Moulis G, Gaudin C, Peyrot C, Vellas B, Cesari M, Nourhashemi F (2013) Proinflammatory cytokines, aging, and age-related diseases. J Am Med Dir Assoc S1525–8610(13):00280–00286

Michor F, Hughes TP, Iwasa Y, Branford S, Shah NP, Sawyers CL, Nowak MA (2005) Dynamics of chronic myeloid leukaemia. Nature 435:1267–1270

Scott JG, Basanta D, Anderson AR, Gerlee P (2013) A mathematical model of tumour self-seeding reveals secondary metastatic deposits as drivers of primary tumour growth. J R Soc Interface 10(82):20130011. doi:10.1098/rsif.2013.0011

Sehl ME, Sinsheimer JS, Zhou H, Lange KL (2009) Differential destruction of stem cells: implications for targeted cancer stem cell therapy. Cancer Res 69:9481–9489

Sharpless NE, DePinho RA (2004) Telomeres, stem cells, senescence, and cancer. J Clin Invest 113:160–168

Shepherd BE, Kiem HP, Lansdorp PM, Dunbar CE, Aubert G, LaRochelle A, Seggewiss R, Guttorp P, Abkowitz JL (2007) Hematopoietic stem-cell behavior in nonhuman primates. Blood 110(6):1806–1813

Sun Z, Komarova N (2012) Stochastic modeling of stem-cell dynamics with control. Math Biosci 240:231–240

Tang M, Gonen M, Quintas-Cardama A, Cortes J, Kantarjian H, Field C, Hughes TP, Branford S, Michor F (2011) Dynamics of chronic myeloid leukemia response to long-term targeted therapy reveal treatment effects on leukemic stem cells. Blood 118(6):1622–1631

Teicher BA (1994) Hypoxia and drug resistance. Cancer Metastasis Rev 13:139–168

Teschendorff AE, Menon U, Gentry-Maharaj A, Ramus SJ, Weisenberger DJ, Shen H, Campan M, Noushmehr H, Bell CG, Maxwell AP, Savage DA, Mueller-Holzner E, Marth C, Kocjan G, Gayther SA, Jones A, Beck S, Wagner W, Laird PW, Jacobs IJ, Widschwendter M (2010) Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. Genome Res 20:440–446

Till JE, McCulloch EA, Siminovitch L (1964) A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. Proc Natl Acad Sci U S A 51(1):29–36

Role of Stem Cells in Angiogenesis

8

Shentong Fang and Petri Salven

Contents

S. Fang (✉)
Translational Cancer Biology Research Program, University of Helsinki, 00014 Helsinki, Finland
e-mail: Shentong.fang@helsinki.fi

P. Salven
Department of Pathology, Haartman Institute, University of Helsinki, 00014 Helsinki, Finland

Abstract

Diverse tissue-specific stem cells/progenitors are required to create the cell populations necessary for angiogenesis switch-on and neovascular growth, and contribute to the angiogenic microenvironment directly or indirectly. The definite cellular origin of the new ECs necessary for angiogenesis remained largely indefinite until recent advances showing that the endothelial stem/progenitor cells involved in adult angiogenesis must be local, non-hematopoietic, and non-circulating tissue resident cells. Further findings have now provided evidence for rare adult vascular endothelial stem cells (VESCs) that reside in the blood vessel wall and are capable of producing very high numbers of endothelial daughter cells and that are responsible for neovascular growth in adults. Stem and progenitor cell subsets for also other vascular or perivascular cell types such as pericytes or smooth muscle cells may provide critical and direct contributions to the growing neovasculature. Tissue-infiltrating hematopoietic cells produced by hematopoietic stem cells (HSCs) contribute to angiogenesis in a paracrine manner or by remodelling the extracellular matrix. Identification of the various tissue resident or hematopoietic stem and progenitor cell subpopulations critical for angiogenesis and better understanding of their proangiogenic functions and mechanisms of action provide completely novel approaches

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and therapeutic targets both for antiangiogenic treatments and for therapies for cardiovascular repair.

Introduction

Vascular development and its maintenance are essential during the lifetime. Angiogenesis mostly refers to the growth of the new blood vessels sprouting from existing vasculature and subsequent stabilization. When forming new vessels, ECs activate and proliferate upon stimulation, migrate towards the angiogenic stimuli. The endothelial cells (ECs) form small vessels such as microvessels, while in larger vessels endothelial cells are surrounded by perivascular supporting and mural cells such as pericytes and smooth muscle cells. Also non-vascular cell types including bone marrow (BM) -derived hematopoietic cells and stromal cells may provide important contribution to angiogenesis, for example in a paracrine manner by expressing various factors that promote the growth and expansion of de novo blood vessels (Murdoch et al. 2008). In adulthood, angiogenesis is turned on transiently during some physiologic processes including wound healing and female reproductive cycling. It is also an important and crucial component in many pathological processes, such as ischemic diseases and tumor progression. As early as 1971, Judah Folkman realized that tumor growth is dependent on the formation of new blood vessels, and thus inhibiting tumor angiogenesis would be an effective strategy to treat cancer. Various stem and progenitor cells could contribute to angiogenesis either directly by producing differentiated daughter cells (such as ECs and other vascular mural cells including pericytes) that constitute the growing blood vessel wall or by producing progeny that act as vascular modulators, for example by producing paracrine growth factors (Fang and Salven 2011; Goligorsky and Salven 2013).

Endothelial Stem Cells Contributing Directly to Angiogenesis by Producing Differentiated Endothelium

During embryogenesis, ECs rise from mesodermal endothelial precursors called angioblasts. However, in adults, the origin of bona fide endothelial cells during neovascular growth remained largely undefined until recent advances. A previously predominant concept of circulating bone marrow (BM) -derived endothelial progenitor cells (EPCs) was originally introduced following observations of putative EPCs from human peripheral blood (Asahara et al. 1997, 1999). These circulating cells in peripheral blood were further described as vascular endothelial growth factor receptor-2 (VEGFR-2) expressing cells mobilized from the BM by angiogenic factors or tumors (Rafii et al. 2002). Later, numerous studies utilizing various carefully controlled experiments and abundant physiologically relevant angiogenic settings including cancer growth showed undetectable or non-significant incorporation of BM-derived cells to vascular endothelium, and also demonstrated that the circulating cells previously interpreted as EPCs were ordinary hematopoietic cells such as monocyte/macrophages and T-lymphocytes (Horrevoets 2009; Lin et al. 2000; Purhonen et al. 2008; Rehman et al. 2003; Salven et al. 2008; Ziegelhoeffer et al. 2004). Therefore, Richardson and Yoder recently proposed that that the term EPC should be retired and the circulating cell subsets contributing to angiogenesis be referred to according to the terms already existent for each subset (Richardson and Yoder 2011).

Earlier, cells with both endothelial characteristics and high proliferative capacity had been observed in umbilical cord blood or in peripheral blood (Ingram et al. 2004, 2005; Lin et al. 2000; Timmermans et al. 2007; Yoder et al. 2007). These observations hinted for a possible existence of a rare vascular endothelial stem cell (VESC) population that responds to angiogenic

stimulus and is capable of generating endothelial cells during vascular growth in adults (Fang and Salven 2011). New advances in the field have now shifted the focus to local stem and progenitor cells for endothelium (Goligorsky and Salven 2013; Purhonen et al. 2008; Rinkevich et al. 2011; Sedwick 2012). However, until recently, candidates for local, tissue residing endothelial stem cells had not been identified by location or marker expression. By using genetically tagged universal or endothelial cell-specific chimeric mouse models, genetically marked parabiotic systems, and by performing genetic fate mapping and clonal analysis of individual cells, Salven and Weissman and coworkers have now showed that the endothelial stem cells involved in adult angiogenesis must be non-circulating, non-hematopoietic, and local tissue resident cells (Purhonen et al. 2008; Rinkevich et al. 2011; Salven et al. 2008). The most recent studies by Salven and coworkers describe a small subpopulation of c-Kit-expressing adult ECs that reside in the blood vessel endothelium and are capable of undergoing clonal expansion in vivo and in vitro, while other ECs have a very limited proliferative capacity (Fang et al. 2012; Sedwick 2012). These lin − CD31 + CD105 + Sca1 + CD117/c-Kit + adult vascular endothelial stem cells (VESCs) make up only 0.4 % of all adult vessel wall lin − CD31 + CD105+ ECs. In cell transplantations using isolated blood vessel wall VESCs a single c-kit + VESC can generate in vivo functional blood vessels that connect to host circulation. VESCs also display long-term self-renewal capacity, a defining functional property of stem cells, which therefore have the ability to repeatedly respond to growth stimulus by giving rise to extensive numbers of daughter cells. VESCs thus constitute a central cellular target for future—and also present— therapies that aim to restrain angiogenesis by inhibiting endothelial-cell proliferation. Their discovery also suggests for the possibility of cell-based therapies for cardiovascular repair using isolated, highly enriched VESCs to restore tissue vascularization.

Progenitor Cells for Vascular Mural Pericytes

Endothelial cells are often lined by vascular mural cells such as pericytes and vascular smooth muscle cells (vSMCs). It is believed that pericytes are involved in affecting EC functions in a paracrine manner or through cell-cell contact. Some observations support the idea that perivascular hematopoietic cells are closely associated with the vascular wall. Rajantie et al. (2004) found abundant bone marrow -derived periendothelial vascular cells in close contact with the underlying endothelial cells. Subpopulations of these cells expressed a developing pericyte specific marker NG2 proteoglycan. However, these perivascular hematopoietic cell populations did not detectably express the smooth muscle markers smooth muscle alpha-actin or desmin. More recently, Song et al. (2005) described a subset of BM-derived PDGFR-ß + perivascular cells in an endogenous mouse model of pancreatic tumorigenesis. When cultured in vivo, these hematopoietic cells also expressed NG2 and α-SMA.

Hematopoietic Stem Cells Contribute to Angiogenesis in a Paracrine Manner or by Remodelling the Extracellular Matrix

Pluripotent hematopoietic stem cells (HSCs) residing in the BM are the source of all blood cells through a series of lineage restricted differentiation. Ample evidence show that BM-derived myeloid cells have proangiogenic functions, playing an important role during angiogenesis especially tumor angiogenesis (Murdoch et al. 2008). Hematopoietic cells originating from the BM HSCs such as tumor associated macrophages (TAM), Tie2 expressing mononuclear (TEM) cells, mast cells, and platelets may promote angiogenesis by delivering various cytokines that promote the growth and sprouting of neovessels

from the pre-existing vasculature (Fang and Salven 2011; Salven et al. 2002).

Macrophages and Mononuclear Cells

Already in the 1970s, initial evidence of macrophages in promoting vascular proliferation was demonstrated by Polverini et al. (1997). Tumor-infiltrating macrophages are often referred to tumor-associated macrophages (TAMs), derived from circulating monocytes. TAMs constitute a significant proportion of bone marrow derived cells homing to tumor areas. Studies in men and mice have suggested that various monocytic cell types act as modulators of neovascular growth by producing angiogenic cytokines such as VEGF (Murdoch et al. 2008).

Tumor Associated Macrophages (TAMs)

A correlation between high numbers of TAMs in human tumors and increased microvessel density, tumor stage and angiogenesis has been shown suggesting a direct proangiogenic function of TAMs (Murdoch et al. 2008). A current accepted mechanism that TAMs involve in supporting tumor growth and stimulating angiogenesis is that they produce a plethora of angiogenic factors, such as VEGF and CSF-1. Furthermore, TAMs secret many extracellular matrix-degrading proteases and matrix remodeling enzymes to the ECM, which then mobilize various angiogenic factors to the microenvironment (Fang and Salven 2011; Murdoch et al. 2008).

There exists a subpopulation in TAMs expressing Tie2 called Tie2-expressing mononuclear/macrophages (TEM) cells. Human TEMs constitute a prominent monocyte population distinct from TAMs (De Palma et al. 2005), while there is no appearance of this specific population in normal tissues (Venneri et al. 2007). Accordingly, Venneri et al. (2007) reported the identification in human peripheral blood of a novel subset of Tie-2-expressing monocytes (TEMs) that promote angiogenesis in paracrine manner. De Palma et al. (2003) also showed that TEMs are recruited to perivascular areas in tumors and specific depletion of TEMs causes impairment in both tumor angiogenesis and tumor growth. TEMs are believed to support tumor angiogenesis by a paracrine manner via a cross-talk with endothelial cells (ECs) and account for major proangiogenic activity of myeloid cells in tumors (De Palma et al. 2005). Furthermore, angiopoietin-2 (ANG2), a ligand of the TIE2 receptor, activates TIE2 expression in TEMs and enhances their proangiogenic activities (Mazzieri et al. 2011). ANG2 specific blockade by a fully humanized ANG2-specific monoclonal antibody impedes tumor angiogenesis and MRC1-expressing TEMs' association with the vasculature in MMTV-PyMT and RIP1-Tag2 tumors (Mazzieri et al. 2011). Thus targeting ANG2/Tie2 pathway may block the functions of TEMs in tumor angiogenesis (Augustin et al. 2009).

Myeloid Derived Suppressor Cells (MDSCs)

Another heterogeneous population involved in angiogenesis is myeloid-derived suppressor cells (MDSCs), including myeloid precursors and immature myeloid cells. These immature myeloid cells are generated from the bone marrow and later differentiate into neutrophils, monocytes and dendritic cells (DCs). The MDSCs could be found in the bone marrow, spleen and tumor microenvironment. These cells are characterized by the expression of CD11b, mostly expressed by myeloid lineage, and Gr1, markers for neutrophils. In mice, MDSCs are termed as CD11b+Gr1+ cells and could be broadly separated into 2 groups which are CD11b+Gr1 high and CD11b+Gr1low respectively (Yang et al. 2004). MDSCs secrete proangiogenic factors such as VEGF and MMP-9 and accelerate vessel remodeling to promote tumor progression (Ferrara 2010).

Neutrophils, Eosinophils, Mast Cells and Dendritic Cells (DCs)

Neutrophils, differentiated from the myeloid progenitor cells in the bone marrow, are the most plentiful leukocytes in bloodstream. Neutrophils play an important role in physiological angiogenesis as a source of VEGF (Fang and Salven 2011; Murdoch et al. 2008). Evidence for involvement of neutrophils in tumor angiogenesis come from studies describing elevated numbers of neutrophils observed in cancer patients. CXC chemokines stimulate neutrophils to migrate across the vasculature and recruit them into the tumor microenvironment. Benelli et al. (2002) have shown that the depletion of Gr-1 mediated neutrophils strongly reduce angiogenesis in Matrigel with CXCL1 or CXCL8 compared to controls, indicating a positive role of neutrophils in stimulating neo-vascularization. Furthermore, Nozawa et al. (2006) have by using a transient depletion of neutrophils shown that infiltrating neutrophils have a decisive role in activating angiogenesis during the early stage of carcinogenesis. Increased levels of eosinophils have been found in many human tumors, indicating their possible association with tumor angiogenesis.

Mast cells, originating from pluripotent progenitors in the bone marrow, circulate as precursors and immature cells in small numbers and migrate to peripheral tissues where to finally mature. Mast cells are frequently observed in close contact with vasculature within tumor microenvironment, indicating a potential role in tumor angiogenesis and tumor progression. There appears to be a positive correlation between mast cell numbers and microvessel density in many human tumors. Starkey et al. (1988) observed a reduced angiogenic response in mast cell deficient W/Wv B16-BL6 tumor bearing mice compared to wild type mice. Furthermore, angiogenic response could be fully restored via bone-marrow repair of the mast cell deficiency (Starkey et al. 1988). Studies have shown that mast cells are a rich source of angiogenic factors and growth factors, such as VEGF, bFGF, MMP-9, and TNFα (Fang and Salven 2011).

Dentritic cells (DCs) are important regulators of adaptive immune responses and major antigen presenting cells, able to induce both primary and secondary T- and B-cell responses. Tumor – derived factors, such as VEGF, may restrict the maturation of myeloid DCs and their subsequent accumulation to tumor tissues. Some in vivo studies have shown that tumor associated DCs may also possess proangiogenic properties by their secretion of pro-angiogenic cytokines (Murdoch et al. 2008). For example, Curiel et al. (2004) showed that tumor associated plasmacytoid dendritic cells could induce angiogenesis by producing TNFα and IL-8, while myeloid dendritic cells were absent from malignant ascites.

Platelets

Maintaining a normal platelet count level in the blood is very crucial to hemostasis on vasculature. Platelets are also involved in multiple physiological processes such as angiogenesis and tissue regeneration. During angiogenesis, platelets store various growth factors, cytokines, chemokines, protease and some other small molecules involving in angiogenesis and act as a rich source of angiogenic, including VEGF, ANG-1, FGF, EGF and PDGF, and antiangiogenic factors, such as angiostatin, thrombospondin-1 and endostatin, upon activation (Jain et al. 2010; Wartiovaara et al. 1998). Studies have been shown in soft tissue tumors; elevated numbers of platelets are associated with VEGF expression and ongoing angiogenesis. Besides, platelet-derived lipid-lysophosphatidic acid (LPA) was found to exert its activity either in an autocrine or paracrine way. LPA could activate downstream signaling pathways to induce cell proliferation, survival, and migration, and thus affect angiogenesis. Recently, Zaslavsky et al. (2010) found that platelet derived thrombospondin-1, a negative regulator of angiogenesis, is increased in tumor bearing mice after tumor resection, indicating a potential role of platelet derived anti-angiogenic factors in therapeutic applications.

Bone Marrow Derived Mesenchymal Stem/Stromal Cells

Mesenchymal stem cells (MSCs) have been described as a rare population of non-hematopoietic precursors residing in the BM and possessing the capacity to generate mesenchymal daughter cells and thus contribute to maintaining and regenerating connective tissues. Human and murine BM-derived MSCs have been described as cells expressing surface markers such as CD73, CD105 and CD44 in the absence of hematopoietic markers and the endothelial markers (Pittenger et al. 1999; Prockop 1997). MSCs may contribute both directly and indirectly to angiogenesis process supporting cells or paracrine effectors. There are also observations suggesting that BM-derived mesenchymal cells could contribute to angiogenesis indirectly by providing a supportive role by differentiating into fibroblast-like cells (Mishra et al. 2008). MSC-derived fibroblasts could also promote angiogenesis by producing proangiogenic factors and cytokines, such as VEGF, PDGF, FGF and SDF-1 (Kinnaird et al. 2004).

Conclusions

It is evident that hematopoietic cells originating from the BM-residing hematopoietic stem cells contribute to angiogenesis. Tissue-infiltrating hematopoietic cells can release proangiogenic/angiogenic factors or create a permissive microenvironment that induces the growth of locally derived blood vessels (Murdoch et al. 2008). Future studies should aim to better identify the particular hematopoietic cell subpopulations that are critical for tumor angiogenesis, to dissect their cellular and molecular proangiogenic functions, and to evaluate their potential as therapeutic targets. Local, tissue resident stem cell populations that are crucial to adult neoangiogenesis have until recently been poorly understood or characterized. However, very recent studies on the stem cell origins of regenerating or angiogenic tissues have provided solid evidence in support of the role of the resident stem cells in neovascular growth. These discoveries offer a novel opportunity to understand of the therapeutic potential of transplanted vascular progenitor cells, as well as an opportunity to study these tissue resident stem cells and their molecular mechanisms as completely novel, direct targets for future anti-angiogenic therapies.

References

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Cannon RO 3rd (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967

Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 18:3964–3972

Augustin HG, Koh GY, Thurston G, Alitalo K (2009) Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. Nat Rev Mol Cell Biol 10:165–177

Benelli R, Morini M, Carrozzino F, Ferrari N, Minghelli S, Santi L, Cassatella M, Noonan DM, Albini A (2002) Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. FASEB J 16:267–269

Curiel TJ, Cheng P, Mottram P, Alvarez X, Moons L, Evdemon-Hogan M, Wei S, Zou L, Kryczek I, Hoyle G et al (2004) Dendritic cell subsets differentially regulate angiogenesis in human ovarian cancer. Cancer Res 64:5535–5538

De Palma M, Venneri MA, Roca C, Naldini L (2003) Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 9:789–795

De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, Naldini L (2005) Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 8:211–226

Fang S, Salven P (2011) Stem cells in tumor angiogenesis. J Mol Cell Cardiol 50:290–295

Fang S, Wei J, Pentinminkko N, Leinonen H, Salven P (2012) Generation of functional blood vessels from a single c-kit+ adulat vascular endothelial stem cell. PLos Biol 10(10):e1001407

Ferrara N (2010) Role of myeloid cells in vascular endothelial growth factor-independent tumor angiogenesis. Curr Opin Hematol 17:219–224

Goligorsky MS, Salven P (2013) Concise review: endothelial stem and progenitor cells and their habitats. Stem Cells Transl Med 2(7):499–504

Horrevoets AJ (2009) Angiogenic monocytes: another colorful blow to endothelial progenitors. Am J Pathol 174:1594–1596

Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC (2004) Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 104:2752–2760

Ingram DA, Mead LE, Moore DB, Woodard W, Fenoglio A, Yoder MC (2005) Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. Blood 105:2783–2786

Jain S, Harris J, Ware J (2010) Platelets: linking hemostasis and cancer. Arterioscler Thromb Vasc Biol 30:2362–2367

Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 94:678–685

Lin Y, Weisdorf DJ, Solovey A, Hebbel RP (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 105:71–77

Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, Politi LS, Gentner B, Brown JL, Naldini L et al (2011) Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. Cancer Cell 19:512–526

Mishra PJ, Humeniuk R, Medina DJ, Alexe G, Mesirov JP, Ganesan S, Glod JW, Banerjee D (2008) Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. Cancer Res 68: 4331–4339

Murdoch C, Muthana M, Coffelt SB, Lewis CE (2008) The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 8:618–631

Nozawa H, Chiu C, Hanahan D (2006) Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci U S A 103:12493–12498

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Polverini PJ, Cotran PS, Gimbrone MA Jr, Unanue ER (1997) Activated macrophages induce vascular proliferation. Nature 269:804–806

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74

Purhonen S, Palm J, Rossi D, Kaskenpaa N, Rajantie I, Yla-Herttuala S, Alitalo K, Weissman IL, Salven P (2008) Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. Proc Natl Acad Sci U S A 105:6620–6625

Rafii S, Lyden D, Benezra R, Hattori K, Heissig B (2002) Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2:826–835

Rajantie I, Ilmonen M, Alminaite A, Ozerdem U, Alitalo K, Salven P (2004) Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood 104:2084–2086

Rehman J, Li J, Orschell CM, March KL (2003) Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 107:1164–1169

Richardson MR, Yoder MC (2011) Endothelial progenitor cells: quo vadis? J Mol Cell Cardiol 50:266–272

Rinkevich Y, Lindau P, Ueno H, Longaker MT, Weissman IL (2011) Germ-layer and lineage-restricted stem/progenitors regenerate the mouse digit tip. Nature 476:409–413

Salven P, Hattori K, Heissig B, Rafii S (2002) Interleukin-1 alpha promotes angiogenesis in vivo via VEGFR-2 pathway by inducing inflammatory cell VEGF synthesis and secretion. FASEB J 16:1471–1473

Salven P, Purhonen S, Rossi D, Yla-Herttuala S, Alitalo K, Weissman IL (2008) Reply to Kerbel et al.: EPCs are again claimed to be essential in yet other models despite the irreproducibility of the original experiments introducing them. Proc Natl Acad Sci U S A 105:E55

Sedwick C (2012) On the hunt for vascular endothelial stem cells. PLoS Biol 10, e1001408

Song S, Ewald AJ, Stallcup W, Werb Z, Bergers G (2005) PDGFR beta + perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat Cell Biol 7:870–879

Starkey JR, Crowle PK, Taubenberger S (1988) Mast-cell-deficient W/Wv mice exhibit a decreased rate of tumor angiogenesis. Int J Cancer 42(48-52):0020–7136

Timmermans F, Van Hauwermeiren F, De Smedt M, Raedt R, Plasschaert F, De Buyzere ML, Gillebert TC, Plum J, Vandekerckhove B (2007) Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arterioscler Thromb Vasc Biol 27:1572–1579

Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C, Naldini L (2007) Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. Blood 109:5276–5285

Wartiovaara U, Salven P, Mikkola H, Lassila R, Kaukonen J, Joukov V, Orpana A, Ristimaki A, Heikinheimo M, Joensuu H et al (1998) Peripheral blood platelets express VEGF-C and VEGF which are released during platelet activation. Thromb Haemost 80:171–175

Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC (2004) Expansion of myeloid immune suppressor

Gr+CD11b+cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell 6:409–421

Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA (2007) Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 109:1801–1809

Zaslavsky A, Baek KH, Lynch RC, Short S, Grillo J, Folkman J, Italiano JE Jr, Ryeom S (2010) Platelet-derived thrombospondin-1 is a critical negative regulator and potential biomarker of angiogenesis. Blood 115:4605–4613

Ziegelhoeffer T, Fernandez B, Kostin S, Heil M, Voswinckel R, Helisch A, Schaper W (2004) Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ Res 94:230–238

Glioblastoma Multiforme Stem Cell Characteristics

9

Mark J. Knights

Contents

Abstract

Glioblastoma Multiforme (GBM) is the most common and most aggressive malignant primary brain tumour in humans. There is a poor prognosis associated with GBM tumours which is due to therapeutic resistance and tumour recurrence after surgical removal. GBM are comprised of a heterogeneous population of cells and it has become clear that a subpopulation of stem cells are responsible for tumour initiation and maintenance. There is a wide variety of GBM stem cells markers, although as yet, there is no single marker that can be used to identify all stem cells with complete specificity and sensitivity. A number of signalling pathways and genetic mutations have also been distinguished in GBM stem cells, although their use in translational therapeutics remains in their infancy.

Introduction

Glioblastoma Multiforme (GBM)/Glioma Grade IV is the commonest type of primary brain tumour in adults and is also the least successfully treated solid tumour. GBM can develop de novo (primary GBM) or from a pre-existing lower grade glioma (secondary GBM). Although Glioblastomas are relatively rare, with an incidence of 2–3 cases per 100,000 people in Europe

M.J. Knights (✉)
Leeds School of Medicine, University of Leeds, Leeds, UK
e-mail: markknights@doctors.org.uk

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and North America, the median survival time of patients following surgical resection, radiation and temozolomide (TMZ) therapy remains stubbornly low at 14 months (Johnson and O'Neill 2012). Compared with the advances in treatment of other solid tumour types, the prognosis for GBM patients has improved minimally over the past decades, thus underscoring the challenges and complexities in early detection and effective treatment.

Cellular Markers

Early evidence for the existence of GBM stem cells came from isolating the cell fraction expressing the neural stem cell surface marker CD133 (Singh et al. 2003). The CD133+ cells could differentiate in culture into tumour cells that phenotypically resembled the tumour from the patient from which the cells originated. The CD133+ stem cell was the first marker of its type for GBM and continues to be the primary cell marker used in research. Over time there have been a variety of other cell markers investigated, many of them cell surface markers (L1CAM, SSEA-1, intregrin α-6, A2B5, CXCR4) but also intracellular markers (nestin, sox-2) have also been used to locate and target the GBM stem cells with varying degrees of success.

CD133 is a pentaspan transmembrane glycoprotein cell surface molecule, whose expression on cancer stem cells can be found in a variety of human malignancies including renal, prostate, colon, hepatocellular, and pancreatic as well as neural tumours including GBM (Mizrak et al. 2008). The cell marker is located in the membranous protrusions of the plasma membrane and whose role, although not fully understood, is speculated as being a regulator of the plasma membrane structure, specifically maintaining an appropriate lipid composition within the plasma membrane (Mizrak et al. 2008). Evidence illustrates that CD133+ GBM stem cells are capable of multi-lineage differentiation, can form neurospheres and imitate tumours which closely resembles the patient's tumour, as well as express neural stem cell genes nestin, Msi-1, CXCR4 and maternal embryonic leucine zipper kinase.

CD133 remains the most popular cell marker for GBM stem cell location and isolation. Despite this, the sensitivity of this marker remains unclear. Research has shown that not all cells in GBM specimens are CD133+, nor does every GBM specimen contain CD133+ cells. In CD133+ GBM tissue samples, the percentage of all cells which are CD133+ is varied. In one study, analysis between GBM samples showed that between 2 and 60 % of the total cells were CD133+ (Ogden et al. 2008). However, such variability between samples was noticed in the earliest studies by Singh and colleagues who showed that immunestainting of GBM xenografts revealed islands of CD133+ cells amid large groups of CD133− cells; indicating that not every cell in the xenograft is CD133+ (Singh et al. 2004). Furthermore, when a small number of purified CD133+ cells were transplanted, a heterogeneous primary xenograft grew, which consisted of a minority of CD133+ with the remaining cells being CD133−. Therefore showing that CD133+ cells can generate CD133− cells and thus suggesting a cell hierarchy exists within a tumour (Singh et al. 2004).

Evidence therefore shows that CD133 does not have to be present nor is it likely to be present in every cell of a GBM for the marker to still remain highly specific as a stem cell marker. However, in an analysis of 16 primary GBM obtained directly from patients, three of the specimens did not contain any detectable levels of CD133+ cells (Ogden et al. 2008). Other studies have shown greater consistency of CD133+ cells being present in GBM tumours, including an analysis of 47 patients with GBM of which 45 contained at least 1 % of CD133+ cells (Zeppernick et al. 2008). It is likely then, that CD133 is not present in every GBM and therefore its use a stem cell marker in this particular type of tumour is not absolute.

The overall relevance of CD133 is not yet understood, and there is growing evidence that the marker may have significant limitations as well, including that CD133+ cells are found in normal brain tissue (Zhang et al. 2008). A reliable

cell marker has the potential to be used as a target for therapeutic purposes, which could not be the case for CD133 if significant amounts are found outside of tumour tissue. Furthermore, the exact association of CD133 and GBM stem cells remains a concern. A study looked at GBM tumour samples from 20 patients who were divided into two groups, CD133+ high group (CD133+ cell ratio >3 %) and a CD133+ low group (CD133+ ratio <3 %), whereby the patients' clinical characteristics were analysed using MRI scan data (Joo et al. 2008). It was found that as compared to CD133-high GBMs, CD133-low GBMs had a tendency to be localised within the deeper structures of the brain and show more invasive growth patterns and ventricle involvement and also the rate of disease progression after chemotherapy and radiotherapy was relatively higher in the CD133-low GBMs compared to the CD133-high GBMs. Indicating that the relationship between CD133 as a marker and GBM tumours is not straight forward.

A cellular marker which has an intimate relationship with CD133 is L1CAM. This marker is a transmembrane protein, specifically a neuronal cell adhesion molecule and a member of the L1 protein family. It is known to be involved in axon guidance and cell migration. In addition, abnormal expression of this protein has been found in several different human cancer types including colon, melanoma, breast, ovarian, renal and neuroblastomas. Analysis of three human glioma surgical specimens through fluorescence-activated cell sorting (FACS) indicated a strong correlation between CD133+ cells and L1CAM+ cells, in that where one marker was present, so was the other (Bao et al. 2008). In contrast the vast majority of CD133− cells were L1CAM negative (>99 %). L1CAM could therefore be as useful a marker as CD133.

Furthermore, when L1CAM is targeted using lentivirus expressing shRNA, there is a 90 % reduction in its expression, and a significant decreased in the ability of CD133+ GBM cells to form neurospheres occurs, with no effect on CD133− GBM cells and their ability to form neurospheres (Bao et al. 2008). Interestingly, L1CAM over expression in a number of solid tumours has been linked to cancer invasion. When primary GBMs have been examined for L1CAM expression using immunohistochemical staining, it has been found that L1CAM expression is enriched in the population of cancer cells in the invasive fronts of tumours (Cheng et al. 2011). Although a subpopulation of cancer cells inside tumours also expressed L1CAM, the invasive fronts showed many more cells with high levels of L1CAM. This suggests that preferential expression of L1CAM in GBM stem cells may be closely associated with the elevated invasive potential of stem cells in GBM tumours. This indicates that cell markers may not only discern stem cells from non-stem cells but also specific characteristics/roles of that stem cell.

The antigen Stage-Specific Embryonic Antigen-1 (SSEA-1/CD15/LeX) is a fructose-containing trisaccharide adhesion molecule that can be expressed on glycoproteins, glycolipids and proteoglycans. SSEA-1 has been used in the recognition of certain leucocyte malignancies including Hodgkin's disease and histiocytosis. Research into its use as a potential GBM stem cell marker is extremely limited but remains promising. Analysis of GBM tumour cell lines has found SSEA-1 expression in 23 of the 24 evaluated tumours, with the percentage of expression ranging from 0.7 to 87.5 % (Son et al. 2009). When the SSEA-1 cells were grown they formed a population of cells which contained the properties to meet tumour stem cell criteria (formed neurospheres, were clonogenic in agar, were highly tumourgenic in vivo) and secondary tumours could be produced when SSEA-1+ cells were extracted from the SSEA-1+ cell-generated orthotopic tumours with the same characteristics as the primary tumour.

Integrin α6 is another cellular marker that although has not been researched extensively in the GBM stem cells evidence as a potential marker is intriguing. Integrin α6 is the receptor for the extracellular matrix protein laminin, and in the brain both of these molecules regulate neural stem cell growth, as well as playing a pivotal role in maintaining adhesion to the ventricular zone, ensuring proper neural stem cell division (Lathia et al. 2010). Previous evidence has shown

that laminin-coated flasks in neural stem cell culture media has been used to isolate and expand GBM stem cells successfully (Pollard et al. 2009) which indicated a possible role for the laminin-integrin relationship in GBM development. In an assessment of GBM surgical biopsy specimens labelled with antibodies against integrin α6, 60 % of the integrin-positive GBM cells were located within 5 μm of a blood vessel (Lathia et al. 2010) indicating invasive properties, especially in the peri-vascular areas. Furthermore, functional assays to define GBM stem cells, which included self-renewal assays, expression of stem cell markers, and tumour propagation, showed that the stem cells expressed significantly higher levels of intregrin α6 compared to matched non-stem cell glioblastoma cells (Lathia et al. 2010).

Difficulties arise in being able to narrow down the most useful GBM stem cell markers because it is uncommon for cell markers to be directly compared to one another. Interestingly, when A2B5 (a cell surface ganglioside that marks a fraction of human subcortical white matter cells that have neural stem cell properties) was compared CD133 in 16 GBM specimens the percentage of cells containing the marker was found to be higher (between 33–90 % vs. 2–60 %) and unlike CD133, A2B5+ cells were found in every specimen (Ogden et al. 2008). When the GBM specimens where FAC-sorted by cell marker (A2B5+CD133−, A2B5+CD133+, A2B5−CD133−) 92 % of the A2B5+CD133− grafts formed xenografts once injected into rats, indicating a clear subset of GBM cells with tumourigenic properties. It is unfortunate that A2B5-CD133+ were so rare that they could not be retrieved by FACS in this study. Despite this the results suggest that A2B5 has promise as an additional or even alternative GBM stem cell marker.

An interesting receptor which has been shown to be over expressed in primary Glioblastoma progenitor cells and is also associated with the recruitment of GBM stem cells is CXCR-4. This receptor is a seven transmembrane G-coupled protein alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF1) and plays an important role in lymphocyte trafficking. CXCR-4 receptors have not only been found to be over expressed in GBM progenitor cells but also its protein ligand, CXCL-12, promotes a proliferative response in these cells (Ehtesham et al. 2009). Further evidence indicates that through SDF-1-CXCR-4 signalling GBM stem cells are recruited to perivascular niches and are induced to become pericytes, predominantly by transforming growth factor β (Cheng et al. 2011). CXCR-4 and SDF1 are also specifically expressed on neo-vessel endothelial cells within the tumour and levels of both increase with increasing grade of astrocytoma (Rempel et al. 2000). The location of SDF1 has led to the hypothesis that the interaction of SDF1 in the GBM tumour and CXCR-4 which is also present on both macrophages and CD8+ T cells, may explain why the immune system fails to mount a successful immune response. SDF1 binds to the CXCR-4 receptor on both macrophages and CD8+ T cells, in which both cells secrete TNF and TNF-R respectively; macrophages and T cells bind to each other through TNF/TNF-R interaction which induces a death signal in the T cell, thereby limiting an immune response to the tumour (Rempel et al. 2000).

Intracellular molecules have also been used as GBM stem cell markers with two of the more successful ones being Nestin and Sex determining region Y Box-2 (Sox-2), although, for obvious reasons the study of live cells is more difficult for intracellular markers when compared to their extracellular counterparts. Sox-2 is a member of the Sry-related High Mobility Group Box family of transcription factors. Like many of the other GBM stem cell markers it is not specific for grade IV gliomas and has been found in all WHO glioma grades (Knights et al. 2012). Interestingly, studies have shown that silencing of Sox-2 expression by RNA interference lead to the loss of tumourigenicity and proliferation of GBM stem cell lines and also it has been found that arsenic trioxide inhibits self-renewal and induces apoptosis in GBM stem cells through the down-regulation of SOX-2 (Knights et al. 2012). Nestin, an intermediate filament protein involved in the organisation of the cytoskeleton, appears to be a more reliable intracellular marker than SOX-2. Nestin has been shown to be positively correlated with higher grade gliomas and higher expression

levels have also been predictive of significantly lower 5-year survival rates (Knights et al. 2012).

Signalling Pathways

A commonly activated signalling cascade in many human malignancies including GBM, is the Akt pathway. Akt, also known as Protein Kinase B (PKB), is a serine/threonine-specific protein kinase which regulates numerous tumour-associated processes, including cell growth, cell cycle progression, survival, migration, and angiogenesis. Upstream regulators of this pathway include epidermal growth factor, PTEN deletion and PIK3CA mutations. Evidence continues to grow regarding the influence of this signalling pathway on GBM development, with early evidence already showing that the pathway is activated in the majority of GBM; its activation has been associated with reduced patient survival times; and that A-443654 (an Akt inhibitor) when delivered locally either at the time of tumour implantation or in a delayed fashion prolonged survival in an experimental intracranial rodent Glioblastoma Multiforme model (Gallia et al. 2009). Importantly, A-443654 also inhibits GBM stem-like cells with similar efficacy compared with traditionally cultured GBM cell lines indicating no observable stem cell specific resistance (Gallia et al. 2009). Other molecules which target the Akt pathway include KP-372-1 and KP-372-2, which been shown to inhibit the *in vitro* growth of six GBM cell lines, where the reduced activation of Akt downstream targets including GSK-3β and p70s6k, caused a decrease in cell growth which stemmed from the induction of apoptosis (Koul et al. 2006).

Notch signalling pathway is a highly conserved signalling network, which is critical for a series of processes in stem cells, including cell fate specification, differentiation, proliferation, and survival. The deregulation of this pathway is found in many cancers including, acute lymphoblastic leukaemia, ovarian and colorectal cancer as well as GBM. Notch signalling is initiated when transmembrane ligands on one cell bind Notch receptors on an adjacent cell and cause the γ-secretase-mediated proteolytic release of the Notch intracellular domain (NICD). NICD then translocates into the nucleus where it interacts with the transcriptional cofactor CBF1 and activates targets such as HES and HEY genes, which modulate neuronal and glial differentiation (Fan et al. 2010).

In Notch pathway blockade through γ-secretase inhibitor (GSI-18) tumour growth was slowed in GBM neurosphere cultures and it was additionally found that Notch pathway blockade with GSI-18 also reduced the percentage of cells expressing the stem/progenitor cell markers CD133 and Nestin in GBM neurospheres and that these cells were no longer able to efficiently form colonies in *vitro* or engraft in *vitro*, consistent with the concept that a key subpopulation of cells required for efficient tumour propagation was no longer present (Fan et al. 2010). Retinoic acid (RA) also shows potential in affecting GBM stem cell growth following its successful use in the treatment of acute promyelocytic leukaemia. Retinoic acid treatment on GBM stem cell neurospheres resulted in rapidly induced morphological changes, induced growth arrest, decreased cyclin D1 expression and increased p27 expression, as well as reduced stem cell markers including CD133, Msi-1, nestin and Sox-2, and overall decreased neurospheres-forming capacity (Ying et al. 2011). There is further evidence to suggest that RA can also impair the secretion of angiogenic cytokines and disrupt GBM stem cell motility (Campos et al. 2010).

Recent studies point to a role for the Hedgehog signalling pathway in regulating adult stem cells involved in maintenance and regeneration of adult tissues. Sonic Hedgehog (SHH) signalling specifically plays a key role in regulating vertebrate organogenesis including the growth of digits on limbs and the organisation of the brain. In adults the SHH-Gli1 pathway controls cell division of stem cells and when it is activated or maintained inappropriately, various tumours can develop, including those in the skin, muscle and brain (Ruiz et al. 2002). Early results have shown that SHH-Gli pathway has a general role in controlling progenitor cell number in the developing dorsal brain (Dahmane et al. 2001).

Furthermore, it has been demonstrated that deregulation of SHH-Gli signalling in the CNS

leads to hyper-proliferation of the precursor cells and suggests its involvement in the initiation and maintenance of brain tumourigenesis (Dahmane et al. 2001). This raises the possibility that tumours are derived from such cells which have abnormal SHH-Gli signalling, possibly even stem cells, which are unable to differentiate and/or stop proliferating (Ruiz et al. 2002). More recently, it has been demonstrated that GBM and their cancer stem cells require SHH-Gli pathway activity for proliferation, survival, self-renewal, and tumourigenicity (Clement et al. 2007). It has also been found that cyclopamine, an antagonist of Gli1, can induce cell death in GBM cell lines, although after its removal culture recovery occurred, indicating a temporary cytostatic and cytotoxic effect (Clement et al. 2007), moreover cyclopamine in combination with TMZ has shown a synergistic effect in GBM stem cell cytotoxicity.

Signal transducer and activator of transcription 3 (STAT3) regulates diverse cellular processes, including cell growth, differentiation and apoptosis and is frequently activated during tumourigenesis. Constitutive activation of STAT3 has been observed in many human cancers including; breast, head and neck, prostate, melanoma and thyroid cancer (Sherry et al. 2009). Inhibition of STAT3 in GBM stem cells irreversibly abrogates neurosphere formation and inhibits proliferation and also causes the down-regulation of genes associated with the neural stem cell phenotype, providing evidence that STAT3 regulates multipotency in these cells (Sherry et al. 2009). GBM stem cells treated with STAT3 inhibitors (SAT-21 and S31-201) have shown a reduction in stem cell markers olig2 and nestin, and have failed to proliferate, although the cells did not undergo apoptosis, nor is there evidence that STAT3 inhibition alone is sufficient to induce complete differentiation of GBM stem cells (Sherry et al. 2009).

Genetics

The Cancer Genome Atlas data reports that *p53* as one of the most commonly mutated tumour suppressor genes. Lower estimates suggest that *p53* mutations are found approximately 30 % of the time in all grades of gliomas (Li et al. 2009) although higher estimates argue that *p53* is nearly invariably altered in sporadic gliomas through either point mutations that prevent DNA binding or loss of chromosome 17p (Furnari et al. 2007). The importance of *p53* in gliomagenesis is also underscored by the increased incidence of gliomas in Li-Fraumeni syndrome, a familial cancer-predisposition syndrome associated with germline *p53* mutations (Furnari et al. 2007). Furthermore, it has also been found that concomitant central nervous system specific deletion of *p53* in the mouse generates a penetrant acute-onset high grade malignant glioma phenotype with notable clinical, pathological and molecular resemblance to primary GBM in humans (Zheng et al. 2008). It has also been found that through inhibiting *p53* function via RNAi in stem cells derived from GBM specimens, these stem cells became significantly more sensitive to TMZ (Blough et al. 2011). Although the molecular basis for the differing effects of *p53* status on sensitivity to TMZ is as yet unknown.

The *Bmi-1* gene was originally identified as a collaborating oncogene in c-Myc induced lymphomagenesis, and is a member of the Polycomb group (PcG) gene family of chromatin modifiers and transcriptional repressors (Bruggeman et al. 2005). When *Bmi-1* is over-expressed in adult forebrain, NSCs expand dramatically and continue to proliferate aggressively. It has been shown that the polycomb factor *Bmi-1* represses cell-cycle inhibitors *p16*, *p19*, and *p21* which are necessary for NSC self-renewal (Cui et al. 2010). Also it has been argued that the up regulating of *Bmi-1* coordinates the down regulation of miRNA-128 which is also found to inversely correlate with WHO tumour grade (Cui et al. 2010).

The phosphatase and tensin homolog (*Pten*) gene mutation is a step in the development of many cancers. *Pten* acts as a tumour suppressor gene through the action of its phosphatase protein product which is involved in the regulation of the cell cycle, particularly in preventing cells from dividing and growing too rapidly. In analysis of 35 clinically annotated human primary GBM samples 40 % had *Pten* missense mutations,

insertions, deletions or splicing mutations (Zheng et al. 2008). *Pten* mutation results in uncontrolled PI3K signalling. More recently, integrated transcriptomic profiling, in *silico* promoter analysis and functional studies of murine neural stem cells established that dual, but not singular, inactivation of *p53* and *Pten* promotes an undifferentiated state with high renewal potential and drives increased Myc protein levels and its associated signature (Zheng et al. 2008).

Amplifcation of the *EGFR* gene and the subsequent over-expression of EGFR protein is one of the most common genetic alteration in GBM, with a frequency of about 40 %, with the most common of the EGFR mutations is the EGFRvIII (Gan et al. 2009). The mutation results in the loss of exons 2 to 7 of the *EGFR* gene, resulting in an in-frame deletion of 267 amino acids in the extracellular domain (Gan et al. 2009). EGFRvIII appears to enhance tumourigenicity through multiple mechanisms including; enhancing cell proliferation by promoting P13K/Akt signalling, Shc and Grb2 association and Ras activity; inhibiting cell cycle regulators such as $p27^{KIP1}$; promoting survival in cells by increasing expression of anti-apoptotic proteins such as Bcl-X_L; and enhancing angiogenesis and cell invasion by unregulating vascular endothelial growth factor, interleukin-8 and matrix metalloproteinase 13 expression (Gan et al. 2009).

More recently, High-mobility group A1 (*HMGA1*) transcriptional and translational expression in GBM stem cells (determined by the presence of NSC markers CD133 and nestin) was found to be significantly higher than in GBM non-stem cells (determined by the presence of glia fibrillary acidic protein but not CD133 or nestin) (Fan et al. 2011) implying that its expression may be correlated with malignant proliferation, invasion and differentiation. The underlying mechanisms that *HMGA1* plays in tumourgenesis is not yet known. The gene codes for a non-histone chromatin protein which takes part in regulation of inducible gene transcription, DNA replication, heterochromation organisation, integration of retroviruses into chromosomes and metastatic progression of cancer cells.

There is growing evidence that GBM can be sub-classified based on their gene expression signature and that expression profile predict better outcome than histological class (Phillips et al. 2006). In one report GBM tissue samples could be separated into three molecular subtypes (proneural, proliferative and mesenchymal), through the analysis of 35 signature genes. The proneural subtype is distinguished by markedly better prognosis and expressed genes associated with normal brain tissue and processes of neurogenesis. Two poor prognosis subtypes, characterised by a resemblance to either highly proliferative cell lines or tissues of mesenchymal origin, show activation of gene expression programs indicative of cell proliferation or angiogenesis, respectively (Phillips et al. 2006). In an analysis of nine different GBM cultures established under neural stem cell conditions, two major phenotypes could be established (Gunther et al. 2008). One group of four cell lines were characterised by the expression of neurodevelopmental genes, showed a multipotent differentiation profile along neuronal, astroglial and oligodendroglial lineages, grew spherically *in vitro*, expressed CD133 and formed highly invasive tumours *in vivo* (Gunther et al. 2008). The other group of five cell lines shared expression signatures enriched for extracellular matrix-related genes, had a more restricted differentiation capacity, contained fewer or no CD133+ cells, grew semi-adherent or adherent *in vitro* and displayed reduced tumourigenicity and invasion *in vivo* (Gunther et al. 2008).

References

Bao S, Wu Q, Li Z, Sathornsumetee S, Wang H, Mclendon RE, Hjelmeland AB, Rich JN (2008) Targeting cancer stem cells through L1CAM suppresses glioma growth. Cancer Res 68:6043–6048

Blough MD, Beauchamp DC, Westgate MR, Kelly JJ, Cairncross JG (2011) Effect of aberrant p53 function on temozolomide sensitivity of glioma cell lines and brain tumor initiating cells from glioblastoma. J Neurooncol 102:1–7

Bruggeman SWM, Valk-Lingbeek ME, Van Der Stoop PPM, Jacobs JJL, Kieboom K, Tanger E, Hulsman D, Leung C, Arsenijevic Y, Marino S, Van Lohuizen M

(2005) Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. Genes Dev 19:1438–1443

Campos B, Wan F, Farhadi M, Ernst A, Zeppernick F, Tagscherer KE, Ahmadi R, Lohr J, Dictus C, Gdynia G, Combs SE, Goidts V, Helmke BM, Eckstein V, Roth W, Beckhove P, Lichter P, Unterberg A, Radlwimmer B, Herold-Mende C (2010) Differentiation therapy exerts antitumor effects on stem-like glioma cells. Clin Cancer Res 16:2715–2728

Cheng L, Wu Q, Guryanova OA, Huang Z, Huang Q, Rich JN, Bao S (2011) Elevated invasive potential of glioblastoma stem cells. Biochem Biophys Res Commun 406:643–648

Clement V, Sanchez P, De Tribolet N, Radovanovic I, Ruiz I, Altaba A (2007) HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. Curr Biol 17:165–172

Cui JG, Zhao Y, Sethi P, Li YY, Mahta A, Culicchia F, Lukiw WJ (2010) Micro-RNA-128 (miRNA-128) down-regulation in glioblastoma targets ARP5 (ANGPTL6), Bmi-1 and E2F-3a, key regulators of brain cell proliferation. J Neurooncol 98:297–304

Dahmane N, Sanchez P, Gitton Y, Palma V, Sun T, Beyna M, Weiner H, Ruiz I, Altaba A (2001) The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. Development 128:5201–5212

Ehtesham M, Mapara KY, Stevenson CB, Thompson RC (2009) CXCR4 mediates the proliferation of glioblastoma progenitor cells. Cancer Lett 274:305–312

Fan X, Khaki L, Zhu TS, Soules ME, Talsma CE, Gul N, Koh C, Zhang J, Li YM, Maciaczyk J, Nikkhah G, Dimeco F, Piccirillo S, Vescovi AL, Eberhart CG (2010) NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells 28:5–16

Fan HT, Guo H, Zhang IY, Liu B, Luan LM, Xu SC, Hou XZ, Liu W, Zhang R, Wang XS, Pang Q (2011) The different HMGA1 expression of total population of glioblastoma cell line U251 and glioma stem cells isolated from U251. Brain Res 1384:9–14

Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, Hahn WC, Ligon KL, Louis DN, Brennan C, Chin L, Depinho RA, Cavenee WK (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 21:2683–2710

Gallia GL, Tyler BM, Hann CL, Siu IM, Giranda VL, Vescovi AL, Brem H, Riggins GJ (2009) Inhibition of Akt inhibits growth of glioblastoma and glioblastoma stem-like cells. Mol Cancer Ther 8:386–393

Gan HK, Kaye AH, Luwor RB (2009) The EGFRvIII variant in glioblastoma multiforme. J Clin Neurosci 16:748–754

Gunther HS, Schmidt NO, Phillips HS, Kemming D, Kharbanda S, Soriano R, Modrusan Z, Meissner H, Westphal M, Lamszus K (2008) Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. Oncogene 27:2897–2909

Johnson DR, O'neill BP (2012) Glioblastoma survival in the United States before and during the temozolomide era. J Neurooncol 107:359–364

Joo KM, Kim SY, Jin X, Song SY, Kong DS, Lee JI, Jeon JW, Kim MH, Kang BG, Jung Y, Jin J, Hong SC, Park WY, Lee DS, Kim H, Nam DH (2008) Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. Lab Invest 88:808–815

Knights MJ, Kyle S, Ismail A (2012) Characteristic features of stem cells in glioblastomas: from cellular biology to genetics. Brain Pathol 22:592–606

Koul D, Shen R, Bergh S, Sheng X, Shishodia S, Lafortune TA, Lu Y, De Groot JF, Mills GB, Yung WK (2006) Inhibition of Akt survival pathway by a small-molecule inhibitor in human glioblastoma. Mol Cancer Ther 5:637–644

Lathia JD, Gallagher J, Heddleston JM, Wang J, Eyler CE, Macswords J, Wu Q, Vasanji A, Mclendon RE, Hjelmeland AB, Rich JN (2010) Integrin alpha 6 regulates glioblastoma stem cells. Cell Stem Cell 6:421–432

Li YQ, Guessous F, Zhang Y, Dipierro C, Kefas B, Johnson E, Marcinkiewicz L, Jiang JM, Yang YZ, Schmittgen TD, Lopes B, Schiff D, Purow B, Abounader R (2009) MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. Cancer Res 69:7569–7576

Mizrak D, Brittan M, Alison M (2008) CD133: molecule of the moment. J Pathol 214:3–9

Ogden AT, Waziri AE, Lochhead RA, Fusco D, Lopez K, Ellis JA, Kang J, Assanah M, Mckhann GM, Sisti MB, Mccormick PC, Canoll P, Bruce JN (2008) Identification of A2B5 + CD133- tumor-initiating cells in adult human gliomas. Neurosurgery 62:505–514

Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro JM, Colman H, Soroceanu L, Williams PM, Modrusan Z, Feuerstein BG, Aldape K (2006) Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 9:157–173

Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, Bayani J, Head R, Lee M, Bernstein M, Squire JA, Smith A, Dirks P (2009) Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell 4:568–580

Rempel SA, Dudas S, Ge S, Gutierrez JA (2000) Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. Clin Cancer Res 6:102–111

Ruiz I, Altaba A, Sanchez P, Dahmane N (2002) Gli and hedgehog in cancer: tumours, embryos and stem cells. Nat Rev Cancer 2:361–372

Sherry MM, Reeves A, Wu JK, Cochran BH (2009) STAT3 is required for proliferation and maintenance of multipotency in glioblastoma stem cells. Stem Cells 27:2383–2392

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63:5821–5828

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB (2004) Identification of human brain tumour initiating cells. Nature 432:396–401

Son MJ, Woolard K, Nam DH, Lee J, Fine HA (2009) SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. Cell Stem Cell 4:440–452

Ying M, Wang S, Sang Y, Sun P, Lal B, Goodwin CR, Guerrero-Cazares H, Quinones-Hinojosa A, Laterra J, Xia S (2011) Regulation of glioblastoma stem cells by retinoic acid: role for Notch pathway inhibition. Oncogene 30:3454–3467

Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, Lichter P, Unterberg A, Radlwimmer B, Herold-Mende CC (2008) Stem cell marker CD133 affects clinical outcome in glioma patients. Clin Cancer Res 14:123–129

Zhang M, Song T, Yang L, Chen R, Wu L, Yang Z, Fang J (2008) Nestin and CD133: valuable stem cell-specific markers for determining clinical outcome of glioma patients. J Exp Clin Cancer Res 27:85

Zheng HW, Ying HQ, Yan HY, Kimmelman AC, Hiller DJ, Chen AJ, Perry SR, Tonon G, Chu GC, Ding ZH, Stommel JM, Dunn KL, Wiedemeyer R, You MJJ, Brennan C, Wang YA, Ligon KL, Wong WH, Chin L, Depinho RA (2008) p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. Nature 455:1129–1133

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Stem Cells and Cancer Stem Cells 13, DOI 10.1007/978-94-017-7233-4