Gustav Steinhoff *Editor*

Regenerative Medicine - from Protocol to Patient

2. Stem Cell Science and Technology

Third Edition

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Edition 3: published in five separate volumes:

Vol. 1: 978-3-319-27581-9
Vol. 2: 978-3-319-27608-3
Vol. 3: 978-3-319-28272-5
Vol. 4: 978-3-319-28291-6
Vol. 5: 978-3-319-28384-5

ISBN 978-3-319-27608-3 ISBN 978-3-319-27610-6 (eBook)
DOI 10.1007/978-3-319-27610-6

Library of Congress Control Number: 2016933856

Originally published in one volume:
1st edition: ISBN: 978-90-481-9074-4 (Print) 978-90-481-9075-1 (Online) (2011)
2nd edition: ISBN: 978-94-007-5689-2 (Print) 978-94-007-5690-8 (Online) (2013)

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

Foreword: Regenerative Medicine: From Protocol to Patient

Third Edition

The vision to unravel and develop biological healing mechanisms based on evolving molecular and cellular technologies has led to a worldwide scientific endeavour to establish *regenerative medicine*. This field involves interdisciplinary basic and (pre) clinical research and development on the repair, replacement, regrowth or regeneration of cells, tissues or organs in congenital or acquired diseases. Stem cell science and regenerative biology is prompting the most fascinating and controversial medical development of the twenty-first century. It can be envisaged that this development will establish completely new molecular and cellular techniques for medical diagnosis and therapy. The early rush of scientific development was initiated more than one hundred years ago by the physiology of blood regeneration (Hall and Eubanks 1896) and successful vascular surgical techniques for organ transplantation (Carrel and Guthrie 1905). However, the clinical realization of allogenic blood transfusion lasted until the discovery of the blood group antigens (Landsteiner and Levine 1928) and successful routine allogenic organ and bone marrow transplantation towards the end of the last century.

Similar to the field of allogenic cell and organ transplantation, it seems that *regenerative medicine* again condenses mankind's visions, hopes and fears regarding medicine: Hopes of eternal life and effective treatment of incurable disease, as well as fears of the misuse of technology and uncontrolled modifications of life are polarizing the scientific field. The development and public acceptance of new ethical and regulatory guidelines is a necessary process to support further clinical development. Nevertheless, the vision of a new medicine using the regenerative power of biology to treat disease and restructure the organism is setting the aims for scientific, technological and medical development. Viewing the great expectations to restructure and regenerate tissues, organs or even organisms, the current attempts of both scientists and physicians are still in an early phase of development.

The field of *regenerative medicine* has developed rapidly over the last 20 years with the advent of molecular and cellular techniques. This collection of volumes on *Regenerative Medicine: From Protocol to Patient* aims to explain the scientific knowledge and emerging technology, as well as the clinical application in different organ systems and diseases. The international leading experts from four continents describe the latest scientific and clinical knowledge in the field of *regenerative medicine*. The process of translating the science of laboratory protocols into therapies is explained in sections on basic science, technology development and clinical translation including regulatory, ethical and industrial issues.

This collection is organized into five volumes: (1) *Biology of Tissue Regeneration*; (2) *Stem Cell Science and Technology*, (3) *Tissue Engineering, Biomaterials and Nanotechnology*, (4) *Regenerative Therapies I.;* and (5) *Regenerative Therapies II. Biology of Tissue Regeneration (Volume 1)* focuses on regenerative biology with chapters on the extracellular matrix, asymmetric stem cell division, stem cell niche regulation, (epi)genetics, immune signalling, and regenerative biology in organ systems and model species such as axolotl and zebrafish.

Stem Cell Science and Technology (Volume 2) provides an overview of the classification of stem cells and describes techniques for their derivation, programming and culture. Basic properties of differentiation states, as well as their function are illustrated, and areas of stem cell pathologies in cancer and therapeutic applications for these cells are discussed with the emphasis on their possible use in *regenerative medicine*.

Tissue Engineering, Biomaterials and Nanotechnology (Volume 3) focuses on the development of technologies, which enable an efficient transfer of therapeutic genes and drugs exclusively to target cells and potential bioactive materials for clinical use. The principles of tissue engineering, vector technology, multifunctionalized nanoparticles and nanostructured biomaterials are described with regards to the technological development of new clinical cell technologies. Imaging and targeting technologies, as well as the biological aspects of tissue and organ engineering are described.

Regenerative Therapies I (Volume 4) gives a survey of the history of regenerative medicine and clinical translation including regulation, ethics and preclinical development. Clinical state-of-the-art, disease-specific approaches of new therapies, application technologies, clinical achievements and limitations are described for the central nervous system, head and respiratory systems. Finally, *Regenerative Therapies II (Volume 5)* contains state-of-the-art knowledge and clinical translation of regenerative medicine in the cardiovascular, visceral and musculoskeletal systems.

These volumes aim to provide the student, the researcher, the healthcare professional, the physician and the patient with a complete account of the current scientific basis, therapeutical protocols, clinical translation and practised therapies in *regenerative medicine*. On behalf of the sincere commitment of the international experts, we hope to increase your knowledge, understanding, interest and support by reading the book.

After the successful introduction of the first edition in 2011, this publication has been developed and expanded for the third edition into five volumes.

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October 2015

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Literature

Carrel A, Guthrie CC (1906) Successful transplantation of both kidneys from a dog into a bitch with removal of both normal kidneys from the latter. Science 9, 23(584):394–395
Hall WS, Eubank MD (1896) The regeneration of the blood. J Exp Med 1(4):656–676
Landsteiner K, Levine P (1928) On individual differences in human blood. J Exp Med 47(5):757–775

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Chapter 1
Characterization and Classification of Stem Cells

Ute Bissels, Yvonne Diener, Dominik Eckardt, and Andreas Bosio

Abstract Starting from a zygote, an organism is made up of thousands, highly organized stem cells, progenitor cells and postmitotic cells which are generated in spatio-temporally coordinated proliferation and differentiation steps. The ongoing advancements in cell culture, isolation techniques, and molecular analyses have driven our basic understanding of different cell types and led to a broad classification of stem cells. This chapter outlines the most prominent techniques used for the characterization and classification of stem cells and provides an overview of many different stem cells, their function and their mRNA, miRNA and protein content.

Keywords Classification • Surface molecules • Transcription factors • DNA methylation • miRNA • mRNA • Protein markers

Abbreviations

BM	Bone marrow
BTSC	Brain tumor stem cell
CB	Cord blood
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CSC	Cancer stem cell
EPC	Endothelial progenitor cell
ErP	Erythroid progenitor
ESC	Embryonic stem cell
GMP	Granulocyte-macrophage progenitor
HpSC	Hepatic stem cell
HSC	Hematopoietic stem cell
iPSC	Induced pluripotent stem cell
LT-HSC	Long-term hematopoietic stem cell
MEP	Megakaryocyte-erythroid progenitor

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_1

MkP	Megakaryocyte progenitor
MP	Multipotent progenitors
MSC	Mesenchymal stem cell
NK	Natural killer
NSC	Neural stem cell
PB	Peripheral blood
RBC	Red blood cells
SPC	Spermatogonial progenitor cell
ST-HSC	Short-term hematopoietic stem cell
TSC	Tissue stem cell

1.1 Introduction

The characterization of stem cells helps us to shed light on general cellular processes and to understand the development and senescence of organs and organisms. It is also a prerequisite to use stem cells as tools for drug target discovery, predictive toxicology, or for cellular therapies including tissue regeneration. A classification of stem cells can be done by measuring and quantifying distinct functional properties and/or molecular markers. While the function of self-renewal defines stem cells in general, the degree of "potency", i.e. the range of differentiation options to generate different cell types, is commonly used for a rough hierarchical classification of cells into:

- totipotent cells: generate all cells including extraembryonic cell types, e.g. zygote
- pluripotent cells: generate all body cells including germ cells, e.g. embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), inner cell mass of the blastocyst-stage embryo
- multipotent cells: generate all tissues cells, e.g. tissue stem cells such as hematopoietic stem cells (HSCs)
- unipotent cells: generate a single cell type, e.g. spermatogonial stem cells (SPCs)

The hierarchy is not unidirectional as in certain circumstances a cell can dedifferentiate to form cells with a higher potency.

A further classification subdivides the different multipotent stem cells according to the tissue cells they can generate. It is assumed that almost every tissue has stem cells which are responsible to keep tissue homeostasis and to regenerate or limit injuries. Most prominent multipotent or tissue stem/progenitor cells are those forming the blood (hematopoietic stem cells, HSCs), endothelium (endothelial progenitor cells, EPCs), mesenchyme (mesenchymal stem/stroma cells, MSCs), muscles (satellite stem cells), heart (cardiac stem/progenitor cells), sperm (spermatogonial stem cells), intestine (intestinal stem cells), pancreas (pancreas derived multipotent

precursors), lung (lung stem cells), liver (hepatic stem cells), brain (neural stem cells, NSC), skin and hair (skin stem cells), and mammary glands (mammary stem cells). The borders are not strict as, although rare in vertebrates, a transdifferentiation of one tissue stem cell into another tissue lineage has been reported in vitro and in vivo.

As a certain function of a cell is usually made up by a complex and time dependent interplay of different molecule classes, it is occasionally difficult to measure or even to quantify it. This is why a purely functional classification of stem cells is sometimes not of practical help and molecular markers come into play. Technical limitations in terms of sensitivity, specificity and ease of (parallelized) measuring further define which markers or classes of markers are eventually used for a certain cell type. Let us take for example the definition of embryonic stem cells, or in general pluripotent stem cells. The term "pluripotent cell" has mainly been derived from the properties of an embryonic stem cell. An embryonic stem cell can give rise to all the cells and tissues of an organism with the exception of the extra embryonic tissue. With this definition, it is clear that in order to proof a cell of being pluripotent, one has to show that this cell, when injected into a blastocyst stage embryo is able to generate a whole organism including the germ cells. This is almost only possible with mice, certainly not with human cells. That is why teratoma formation has been introduced as a surrogate test. Here, the potential of a cell to differentiate into any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system) is interrogated. But even this is very time consuming, not really quantifiable and cannot be used as a prospective definition but only as a retrograde proof. Therefore, molecular markers have been defined which are correlated with pluripotency, like certain proteins expressed on the surface of pluripotent cells, transcription factors, microRNAs (miRNAs), messenger RNAs (mRNAs) or the methylation status of genomic sequences. Still, after many years, it is hotly debated which the right pluripotency markers are and whether it is acceptable at all to rely only on markers when referring to pluripotency. In conclusion, a classification of stem cells is based on both, molecular markers for practical reasons and their function for reasons of clarity.

1.2 Methods for the Characterization and Classification of Stem Cells

From a biochemical point of view, stem cells do not differ from other cells and thus all known methods which allow to measure the status and interaction of biomolecules can be used to characterize stem cells. However, for stem cells, the description of some biomolecules using certain techniques has been found to be more instrumental than others.

- DNA methylation: It stably alters the gene expression pattern in cells indicating if a gene is likely to be transcribed (active) or not (silenced). It is measured for instance by Methylation Specific PCR (MSP), or ChIP-on-chip assays.
- mRNA status or transcriptome: It tells which genes are transcribed and therefore are active. As all transcripts in a cell can be measured in parallel using microarrays or library sequencing, a good estimation of all active genomic pathways can be drawn.
- miRNAs: They are analyzed like mRNAs using PCR, blotting techniques, microarrays, sequencing, and *in situ* hybridization and are a relatively young class of molecules which help to understand if corresponding mRNAs are translated into proteins or not. Their expression has been found to be quite robustly correlated to some cell types.
- Cell surface molecules: They can be mainly identified by their reaction with specific antibodies using techniques such as flow cytometry, immunohistochemistry, immunocytochemistry, or different sorts of gel electrophoresis and blotting. In addition, mass spectrometry is used to analyze the cell surface proteome without antibodies. Also, raising new antibodies by immunization of rats and mice with cells has led to the identification of many new markers. Especially adhesion molecules and receptors can also be analyzed using the respective interaction partners and give insights into the "communication status" of a cell. The massive advantage of surface proteins or molecules in general is that they can be used to sort cells very easily e.g. by using flow cytometry based sorting, immunopanning, or magnetic cell sorting. In order to standardize the annotation of surface molecules a CD (cluster of differentiation) nomenclature was established in 1982 at the 1st International Workshop and Conference on Human Leukocyte Differentiation Antigens (HLDA). The CD system originally classifies monoclonal antibodies (mAbs) generated against epitopes on the surface of leukocytes and has then been expanded to many other cell types.
- Transcription factors: They are very indicative for some cell types as they indicate which pathways of a cell are activated and which not. Many of them are a master switch deciding which lineage a cell is following. Their importance has been proofed by the fact that the ectopic expression of single transcription factors can redirect (or reprogram) the differentiation fate of a cell.
- Cell surface membrane transporter: At least some stem cells differ from non-stem cells in their ability to transport Hoechst stains (Hoechst 33342) out of the cell. Hoechst 33342 is a DNA-binding fluorescent dye, excitable by ultraviolet light at 350 nm and emitting at 461 nm. A multidrug-like transporter in stem cells causes an increased efflux of Hoechst 33342 by an active biological process. This can be used to identify stem cells by flow cytometry as a "side population" (Goodell et al. 1996).
- Enzymes: Stem and progenitor cells also possess a different aldehyde dehydrogenase (ALDH) activity compared to other cells. This enzyme converts a nonfluorescent substrate (an aminoacetaldehyde) into a fluorescent product (an aminoacetate) that is retained within living cells with an intact membrane. Cells with different ALDH enzyme activity can thus be differentially stained with the

fluorescent product, and stem cells can be isolated by flow cytometry based on their enzyme activity (Jones et al. 1995; Storms et al. 1999).

The analysis of most of the above mentioned molecules is optimally done on highly purified stem cells rather than mixtures of different cell types. A detailed description of techniques for the enrichment of stem cells has been reviewed by Bosio et al. (2009).

Interestingly, although it is an absolute prerequisite for single cell based isolation and characterization of stem cells, we noticed a lack of standardized protocols for proper dissociation of tissues. Solid organs consist of a mixture of cell types which are interconnected in multiple ways. Specific transport proteins as well as gap junctions connect cells and allow the transport of molecules, whereas tight junctions build up a barrier to avoid free transport across cell layers. In addition, cell adhesion molecules like cadherins are important for stability of the tissue and localization of the cells. All cells in these tissues are surrounded by a complex extracellular matrix composed of a variety of proteins and polysaccharides. The most important components are collagens, hyaluronan, and glycosaminoglycan (Iozzo 1998). The major goal of tissue dissociation is the disruption of the extracellular matrix and cell adhesion components without harming the integrity of the cell membrane and the surface epitopes. We have established automated procedures for the enzymatic and mechanical dissociation of solid tissues and optimized them according to the specific needs of a given tissue or cell type (Jungblut et al. 2008, 2009; Pennartz et al. 2009).

An interesting approach combining the knowledge of stem cell type specific gene expression with the convenience of surface markers is the use of genetically modified stem cells to label or enrich these cells. Here, the promoter of a gene specifically expressed in a cell type is used to drive the expression of a selection marker such as the green fluorescence protein (GFP), an antibiotic resistance gene, or an artificial surface epitope like the human CD4 molecule lacking its intracellular domain.

In vitro and in vivo assays to functionally characterize stem cells are partially dependent on the respective stem cell but some assays are used for multiple stem cell types. For example, measuring the replication of cells by incorporating detectable molecules like BrdU into the DNA, or proliferation of cells by CFSE via staining of intracellular proteins. This allows to distinguish non dividing (postmitotic) cells from proliferating or differentiating ones. In vitro culturing and differentiation of cells as well as the transplantation of cells into animal models are methods used to track the differentiation potential, the regenerative power or malignancy of stem cells. The differentiation behavior of single stem cell clones can be analyzed by cellular barcoding, in which genetic marks are introduced into each cells' genome via retroviral vectors. The unique DNA-sequences (barcodes) can be easily identified in the clonal progeny using sequencing-based detection systems (Gerrits et al. 2010). Culturing of stem cells in semi-solid media (colony forming unit (CFU) assays) offers the opportunity to analyze the lineages and to quantify the number of colonies derived from stem cells and is especially used for hematopoietic stem cells.

1.3 Protein Markers of Stem Cells

Protein markers are widely used for classification of stem cells. This is due to the fact that the expression of proteins is less variable than for example mRNA expression and that, especially for proteins expressed on the cell surface, it is possible to use them for the isolation of the respective cells by e.g. immunopanning, flow cytometric sorting, or magnetic sorting. Once the cells are isolated, they can be further analyzed which allows a clear decision to which extend a protein marker is reflecting a stem cell function. Figures 1.1 and 1.2 summarize the most commonly used markers for the different types of human stem/progenitor and cancer stem cells. Just as the cells which make up a tissue, tumor cells are functionally heterogeneous. They are organized in a hierarchy of cell populations with different biological properties. Only a minority of tumor cells have the capacity to regenerate a tumor and sustain its growth when injected into an immune-compromised mouse model which is the functional definition of a cancer stem cell (Tang et al. 2007).

For mouse **pluripotent cells** such as ESCs and iPSCs, mainly E-cadherin (CD324), EpCAM (CD326) and SSEA-1 (CD15) have been used as surface marker. Different proteomic strategies like mass spectrometry of mouse ESCs revealed further details about the cell surface signature of pluripotent mouse stem cells (Nunomura et al. 2005; Wollscheid et al. 2009). Mostly EpCAM (CD326), E-cadherin (CD324), CD90, SSEA-3, SSEA4, SSEA-5, CD9, TRA-1-60, and

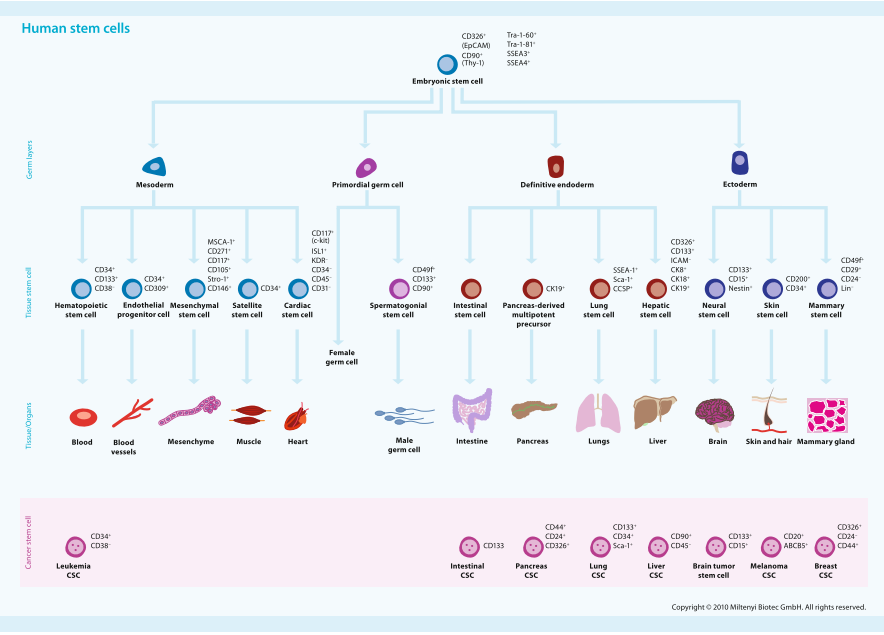

Fig. 1.1 Hierarchical illustration of human stem cells and their cell surface markers

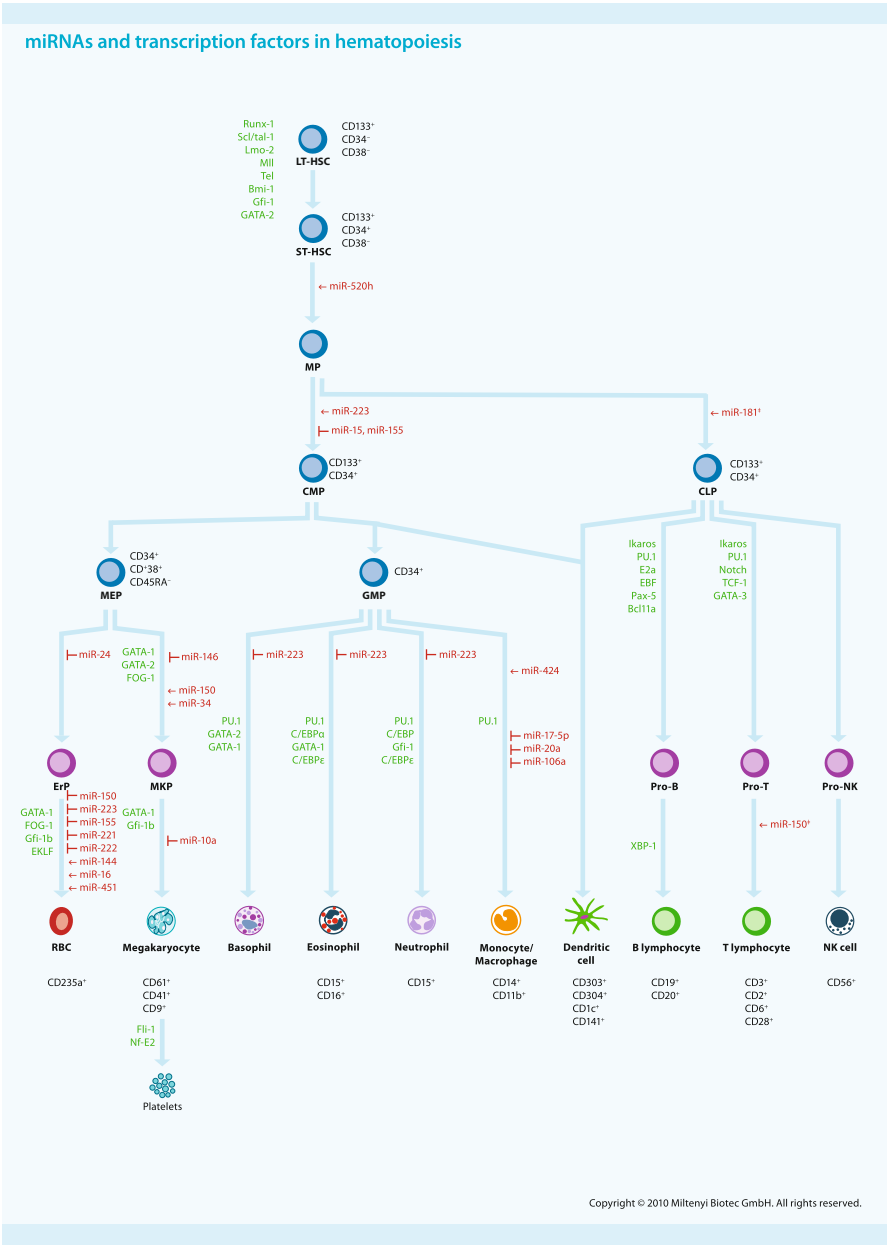

Fig. 1.2 Prominent miRNAs, transcription factors and cell surface markers in hematopoiesis. The miRNAs that regulate the different steps of hematopoiesis are shown in red. The depicted miRNAs were mainly identified in in-vitro assays with human cells. The role of the miRNAs labelled with ‡, e.g. miR-181‡ that drives differentiation towards CLPs, were identified in mouse experiments. The transcription factors are selected according to Orkin and Zon (2008). Abbreviations: *LT-HSC* long-term hematopoietic stem cell, *ST-HSC* short-term hematopoietic stem cell, *MP* multipotent progenitors, *CMP* common myeloid progenitor, *CLP* common lymphoid progenitor, *MEP* megakaryocyte-erythroid progenitor, *GMP* granulocyte-macrophage progenitor, *ErP* erythroid progenitor, *MkP* megakaryocyte progenitor, *RBC* red blood cells, *NK* natural killer

TRA-1-81 have been used to characterize human ESCs and iPSCs (Adewumi et al. 2007; Tang et al. 2007). Interestingly, the carbohydrate SSEA-1 is a pluripotency marker in case of mouse pluripotent stem cells, whereas in the human system, SSEA-1 is indicative of pluripotent stem cell differentiation. More than 200 cell surface proteins of the human embryonic stem cell line HUES-7 have been identified by Dormeyer et al. (2008).

Murine **hematopoietic stem and progenitor cells**, HSCs, have been defined by absence of lineage commitment markers such as CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), and Ter-119, and high expression of CD117 (c-kit/SCFR) and Sca-1 (Hubin et al. 2005; Schiedlmeier et al. 2007). CD34 is expressed on HSCs of the murine fetus and neonate, but decreases with age (Ogawa 2002) and is not or only weakly expressed on mature mouse HSCs (Osawa et al. 1996). Another way of defining hematopoietic stem and progenitor cells is the use of SLAM markers (Kiel et al. 2005). Accordingly, multipotent HSCs are CD150+CD48−CD244−; multipotent progenitor cells (MPPs) are CD150−CD48−CD244+, and lineage-restricted progenitor cells (LRPs) are CD150−CD48+CD244+. CD34 and CD133 label human HSCs with long-term engraftment in NOD/SCID mice. However, about 95 % of the CD34+ cells and 70 % of the CD133+ cells have a progenitor status, identified by co-expression of CD38. Therefore, CD34+CD38−CD133+ is mostly used as the surface signature of human HSCs (Buhring et al. 1999; Copland et al. 2006; Giebel et al. 2006). A detailed description of protein markers for murine and human stem cells can be found in chapter 10.2.2.1 and 10.2.2.2 of this book, respectively. **Leukemic stem cells** (LSCs) were identified to be CD34+CD38− and can be isolated from human AML samples by FACS. John Dick and colleagues demonstrated that these cells initiated leukemia in NOD-SCID mice compared with the CD34+CD38+ and CD34− fractions (Bonnet and Dick 1997). Such xenotransplantations are an important criterion in defining cancer stem cells (Tang et al. 2007).

Several cell surface antigens have been suggested for the isolation of **mesenchymal stem/stromal cells**, MSCs, such as antifibroblast antigen (Jones et al. 2002), CD117 (Huss and Moosmann 2002), CD105 (Aslan et al. 2006; Majumdar et al. 2003), Stro-1 and CD146 (Shi and Gronthos 2003), CD133 (Tondreau et al. 2005), CD271 (Quirici et al. 2002) and MSCA-1 (W8B2) (Buhring et al. 2007). A comprehensive cell surface proteome analysis of human plastic adherent MSCs has been published recently by Niehage et al. (2011), describing even among the 41 identified CD markers, 5 epitopes previously not linked to the MSC cell surface. MSCs expanded from mouse bone marrow culture are described to be positive for Sca-1, CD117 (c-kit), and CD105 (Sun et al. 2003).

Molecular markers including surface molecules, intracellular proteins and microRNAs for ESCs, HSCs and MSCs are reviewed by Calloni et al. (2013).

Another stem cell type which is found in the bone marrow and mobilized to the blood stream by environmental stimuli for physiological and pathological tissue regeneration are the **endothelial progenitor cells** (EPCs) which form new blood vessels and contribute to vascular repair (Asahara et al. 2011). In humans, these cells have been defined by the expression of the markers CD34, CD133, CD309

(VEGFR2/KDR/Flk-1), CD184 (CXCR4), CD105 (Endoglin), and in the mouse by Lin−Sca-1+c-kit+CD34+ CD309+ (VEGFR-2/KDR/Flk-1) (Rafii and Lyden 2003; Timmermans et al. 2009). Nevertheless, the identification of a unique combination of receptors specific and selective for primary EPCs, enabling an unambiguous distinction between EPCs and HSCs, is still missing.

Neural stem cells (NSCs) share many characteristics with astrocytes and show expression of typical astrocyte proteins, like GFAP, or GLAST (Merkle and Alvarez-Buylla 2006; Mori et al. 2005). Furthermore, CD133/Prominin, EGFreceptor, CD15, and Nestin have been described as markers for neural stem cells (Conti and Cattaneo 2010), but isolation of these cells from primary neural tissue with high purity has been difficult. Therefore, a combination of markers has been used to increase the purity. Beckervordersandforth et al. (2011) followed a dual labeling strategy to isolate GFAP/prominin1 double positive self-renewing multipotent stem cells from adult hGFAP-GFP mice in combination with prominin labeling. In another approach GFAP/EGFR+ cells were successfully isolated and identified as activated stem cell astrocytes (Pastrana et al. 2009). In a recent study, Mich et al. (2014) investigated the expression of different markers on quiescent NSCs and neurosphere-initiating cells (NICs) and found PlexinB2 to be moderately expressed on the quiescent NSCs. Furthermore, Kokovay et al. (2012) described CD106 to be expressed on the apical endfeet of NSCs. Many more cell surface proteins have been described and used for sorting of **neural progenitor cells** like PSA-NCAM (neuronal precursors) (Boutin et al. 2010; Pennartz et al. 2004), and A2B5 (glial precursors) (Seidenfaden et al. 2006). Singh et al. (2003, 2004a) reported the identification and purification of **cancer stem cells** from human brain tumors of different phenotypes that possess a marked capacity for proliferation, self-renewal, and differentiation.

The increased self-renewal capacity of the **brain tumor stem cell** (BTSC) was highest among the most aggressive clinical samples of medulloblastoma compared with low-grade gliomas. Several other reports demonstrated that isolation of cells expressing the surface marker CD133 leads to enrichment of the BTSC population (Bao et al. 2006; Piccirillo and Vescovi 2006; Singh et al. 2004b), whereas Son et al. (2009) showed that SSEA-1 (CD15) enriches for tumorigenic subpopulations in human glioblastoma.

The existence of various resident populations of **cardiac progenitor/stem cells** in postnatal hearts has been claimed (Sturzu and Wu 2011). CD117 (c-kit)+/lin – cells isolated from the adult mouse heart were described to be clonogenic and self-renewing, capable of differentiating into cardiomyocytes, vascular smooth muscle cells, and endothelial cells, although this population only heterogeneously expresses early cardiac transcription factors such as GATA4, Mef2c, and Nkx2.5 (Beltrami et al. 2003). A more recent study analyzing various genetic mouse models came to the conclusion that in the adult mouse heart CD117+ cells mainly form endothelial cells, while CD117+ cell-derived cardiomyocytes were only found at a ratio below 0.03 %, calling the relevance of CD117+ cardiomyocyte progenitors for cardiomyocyte regeneration into question (van Berlo et al. 2014). Nevertheless, a clinical

phase I study testing safety and feasibility of autologous CD117+ cells as an adjunctive treatment for patients undergoing coronary bypass surgery was initiated (Bolli et al. 2011), based on data by Bearzi et al. (2007) who described a CD117+ population of cardiac cells in the human heart exhibiting key characteristics of stem cells in vitro and in vivo. Two other publications (Oh et al. 2003; Pfister et al. 2005) referred to the Sca-1+ population as putative adult cardiac progenitors. Expression of early cardiac transcription factors GATA4 and Mef2c, as well as telomerase activity, associated with self renewal potential, were detected in Sca-1+ cells. However, fusion between Sca-1+ cells and host cardiomyocytes was frequently detected, leaving some uncertainty about the true in vivo differentiation potential of Sca-1+ progenitors (Oh et al. 2003). Expression of the transcription factor Isl-1 in multipotent heart progenitors found in fetal mouse and human heart has not yet been correlated with a distinct surface marker which would allow for antibody-based enrichment (Bu et al. 2009). In addition, several groups have described in vitro cardiomyogenic potential of human cardiac cells reactive to an antibody against the mouse Sca-1 epitope. Lastly, a heterogeneous cell population isolated from human atrium forms so called cardiospheres in suspension culture. Cardiosphere containing CD117+, Sca-1-like+ and CD309 (KDR)+ cells have been ascribed stem cell characteristics (Messina et al. 2004) and as well cardiospheres have been used for a phase I clinical trial of patients with left ventricular dysfunction (Makkar et al. 2012). To date there is no consensus on the best marker (set) for unambiguous identification of cardiac stem cells.

Several surface markers have been described and used for isolation of murine **spermatogonial stem cells** (SSC). In 2004 Kubota (Kubota et al. 2004) described a Thy-1 (CD90) antibody-based enrichment of murine SSCs, further expansion on STO feeder cells in serum-free medium and in vivo proof of an SSC phenotype after transplantation. Seandel et al. (Seandel et al. 2007) showed that SPCs express GPR125, an orphan adhesion-type G-protein-coupled receptor, and can be efficiently obtained by cultivation on mitotically inactivated testicular feeders containing CD34+ stromal cells. Recently, Kanatsu-Shinohara et al. (2011) showed that SSCs have an unstable side population phenotype and provide evidence that SSCs change their phenotype characteristics in response to their microenvironment. A study by Conrad et al. (Conrad et al. 2008) described the isolation and characterization of human germline stem cells (GSCs) using defined cultivation techniques, SPC adhesion properties and a positive selection using CD49f, CD133, or CD90.

According to Schmelzer et al., **human hepatic stem cells** (hHpSCs) (Schmelzer et al. 2007; Schmelzer and Reid 2008) can be isolated by positive immunoselection for the epithelial cell adhesion molecule CD326 (EpCAM +). The hHpSCs express cytokeratins 7 and 19, CD133/1, telomerase, CD44H, claudin 3, and albumin (weakly). They are negative for alpha-fetoprotein (AFP), intercellular adhesion molecule 1 (ICAM-1), and for markers of adult liver cells (cytochrome P450s) and hematopoietic (progenitor) cells (CD45, CD34, CD14, CD38, CD90 (Thy1), CD235a (Glycophorin A)). As for rodent HpSCs, Yovchev et al. compared hepatic cells isolated by two surface markers, EpCAM and Thy-1 (CD90). It was shown

that Thy-1 + cells are mesenchymal cells with characteristics of myofibroblasts/activated stellate cells whereas transplantation experiments revealed that EpCAM+cells are true progenitors capable of repopulating injured rat liver (Yovchev et al. 2007; Yovchev et al. 2008).

Yang et al. (2008) have delineated **liver cancer stem cells** serially from HCC cell lines, human liver cancer specimens, and blood samples, using CD90 as a marker. CD45−CD90+cells were detected in all the tumor specimens, but not in the normal, cirrhotic, and parallel non-tumorous livers. Cheung et al. (2011) have shown that expression of ABCB5 (ATP-dependent binding cassette B5) in liver cancer stem cells is associated with chemoresistance and reduced survival times of patients with hepatocellular carcinoma. **Mammary stem cells** have been characterized by the markers CD49f, CD29 (also known as α6 and β 1 integrins) and CD24 when showing a CD24 low CD49f high or CD24 low CD29 high molecular signature (Shackleton et al. 2006; Stingl et al. 2006). In contrast to their differentiated progeny, mammary stem cells are negative for estrogen receptor (ERα), progesterone receptor (PR) and the tyrosine kinase receptor HER2 – three molecular markers that define different populations of differentiated luminal epithelial cells – but are highly positive for the transcription factor p63, the epidermal growth factor receptor (EGFR) and cytokeratin 14 (CK14), confirming their basal origin (Asselin-Labat et al. 2006; Pontier and Muller 2009). **Breast cancer stem cells** have been reported to be ESA+CD44+CD24−Lineage – (Al-Hajj et al. 2003). ESA (epithelial specific antigen) is also known as EpCAM (CD326). O'Brien et al. (O'Brien et al. 2007) and Ricci-Vitiani et al. (Ricci-Vitiani et al. 2007) showed that the tumorigenic population in **colon cancer** is restricted to CD133+cells, which are able to reproduce the original tumor in permissive recipients. Additionally, the surface marker pattern CD326 (EpCam)+CD44+ CD166+ has been described by Du et al. (2008) and Dalerba et al. (2007). Pang et al. (2010) have described CD26 as marker for the tumorigenic population in colon cancer.

Li et al. (2007) identified a highly tumorigenic subpopulation of **pancreatic cancer cells** expressing the cell surface markers CD44, CD24, and epithelial- specific antigen (ESA; EpCAM; CD326). Pancreatic cancer cells with the CD44+CD24+ESA+phenotype (0.2–0.8 % of pancreatic cancer cells) had a 100-fold increased tumorigenic potential compared to non-tumorigenic cancer cells, with 50 % of animals injected with as few as 100 CD44+CD24+ESA+cells forming tumors that were histologically indistinguishable from the human tumors from which they originated.

As a conclusion, protein markers correlated to functional properties of the respective stem/progenitor cell types which have been defined for most tissues and pluripotent cells. However some of the markers have only recently been reported and are still intensively debated. It can be estimated that sorting of pluripotent and tissue stem cells will increase in the future as it offers the option for a detailed analysis and understanding of malignant and disease-causing cells, as well as of cell types urgently needed for tissue regeneration and tissue engineering approaches.

1.4 miRNAs in Stem Cells

MicroRNAs (miRNAs), short noncoding RNAs of 21–23-nucleotides (nt) in length, regulate target mRNAs post-transcriptionally. miRNAs in stem cells are not as well characterized as proteins. However, they have been shown to play an important role in many different cellular, developmental, and physiological processes as divergent as cell lineage decisions, cell proliferation, apoptosis, morphogenesis, fat metabolism, hormone secretion, neuronal synaptic plasticity, and long-term memory (Aravin and Tuschl 2005).

In 2004, it was shown for the first time that miRNAs are involved in hematopoietic lineage differentiation (Chen et al. 2004). For example, ectopic expression of miR-181 in lineage negative (Lin-) hematopoietic progenitor cells from mouse bone marrow increased the fraction of B-lineage cells (CD19 +) in vitro and in vivo. As summarized in Fig. 1.2, further analysis showed that miRNAs fine tune essentially each step in hematopoiesis. It was demonstrated, for instance, that miR-150 drives megakaryocyte-erythrocyte progenitor (MEP) differentiation towards megakaryocytes at the expense of erythroid cells (Lu et al. 2008). Erythropoiesis was reported to be promoted by miR-451, miR-16 and miR-144 and negatively regulated by miR-150, miR-155, miR-221, miR-222 and miR-223 (Bruchova et al. 2007; Dore et al. 2008; Felli et al. 2005; Zhan et al. 2007). Furthermore, it was shown that the miRNA cluster miR-17-5p-92 controls monocytopoiesis (Fontana et al. 2007) and that miR-424 is upregulated during monocyte/macrophage differentiation. Within the lymphoid lineage, the decision between T cells and B cells is regulated by miR-150 (Xiao et al. 2007; Zhou et al. 2007). In a recent study, Raghavachari et al. (2014) performed an integrated analysis of miRNAs and mRNAs during erythropoietic, granulopoietic and megakaryopoietic differentiation of CD34 + cell from mobilized peripheral blood. They found miR-18a and miR-145 to be specifically upregulated during erythropoiesis and granulopoiesis, respectively. For further reading about miRNAs in hematopoiesis, we recommend the following reviews: Undi et al. (2013); Lazare et al. (2014) and Hong et al. (2015).

The early steps of HSC differentiation, e.g. the role of miRNAs in self-renewal of LT-HSCs and ST-HSC, as well as the function of miRNAs in multipotent progenitors, are currently mostly unknown due to the difficulty to perform whole genome miRNA screens of small numbers of cells. Up to now, expression of miRNAs has been analyzed in human primitive Lin-CD34+CD38-CD90 + CD45RA-cells (Han et al. 2010; Ooi et al. 2010), CD34 + CD38– cells (Liao et al. 2008), CD133+ cells (Bissels et al. 2011; Jin et al. 2008) and murine HSCs (Guo et al. 2010; O'Connell et al. 2010; Petriv et al. 2010). Liao and coworkers found miR-520 h to be overexpressed in CD34 + CD38 – cells compared to more committed CD34+ cells. Ooi et al. (2010) compared HSCs (Lin – CD34 + CD38 – CD90 + CD 45RA–) and MPPs (Lin – CD34 + CD38 – CD90 – CD45RA–) to more committed progenitor populations and found miR-125b to be highly expressed in the stem cell fractions. Recently, we presented the first relative and absolute miRNA copy number profile of CD133+ bone marrow cells and directly compared donor-matched

CD133+ cells with the more differentiated CD34+CD133- and CD34-CD133- cells on miRNA and mRNA level (Bissels et al. 2009; Bissels et al. 2011b). 18 miRNAs were significantly differentially expressed between CD133+ and CD34+CD133- cells. These differentially expressed miRNAs are involved in inhibition of differentiation, prevention of apoptosis, and cytoskeletal remodeling. miRNA expression profiles are further available for CD34+progenitor cells from bone marrow and mobilized peripheral blood (Georgantas et al. 2007) as well as from cord blood (Merkerova et al. 2009). Furthermore, Mintz et al. (2012) performed miRNA profiling in adherent and suspension CD34+cells from mobilized peripheral blood. They found miR-181a*, which targets the stem cell-associated gene Nanog, to be highly expressed in the adherent CD34+ subpopulation. A study by Arnold et al. (2011) identified miRNAs shared by multiple tissue-specific stem cells and miRNAs unique to various tissue-specific murine stem cells. miR-192 was identified as specific for LT-HSCs ($Endoglin^{+}Rho^{low}Sca\text{-}1^{+}Lin^{-}$) and absent from all other analyzed cell types.

Regarding the function of miRNAs in the HSC compartment, several studies showed that miRNAs regulate HSC proliferation and differentiation. This can occur e.g. through targeting of pro-apoptotic proteins (Gerrits et al. 2012; Guo et al. 2010; Ooi et al. 2010)) or modulation of responsiveness to extrinsic signals by targeting the PI3K/AKT/GSK3ß pathway (Lechman et al. 2012). These data indicate that miRNAs harbor the potential to expand HSCs for clinical approaches. Moreover, miRNAs have been shown to be involved in the pathogenesis of hematologic malignancies by acting as oncomiRs (Chaudhuri et al. 2012; Gordon et al. 2013; Li et al. 2012; Wang et al. 2012).

While the different cell types of the hematopoietic system express a multitude of miRNAs, five were reported to be common hematopoietic miRNAs, namely miR-142, miR-144, miR-150, miR-155 and miR-223. Those miRNAs were identified as highly specific for hematopoietic cells within a large-scale study to identify miRNAs and to assess their expression patterns in >250 small RNA libraries from >26 different organ systems (Landgraf et al. 2007).

Specifically expressed miRNAs are also known for other types of stem cells e.g. cancer stem cells (CSCs) and human embryonic stem cells (hESCs) . Breast cancer stem cells (BCSCs) are characterized among others by downregulation of miR-200c. Importantly, miR-200c suppresses tumorigenicity of BCSCs (Shimono et al. 2009). In hESCs the miR-302~367 cluster is specifically expressed (Landgraf et al. 2007; Suh et al. 2004) and may therefore serve as a marker for hES cells. The first miRNA profile of induced pluripotent stem cells (iPSC) revealed that the miR-302~367 cluster is also highly expressed in the reprogrammed cells (Wilson et al. 2009). However, Zhao et al. (2014) showed that miRNA expression differs in ESCs and induced pluripotent stem cells reprogrammed by different methods. Recently, Gruber et al. (2014) investigated another ESC-specific miR-cluster (miR-290~295) and its downstream regulatory network. By computational analysis of existing data sets, they found several transcription factors, which are involved in ESC differentiation to be targets of the miR-290~295 cluster. Regarding the application of miRNAs in tissue engineering, it has been shown that expression of the miR-302/367

cluster can directly reprogram mouse and human somatic cells to a pluripotent stem cell state in the absence of the commonly used transcription factors Oct4, Sox2, Klf4 and Myc (Anokye-Danso et al. 2011). This miRNA-based reprogramming approach is two orders of magnitude more efficient than standard methods. Miyoshi et al. (2011) showed that reprogramming of murine and human cells is even feasible by direct transfection of mature miRNAs with a non-viral approach. Recent advances in miRNA-based reprogramming and tissue-engineering are reviewed by Moradi et al. (2014) and Ribeiro et al. (2014).

Taken together, the characterization of stem cells with respect to miRNAs is well advanced for some stem cell types and has almost not been addressed for some other stem and progenitor cell types. This is partly due to difficulties in isolating enough stem cells for a proper miRNA analysis, and it is likely to be solved in the next years. Only then it will, if at all, be possible to speculate on common miRNA signatures of stem cells and to shed light on the miRNA-based regulation of stem cell-relatedcellular functions. For further reading about the role of miRNAs in stem cells, we recommend the following reviews: Hatfield and Ruohola-Baker (2008), Gangaraju and Lin (2009), Mallanna and Rizzino (2010), Bissels et al. (2012).

1.5 The mRNA of Stem Cells

In 2002, two independent studies (Ivanova et al. 2002; Ramalho-Santos et al. 2002) tried to identify a general stem cell signature by comparing the expression profiles of embryonic, hematopoietic and neural stem cells. However, the two lists of "stemness" enriched transcripts yielded only 15 common genes (Burns and Zon 2002) which was kind of disappointing. Later on, a third independent expression profiling study (Fortunel et al. 2003) reduced the list of commonly expressed genes to just one: integrin alpha-6. Thus, a universal stem cell signature may not exist, but each stem cell type may have its own transcriptional network responsible for certain unique stem cell properties (Gerrits et al. 2008). A comprehensive transcriptome analysis of human hematopoiesis was carried out by Novershtern et al. (2011) and revealed dense transcriptional circuits in HSCs, that gradually disappear during differentiation, while new but less intricate circuits emerge. Recently, Cabezas-Wallscheid et al. (2014) investigated the early differentiation steps of murine HSCs at the epigenetic, transcriptional and translational level and found coordinated alterations between HSCs and different multipotent progenitor populations. A study by Klimmeck et al. (2014) compared the transcriptome of murine HSC and myeloid committed progenitors and identified a stem/progenitor expression pattern marked by genes involved in immune response and cell adhesion. Combined analysis of transcriptome and proteome data indicated that posttranscriptional regulation is especially involved in metabolic processes and stress response of HSCs.

With respect to HSCs, a lot of gene expression profiling studies have been carried out. Most of them compared either CD34+CD38 – Lin - cells with

CD34+CD38+Lin+cells (Georgantas et al. 2004; Ivanova et al. 2002) or CD133+with CD133 – cells (He et al. 2005; Hemmoranta et al. 2006; Jaatinen et al. 2006; Toren et al. 2005). These studies revealed a number of transcripts overexpressed in HSCs, such as CD133, CD34, the RNA processing protein RBPMS and the receptor tyrosine kinase c-kit. Furthermore transcription factors as Gata-2, Gata-3, ERG and HLF are overrepresented in HSCs. The transcript BAALC, whose function is unknown, is highly enriched in CD133+cells (Baldus et al. 2003; Jaatinen et al. 2006). The homolog of the *Drosophila* Dlg1 tumor suppressor gene Dlg7 was identified as a potential stem cell gene by Gudmundsson et al. (2007). However, although the described transcripts have been found as overrepresented in HSCs in most of the studies, it is difficult to name specific mRNA markers for HCSs. The reasons are among others the variability of gene expression profiles due to varying stem cell sources, e.g. BM, CB, and PB (Ng et al. 2004; Steidl et al. 2002), and donor age (Nijnik et al. 2007; Rossi et al. 2005). Table 1.1 summarizes the mRNAs found in HSPCs.

1.6 Conclusion and Future Developments

The characterization of stem cells is currently rapidly moving forward. While some stem cells like HSCs are already routinely used in clinical settings, many new stem cells have just been described in the last years and many more will be defined in the near future.

Although molecular markers have been named for most of the stem cells, it is also true that many of these markers are not exclusive and certainly not highly specific with respect to a distinct function. This points to essentially three major tasks which need to be addressed: First, a better classification of stem cells with respect to robust molecular markers and especially those markers which can be used for purification of cells. This goes along with technical improvements of sorting techniques, culturing protocols and moreover highly sensitive molecular analysis tools. It is challenging as the nature of stem cells includes that they are proliferating slowly and that the cell numbers are small. Second, a harmonization of markers and isolation procedures, following the example of the CD nomenclature in the field of immunology. This would improve the exchange and gathering of data about stem cells, which is needed before more stem cell types are entering clinical applications. Third, we need a better understanding of stem cells with respect to their regenerative potential. The reports about reprogramming, dedifferentiation and transdifferentiation of cells and stem cells have raised the notion that essentially all cells can be engineered to generate every type of tissue. This is appealing from a research point of view but raises also some concerns about the predictability of stem cell differentiation when used for tissue regeneration or cellular therapies in general. Solving these issues will broaden our understanding in the exciting field of stem cell biology.

Table 1.1 mRNAs overrepresented in human HSPCs

Publication	Ivanova	Georgantas	Jaatinen	Hemmoranta	Toren	Huang	He	Wagner	Wagner
Stem cell fraction	CD34+CD38–Lin–		CD133+			CD133+	CD133+CD34+	CD34+ CD38–	CD34+ CD38–SDF
Control fraction	Lin+		CD133–			MSCs, NSCs	CD133–CD34–	CD34+CD38+	CD34+CD38– FDF
CD133	–	–	x	x	x	x	x	–	x
RBPMS	x	x	x	–	x	–	x	x	–
CD34	x	–	x	x	x	x	x	–	–
KIT	–	–	x	x	x	x	x	–	–
Gata2	x	–	x	–	x	x	x	–	–
FLJ14054	–	x	x	–	x	–	x	–	x
SPINK2	–	x	x	x	x	–	x	–	–
NRIP1	x	x	x	–	x	–	x	–	–
HOXA9	x	–	x	–	x	–	x	–	x
MEIS1	x	–	x	–	x	x	x	–	–
FHL1	–	–	x	–	x	–	x	–	x
KIAA0125	–	x	x	–	x	–	–	–	x
SOCS2	x	x	x	–	x	–	–	–	–
MLLT3	–	x	x	–	x	x	–	–	–
PLS3	x	x	x	–	x	–	–	–	–
TFP1	x	x	x	–	x	–	–	–	–
ERG	–	x	x	–	x	–	–	–	x
HLF	x	x	–	–	–	x	–	–	–
GUCY1A3	–	x	x	–	x	–	–	–	–
HLF	–	x	x	–	x	–	–	–	–
NPR3	–	x	x	–	x	–	–	–	–
SEPP1	–		x	–	x	–	–	–	–

H2BFQ	–	x	–	–	–	–	x	x	–
GUCY1B3	–	x	–	–	–	–	x	–	x
BAALC	–	–	x	–	x	x	–	–	–
TIE	x	–	–	–	x	–	–	–	–
FLT3	x	–	–	–	x	x	–	–	–
MPL	x	x	–	–	x	–	–	–	–
EVI1	x	x	–	–	x	–	–	–	–
LAPTM4B	x	–	x	–	x	–	–	–	–
GATA3	x	x	–	–	–	–	–	–	–
HOXB6	x	x	–	–	–	–	–	–	–
MDS1	x	x	–	–	–	–	–	–	–
TRAIL	x	x	–	–	–	–	–	–	–
CEBPB	x	x	–	–	–	–	–	–	–
HOXA3	–	x	x	–	–	–	–	–	–
KIAA1102	–	x	x	–	–	–	–	–	–
D2S448	–	–	x	–	–	–	–	–	x
PLCB1	–	–	x	–	–	–	–	–	x
CRFBP	–	x	–	–	–	–	x	–	–
HIF2	–	x	–	–	–	–	x	–	–
H2A	–	x	–	–	–	–	x	–	–
H2AFO	–	x	–	–	–	–	x	–	–
NPIP1	–	x	–	–	–	–	x	–	–
FZD6	–	–	–	–	x	–	–	–	x
C17	–	–	x	–	x	–	–	–	–
HOXA5	x	–		–	x	–	–	–	–
PKD2	–	x	–	–	x	–	–	–	–

The table encompasses the following publications: Ivanova et al. (2002), Georgantas et al. (2004), Jaatinen et al. (2006), Hemmoranta et al. (2006), Toren et al. (2005), Huang et al. (2008), He et al. (2005) and Wagner et al. (2004)

References

Adewumi O, Aflatoonian B, Ahrlund-Richter L et al (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25:803–816

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100:3983–3988

Anokye-Danso F, Trivedi CM, Juhr D et al (2011) Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 8:376–388

Aravin A, Tuschl T (2005) Identification and characterization of small RNAs involved in RNA silencing. FEBS Lett 579:5830–5840

Arnold CP, Tan R, Zhou B et al (2011) MicroRNA programs in normal and aberrant stem and progenitor cells. Genome Res 21:798–810

Asahara T, Kawamoto A, Masuda H (2011) Concise review: circulating endothelial progenitor cells for vascular medicine. Stem Cells 29:1650–1655

Aslan H, Zilberman Y, Kandel L, Liebergall M, Oskouian RJ, Gazit D, Gazit Z (2006) Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105+ cells. Stem Cells 24:1728–1737

Asselin-Labat ML, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE, Lindeman GJ (2006) Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst 98:1011–1014

Baldus CD, Tanner SM, Kusewitt DF, Liyanarachchi S, Choi C, Caligiuri MA, Bloomfield CD, de la Chapelle A (2003) BAALC, a novel marker of human hematopoietic progenitor cells. Exp Hematol 31:1051–1056

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444:756–760

Bearzi C, Rota M, Hosoda T et al (2007) Human cardiac stem cells. Proc Natl Acad Sci U S A 104:14068–14073

Beckervordersandforth R, Tripathi P, Ninkovic J et al (2011) In vivo fate mapping and expression analysis reveals molecular hallmarks of prospectively isolated adult neural stem cells. Cell Stem Cell 7:744–758

Beltrami AP, Barlucchi L, Torella D et al (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114:763–776

Bissels U, Wild S, Tomiuk S, Holste A, Hafner M, Tuschl T, Bosio A (2009) Absolute quantification of microRNAs by using a universal reference. RNA 15:2375–2384

Bissels U, Wild S, Tomiuk S, Hafner M, Scheel H, Mihailovic A, Choi YH, Tuschl T, Bosio A (2011) Combined Characterization of MicroRNA and mRNA profiles delineates early differentiation pathways of CD133(+) and CD34(+) hematopoietic stem and progenitor cells. Stem Cells 29(5):847–857

Bissels U, Bosio A, Wagner W (2012) MicroRNAs are shaping the hematopoietic landscape. Haematologica 97(2):160–167

Bolli R, Chugh AR, D'Amario D et al (2011) Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet 378:1847–1857

Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730–737

Bosio A, Huppert V, Donath S, Hennemann P, Malchow M, Heinlein UA (2009) Isolation and enrichment of stem cells. Adv Biochem Eng Biotechnol 114:23–72

Boutin C, Hardt O, de Chevigny A, Core N, Goebbels S, Seidenfaden R, Bosio A, Cremer H (2010) NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. Proc Natl Acad Sci U S A 107(3):1201–1206

Bruchova H, Yoon D, Agarwal AM, Mendell J, Prchal JT (2007) Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. Exp Hematol 35:1657–1667

Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, Roberts DJ, Huang PL, Domian IJ, Chien KR (2009) Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 460:113–117

Buhring HJ, Seiffert M, Bock TA, Scheding S, Thiel A, Scheffold A, Kanz L, Brugger W (1999) Expression of novel surface antigens on early hematopoietic cells. Ann N Y Acad Sci 872:25–38; discussion 38–29

Buhring HJ, Battula VL, Treml S, Schewe B, Kanz L, Vogel W (2007) Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262–271

Burns CE, Zon LI (2002) Portrait of a stem cell. Dev Cell 3:612–613

Cabezas-Wallscheid N, Klimmeck D, Hansson J et al (2014) Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA Methylome analysis. Cell Stem Cell 15:507–522

Calloni R, Cordero EA, Henriques JA, Bonatto D (2013) Reviewing and updating the major molecular markers for stem cells. Stem Cells Dev 22:1455–1476

Chaudhuri AA, So AY, Mehta A, Minisandram A, Sinha N, Jonsson VD, Rao DS, O'Connell RM, Baltimore D (2012) Oncomir miR-125b regulates hematopoiesis by targeting the gene Lin28A. Proc Natl Acad Sci U S A 109:4233–4238

Chen CZ, Li L, Lodish HF, Bartel DP (2004) MicroRNAs modulate hematopoietic lineage differentiation. Science 303:83–86

Cheung ST, Cheung PF, Cheng CK, Wong NC, Fan ST (2011) Granulin-epithelin precursor and ATP-dependent binding cassette (ABC)B5 regulate liver cancer cell chemoresistance. Gastroenterology 140:344–355

Conrad S, Renninger M, Hennenlotter J et al (2008) Generation of pluripotent stem cells from adult human testis. Nature 456:344–349

Conti L, Cattaneo E (2010) Neural stem cell systems: physiological players or in vitro entities? Nat Rev Neurosci 11:176–187

Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK, Jordanides N, Barow M, Mountford JC, Holyoake TL (2006) Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood 107:4532–4539

Dalerba P, Dylla SJ, Park IK et al (2007) Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 104:10158–10163

Dore LC, Amigo JD, Dos Santos CO et al (2008) A GATA-1-regulated microRNA locus essential for erythropoiesis. Proc Natl Acad Sci U S A 105:3333–3338

Dormeyer W, van Hoof D, Braam SR, Heck AJ, Mummery CL, Krijgsveld J (2008) Plasma membrane proteomics of human embryonic stem cells and human embryonal carcinoma cells. J Proteome Res 7:2936–2951

Du L, Wang H, He L et al (2008) CD44 is of functional importance for colorectal cancer stem cells. Clin Cancer Res 14:6751–6760

Felli N, Fontana L, Pelosi E et al (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci U S A 102:18081–18086

Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, Croce CM, Brunetti E, Grignani F, Peschle C (2007) MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. Nat Cell Biol 9:775–787

Fortunel NO, Otu HH, Ng HH et al (2003) Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science 302:393; author reply 393

Gangaraju VK, Lin H (2009) MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 10:116–125

Georgantas RW 3rd, Tanadve V, Malehorn M, Heimfeld S, Chen C, Carr L, Martinez-Murillo F, Riggins G, Kowalski J, Civin CI (2004) Microarray and serial analysis of gene expression analyses identify known and novel transcripts overexpressed in hematopoietic stem cells. Cancer Res 64:4434–4441

Georgantas RW 3rd, Hildreth R, Morisot S, Alder J, Liu CG, Heimfeld S, Calin GA, Croce CM, Civin CI (2007) CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. Proc Natl Acad Sci U S A 104:2750–2755

Gerrits A, Dykstra B, Otten M, Bystrykh L, de Haan G (2008) Combining transcriptional profiling and genetic linkage analysis to uncover gene networks operating in hematopoietic stem cells and their progeny. Immunogenetics 60:411–422

Gerrits A, Dykstra B, Kalmykowa OJ, Klauke K, Verovskaya E, Broekhuis MJ, de Haan G, Bystrykh LV (2010) Cellular barcoding tool for clonal analysis in the hematopoietic system. Blood 115:2610–2618

Gerrits A, Walasek MA, Olthof S, Weersing E, Ritsema M, Zwart E, van Os R, Bystrykh LV, de Haan G (2012) Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. Blood 119:377–387

Giebel B, Zhang T, Beckmann J, Spanholtz J, Wernet P, Ho AD, Punzel M (2006) Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. Blood 107:2146–2152

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806

Gordon JE, Wong JJ, Rasko JE (2013) MicroRNAs in myeloid malignancies. Br J Haematol 162:162–176

Gruber AJ, Grandy WA, Balwierz PJ, Dimitrova YA, Pachkov M, Ciaudo C, Nimwegen E, Zavolan M (2014) Embryonic stem cell-specific microRNAs contribute to pluripotency by inhibiting regulators of multiple differentiation pathways. Nucleic Acids Res 42:9313–9326

Gudmundsson KO, Thorsteinsson L, Sigurjonsson OE, Keller JR, Olafsson K, Egeland T, Gudmundsson S, Rafnar T (2007) Gene expression analysis of hematopoietic progenitor cells identifies Dlg7 as a potential stem cell gene. Stem Cells 25:1498–1506

Guo S, Lu J, Schlanger R et al (2010) MicroRNA miR-125a controls hematopoietic stem cell number. Proc Natl Acad Sci U S A 107:14229–14234

Han YC, Park CY, Bhagat G, Zhang J, Wang Y, Fan JB, Liu M, Zou Y, Weissman IL, Gu H (2010) microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. J Exp Med 207:475–489

Hatfield S, Ruohola-Baker H (2008) microRNA and stem cell function. Cell Tissue Res 331:57–66

He X, Gonzalez V, Tsang A, Thompson J, Tsang TC, Harris DT (2005) Differential gene expression profiling of CD34+ CD133+ umbilical cord blood hematopoietic stem progenitor cells. Stem Cells Dev 14:188–198

Hemmoranta H, Hautaniemi S, Niemi J, Nicorici D, Laine J, Yli-Harja O, Partanen J, Jaatinen T (2006) Transcriptional profiling reflects shared and unique characters for CD34+ and CD133+ cells. Stem Cells Dev 15:839–851

Hong SH, Kim KS, Oh IH (2015) Concise review: exploring miRNAs–toward a better understanding of hematopoiesis. Stem Cells 33:1–7

Huang TS, Hsieh JY, Wu YH et al (2008) Functional network reconstruction reveals somatic stemness genetic maps and dedifferentiation-like transcriptome reprogramming induced by GATA2. Stem Cells 26:1186–1201

Hubin F, Humblet C, Belaid Z, Lambert C, Boniver J, Thiry A, Defresne MP (2005) Murine bone marrow stromal cells sustain in vivo the survival of hematopoietic stem cells and the granulopoietic differentiation of more mature progenitors. Stem Cells 23:1626–1633

Huss R, Moosmann S (2002) The co-expression of CD117 (c-kit) and osteocalcin in activated bone marrow stem cells in different diseases. Br J Haematol 118:305–312

Iozzo RV (1998) Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem 67:609–652

Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR (2002) A stem cell molecular signature. Science 298:601–604

Jaatinen T, Hemmoranta H, Hautaniemi S, Niemi J, Nicorici D, Laine J, Yli-Harja O, Partanen J (2006) Global gene expression profile of human cord blood-derived CD133+ cells. Stem Cells 24:631–641

Jin P, Wang E, Ren J, Childs R, Shin JW, Khuu H, Marincola FM, Stroncek DF (2008) Differentiation of two types of mobilized peripheral blood stem cells by microRNA and cDNA expression analysis. J Transl Med 6:39

Jones R, Barber J, Vala M (1995) Assessment of Aldehyde Dehydrogenase in Viable Cells. Blood 85:2742–2746

Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM, Markham AF, Jack A, Emery P, McGonagle D (2002) Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 46:3349–3360

Jungblut M, Oeltze K, Zehnter I, Hasselmann D, Bosio A (2008) Preparation of single-cell suspensions from mouse spleen with the gentleMACS Dissociator. J Vis Exp. doi:10.3791/1029

Jungblut M, Oeltze K, Zehnter I, Hasselmann D, Bosio A (2009) Standardized preparation of single-cell suspensions from mouse lung tissue using the gentleMACS Dissociator. J Vis Exp. doi:10.3791/1266

Kanatsu-Shinohara M, Takashima S, Ishii K, Shinohara T (2011) Dynamic changes in EPCAM expression during spermatogonial stem cell differentiation in the mouse testis. PLoS One 6:e23663

Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121:1109–1121

Klimmeck D, Cabezas-Wallscheid N, Reyes A, von Paleske L, Renders S, Hansson J, Krijgsveld J, Huber W, Trumpp A (2014) Transcriptome-wide profiling and posttranscriptional analysis of hematopoietic stem/progenitor cell differentiation toward myeloid commitment. Stem Cell Rep 3:858–875

Kokovay E, Wang Y, Kusek G, Wurster R, Lederman P, Lowry N, Shen Q, Temple S (2012) VCAM1 is essential to maintain the structure of the SVZ niche and acts as an environmental sensor to regulate SVZ lineage progression. Cell Stem Cell 11:220–230

Kubota H, Avarbock MR, Brinster RL (2004) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101:16489–16494

Landgraf P, Rusu M, Sheridan R et al (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129:1401–1414

Lazare SS, Wojtowicz EE, Bystrykh LV, de Haan G (2014) microRNAs in hematopoiesis. Exp Cell Res 329:234–238

Lechman ER, Gentner B, van Galen P et al (2012) Attenuation of miR-126 activity expands HSC in vivo without exhaustion. Cell Stem Cell 11:799–811

Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM (2007) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037

Li Y, Vecchiarelli-Federico LM, Li YJ, Egan SE, Spaner D, Hough MR, Ben-David Y (2012) The miR-17-92 cluster expands multipotent hematopoietic progenitors whereas imbalanced expression of its individual oncogenic miRNAs promotes leukemia in mice. Blood 119:4486–4498

Liao R, Sun J, Zhang L, Lou G, Chen M, Zhou D, Chen Z, Zhang S (2008) MicroRNAs play a role in the development of human hematopoietic stem cells. J Cell Biochem 104:805–817

Lu J, Guo S, Ebert BL et al (2008) MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors. Dev Cell 14:843–853

Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, Mosca JD (2003) Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci 10:228–241

Makkar RR, Smith RR, Cheng K et al (2012) Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 379:895–904

Mallanna SK, Rizzino A (2010) Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells. Dev Biol 344:16–25

Merkerova M, Vasikova A, Belickova M, Bruchova H (2009) MicroRNA expression profiles in umbilical cord blood cell lineages. Stem Cells Dev 19(1):17–26
Merkle FT, Alvarez-Buylla A (2006) Neural stem cells in mammalian development. Curr Opin Cell Biol 18:704–709
Messina E, De Angelis L, Frati G et al (2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95:911–921
Mich JK, Signer RA, Nakada D, Pineda A, Burgess RJ, Vue TY, Johnson JE, Morrison SJ (2014) Prospective identification of functionally distinct stem cells and neurosphere-initiating cells in adult mouse forebrain. eLife 3:e02669
Mintz PJ, Saetrom P, Reebye V et al (2012) MicroRNA-181a* targets nanog in a subpopulation of CD34(+) cells isolated from peripheral blood. Mol Ther Nucleic Acids 1:e34
Miyoshi N, Ishii H, Nagano H et al (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8:633–638
Moradi S, Asgari S, Baharvand H (2014) Concise review: harmonies played by microRNAs in cell fate reprogramming. Stem Cells 32:3–15
Mori T, Buffo A, Gotz M (2005) The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. Curr Top Dev Biol 69:67–99
Ng YY, van Kessel B, Lokhorst HM, Baert MR, van den Burg CM, Bloem AC, Staal FJ (2004) Gene-expression profiling of CD34+ cells from various hematopoietic stem-cell sources reveals functional differences in stem-cell activity. J Leukoc Biol 75:314–323
Niehage C, Steenblock C, Pursche T, Bornhauser M, Corbeil D, Hoflack B (2011) The cell surface proteome of human mesenchymal stromal cells. PLoS One 6:e20399
Nijnik A, Woodbine L, Marchetti C et al (2007) DNA repair is limiting for haematopoietic stem cells during ageing. Nature 447:686–690
Novershtern N, Subramanian A, Lawton LN et al (2011) Densely interconnected transcriptional circuits control cell states in human hematopoiesis. Cell 144:296–309
Nunomura K, Nagano K, Itagaki C, Taoka M, Okamura N, Yamauchi Y, Sugano S, Takahashi N, Izumi T, Isobe T (2005) Cell surface labeling and mass spectrometry reveal diversity of cell surface markers and signaling molecules expressed in undifferentiated mouse embryonic stem cells. Mol Cell Proteomics 4:1968–1976
O'Brien CA, Pollett A, Gallinger S, Dick JE (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445:106–110
O'Connell RM, Chaudhuri AA, Rao DS, Gibson WS, Balazs AB, Baltimore D (2010) MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. Proc Natl Acad Sci U S A 107:14235–14240
Ogawa M (2002) Changing phenotypes of hematopoietic stem cells. Exp Hematol 30:3–6
Oh H, Bradfute SB, Gallardo TD et al (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 100:12313–12318
Ooi AG, Sahoo D, Adorno M, Wang Y, Weissman IL, Park CY (2010) MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. Proc Natl Acad Sci U S A 107:21505–21510
Orkin SH, Zon LI (2008) Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132:631–644
Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245
Pang R, Law WL, Chu AC et al (2010) A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell 6:603–615
Pastrana E, Cheng LC, Doetsch F (2009) Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. Proc Natl Acad Sci U S A 106:6387–6392
Pennartz S, Belvindrah R, Tomiuk S, Zimmer C, Hofmann K, Conradt M, Bosio A, Cremer H (2004) Purification of neuronal precursors from the adult mouse brain: comprehensive gene expression analysis provides new insights into the control of cell migration, differentiation, and homeostasis. Mol Cell Neurosci 25:692–706

Pennartz S, Reiss S, Biloune R, Hasselmann D, Bosio A (2009) Generation of single-cell suspensions from mouse neural tissue. J Vis Exp. doi:10.3791/1267
Petriv OI, Kuchenbauer F, Delaney AD et al (2010) Comprehensive microRNA expression profiling of the hematopoietic hierarchy. Proc Natl Acad Sci U S A 107(35):15443–15448
Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, Colucci WS, Liao R (2005) CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res 97:52–61
Piccirillo SG, Vescovi AL (2006) Bone morphogenetic proteins regulate tumorigenicity in human glioblastoma stem cells. Ernst Schering Found Symp Proc 5:59–81
Pontier SM, Muller WJ (2009) Integrins in mammary-stem-cell biology and breast-cancer progression—a role in cancer stem cells? J Cell Sci 122:207–214
Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 30:783–791
Rafii S, Lyden D (2003) Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 9:702–712
Raghavachari N, Liu P, Barb JJ, Yang Y, Wang R, Nguyen QT, Munson PJ (2014) Integrated analysis of miRNA and mRNA during differentiation of human CD34+ cells delineates the regulatory roles of microRNA in hematopoiesis. Exp Hematol 42:14–27, e11–12
Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298:597–600
Ribeiro AO, Schoof CR, Izzotti A, Pereira LV, Vasques LR (2014) MicroRNAs: modulators of cell identity, and their applications in tissue engineering. MicroRNA 3:45–53
Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445:111–115
Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R, Wagers AJ, Weissman IL (2005) Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A 102:9194–9199
Schiedlmeier B, Santos AC, Ribeiro A et al (2007) HOXB4's road map to stem cell expansion. Proc Natl Acad Sci U S A 104:16952–16957
Schmelzer E, Reid LM (2008) EpCAM expression in normal, non-pathological tissues. Front Biosci 13:3096–3100
Schmelzer E, Zhang L, Bruce A et al (2007) Human hepatic stem cells from fetal and postnatal donors. J Exp Med 204:1973–1987
Seandel M, James D, Shmelkov SV et al (2007) Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature 449:346–350
Seidenfaden R, Desoeuvre A, Bosio A, Virard I, Cremer H (2006) Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain. Mol Cell Neurosci 32:187–198
Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE (2006) Generation of a functional mammary gland from a single stem cell. Nature 439:84–88
Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18:696–704
Shimono Y, Zabala M, Cho RW et al (2009) Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 138:592–603
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, Clarke ID, Hide T, Dirks PB (2004a) Cancer stem cells in nervous system tumors. Oncogene 23:7267–7273
Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB (2004b) 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

Steidl U, Kronenwett R, Rohr UP et al (2002) Gene expression profiling identifies significant differences between the molecular phenotypes of bone marrow-derived and circulating human CD34+ hematopoietic stem cells. Blood 99:2037–2044

Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439:993–997

Storms R, Trujillo A, Springer J (1999) Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci U S A 96:9118–9123

Sturzu AC, Wu SM (2011) Developmental and regenerative biology of multipotent cardiovascular progenitor cells. Circ Res 108:353–364

Suh MR, Lee Y, Kim JY et al (2004) Human embryonic stem cells express a unique set of microRNAs. Dev Biol 270:488–498

Sun S, Guo Z, Xiao X, Liu B, Liu X, Tang PH, Mao N (2003) Isolation of mouse marrow mesenchymal progenitors by a novel and reliable method. Stem Cells 21:527–535

Tang C, Ang BT, Pervaiz S (2007) Cancer stem cell: target for anti-cancer therapy. FASEB J 21:3777–3785

Timmermans F, Plum J, Yoder MC, Ingram DA, Vandekerckhove B, Case J (2009) Endothelial progenitor cells: identity defined? J Cell Mol Med 13:87–102

Tondreau T, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, Mortier C, Bron D, Lagneaux L (2005) Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 23:1105–1112

Toren A, Bielorai B, Jacob-Hirsch J et al (2005) CD133-positive hematopoietic stem cell "stemness" genes contain many genes mutated or abnormally expressed in leukemia. Stem Cells 23:1142–1153

Undi RB, Kandi R, Gutti RK (2013) MicroRNAs as Haematopoiesis Regulators. Adv Hematol 2013:695754

van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marban E, Molkentin JD (2014) c-kit+ cells minimally contribute cardiomyocytes to the heart. Nature 509:337–341

Wagner W, Ansorge A, Wirkner U et al (2004) Molecular evidence for stem cell function of the slow-dividing fraction among human hematopoietic progenitor cells by genome-wide analysis. Blood 104:675–686

Wang XS, Gong JN, Yu J et al (2012) MicroRNA-29a and microRNA-142-3p are regulators of myeloid differentiation and acute myeloid leukemia. Blood 119:4992–5004

Wilson KD, Venkatasubrahmanyam S, Jia F, Sun N, Butte AJ, Wu JC (2009) MicroRNA profiling of human-induced pluripotent stem cells. Stem Cells Dev 18:749–758

Wollscheid B, Bausch-Fluck D, Henderson C, O'Brien R, Bibel M, Schiess R, Aebersold R, Watts JD (2009) Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. Nat Biotechnol 27:378–386

Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, Rajewsky N, Bender TP, Rajewsky K (2007) MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 131:146–159

Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, Chu PW, Lam CT, Poon RT, Fan ST (2008) Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 13(2):153–166. doi:10.1016/j.ccr.2008.01.013

Yovchev MI, Grozdanov PN, Joseph B, Gupta S, Dabeva MD (2007) Novel hepatic progenitor cell surface markers in the adult rat liver. Hepatology 45:139–149

Yovchev MI, Grozdanov PN, Zhou H, Racherla H, Guha C, Dabeva MD (2008) Identification of adult hepatic progenitor cells capable of repopulating injured rat liver. Hepatology 47:636–647

Zhan M, Miller CP, Papayannopoulou T, Stamatoyannopoulos G, Song CZ (2007) MicroRNA expression dynamics during murine and human erythroid differentiation. Exp Hematol 35:1015–1025

Zhao B, Yang D, Jiang J, Li J, Fan C, Huang M, Fan Y, Jin Y, Jin Y (2014) Genome-wide mapping of miRNAs expressed in embryonic stem cells and pluripotent stem cells generated by different reprogramming strategies. BMC Genomics 15:488

Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF (2007) miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. Proc Natl Acad Sci U S A 104:7080–7085

Chapter 2
Human Embryonic Stem Cells

Terri Gaskell, Mikael C.O. Englund, and Johan Hyllner

Abstract Stem cells can be isolated from a variety of sources and they are typically classified based on their tissue of origin. Embryonic stem cells are, as the name indicates, derived from the inner cell mass of pre-implantation stage blastocysts at day 5–7 post fertilisation. These cells possess qualities such as pluripotency and a seemingly limitless capacity to proliferate *in vitro* in their undifferentiated state. Embryonic stem cells were first derived from mouse embryos in the early 1980s but have now been derived from a number of different species including rat, rabbit, sheep, pig, horse and human. This chapter focuses on human embryonic stem cells and describes techniques used for their derivation and culture. In addition, the basic properties of these cells are illustrated, including some examples of their capacity to differentiate to various precursors and functional cell types. Finally, some areas of applications for these cells are discussed with emphasis on their possible future use in regenerative medicine including current clinical trials.

Keywords Embryonic stem cells • Blastocysts • Surface markers • Transcription factors • Enzyme mediated passage • Pluripotency • Xeno-free culture

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_2

2.1 Introduction

The developments in the human pluripotent stem cell field during the last 15 years are remarkable, and the scientific achievements made have substantially furthered our understanding of the opportunities that these cells provide for basic and applied research as well as for future regenerative medicine applications. There has been rapid progress in the development of improved derivation and culture technologies for human embryonic stem (hES) cells since the initial derivation of stable cell lines in 1998 (Thomson et al. 1998). One of the main driving forces behind this is the aim to generate high quality, clinically compliant, hES cell lines which can be used for cell therapy in humans. As such, the cell lines need to be manufactured according to good manufacturing practice (GMP) in order to comply with good clinical practice (GCP) which is a set of internationally recognised ethical and scientific quality requirements that must be observed for designing, conducting, recording and reporting clinical trials that involve the participation of human subjects (2001/20/EC 2001; 2005/28/EC 2005). In addition, there are more immediate opportunities to use hES cells and their derivatives as *in vitro* tools to study, for example, human development and genetic diseases. Furthermore, the cells are also expected to contribute to improvements of the models currently used in drug discovery and by providing a source of a variety of human specialised cells which then can be manufactured under standardised conditions. However, in order to realise the opportunities that human pluripotent stem cells provide, there are a number of challenges that need to be addressed. Cost-efficient culture conditions which allow large scale production of the undifferentiated cells is one, efficient and robust protocols for the process of differentiation of the pluripotent cells to the desired end point is another. Characterisation and quality control also require specific attention and monitoring the phenotype and genomic stability of the cells during expansion and propagation is critical. For any kind of therapeutic applications, regulatory compliance needs to be factored in as well. Ideally culture systems would be based around fully defined components, using small molecules with a decreased dependency on biologics. Much effort has been spent on genetically modifying the cells, e.g. to make cells overexpress genes linked to desirable functionality such as metabolising enzymes and also to generate reporter lines. With the last years' advancements in the generation of induced pluripotent stem (iPS) cells, the technologies used for genetically modifying cells has certainly fuelled further research in this area of engineering stem cells. Further details on the iPS cells and the opportunities and challenges they provide are reviewed in a separate chapter of this book and will not be discussed further here. Below, we will cover basic aspects on the derivation process of hES cells and highlight different culture conditions for maintenance of the undifferentiated cells. We will also describe various ways to characterise hES cells in order to verify their unique properties. The pluripotency of the cells will be illustrated with some examples of differentiated cell types which can be generated from the hES cells, and applications in regenerative medicine will be discussed.

2.2 Derivation/Classification

2.2.1 Derivation

The different stages of human embryos which have been successfully used for derivation of new hES cell lines are blastocysts (Thomson et al. 1998), morulae (Strelchenko et al. 2004), late-arrested embryo (Feki et al. 2008; Gavrilov et al. 2009; Zhang et al. 2006) (Gavriolov 2009) or blastomere (Geens et al. 2009; Klimanskaya et al. 2006).

Initially, the method was adapted from the previously developed protocol for mouse ES cells (Evans and Kaufman 1981; Martin 1981). The substantial species differences and the lack of appropriate culture medium for human embryos were likely a large part of reason why it was not until the late 1990s that the first human embryonic stem cell line was isolated. Divergences in intracellular pathway signalling between mice and human have been demonstrated as one explanation to the differences in culture requirements (Brandenberger et al. 2004; Rho et al. 2006; Xu et al. 2002). In 1994, Bongso and co-workers managed to isolate and, to some extent, propagate inner cell masses (ICMs) from human blastocysts and these cells displayed stem-cell like properties (Bongso et al. 1994). This achievement in concert with the successful derivation of non-human primate ES cell lines in the mid-1990s (Thomson et al. 1995, 1996) paved the way for the subsequent derivation of stable hES cell lines in 1998 (Thomson et al. 1998). Since then, the generation of a large number of hES cell lines have been reported, numbering well over 1000 different lines (Loser et al. 2010) with more being added every year. Registries of currently available hESC lines are maintained by ISCF (http://www.stem-cell-forum.net/initiatives/isci/stem-cell-registry/), UK Stem Cell Bank (http://www.nibsc.org/), NIH (http://grants.nih.gov/stem_cells/registry/current.htm) and EU hESCreg (http://www.hescreg.eu/).

The majority of hES cell lines derived thus far have utilised donated surplus embryos from assisted conception laboratories, i.e. from the procedure of *in vitro* fertilisation (IVF). Both fresh and previously frozen material can be used for stem cell derivation. The embryos are cultured to the blastocyst stage before measures are taken to isolate the ICM cells. The first step is removal of the zona pellucida which can be achieved by the use of pronase (xeno-derived) or alternatively Tyrode's acid solution or mechanical dissection is a xeno-free method is preferred. Spontaneously hatched blastocysts can also be processed further without the need for zona pellucida removal (Heins et al. 2004). Subsequently, the blastocyst is treated with mouse antibodies directed against human trophoectoderm cells and guinea pig complement components. This process, called immunosurgery, lyses the cells by an antibody/complement reaction leaving the inner cell mass cell mostly intact, and these cells can then be sub-cultured further on a layer of mitotically inactivated mouse embryonic fibroblast feeder cells (mEF cells). The initial outgrowth from the inner cell mass cells is usually dissected mechanically under the microscope and transferred to new culture dishes after 1–2 weeks. An established hES

cell line is typically passaged every 5–10 days, depending on culture method and population doubling time. As soon as the hES cells are growing in a way allowing culture expansion, low passage samples should be cryopreserved as a seed bank, and also as larger well characterised Master Cell Banks (MCB). From these MCBs, Working Cell Banks (WCB) can be established, for further expansion and various endpoint applications. If the aim is to derive hES cells for downstream clinical use, i.e. to generate therapeutic cells, then the whole procedure needs to comply with regulatory demands, such as current Good Manufacturing Practice (cGMP), but also comply with ethical regulations (Crook et al. 2007; Murdoch et al. 2012).

The methodology for ICM isolation briefly described here is dependent on careful monitoring and execution for successful results since the viability of the resulting ICM isolate is fragile. A success rate in hES cell line generation of at least 5–10 % is reasonable to expect but greater than 30 % success rate has been reported (Chen et al. 2009; Sjogren et al. 2004). Negative results could be related to sub optimal culture conditions or other technical issues. Blastocyst quality is also an influencing factor for successful derivation of hES cell lines, and the use of higher quality embryos leads to a greater success rate (Lerou et al. 2008b). However hES cell lines have been established from blastocysts graded as of low quality (Dokras et al. 1993; Heins et al. 2004; Lerou et al. 2008a) and even from non-viable embryos, albeit with questionable quality (Feki et al. 2008). Assuming embryo culture is well conducted it appears that derivation from previously cryopreserved embryos or blastocysts should be as successful as derivation from fresh material (Sjogren et al. 2004). The proportion of cryopreserved embryos eligible and donated for research activities varies, however, there seems to be a positive correlation between the level of information and support from the IVF clinic staff and the willingness of patients to donate surplus material for research (Brett et al. 2009).

2.2.2 Classification

The essence of a hES cell is its ability to both self-renew and differentiate into other, more specialised cell types, such as muscle cells, neurons, connective tissue and epithelial cells, to mention a few. The hES cells are pluripotent, i.e. they can form cells representing the three germ layers endoderm, ectoderm, and mesoderm. To assess the quality of a recently derived hES cell line, the expression or selective absence of various surface markers, transcription factors, and other properties of the assumed pluripotent hES cells are monitored. Several of these markers have been found closely associated to the pluripotent state of hES cells and researchers normally apply a panel of several markers for the characterisation procedure (Heins et al. 2004; Thomson et al. 1998). Briefly, they include

- Cell membrane bound surface markers like the glycol lipids SSEA-1,-3, -4; and the keratin sulphate molecules TRA-1-60, -1-81.
- Transcription factors such as Oct-4, Sox2, and Nanog.

- Telomerase activity, as a measure of the hES cells ability to continuously go through mitosis.
- Alkaline phosphatase activity.
- *In vitro* pluripotency, assayed for by using markers for endo-, ecto-, and mesoderm on spontaneously differentiated cell material. Commonly used markers are the transcription factor forkhead box A2 (Foxa2) transcription factor for endoderm, β-III-tubulin for ectoderm and Arterial Smooth Muscle Actin (ASMA) as a marker for Mesoderm.
- *In vivo* pluripotency, assayed for by xenografting hES cells into an immune-deficient mouse and subsequently analysing the resulting teratoma for endo-, ecto-, and mesoderm derivatives. Typically, the cells are placed under the kidney capsule but other areas have also been used such as testis and skeletal muscle.

In Fig. 2.1a, the nuclei of a confluent layer of hES cells have been stained with an antibody for the transcription factor Nanog to illustrate its presence. Nanog was simultaneously discovered in 2003 as a pluripotency sustaining factor by Chambers and Mitsui (Chambers et al. 2003; Mitsui et al. 2003). Flow Cytometry (FC) allows a more exact quantification of markers than microscopic observations of immunostaining, and several markers could be analysed simultaneously, e.g. to asses co-expression of more than marker. In Fig. 2.1b, an FC plot illustrates a population of Oct-4 positive cells as an indication of pluripotency, with Fig. 2.1c illustrating the isotype negative control for the assay as a comparison. In addition to the above examples of properties linked to pluripotency, the genomic stability of the hES cells is normally assessed, employing standard G-banding karyotyping or more high resolution techniques such as high density array based SNP analysis. The absence of pathogens is also normally confirmed as well as the thawing recovery rate after cryo-preservation.

The International Stem Cell Forum launched a characterisation initiative a few years ago (The International Stem Cell Initiative or ISCI) as a global collaborative effort to reach consensus on basic methodology and criteria for the derivation,

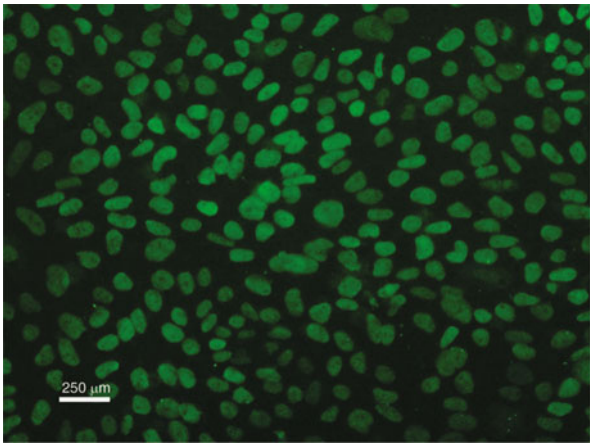

Fig. 2.1a Nanog nuclear staining of a confluent layer of hES cells. This methodology, known as immunocytochemistry, is commonly used to illustrate the presence of various factors and markers linked to certain properties of cells, e.g. pluripotency of hES cells

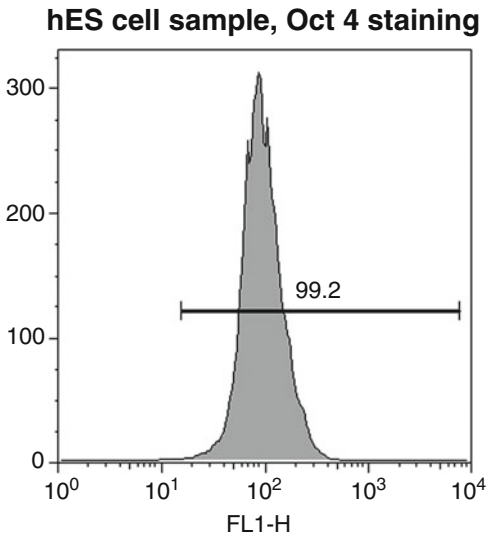

Fig. 2.1b Flow Cytometry (FC) diagram of hES cells positive to 9X, x % for Oct-4 transcription factor. By using quantitative methods like FC a more exact determination of the characteristics of stem cells is possible, compared to qualitative methods

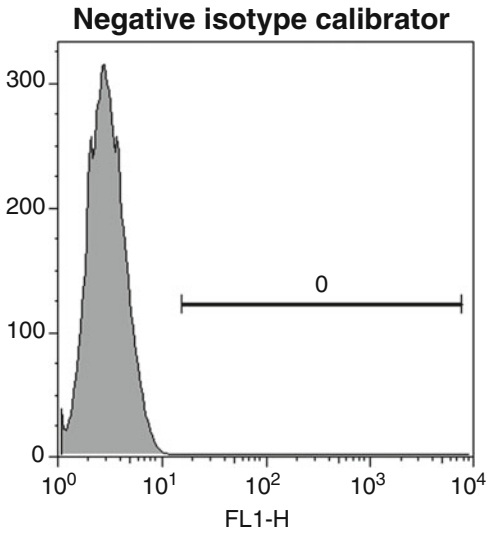

Fig. 2.1c FC isotype negative control. This illustrates how cells negative for the marker appear in the FC analysis

characterisation and maintenance of hES cells (Adewumi et al. 2007), also discussed by Stephenson et al. (Stephenson et al. 2007). The consensus of the scientific community in concert with the regulatory bodies for the major markets will be central to the eventual development and adoption of medical applications of hES cells.

2.3 Culture Conditions

2.3.1 General

It is anticipated that hES cells are more sensitive to sub optimal culture conditions than common somatic cell lines, hence demanding a more stable and controlled environment and a precise culture medium formulation to maintain the undifferentiated and pluripotent state during long term culture. A number of critical parameters should be accounted for when setting up a hES cell culture laboratory, including:

- Laboratory facilities – there should be a dedicated, fit for purpose, and secluded cell culture area with
  - Clean filtered air and stable temperature, preferably positive pressure.
  - A minimum of consumables storage in the direct cell culture area.
  - Good cleaning routines and easily cleaned equipment and surfaces.
  - High quality equipment that is regularly serviced and calibrated where appropriate, cell culture incubators maintaining a stable climate to avoid changes in pH, temperature and osmolality of the culture medium.
  - Heated stages fitted to the microscopes to avoid a decrease in culture temperature when outside of the incubator for inspection or manipulation.
- Aseptic handling – especially important if cultures are not supplemented with antibiotics. The staff should be trained and audited accordingly.
- Contact materials – all materials should preferably be tested for embryotoxicity or be of IVF grade. Surface cleaning detergents should be non-abrasive, non-volatile and non-toxic.
- High quality culture reagents, such as medium, growth factors and other reagents and solutions.
- Good cell culture laboratory routines in general, regular testing for mycoplasma, quarantine routines and preferable a quality system in place that regulates version control of protocols and deviations.

2.3.2 Culture Propagation Techniques – Manual Dissection

The classic method for culture of hES cells is in co-cultures with supporting feeder cells, typically mitotically inactivated mouse embryonic fibroblasts, that provide support in terms of conditioning of the culture medium, surface matrix component deposition and other direct cell-cell interactions (Ellerstrom et al. 2007; Heins et al. 2004; Thomson et al. 1998). Critical parameters for high quality hES cell colonies are the quality of the feeder cells, as well as their density and capacity to condition the culture medium. As further discussed below, other hES cell culture systems free of a feeder layer per se could still rely on medium being conditioned by feeder cells,

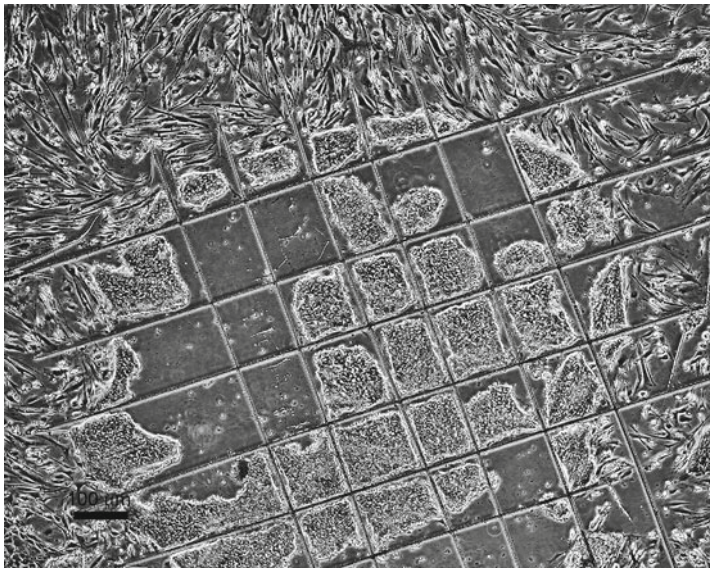

Fig. 2.2 Manually dissected hES cell colony, demonstrating the principles of this propagation method. The mature hES cell colony is sliced up in small fragments using a fine sharp object like a pulled-out glass capillary or a micro scalpel. The colony fragments are subsequently detached from the culture dish and transferred to a new dish with a fresh feeder cell layer. This procedure is typically repeated every 5–7 days, and culture medium is refreshed in between

thus the medium contain factors favourable for maintaining the hES cells in a pluripotent state (Prowse et al. 2005; Lim and Bodnar 2002), The hES cell culture medium could also be totally free of feeder cell influence and have more or less defined components as discussed below and reviewed in Amit and Itskovitz-Eldor 2006 and Amit 2007. The original method for propagation of hES cell cultures on feeder cells is by mechanical dissection. A fine sharp object, e.g. a capillary drawn out over a flame, micro scalpels or other bespoke stem cell cutting tools are used for slicing up a mature colony of hES cells into smaller pieces that is subsequently transferred to a new culture vessel with feeder cells. In Fig. 2.2, a hES cell colony has been sectioned and some pieces removed to illustrate the technique. hES cell colonies typically grow to rounded flat and homogenous colonies, a layer just a single, or a few cells thick. The colonies are clearly visible by eye and can be sizeable in diameter (mm scale). The culture schedules for individual hES cell line often have to be determined empirically, however, the colonies generally need to be passaged every 5–7 days. If allowed to overgrow, the hES cell colony will eventually start to show signs of differentiation, with random 3D structures appearing, migration of fibroblast-like cells and even formation of spontaneously beating cells and neural-like outgrowths. In addition, the feeder layer will deteriorate. Typically, the culture vessels with the feeder layer are prepared some time in advance, allowing conditioning of the culture medium. During the course of culture, culture medium is normally replaced, either fully or partially, at intervals. Centre well dishes like the

ones used for IVF are commonly used since they are designed to allow manipulation of their content, have a rim compartment that should contain culture medium or sterile buffered solution in order to minimise changes in osmolality of the centre well, and not the least, they are subjected to a rigorous quality control. Nevertheless, a number of different culture vessels can be used. When simultaneously maintaining more than one hES cell line in culture, routines should be established so the risk of cross contamination between lines is eliminated.

2.3.3 Enzyme Mediated Passage

The manual dissection method for hES cell passage described above is very labour intensive and requires staff skilled in micro dissection. It is also practically impossible to acquire enough cells for large scale experiments, not to mention sufficient number of cells for use in compound screening or future regenerative medicine applications. As a consequence, protocols for enzymatic digestion of hES cell colonies have been developed and reported by several groups (Ellerstrom et al. 2007; Richards and Bongso 2006; Sjogren-Jansson et al. 2005). There are several commercially available culture systems, with more or less defined components, allowing feeder cell independent stem cell culture in confluent monolayers with enzyme mediated passage. The important aspect of these culture systems is their ability to support hES cell growth on either a biologic or synthetic matrix without involvement of feeder cells and their inherent variability and undefined contribution. The advantages are apparent; it is less time consuming to passage the hES cells and a larger number of cells can be cultured by a single person. After establishment and initial mechanical passage, hES cells can be made progressively tolerant to enzymatic digestion, eventually allowing the cell colonies to be dissociated from old culture vessels and transferred into new ones with good viability. This requirement for adaptation may vary between lines. Colonies can be dissociated by enzymes, such as trypsin and collagenase IV, and also by treatment with EDTA. Needless to say, a system based on enzymatic digestion and large scale cultures need to be robust and validated to support long term pluripotency. Such a system is also ideal for automation of stem cell cultures. A certain level of control is lost when transferring from mechanical to enzymatic passaging methods, i.e. the selection of morphologically perfect specimens over differentiated, or partly differentiated cells. However many commercially available culture systems now maintain hESC with minimal differentiation which mitigates the impact of this change. Nonetheless, until there are more data and therefore more confidence in non-mechanical passaging methods it is likely that manual passaging will continue to be practised in many laboratories to generate and bank high quality starting material.

When hES cell colonies or confluent layers are enzymatically digested, two principles of passage can be applied. Either the cells can be dissociated to clusters of a few hundred cells or to a single cell suspension. Cell viability is largely preserved in the former procedure, however, the stress inflicted on the hES cells in the latter

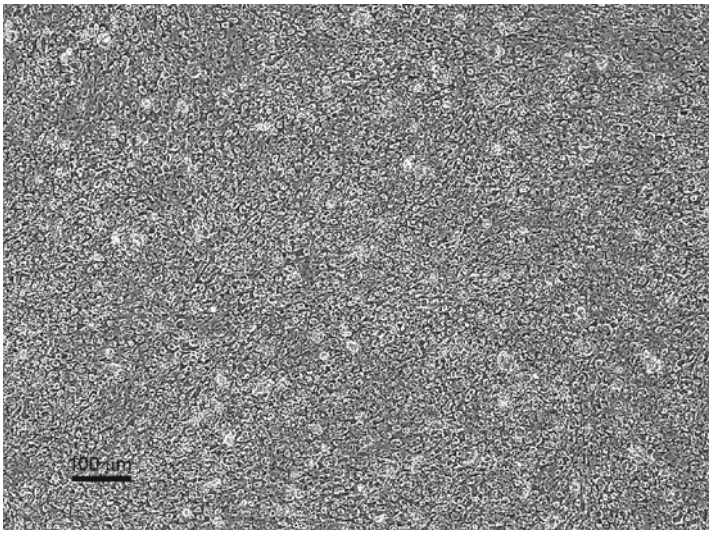

Fig. 2.3 A confluent monolayer of hES cells cultured without the presence of feeder cells. This technique allows scaled up, and also automated hES cell culture

will lead to apoptosis (Watanabe et al. 2007). Watanabe and co-workers made the significant finding that addition of a rho-kinase inhibitor to the medium that prevents apoptosis, thus allowing viable single cell suspensions of hES cells to be achieved. The molecule also improved cell recovery after cryopreservation (Li et al. 2009). Viable single cell passaging can also be achieved, in the absence of rho-kinase inhibitor, by the use of novel substrates such as laminin-521, these can also be used in conjunction with defined media formulations presenting an attractive proposition (Rodin et al. 2014). The ability to produce viable single cell suspension allows hES cells to be seeded in a reproducible and defined manner, i.e. to seed them into culture vessels at exact numbers, which is a prerequisite for e.g. compound screening campaigns or other applications that require absolute control over seeding densities. Single cell passaging also paves the way for easier automated cell culture according to specified protocols and seeding densities which result in more predictable cell growth. Figure 2.3 illustrates a confluent layer of hES cells in a feeder-free culture system.

Concerns were raised in 2004 by Draper and co-workers that prolonged exposure of hES cell cultures to enzymes would cause chromosomal aberrations (Draper et al. 2004), however other studies have later demonstrated that hES cells can be enzymatically propagated over longer periods of time with stable characteristics (Sjogren-Jansson et al. 2005; Suemori et al. 2006). As Catalina and colleagues suggest, the individual hES cell lines may have different pre-dispositions to chromosomal instability independent of culture conditions (Catalina et al. 2008). To address the concern of chromosomal changes, all hES cell cultures should either be regularly karyotyped or only cultured in a passage window that is unlikely to allow

chromosomal deviations to appear and propagate. hES cells not regularly subjected to enzymatic digestion have been shown to sustain a stable karyotype for up to almost 2 years in continuous culture but should still be regularly monitored (Caisander et al. 2006).

2.4 Scaling Up Cultures

For any clinical or industrial application of either undifferentiated hES cells or derivatives thereof, a scaled up, possibly even automated, production methodology is required. Protocols for directed differentiation of naïve hES cells to a specialised cell fate are often inefficient in terms of conversion from starting material, often due to extensive cell loss at early stages. It has been estimated that for a hES cell based repair of a heart damaged by ischemia, hES cells in the range of billions would be required (Passier and Mummery 2005). This is just one example and clearly the number of cells required at the start of a certain differentiation protocol will depend on the level of loss and number of differentiated cells required. At either end of this spectrum would be hESC derived RPE (retinal pigment epithelium) for ophthalmic diseases and hESC derived RBC (red blood cells) for transfusion. Efficacy is one challenge as well as efficiency, the cost of the process and purity of cell populations are two examples. To achieve billions of stem cells as a starting point is not practical with labour intensive protocols so the need for massive amounts of starting material has been a clear driver for the development of scalable culture systems, such as these discussed in the previous section. The scaled up culture process for hES cells needs to be as robust and straightforward as possible, to generate the necessary reproducibility and to be cost effective. Thus, the number of undefined components in the process should be kept to a minimum and ideally a hES cell culture system should be feeder independent and based on defined media and matrix, synthetic or recombinant. The culture procedure must also be subject to quality control, and preferably GLP procedures should apply. The application of MCB (master cell bank) and WCB (working cell bank) approaches will contribute to reproducibility and robustness in the culture system.

Recent progress has been reported where hES cells have been successfully cultured in amounts required for conducting compound screening campaigns in multiwell plate formats (Desbordes et al. 2008; Thomas et al. 2009) (Andrews et al. 2010) These achievements are essential for the use of hES cells in high throughput applications e.g. drug discovery and toxicology screening.

The definition and development of industrial cell production standards needs to be addressed, since scaled up hES cell culture is still a relatively new technology. The ability to manipulate hES cell cultures as single cell suspensions is a first critical step towards reproducible scaled up culture, and not the least, distribution of even cell numbers in multiwell plates for screening. The possibility of adding wholly or partly automated cell culture technology will further strengthen the capability to supply a consistent quality of cells in useful numbers (Thomas et al. 2009).

Future widespread use of hES cell derivatives in bio reactors will also demand cell numbers of an industrial scale.

2.5 Deriving Xeno Free hES Cells and hES Cells for Clinical Use

Routinely, all mammalian cell culture relies heavily on reagents sourced from animals, e.g. serum, amino acids, albumin, various matrices (e.g. collagen) and other factors. This is cost effective and gives enough consistency for the majority of applications. However, the undefined culture condition that is a consequence of the common use of bovine serum as a source of growth factors may be of concern for a number of reasons. One practical consideration is that of reproducibility due to batch-to-batch variation which impacts on the robustness of any culture system. Maybe of more concern in the context of the stem cell therapy area, is the risk of contamination by prions, viruses or other zoonoses of cells cultured in contact with animal derived material. Concerns were raised when it was observed that hES cells cultured in contact with animal material incorporated and expressed animal sialic acid on their cell surface, however, this has also been demonstrated to be reversible (Heiskanen et al. 2007; Martin et al. 2005; Nasonkin and Koliatsos 2006). To address this potential problem, derivation, propagation and banking of hES cell lines strictly without any contact with animal-sourced material, i.e. under "xeno-free" conditions, has been reported by several groups (Ellerstrom et al. 2006; Ludwig et al. 2006; Rajala et al. 2007; Richards et al. 2004) (Ilic et al. 2012). Desai and colleagues have produced a comprehensive review of the evolution of xeno-free hESC culture (Desai et al. 2015).

Xeno-free culture is still substantially more expensive than regular hES cell culture due to the high costs of the reagents, but also the need for dedicated plasticware and equipment as well as routines separated from the non-xeno free cell cultures. The International Stem Cell Banking Initiative is a group of stake holders striving to harmonise guidelines for stem cell banking worldwide, with the goal of delivering the best practice for clinical grade stem cell delivery (Crook et al. 2010).

In order to minimise the dependency on undefined contributions from feeder cells, serum and complex culture matrices like Matrigel (BD Biosciences), efforts have been made to elucidate the hES cell culture requirements for defined culture systems, resulting in commercially available defined culture systems for hES cells and iPS cells. For clinical use of hES cell or hES cell derived cells, cultures free of any animal components would desirably decrease the number of unknowns in the equation as would replacing biologics with small molecules. In addition to the biological and regulatory challenges, the industry and institutions need to generate enough cells suitable for therapeutic use and at the same time make it financially sound.

One of the great hopes of hES cells is to utilise them as raw materials for tissue engineering and replacement for damaged organs or tissues. A number of clinical situations have been identified as potential targets for this kind of approach. A therapy where insulin-dependent type-1 diabetics could receive functional beta cells that would integrate and normalise blood glucose levels would revolutionise the lives of thousands of those affected by this auto immune disease that targets and destroys the endogenous insulin producing cells. Replacing damaged neurons in patients suffering from spinal cord injuries could mean the difference between confinement to a wheel chair and normal mobility. The examples are plenty and great hope for future development is held by many patient groups and clinicians.

Since the above described scenarios include the transplantation of living cells into patients, the regulatory framework is comprehensive. For any future cell-based therapy, Good Clinical Practice (GCP) is a requirement for clinical trials, and this includes that the cells have been sourced and produced according to cGMP. This is a regulatory framework ensuring that an end product meets pre-set specifications. For a putative therapy based on hES cell derived functional cells, it includes the regulation of processes for

- Donation and procurement of the starting materials, e.g. blastocysts and feeder cells
- Testing and quality control
- Processing and manufacturing of the undifferentiated cells as well as their differentiated derivatives.
- Traceability of all reagents and materials as well as release criteria.

In 2007, Crook and co-workers published the first six hES cell lines derived and further processed in line with GMP regulations (Crook et al. 2007) and the most recent efforts in this area was published in 2012 (Ilic et al. 2012). These are important steps towards the clinic; however, there were and are still big challenges to overcome; to develop differentiated functional cells, verified and validated, of clinical value. Also, the six GMP compliant hES cell lines derived by Crook and co-workers were not derived in absence of animal material, i.e., they cannot be defined as xeno-free. However, from a GMP perspective, the use of reagents sourced from animals, like serum, is acceptable, although it has been argued that clinical grade hES cells, or hES cell derived cells need to be derived xeno-free (Unger et al. 2008). Nevertheless, in 2009, Geron Corporation was granted permission from the US Food and Drug Administration (FDA) to initiate the first phase 1 clinical trials for a spinal cord injury therapy, based on specialised cells derived from the hES cell line H1 (Alper 2009; Barde 2009). This hES cell line was originally cultured in contact with animal components and without all the intricate documentation and assurance that GMP compliance gives (Geron 2009; Thomson et al. 1998). Demonstration that some groups have overcome these constraints can be found in the completed, ongoing and approved clinical trials using hESC derived cells which are discussed briefly later in this chapter.

2.6 Differentiation Capacity

The ability of hES cells to differentiate into virtually any specialised cell type present in the adult body is one of the key features of these cells. Spontaneous differentiation occurs *in vitro* when the cells are cultured in conditions lacking the appropriate components that sustain pluripotency. In addition, several protocols for directed differentiation of hES cells into various specialised cell types have been reported in which the cells are guided along lineage restricted pathways to generate relatively pure populations of cells. A detailed review on this topic is beyond the scope of the present chapter and here we only exemplify the differentiation capacity of hES cells using two brief examples; cardiomyocytes and hepatocytes.

The differentiation of hES cells towards the cardiac lineage can be observed through the appearance of clusters of spontaneously contracting cells, as originally reported in 2000 (Itskovitz-Eldor et al. 2000). Different approaches have been developed to induce hES cells to differentiate to cardiomyocytes in culture. One is based on the formation of embryoid body-like structures under the influence of various cocktails of growth factors or small molecules in attempts to recapitulate heart development *in vivo* (Kehat et al. 2001; Yang et al. 2008). Another method is to utilise a co-culture system with hES cells and END-2 cells (a visceral endoderm mouse cell line), in which the pluripotent stem cells are directly exposed to cell-cell interactions as well as the secretome of the END-2 cells (Mummery et al. 2003). In recent years, derivation of cardiomyocytes from monolayers of hES and iPS cells has been demonstrated (Laflamme et al. 2007; Uosaki et al. 2011) and from hES cells cultured under defined conditions using a directed differentiation approach using small molecules (Parsons et al. 2011). Molecular, pharmacological, and electrophysiological studies have characterised hES cell-derived cardiomyocytes to various extents and these cells express many cardiac markers, including transcription factors, structural proteins, ion-channels, and different junction proteins (Beqqali et al. 2006; Synnergren et al. 2008). Despite the similarities with their adult counterparts, hES cell-derived cardiomyocytes still seem to mainly display a foetal cardiac phenotype, and future research is needed to develop protocols which can sustain *in vitro* maturation of the cells towards a phenotype more close to the adult human cardiomyocyte. For drug discovery applications it would be desirable to generate pure populations of e.g. ventricular cardiomyocytes, to study specific targets.

In 2003, the first report appeared which described the generation of hepatic-lineage cells from hES cells, with several publications following (Agarwal et al. 2008; Baharvand et al. 2008; Cai et al. 2007; Rambhatla et al. 2003) and more recently by Medine and co-workers (Medine et al. 2011). It is however a major challenge to generate truly metabolically competent cells, i.e., cells expressing relevant enzymatic activities. No studies to date have shown activity levels of different Cytochrome P450 enzymes that resembles those of freshly isolated human primary hepatocytes. Rather, the accumulated published work implicates that obtaining fully functional cells from hES cells is a major challenge (D'Amour et al. 2006),

(Guguen-Guillouzo et al. 2010). Progress has been made however, as demonstrated by recent publications on the derivation of insulin producing beta cells from hES cells (Bruin et al. 2014; Pagliuca et al. 2014).

2.7 Potential Applications for Therapies

Therapeutic applications based on hES cells add several dimensions to the challenges regarding hES cell culture, differentiation, and purification as discussed above. In addition, critical aspects such as safety and efficacy need to be clarified in detail before such cells can routinely progress to clinical trials, this has been achieved (for PhI trials) in some cases and there are reports of compassionate use of hESC derived cell products (Menasché et al. 2015). Indeed, much advancement has been made towards these ends and there are many diseases that are envisioned as suitable for targeting with stem cell therapies.

For example, despite important advances in pharmacological therapies and organ transplantation, heart failure represents an enormous clinical problem. The limitations of the current interventions have driven the search for stem cell-based techniques to repair and regenerate heart muscle. The opportunity to create the major cell types present in the human heart (i.e., cardiomyocytes, smooth muscle cells, and endothelial cells) from pluripotent stem cells and subsequently transplanting these into the site of injury is an appealing strategy. Pre- clinical studies have begun to investigate hES cell-based heart regeneration, and initial studies provided positive encouragement and demonstrated short term (4 weeks) functional improvement following transplantation of hES cell-derived cardiomyocytes to injured myocardium (Caspi et al. 2007; Laflamme et al. 2007; Leor et al. 2007). However, it was later demonstrated that the functional improvement was transient and no differences were observed between the cardiomyocyte transplanted group and the control animals at 12 weeks post-myocardial infarction (van Laake et al. 2007). Also, formation of fibrous tissue around the graft has been observed, hampering electrophysiological integration. However when hES cell derived cardiomyocytes were co-transplanted with hES cell derived endothelial cells and/or cardiac progenitor cells into mouse hearts, functional capillaries were formed and the cardiomyocytes were found to survive for up to 24 weeks. The formation of capillaries suggests the possibility of an increased blood supply to the graft area. Although the understanding of the mechanism of action is limited at present, it appears that transplantation of cells to the injured heart has some beneficial effects but there are other possibly prohibitive mechanisms that need to be elucidated further (van Laake et al. 2009, 2010). This hypothesis is being tested in a current clinical trial which is outlined below.

The possibility of generating relevant numbers and quality of hepatocytes for bio-artificial liver support technology and possibly also for *in vivo* liver regeneration is a thrilling prospect (Dalgetty et al. 2009), (Medine et al. 2011). In addition, achieving an unlimited access to competent liver cells would be of exceptional use

for studying drug targets, metabolism and toxicity (Jensen et al. 2009). On the other hand, *in vitro* differentiation of hES cells towards the hepatic lineage clearly is a challenging task (Snykers et al. 2009). A number of neurological disorders, such as Alzheimer and Parkinson's diseases, amyotrophic lateral sclerosis and multiple sclerosis (ALS and MS respectively) as well as spinal cord injury have been put forward as targets for hES cell derived therapies and are the subject of extensive research (Kim and Vellis 2009). Although many of the hESC derived therapies in development are still many years from the clinic great progress has been made more recently resulting in a number of clinical trials at various stages of approval and execution.

A comprehensive description of hESC derived cells in clinical trials is not possible here however it is useful to consider which types of therapy have already made it to clinical trial, although many are still to publish data or even recruit. Very preliminary data have been published from ACTs hESC derived RPE for Stargardt's macular dystrophy trial, these data are promising but preliminary and should not be extrapolated at this stage (Schwartz et al. 2015). A French academic led trial to assess the feasibility and safety of hESC derived cardiac-committed progenitor cells in patients with severe heart failure is currently recruiting with an estimated study completion date of June 2017 (clinicaltrials.gov Identifier NCT02057900). Viacyte have FDA approval for a trial of their device encapsulated hESC derived 'beta –like cells' in patients with TypeI diabetes (Borowiak and Melton 2009). The aim is to enrol 40 subjects in this trial and is estimated to collect the final data August 2017 (clinicaltrials.gov NCT02239354). Pfizer are sponsoring a trial to test a hESC derived RPE delivered on a patch that was developed at University College London; this trial is listed with an estimated enrolment of 10 subjects however is not yet open for recruitment (clinicaltrials.gov NCT01691261).

2.8 Conclusions and Future Development in Research

We have now had access to hES cells for over 15 years, and the progress of the scientific field has been spectacular. One could only speculate about what the next decade will generate in terms of greater understanding of human developmental biology, and all aspects of the generation of functional cells from hES cells for therapy as well as drug discovery applications. The field has started to transform from research and development phase into a state where therapeutic and industrial applications begin to be tangible and indeed have reached clinical trials despite the technical and ethical challenges. In terms of the therapeutic area, the regulatory bodies are fundamental in developing and harmonising the legal framework for the use of stem cells in the clinic, and also when it comes to drug safety assessment legislation.

The hES cell field has largely been directed by scientific drivers, however it becomes more obvious that as products mature financial drivers will become more significant. Any application based on hES cells or their derivatives needs to be

proven biologically effective, safe where applicable, but also economically justified, compared to the alternatives. Efficient ways of producing the required quality (GMP for therapeutic use) and quantity of cells need to be further developed and established with an emphasis on defined and/or xeno-free and automation where appropriate. In addition to potential therapeutic use, modified hES cells and/or derivatives thereof are likely to be important tools for further research and development, for example reporter gene containing hES cell lines, immortalised precursors and cells modified to overexpress genes of interest, e.g. ion channels or metabolising enzymes.

In terms of the generation of new hES cell lines, it has been argued that there is a continued need for this. As has been described, technology has moved apace since the early lines were derived and it would be prudent to use lines derived using these new, better defined, techniques as we progress with research in this field, especially in the therapeutic space, A number of the GMP lines currently available have been retrospectively qualified after having been derived as research grade and have been in contact with non-optimal reagents in their history. However there are now a number of true GMP lines which have been derived and maintained under GMP conditions from the outset. One can also argue that there might not be sufficient diversity among the existing hES cell lines to cover the needs for future cell-based therapies as well as for the development of novel drugs for treatment of disease (Civin and Rao 2006). However, studies of renal allograft donor-recipient relations give fuel for speculation that a relatively low number of hES cell lines, in the range of low hundreds or ever less, would cover the larger proportion of the population for future therapies (Nakajima et al. 2007; Taylor et al. 2005), as discussed by Daley and Scadden (Daley and Scadden 2008). Some compounds are known to be metabolised differently between individuals within various ethnic backgrounds as well as between ethnic groups, one example is the well known genetic diversity of the alcohol dehydrogenase. Tissue type matching will obviously be important for regenerative use but these metabolic differences will be of relevance in drug screening strategies. Some of this diversity can be achieved by the generation of new hESC lines whereas more specific modifications can be achieved through the use of the rapidly developing genome modification technologies such as zinc finger nucleases, CRISPR/Cas9, etc. (reviewed in Li et al. 2014).

The advent of hiPSC lines predictably led to the argument that establishing new hESC lines would be redundant. However, the variability in reprogramming methods and the impact these will have on the safety profile of these cells mean that there is still a case for using hESC cells for therapeutic applications. Uncertainty about the equivalence of iPSC to hESC also leaves the question as to whether or not hiPSC can be used as a simple replacement for hESC unanswered. This is discussed in a recent publication from Barad and colleagues (Barad et al. 2014). It the last edition of this book we said that only time, together with high quality peer/reviewed research and development, will contribute to the future directions of stem cells in general and hES cells in particular. Despite the many advances in technology and clinical applicability since then this statement still holds true.

Acknowledgements The authors thank Miss Claire Balfour for providing images for the figures.

References

2001/20/EC, E.D (2001) *EU Directive 2001/20/EC*

2005/28/EC, E.D (2005) *EU Directive 2005/28/EC*

Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, Bello PA, Benvenisty N, Berry LS, Bevan S et al (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25:803–816

Advanced Cell Technology clinical trials. http://www.advancedcell.com/patients/clinical-trial-information/

Agarwal S, Holton KL, Lanza R (2008) Efficient differentiation of functional hepatocytes from human embryonic stem cells. Stem Cells 26:1117–1127

Alper J (2009) Geron gets green light for human trial of ES cell-derived product. Nat Biotechnol 27:213–214

Amit M (2007) Feeder-layer free culture system for human embryonic stem cells. Methods Mol Biol 407:11–20

Amit M, Itskovitz-Eldor J (2006) Feeder-free culture of human embryonic stem cells. Methods Enzymol 420:37–49

Andrews PD, Becroft M, Aspegren A, Gilmour J, James MJ, McRae S, Kime R, Allcock RW, Abraham A, Jiang Z, Strehl R, Mountford JC, Milligan G, Houslay MD, Adams DR, Frearson JA (2010) High-content screening of feeder-free human embryonic stem cells to identify pro-survival small molecules. Biochem J 432(1):21–33

Baharvand H, Hashemi SM, Shahsavani M (2008) Differentiation of human embryonic stem cells into functional hepatocyte-like cells in a serum-free adherent culture condition. Differentiation 76:465–477

Barad L, Schick R, Zeevi-Levin N, Itskovitz-Eldor J, Binah O (2014) Human embryonic stem cells vs human induced pluripotent stem cells for cardiac repair. Can J Cardiol 30(11):1279–1287

Barde Y (2009) Caution urged in trial of stem cells to treat spinal-cord injury. Nature 458:29

Beqqali A, Kloots J, Ward-van Oostwaard D, Mummery C, Passier R (2006) Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells 24:1956–1967

Bongso A, Fong CY, Ng SC, Ratnam S (1994) Isolation and culture of inner cell mass cells from human blastocysts. Hum Reprod 9:2110–2117

Borowiak M, Melton DA (2009) How to make beta cells? Curr Opin Cell Biol 21(6):727–732

Brandenberger R, Wei H, Zhang S, Lei S, Murage J, Fisk GJ, Li Y, Xu C, Fang R, Guegler K et al (2004) Transcriptome characterization elucidates signaling networks that control human ES cell growth and differentiation. Nat Biotechnol 22:707–716

Brett S, Livie M, Thomas G, McConnell A, Rajkhowa M (2009) Report on the donation of supernumerary embryos from fresh IVF and ICSI treatment cycles for human stem cell research. Hum Fertil (Camb) 12:34–39

Bruin JE, Erener S, Vela J, Hu X, Johnson JD, Kurata HT, Lynn FC, Piret JM, Asadi A, Rezania A et al (2014) Characterization of polyhormonal insulin-producing cells derived in vitro from human embryonic stem cells. Stem Cell Res 12:194–208

Cai L, Johnstone BH, Cook TG, Liang Z, Traktuev D, Cornetta K, Ingram DA, Rosen ED, March KL (2007) Suppression of hepatocyte growth factor production impairs the ability of adipose-derived stem cells to promote ischemic tissue revascularization. Stem Cells 25:3234–3243

Caisander G, Park H, Frej K, Lindqvist J, Bergh C, Lundin K, Hanson C (2006) Chromosomal integrity maintained in five human embryonic stem cell lines after prolonged in vitro culture. Chromosome Res 14:131–137

Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L (2007) Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol 50:1884–1893

Catalina P, Montes R, Ligero G, Sanchez L, de la Cueva T, Bueno C, Leone PE, Menendez P (2008) Human ESCs predisposition to karyotypic instability: is a matter of culture adaptation or differential vulnerability among hESC lines due to inherent properties? Mol Cancer 7:76

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113:643–655

Chen AE, Egli D, Niakan K, Deng J, Akutsu H, Yamaki M, Cowan C, Fitz-Gerald C, Zhang K, Melton DA et al (2009) Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. Cell Stem Cell 4:103–106

Civin CI, Rao MS (2006) How many human embryonic stem cell lines are sufficient? A U.S. perspective. Stem Cells 24:800–803

Crook JM, Peura TT, Kravets L, Bosman AG, Buzzard JJ, Horne R, Hentze H, Dunn NR, Zweigerdt R, Chua F et al (2007) The generation of six clinical-grade human embryonic stem cell lines. Cell Stem Cell 1:490–494

Crook JM, Hei D, Stacey G (2010) The International Stem Cell Banking Initiative (ISCBI): raising standards to bank on. In Vitro Cell Dev Biol Anim 46(3–4):169–172. Epub 2010 Mar 3. Review

D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24:1392–1401

Daley GQ, Scadden DT (2008) Prospects for stem cell-based therapy. Cell 132:544–548

Dalgetty DM, Medine CN, Iredale JP, Hay DC (2009) Progress and future challenges in stem cell-derived liver technologies. Am J Physiol Gastrointest Liver Physiol 297:G241–G248

Desai N, Rambhia P, Gishto A (2015) Human embryonic stem cell cultivation: historical perspective and evolution of xeno-free culture systems. Reprod Biol Endocrinol 13(1):9

Desbordes SC, Placantonakis DG, Ciro A, Socci ND, Lee G, Djaballah H, Studer L (2008) High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. Cell Stem Cell 2:602–612

Dokras A, Sargent IL, Barlow DH (1993) Human blastocyst grading: an indicator of developmental potential? Hum Reprod 8:2119–2127

Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, Meisner L, Zwaka TP, Thomson JA, Andrews PW (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22:53–54

Ellerstrom C, Strehl R, Moya K, Andersson K, Bergh C, Lundin K, Hyllner J, Semb H (2006) Derivation of a xeno-free human embryonic stem cell line. Stem Cells 24:2170–2176

Ellerstrom C, Strehl R, Noaksson K, Hyllner J, Semb H (2007) Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. Stem Cells 25:1690–1696

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

Feki A, Bosman A, Dubuisson JB, Irion O, Dahoun S, Pelte MF, Hovatta O, Jaconi ME (2008) Derivation of the first Swiss human embryonic stem cell line from a single blastomere of an arrested four-cell stage embryo. Swiss Med Wkly 138:540–550

Gavrilov S, Prosser RW, Khalid I, MacDonald J, Sauer MV, Landry DW, Papaioannou VE (2009) Non-viable human embryos as a source of viable cells for embryonic stem cell derivation. Reprod Biomed Online 18(2):301–308

Geens M, Mateizel I, Sermon K, De Rycke M, Spits C, Cauffman G, Devroey P, Tournaye H, Liebaers I, Van de Velde H (2009) Human embryonic stem cell lines derived from single blastomeres of two 4-cell stage embryos. Hum Reprod 24:2709–2717

Geron (2009) Geron receives FDA clearance to begin world's first human clinical trial of embryonic stem cell-based therapy. http://ir.geron.com/phoenix.zhtml?c=67323&p=irol-newsArticle&ID=1636192. Press release

Guguen-Guillouzo C, Corlu A, Guillouzo A (2010) Stem cell-derived hepatocytes and their use in toxicology. Toxicology 270(1):3–9. Epub 2009 Oct 6

Heins N, Englund MC, Sjoblom C, Dahl U, Tonning A, Bergh C, Lindahl A, Hanson C, Semb H (2004) Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22:367–376

Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, Mikkola M, Olsson C, Miller-Podraza H, Blomqvist M et al (2007) N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells 25:197–202

Ilic D, Stephenson E, Wood V, Jacquet L, Stevenson D, Petrova A, Kadeva N, Codognotto S, Patel H, Semple M, Cornwell G, Ogilvie C, Braude P (2012) Derivation and feeder-free propagation of human embryonic stem cells under xeno-free conditions. Cytotherapy 14(1):122–128. Epub 2011 Oct 27

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N (2000) Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6:88–95

Jensen J, Hyllner J, Bjorquist P (2009) Human embryonic stem cell technologies and drug discovery. J Cell Physiol 219:513–519

Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108:407–414

Kim SU, de Vellis J (2009) Stem cell-based cell therapy in neurological diseases: a review. J Neurosci Res 87:2183–2200

Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R (2006) Human embryonic stem cell lines derived from single blastomeres. Nature 444:481–485

Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S et al (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25:1015–1024

Leor J, Gerecht S, Cohen S, Miller L, Holbova R, Ziskind A, Shachar M, Feinberg MS, Guetta E, Itskovitz-Eldor J (2007) Human embryonic stem cell transplantation to repair the infarcted myocardium. Heart 93:1278–1284

Lerou PH, Yabuuchi A, Huo H, Miller JD, Boyer LF, Schlaeger TM, Daley GQ (2008a) Derivation and maintenance of human embryonic stem cells from poor-quality in vitro fertilization embryos. Nat Protoc 3:923–933

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

Li X, Krawetz R, Liu S, Meng G, Rancourt DE (2009) ROCK inhibitor improves survival of cryopreserved serum/feeder-free single human embryonic stem cells. Hum Reprod 24:580–589

Li M, Suzuki K, Kim NY, Liu GH, Izpisua Belmonte JC (2014) A cut above the rest: targeted genome editing technologies in human pluripotent stem cells. J Biol Chem 289(8):4594–4599

Lim JW, Bodnar A (2002) Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. Proteomics 2(9):1187–1203

Loser P, Schirm J, Guhr A, Wobus AM, Kurtz A (2010) Human embryonic stem cell lines and their use in international research. Stem Cells 28:240–246

Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS et al (2006) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24:185–187

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

Martin MJ, Muotri A, Gage F, Varki A (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 11:228–232

Medine CN, Lucendo-Villarin B, Zhou W, West CC, Hay DC (2011) Robust generation of hepatocyte-like cells from human embryonic stem cell populations. J Vis Exp 2011:e2969. doi:10.3791/2969

Menasché P, Vanneaux V, Fabreguettes JR, Bel A, Tosca L, Garcia S, Bellamy V, Farouz Y, Pouly J, Damour O, Périer MC, Desnos M, Hagège A, Agbulut O, Bruneval P, Tachdjian G, Trouvin JH, Larghero J (2015) Towards a clinical use of human embryonic stem cell-derived cardiac progenitors: a translational experience. Eur Heart J 36(12):743–750. Epub 2014 May 16

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631–642

Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, de la Riviere AB et al (2003) Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107:2733–2740

Murdoch A, Braude P, Courtney A, Brison D, Hunt C, Lawford-Davies J, Moore H, Stacey G, Sethe S (2012) For the procurement Working Group of the National Clinical hESC Forum. The procurement of cells for the derivation of human embryonic stem cell lines for therapeutic use: recommendations for good practice. Stem Cell Rev 8(1):91–99

Nakajima F, Tokunaga K, Nakatsuji N (2007) Human leukocyte antigen matching estimations in a hypothetical bank of human embryonic stem cell lines in the Japanese population for use in cell transplantation therapy. Stem Cells 25:983–985

Nasonkin IO, Koliatsos VE (2006) Nonhuman sialic acid Neu5Gc is very low in human embryonic stem cell-derived neural precursors differentiated with B27/N2 and noggin: implications for transplantation. Exp Neurol 201:525–529

Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, Peterson QP, Greiner D, Melton DA (2014) Generation of functional human pancreatic β cells in vitro. Cell 159(2):428–439

Parsons XH, Teng YD, Parsons JF, Snyder EY, Smotrich DB, Moore DA (2011) Efficient derivation of human cardiac precursors and cardiomyocytes from pluripotent human embryonic stem cells with small molecule induction. J Vis Exp 2011(57):e3274. doi:10.3791/3274

Passier R, Mummery C (2005) Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 16:498–502

Prowse AB, McQuade LR, Bryant KJ, Van Dyk DD, Tuch BE, Gray PP (2005) A proteome analysis of conditioned media from human neonatal fibroblasts used in the maintenance of human embryonic stem cells. Proteomics 5(4):978–989

Rajala K, Hakala H, Panula S, Aivio S, Pihlajamaki H, Suuronen R, Hovatta O, Skottman H (2007) Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum Reprod 22:1231–1238

Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK (2003) Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant 12:1–11

Rho JY, Yu K, Han JS, Chae JI, Koo DB, Yoon HS, Moon SY, Lee KK, Han YM (2006) Transcriptional profiling of the developmentally important signalling pathways in human embryonic stem cells. Hum Reprod 21:405–412

Richards M, Bongso A (2006) Propagation of human embryonic stem cells on human feeder cells. Methods Mol Biol 331:23–41

Richards M, Fong CY, Tan S, Chan WK, Bongso A (2004) An efficient and safe xeno-free cryopreservation method for the storage of human embryonic stem cells. Stem Cells 22:779–789

Rodin S, Antonsson L, Hovatta O, Tryggvason K (2014) Monolayer culturing and cloning of human pluripotent stem cells on laminin-521-based matrices under xeno-free and chemically defined conditions. Nat Protoc 9(10):2354–2368. Epub 2014 Sep 11

Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, Hubschman JP, Davis JL, Heilwell G, Spirn M, Maguire J, Gay R, Bateman J, Ostrick RM, Morris D, Vincent M, Anglade E, Del Priore LV, Lanza R (2015) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 385(9967):509–516. doi:10.1016/S0140-6736(14)61376-3. Epub 2014 Oct 15

Sjogren A, Hardarson T, Andersson K, Caisander G, Lundquist M, Wikland M, Semb H, Hamberger L (2004) Human blastocysts for the development of embryonic stem cells. Reprod Biomed Online 9:326–329

Sjogren-Jansson E, Zetterstrom M, Moya K, Lindqvist J, Strehl R, Eriksson PS (2005) Large-scale propagation of four undifferentiated human embryonic stem cell lines in a feeder-free culture system. Dev Dyn 233:1304–1314

Snykers S, De Kock J, Rogiers V, Vanhaecke T (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. Stem Cells 27:577–605

Stephenson EL, Braude PR, Mason C (2007) International community consensus standard for reporting derivation of human embryonic stem cell lines. Regen Med 2:349–362

Strelchenko N, Verlinsky O, Kukharenko V, Verlinsky Y (2004) Morula-derived human embryonic stem cells. Reprod Biomed Online 9:623–629

Suemori H, Yasuchika K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N (2006) Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem Biophys Res Commun 345:926–932

Synnergren J, Akesson K, Dahlenborg K, Vidarsson H, Ameen C, Steel D, Lindahl A, Olsson B, Sartipy P (2008) Molecular signature of cardiomyocyte clusters derived from human embryonic stem cells. Stem Cells 26:1831–1840

Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA (2005) Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 366:2019–2025

Thomas RJ, Anderson D, Chandra A, Smith NM, Young LE, Williams D, Denning C (2009) Automated, scalable culture of human embryonic stem cells in feeder-free conditions. Biotechnol Bioeng 102:1636–1644

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP (1995) Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 92:7844–7848

Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP (1996) Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. Biol Reprod 55:254–259

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 282:1145–1147

Unger C, Skottman H, Blomberg P, Dilber MS, Hovatta O (2008) Good manufacturing practice and clinical-grade human embryonic stem cell lines. Hum Mol Genet 17:R48–R53

Uosaki H, Fukushima H, Takeuchi A, Matsuoka S, Nakatsuji N, Yamanaka S, Yamashita JK (2011) Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. PLoS One 6(8):e23657. Epub 2011 Aug 18

van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, den Ouden K, Ward-van Oostwaard D, Korving J, Tertoolen LG et al (2007) Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. Stem Cell Res 1:9–24

van Laake LW, Passier R, den Ouden K, Schreurs C, Monshouwer-Kloots J, Ward-van Oostwaard D, van Echteld CJ, Doevendans PA, Mummery CL (2009) Improvement of mouse cardiac

function by hESC-derived cardiomyocytes correlates with vascularity but not graft size. Stem Cell Res 3:106–112

van Laake LW, van Donselaar EG, Monshouwer-Kloots J, Schreurs C, Passier R, Humbel BM, Doevendans PA, Sonnenberg A, Verkleij AJ, Mummery CL (2010) Extracellular matrix formation after transplantation of human embryonic stem cell-derived cardiomyocytes. Cell Mol Life Sci 67(2):277–290. Epub 2009 Oct 22

Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Muguruma K, Sasai Y (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25:681–686

Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA (2002) BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 20:1261–1264

Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM et al (2008) Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 453:524–528

Zhang X, Stojkovic P, Przyborski S, Cooke M, Armstrong L, Lako M, Stojkovic M (2006) Derivation of human embryonic stem cells from developing and arrested embryos. Stem Cells 24:2669–2676

Chapter 3
Induced Pluripotent Stem Cells in Regenerative Medicine

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Abstract The conversion of somatic cells into pluripotent cells is transforming the way diseases are studied and treated. Owing to their ability to differentiate into any cell type in the body and being patient-specific, induced pluripotent stem cells (iPSCs) hold great promise for disease modeling, drug discovery and regenerative medicine. Since their discovery in 2006, significant efforts have been made to understand the reprogramming process and to generate human iPSCs with potential for clinical use. Additionally, the development of advanced genome-editing platforms to increase homologous recombination efficiency, namely DNA nucleases, is making the generation of gene-corrected patient-specific iPSCs an achievable goal, with potential future therapeutic applications. Here, we review recent developments in the generation, differentiation and genetic manipulation of human iPSCs and discuss their relevance to regenerative medicine and the challenges still remaining for clinical application.

Keywords Induced pluripotent stem cells • Reprogramming • Retrovirus • Lentivirus • Transduction • Zero-footprint method • PiggyBac transposase • Cardiac differentiation • Targeting vector • Macula degeneration

3.1 Introduction

The promise of using pluripotent stem cells (PSCs) for regenerative medicine dates back to 1998 when James Thomson (Thomson et al. 1998) first derived human embryonic stem cells (hESCs) from the inner cell mass of developing embryos.

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_3

Not only do PSCs have the ability to self-renew indefinitely, but in theory they can also differentiate into any cell type in the body, thus providing functional replacement or trophic support to dysfunctional cells and diseased tissues. Ethical concerns surrounding the destruction of human embryos, which occurs in most hESC derivation methods, and safety issues created controversy in developing therapies from hESC lines for many years and stimulated researchers to find alternative approaches to obtain hESC-like cells. In 2006 Kazutoshi Takahashi and Shinya Yamanaka made the seminal discovery that mouse skin fibroblasts can be reprogrammed to an ESC-like state by simple overexpression of master stemness regulators (Takahashi and Yamanaka 2006). They named these cells "induced pluripotent stem cells" (iPSCs). One year later, these same investigators as well as groups headed by James Thomson and George Daley succeeded in converting human fibroblasts into human iPSCs (hiPSCs) (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008). Reprogramming to pluripotency has now been achieved starting with a variety of somatic cell types (Aasen et al. 2008; Hanna et al. 2008; Utikal et al. 2009; Carette et al. 2010; Miyoshi et al. 2010; Seki et al. 2010; Tsai et al. 2010; Kim et al. 2011a) and generation of patient- and disease-specific hiPSCs is now possible, opening new avenues for exploring disease etiology, developing novel drugs, toxicology screening and cell replacement therapies. Overcoming the ethical difficulties regarding the use of human embryos that are related with hESCs and being genetically matched to the donor, hiPSCs are increasingly used in modern medicine. Latest advances in genome editing of hiPSCs enable researchers to investigate the intricacies of the human genome in a dish and expand the possibilities of combining cell and gene therapies for treating congenital and degenerative disorders.

Here, we review recent developments for the generation of hiPSCs and emphasize those attractive for obtaining translational-grade cells. Furthermore, we summarize the latest advances in their differentiation and the challenges for obtaining functional and safe hiPSC derivatives for therapy, with specific focus on cardiac muscle cells. Finally, we give a brief overview on the latest available genome-editing platforms for generation of gene-corrected patient-specific hiPSCs and discuss their relevance for regenerative medicine purposes.

3.2 Discovery of iPSCs

Pioneering work in cellular reprogramming had demonstrated that somatic cells can be reprogrammed by transferring their nuclear contents into oocytes (Wilmut et al. 1997) or by fusion with ESCs (Tada et al. 2001; Cowan et al. 2005), indicating that unfertilized eggs and ESCs contain factors that can confer pluripotency to somatic cells.

In 2006, Yamanaka and Takahashi hypothesized that factors that play important roles in the maintenance of ESC identity also play pivotal roles in the induction of pluripotency in somatic cells. Using a retroviral system, they forced expression of a selected set of 24 candidate genes in mouse embryonic fibroblasts (MEFs) and were successful in establishing clones that possessed ESC-like morphologies, proliferation rates, expressed ESC markers and had demethylated the promoter of pluripo-

tency genes (Takahashi and Yamanaka 2006). These cells were termed as induced pluripotent stem cells (iPSCs). Removing one factor at a time they further demonstrated that a minimum set of only four factors namely, Klf4, cMyc, Oct4 and Sox2 were necessary for reprogramming MEFs as well as tail-tip fibroblasts from adult mice into iPSCs. Later studies showed that the presence of cMyc is not an absolute reprogramming requirement but its absence significantly reduces the efficiency of the process (Nakagawa et al. 2008).

The successful reprogramming of human somatic cells to hiPSCs was reported within 1 year (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008). Takahashi and Yamanaka, as well as Daley's group used KLF4, cMYC, OCT4 and SOX2, the same factors as in the mouse system, to convert human fibroblasts into hiPSCs (Takahashi et al. 2007; Park et al. 2008). Thomson's group achieved the same results using LIN28, NANOG, OCT4 and SOX2 (Yu et al. 2007).

Since then the field of cellular reprogramming has progressed at an unprecedented pace. An increasing number of studies constantly provide new insights into the molecular mechanism of the reprogramming process (Brambrink et al. 2008; Mikkelsen et al. 2008; Li et al. 2010; Samavarchi-Tehrani et al. 2010; Fussner et al. 2011). Moreover, specific advances have been made to facilitate the transition of this technology into the clinic, including the use of various cell types for reprogramming (Aasen et al. 2008; Hanna et al. 2008; Utikal et al. 2009; Carette et al. 2010; Miyoshi et al. 2010; Seki et al. 2010; Tsai et al. 2010; Kim et al. 2011a), and the replacement of individual factors by other regulators (Zhao et al. 2008; Feng et al. 2009; Heng et al. 2010; Nakagawa et al. 2010; Moon et al. 2011), small molecules (Huangfu et al. 2008; Shi et al. 2008; Ichida et al. 2009; Li et al. 2009; Zhu et al. 2010; Moon et al. 2011; Staerk et al. 2011) or a modified culture condition (Marson et al. 2008). Furthermore, hiPSC lines have been derived from patients affected by various diseases (Moretti et al. 2010; Unternaehrer and Daley 2011; Zhu et al. 2011; Jung et al. 2012; Cherry and Daley 2013; Gramlich et al. 2015) and from species other than mice or humans, including rhesus monkey (Liu et al. 2008), marmoset (Tomioka et al. 2010), rat (Liao et al. 2009; Maherali and Hochedlinger 2009), pig (Esteban et al. 2009; Ezashi et al. 2009; Wu et al. 2009), dog (Shimada et al. 2010; Luo et al. 2011), sheep (Bao et al. 2011), horse (Nagy et al. 2011) and cow (Han et al. 2011).

The following section will focus on developed approaches for the generation of iPSCs from human origin.

3.3 Generation of Human iPSCs: Developments Towards Translational-Grade hiPSCs

Cell Source for Reprogramming

Among the different issues to be considered when reprogramming human somatic cells into hiPSCs an important one is the choice of the starting material. In general, each actively dividing somatic cell type can be used for reprogramming (Haase et al. 2009). Takahashi and Yamanaka used fibroblasts as the starting somatic cell

type and to date, owing their easy culture conditions and efficient transduction, dermal fibroblasts are still one of the most commonly used primary cell source. However, the relatively low reprogramming efficiency (0.01–0.5 %) and especially the need of uncomfortable biopsies have stimulated the search for other, "easier accessible" cell sources. Efficient reprogramming has been demonstrated for peripheral blood mononuclear cells (PBMCs) (Loh et al. 2009), exfoliated renal tubular epithelial cells obtained from urine (Zhou et al. 2011), and keratinocytes from plucked hair (Aasen et al. 2008). One advantage of PBMCs is that they can be obtained from routine blood tests or in patient follow- up and can be frozen and stored before reprogramming.

It has been acknowledged that reprogrammed iPSCs can retain specific DNA methylation profiles associated with their parental source cell type (Bar-Nur et al. 2011; Kim et al. 2011b; Lister et al. 2011). Variations in these signatures also appear to account for intra-line variability among different clones originating from the same iPSC line (Kim et al. 2011b; Lister et al. 2011). The long-term effect of epigenetic pattern retention, such as methylation profiles from the originating somatic cell type, is not yet fully understood. However, the somatic source cell type is known to affect differentiation efficiency into specific iPSC derivatives and epigenetic memory is a key determinant of iPSC differentiation into lineages that are distinct from the parental one (Ohi et al. 2011; Sanchez-Freire et al. 2014). For example, cardiac progenitor cell-derived iPSC lines have shown an enhanced ability to differentiate into cardiomyocytes compared to fibroblast-derived iPSC lines (Sanchez-Freire et al. 2014). Prolonged propagation of iPSCs through many passages reduces these effects, suggesting that residual epigenetic memory is attenuated in the course of long-term culture (Ohi et al. 2011; Sanchez-Freire et al. 2014). This issue, clearly important for therapeutic applications, will require further study in order to determine to what extent the ultimate transplantable cell type should influence the source of patient-specific cells for reprogramming. As this remains unclear, the choice of the starting tissue material should be based, first, on the most accessible and least invasive, and then, depending on the future use of the hiPSCs, an epigenetically related cell source should be considered if available.

Reprogramming Methodologies

The common aim of all reprogramming methods is the forced expression of the reprogramming factors. As mentioned above, hiPSCs were initially derived from fibroblasts by retrovirus- and lentivirus-mediated transduction of genes encoding transcriptional regulators of stem cells: OCT4, SOX2, LIN28, and NANOG (OSLN) (Yu et al. 2007) or OCT4, SOX2, KLF4, and c-MYC (OSKM) (Takahashi et al. 2007; Park et al. 2008). However, viral delivery of transgenes results in the integration of vector sequences into the genome, which is a source of potential insertion mutagenesis, residual expressions, and reactivation of transgenes during differentiation. Therefore, cells generated by permanent and random integration of exogenous genes have a certain oncogenic potential and are not suitable for therapeutic applications.

Safer non-integrating reprogramming methods have since been developed using minimal footprint systems, such as excisable viruses (Chang et al. 2009; Soldner et al. 2009; Somers et al. 2010), and zero footprint technologies, including adenovirus (Stadtfeld et al. 2008), Sendai virus (Fusaki et al. 2009), plasmids (Okita et al. 2008; Gonzalez et al. 2009; Si-Tayeb et al. 2010), episomal or minicircle vectors (Yu et al. 2009; Jia et al. 2010), piggyBac transposons (Kaji et al. 2009; Woltjen et al. 2009; Yusa et al. 2009), microRNA mimics (Miyoshi et al. 2011), synthetic mRNAs (Warren et al. 2010), and proteins (Zhou et al. 2009) (Fig. 3.1).

Minimal footprint approaches mostly use lentiviruses containing loxP sites in the 5′ and 3′ LTR of the viral vectors. The presence of loxP sites provides a substrate to remove most of the transgene sequences by Cre-mediated recombination. However, one loxP site flanked by small portions of the 5′ and 3′ LTRs remains in the iPSC

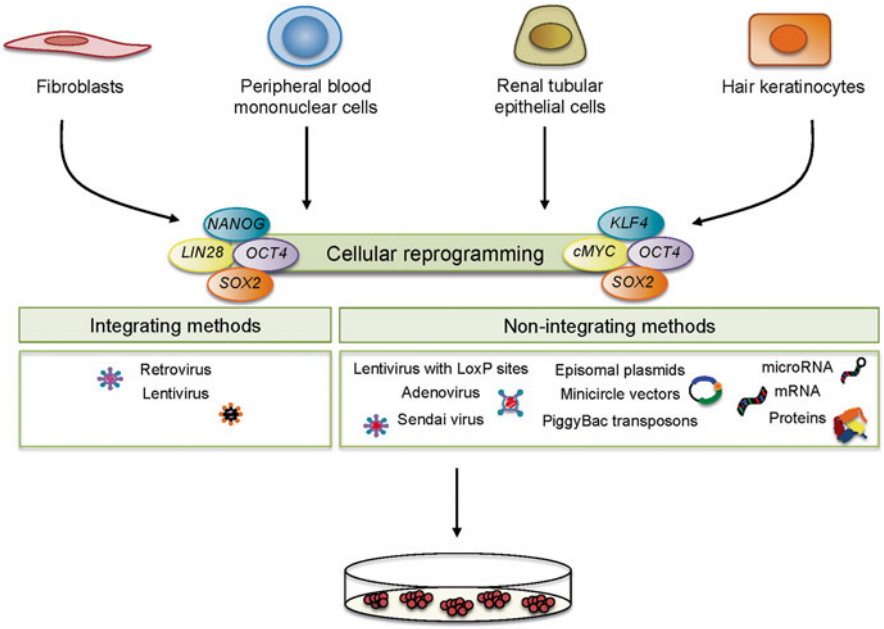


Fig. 3.1 Generation of human iPSCs. Different starting cell types are available for the generation of human induced pluripotent stem cells (hiPSCs). Fibroblasts were the first and still the most commonly used cell source. Amongst others, three easily accessible starting cell type are blood cells (T lymphocytes), exfoliated renal tubular epithelial cells obtained from urine, and keratinocytes from plucked hair. The reprogramming can be obtained through the expression of several combinations of pluripotency regulators (OCT4, SOX2, NANOG, LIN28, cMYC AND KLF4) and different methods are available to induce their expression. They can be divided into two major groups: integrating methods, which consist of retrovirus or lentivirus delivery of transgenes that randomly integrate into the genome, and non-integrating methods that enable the generation of hiPSCs without any permanent genetic modification

genome following Cre-mediated excision (Soldner et al. 2009; Somers et al. 2010). Thus, the continued presence of exogenous transgene sequences (no matter how minimal) could be a concern if differentiated cells derived from these hiPSCs are to be transplanted into a patient.

Zero-footprint methods include adenoviruses, which are non-integrating viruses that infect both replicating and non-replicating cells. Human iPSCs created *via* adenovirus showed no signs of transgene integration (Zhou and Freed 2009), which is a favorable result for translational applications. However, adenovirus-based reprogramming has low efficiency. More suitable are Sendai viruses, which are negative sense, single-stranded RNA viruses that produce large amounts of protein without entering the nucleus of the infected cells, thus being completely lost after several cell passages. Generation of translational-grade hiPSCs from multiple somatic cell types, including PBMCs (Seki et al. 2010; Orban et al. 2015), has been successfully and efficiently achieved with this method (Fusaki et al. 2009; Ban et al. 2011; Seki et al. 2012).

Another way to generate zero-footprint hiPSCs is the overexpression of the reprogramming factors by episomal plasmids. Yu et al. (2009) developed an oriP/EBNA (Epstein–Barr nuclear antigen)-based plasmid that allows their expression for a long enough period of time sufficient to initiate the reprogramming process. The plasmid will be lost from proliferating cells if drug selection is removed, therefore leaving no footprint. Further modifications of the episomal plasmid reprogramming method (Chen et al. 2011; Okita et al. 2011) have made this approach also very attractive for generation of iPSCs that could be used in translational studies. Episomal vectors are particularly appealing because they are easy to manipulate, allow a relatively high efficiency of reprogramming, and have been proven to work for many somatic cell types, including blood cells (Chou et al. 2011).

Additional DNA-based zero-footprint systems that have been tested for cellular reprogramming are minicircle vectors and piggyBac transposons. Minicircle vectors are circularized constructs in which the plasmid backbone has been released leaving only the eukaryotic promoter and cDNA(s) that are to be expressed. A minicircle vector was produced with LIN28, NANOG, SOX2, and OCT4 and used to reprogram human adipose stem cells (Jia et al. 2010; Narsinh et al. 2011). However, more validation is required since this method worked at lower efficiency for neonatal fibroblasts and no data of successful reprogramming exist for any other cell types. On the hand, hiPSCs have been generated at a reasonable reprogramming efficiency using a piggyBac transposon (Mali et al. 2010), which is a mobile genetic element that in the presence of the piggyBac transposase can be integrated into chromosomal TTAA sites. Re-expression of the transposase after the transposon has been stably integrated results in its excision with no sequence vestiges at the integrated site. Limitations of this system that hamper any clinical translation are the additional step required for excision of the transposon plus the dearth of information on successful excision in hiPSCs (Mali et al. 2010).

More recently, new zero-footprint tools, which are virus- and DNA-free, have emerged. Direct expression of reprogramming factors as proteins has been used to successfully generate hiPSCs (Kim et al. 2009; Zhou et al. 2009). However, this method is limited by the lengthy timeline, low efficiency, and special technical

skills required for the synthesis of bioactive reprogramming proteins. Synthetic modified mRNAs have also been explored (Warren et al. 2010). In mRNA reprogramming, cells are transfected with *in vitro*-transcribed mRNAs encoding for the reprogramming factors. Several chemical measures are employed to limit activation of the innate immune system by foreign nucleic acids and, due to the very short half-life of mRNAs, daily transfections are required to induce hiPSCs. Also miRNAs have been proven useful for generation of hiPSCs without genome-integrating DNA elements (Miyoshi et al. 2011). It is worth noting that since miRNA-mediated reprogramming are mostly dependent on endogenous pathways and, hence, maintain a smooth epigenetic modification, it shows certain advantages in producing better, safer hiPSCs, but more studies are needed to tackle this issue. Finally, a recent report by Hou et al. (2013) described a gene-free, small molecule–based method for generation of mouse iPSCs, demonstrating that the field of pluripotency induction continues to evolve at a rapid pace.

It is worth mentioning that, based on a recent systematic evaluation of the three so far most widely used techniques for generating integration-free hiPSCs (Sendai viruses, episomal plasmids, and synthetic modified mRNAs) (Schlaeger et al. 2015), significant differences exist in aneuploidy rates, reprogramming efficiency, reliability and workload, but all methods generate high-quality hiPSCs. Thus the choice of the reprogramming method should depend on each laboratory's particular requirements.

Genomic stability is critical for clinical applications of hiPSCs. There are evidences that hiPSCs may harbor epigenetic and transcriptional abnormalities (Kim et al. 2010; Polo et al. 2010; Stadtfeld et al. 2010; Bar-Nur et al. 2011; Kim et al. 2011b) as well as genomic aberrations that are either pre-existing or generated during reprogramming (Mayshar et al. 2010; Gore et al. 2011; Laurent et al. 2011; Lister et al. 2011; Pera 2011), raising significant concerns about their safety for potential clinical applications. However, most of the hiPSCs described in these studies have genomic abnormalities generated from integrating reprogramming methods. A recent comparative work demonstrated, using high resolution HD genotyping, that hiPSC lines obtained by non-integrating approaches have lower incidences of genomic aberrations (Kang et al. 2015). The use of high-resolution methods to monitor genomic aberrations in hiPSCs intended for clinical applications will be necessary. Moreover, the focus of current technology development efforts should be the identification of novel pathways that can be manipulated to augment the efficiency and completeness of reprogramming (Jiang et al. 2013), possibly leading to improved methodologies for safe clinical translation.

3.4 iPSC Differentiation and Challenges for Translational-Grade Derivatives: Cardiomyocytes as an Example

Owing their potential of differentiating into virtually all cell types found in the human body (neurons, cardiac muscle cells, hepatocytes, chondrocytes, retinal pigment epithelial cells, and many others), hiPSCs serve as an unlimited source of

human cells for both biomedical research and regenerative medicine purposes. There are a number of ways in which the fate of hiPSCs can be directed towards specific cell lineages and the methodologies are being continuously optimized to improve differentiation efficiency and scalability and enable clinical applications. Depending on the tissue of interest, various differentiation approaches have been explored, ranging from two-dimensional (2D) monolayer cultures with specific growth factors/cytokines and signaling inhibitors, co-culture with supporting cells, up to three-dimensional (3D) differentiation systems. In some cases, when organ development and differentiation pathways are well characterized, it is also feasible to isolate precursor cells at intermediate stages and then direct them further to terminal differentiation (Cao et al. 2013; Reinhardt et al. 2013a). More recently, through the development of 3D culture systems, structures exhibiting multiple cell types that self-organize to form an organ-like tissue, termed "organoids", have been generated from hPSCs (Lancaster and Knoblich 2014; Huch and Koo 2015). To date, derivation methods specific for obtaining intestinal (Spence et al. 2011), kidney (Humphreys 2014), brain (Lancaster et al. 2013), and retinal (Nakano et al. 2012) organoids, as well as liver organoid-like tissues called liver buds (Takebe et al. 2013) have been established, making the therapeutic promise of organoids an area of greatest potential for personalized regenerative medicine.

It is important to keep in mind that the differentiation process of PSCs is considered to mimic developmental processes. Therefore, most of the differentiated cells from hPSCs tend to be a reflection of the early stage of development (i.e., embryonic or infant stage). Such immature cells significantly differ from adult cells. Establishment of mature phenotypes is an important challenge for obtaining functional cells for cell therapy. Likewise, the purity and cell number are critical issues for any translational application. Below, we discuss these aspects in details for the differentiation of hiPSCs towards the cardiac lineage.

Cardiac Differentiation of hiPSCs

Historically, the most common method by which cardiomyocytes have been derived from PSCs has involved the formation of three-dimensional aggregates, so-called embryoid bodies (EBs) (Mummery et al. 2003) (Fig. 3.2). Spontaneous EB differentiation relies on a combination of physical and chemical cues to modulate cell signaling pathways and directs PSCs toward various cell types, with 5–70 % of EBs contain beating cardiomyocytes (Kawamura et al. 2012). High variability between experiments, low cardiomyocyte yield (often, 1 %) and immature cardiomyocyte phenotype (Laflamme et al. 2007; Kawamura et al. 2012) have stimulated researchers to explore alternative methods (Moretti et al. 2013). Coculture systems with END-2 stromal cells (Mummery et al. 2003), cardiac fibroblasts (Ou et al. 2011) or human umbilical-vein endothelial cells (Stevens et al. 2009) have been tested in order to mimic microenvironmental factors that are potentially important for cardiac differentiation (Fig. 3.2). In the last decade, knowledge from *in vivo* developmental studies (Garry and Olson 2006; Evans et al. 2010; Noseda et al. 2011) has guided the establishment of novel 2D and 3D cardiomyocyte differentiation approaches that rely on specific temporal and dose dependent modulation of key

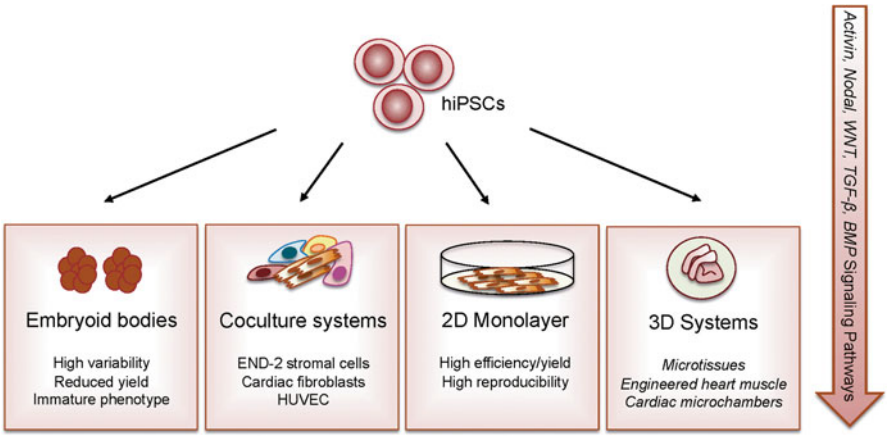


Fig. 3.2 Differentiation of human iPSCs into the cardiac lineage. Methods for differentiation of human iPSCs into cardiomyocytes are based on a combination of physical and chemical cues able to induce temporal and dose dependent modulation of specific signaling pathways with pivotal roles during cardiovascular development (Activin, WNT, transforming growth factor β (TGF-β) and bone morphogenic protein (BMP)). The current differentiation protocols can be divided into three main categories: three-dimensional (3D) systems, which mainly include embryoid bodies and the more recently developed 3D systems (Microtissues, engineered heart muscle and cardiac microchambers); two-dimensional (2D) systems, which include several monolayer directed differentiation protocols; and co-culture systems with cells able to promote cardiogenesis (e.g. END-2 stromal cells, cardiac fibroblasts or human umbilical-vein endothelial cells (HUVEC))

pathways involved in cardiogenesis, such as activin/nodal/transforming growth factor-β, Wnt, and bone morphogenetic protein (Kehat et al. 2001; Mummery et al. 2007; Paige et al. 2010; Burridge et al. 2012; Zhang et al. 2012) (Fig. 3.2). Most recently, specific small molecules have been employed to replace growth factors as modulators of these signaling pathways (Lian et al. 2012; Burridge et al. 2014). Using distinct growth factors and small molecules to specifically direct hPSCs towards the cardiac lineages has allowed to achieve more efficient cardiomyocyte differentiation, with yields as high as 85–95 %, and, thanks to the fully defined culture conditions, more reproducible results (Laflamme et al. 2007; Lian et al. 2012, 2013; Cao et al. 2013; Burridge et al. 2014). Although different hiPSC lines can respond differently to developmental signals because of the intrinsic differences in their genetic background, directed differentiation protocols have been successfully applied to various hiPSCs derived from distinct sources of somatic cells and reprogramming methods (Passier et al. 2005; Paige et al. 2010; Lian et al. 2012; Xu et al. 2012; Okano et al. 2013).

Yet despite the advances in differentiation efficiency, major challenges still remain for safe clinical translation of hiPSC-derived cardiomyocytes. One important issue is their purity and risk of teratomas arising from residual undifferentiated hiPSCs. Several non-genetic methods has been reported to improve cardiomyocyte purity after directed hiPSC differentiation, including cell-surface markers (Mummery et al. 2003; Graichen et al. 2008), mitochondria-specific dyes (Kawamura

et al. 2012), fluorescent probes (Laflamme et al. 2007), and glucose deprivation (Burridge et al. 2014).

Another fundamental concern regarding iPSC cardiac differentiation is the varying degree of heterogeneity achieved in the generated cardiomyocyte population. Current hiPSC differentiation strategies yield a heterogeneous mixture of atrial-like and ventricular-like lineages, as well as pacemaker-like lineages such as atrioventricular node-like, sinoatrial node-like, and Purkinje fiber-like cells (Burridge et al. 2012). A deeper understanding of directed lineage differentiation, followed by its modulation, would facilitate subtype-specific cardiac differentiation. In this respect, recent reports suggest that hiPSCs could be directed either to atrial- or to ventricular-like cardiomyocytes by modulating the retinoic acid (Cao et al. 2013; Lian et al. 2013; Devalla et al. 2015) and Wnt signaling pathways (Kim et al. 2013). Additionally, direct manipulations at the epigenetic level or by achieving mRNA-based delivery of lineage-specific factors have also been tested (Ong et al. 2015).

The most immediate need for potential translational applications of hiPSC-derived cardiomyocytes, however, is to achieve defined culture conditions and standardized protocols that address the issue of cellular maturation. These cells begin contracting in the first 2 weeks of differentiation (Burridge et al. 2014), but have a relative immature phenotype more similar to fetal than to adult cardiomyocytes (Robertson et al. 2013). For instance, at the structural level, hPSC-derived cardiomyocytes have a smaller length-to-width aspect ratio (3:1 compared to 15:1), are mononuclear, have fewer mitochondria, and have poor sarcomere organization (Lundy et al. 2013). Also their global gene expression profile is closer to embryonic than adult cardiomyocytes (Gupta et al. 2010). Finally, from the functional point of view, they show underdeveloped Ca^{2+} handling, low Ca^{2+} buffering capacity in the sarcoplasmic reticulum, slow beat rates (~40 BPM), immature action potential characteristics, abnormal levels of ionic currents, and negative force–frequency relationships (Lundy et al. 2013). Attempts to bypass this limitation have demonstrated that long-term culture enhances the appearance of more mature sarcomeric structural organization and change in global gene expression profile (Otsuji et al. 2010; Lundy et al. 2013). In addition, external cues such as electrical stimulation and mechanical cyclic stretching have been reported to aid in obtaining functionally mature hiPSC-derived cardiomyocytes (Lieu et al. 2013; Hirt et al. 2014a). Improvements in maturation were also achieved via genetic overexpression of distinct factors (Fu et al. 2011; Bett et al. 2013; Lieu et al. 2013) and novel 3D culture methods (Nunes et al. 2013; Rao et al. 2013). Moreover, 3D differentiation systems have also been scaled up to generate three-dimensional microtissues (3D-MTs) (Emmert et al. 2013; Thavandiran et al. 2013), engineered heart muscle (EHM) (Kensah et al. 2013; Hirt et al. 2014a), and more recently cardiac microchambers (Ma et al. 2015). Since low retention rate of transplanted single-cell suspensions remains a major issue for clinical translation, the concept of scaffold-free cellular self-assembly into 3D-MTs or EHMs prior to transplantation may be also beneficial to enhance cellular engraftment and survival. These approaches are currently subjects of intense research (Hirt et al. 2014b).

3.5 Genetic Engineering of iPSCs and Personalized Medicine

With the advent of hPSCs, it has become clear that efficient and precise genome editing is crucial for realizing their full potential in research and therapy.

In particular for hiPSCs, genetic correction of the disease-associated mutation(s) in patient-specific lines serves several purposes (Fig. 3.3). First, it will generate isogenic cells that share a common genotype with the exception of the disease-causing mutation, thereby eliminating confounding effects from genetic heterogeneity. These disease-corrected hiPSCs are the perfect control for any comparative analyses of disease phenotype and allow generation of accurate, reliable, and less expensive *in vitro* human models for understanding diseases and studying genotype/phenotype relationships. Second, genomic modification to directly correct disease-specific point mutations *in vitro* is also valuable for exploring drug development and performing toxicology tests in patient-specific cells. A large majority of identified candidate drugs fail to reach the market because of safety concerns (about one third

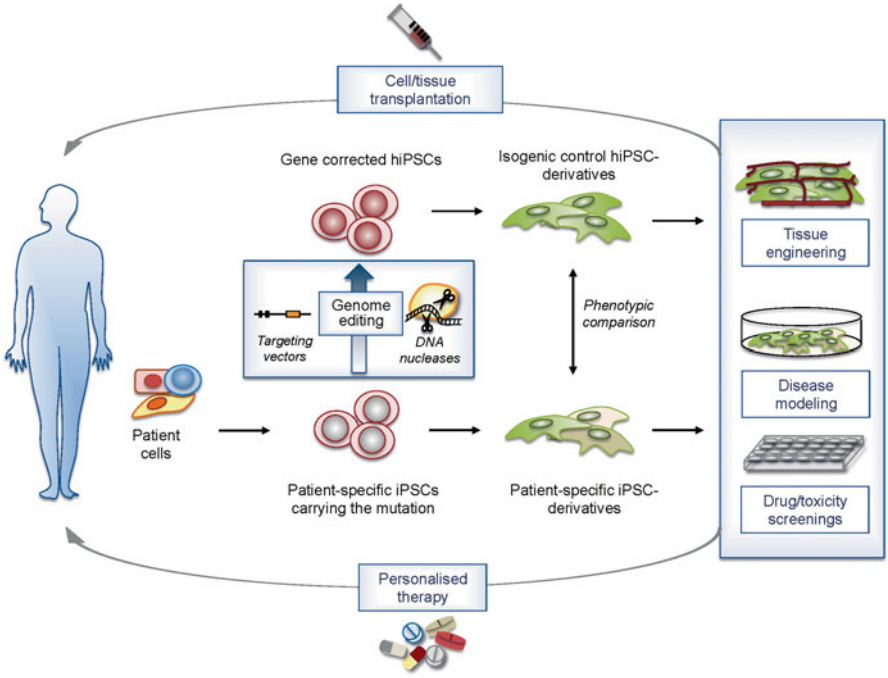


Fig. 3.3 Genetic engineering of hiPSCs and applications in personalized medicine. Patient-specific iPSCs and isogenic control iPSC lines generated through genome editing approaches can be differentiated toward a specific cell type of interest. Patient-specific and corrected iPSC derivatives can then be used for disease modeling studies, tissue engineering approaches, and high-throughput drug/toxicity screenings, thus facilitating personalized therapy and ultimately autologous cell transplantation for regenerative purposes

of pharmaceuticals are withdrawn due to cardiotoxicity (Guo et al. 2011) and efficacy issues). Human iPSC-derived cardiomyocytes are currently being utilized as a system to evaluate novel and existing medications and to test patient-specific drug responses (Liang et al. 2013; Sinnecker et al. 2013; Wang et al. 2014). Finally, genome editing may accelerate the future clinical application of integration-free cell-based gene therapy, including the autologous transplantation of patient-specific, genome-corrected hiPSC-derived target cells. Of note, genetic correction directly in hiPSCs is however not always achievable, because some genetic diseases imply a reprogramming barrier (e.g. Fanconi anemia (Raya et al. 2009)). In those cases, the cells of origin could be corrected before generating patient-specific hiPSCs.

3.5.1 Genetic Manipulation of hiPSCs and Gene Correction Approaches

Owing to the fragile nature of hPSCs when dissociated into single cells and their low transfection frequency, gene targeting in hPSCs present a bigger challenge than in the mouse counterparts. An important contribution to improving the handling of hPSCs was made by Yoshiki Sasai's team whit the discovery of a selective inhibitor of Rho-associated kinase (ROCKi) Y-27632 (Watanabe et al. 2007). The inhibitor significantly suppressed the apoptosis of hPSCs when dissociated, enabling cells to be electroporated and subcloned more easily.

At present, various strategies have been tested and proven for genetic manipulation of patient-specific hiPSCs (Hotta and Yamanaka 2015). Below, we focus exclusively on the approaches that have been used for site-specific genome modification *via* homologous recombination (HR) and emphasize their advantages and limitations.

Targeting Vector Approach

Initial triumphs in gene targeting of hPSCs were achieved by using a targeting vector, which employs long (5–10 kb) and short (1–4 kb) homology arms on both sides (Zwaka and Thomson 2003). Owing to the low frequency of targeting events in general, the classical targeting vector also contained a drug-selection cassette, such as a neomycin resistance gene derived from a ubiquitous PGK gene promoter, for positive selection. Flanking of the selection cassette by two loxP sequences allowed, after successful targeting, its excision by a Cre recombinase. Using this targeting strategy, only few disease-causing mutations have been corrected in patient-specific hiPSCs (Yusa et al. 2011; Bellin et al. 2013), merely due to the inherently low HR efficiencies. In fact, the propensity of a genomic region to undergo HR is dependent on the local chromatin structure and the generation of a double strand break (DSB) at the specific target site (Carroll 2011b), as well as on transit through the S–G2 phase of the cell cycle (Delacote and Lopez 2008). Thus, the nonhomologous end-joining pathway (NHEJ), which is several orders of magnitude more efficient than HR, is responsible for random integration of targeting vectors. Improved HR

efficiency in hPSCs has been achieved by using viral vector-mediated targeting approaches (adeno and adeno-associated viruses) (Mitsui et al. 2009; Khan et al. 2010), which have the advantage of high transduction efficiency, and bacterial artificial chromosome-based strategy (Song et al. 2010), which allows increasing the length of homology between targeting vectors and endogenous loci.

Engineered DNA Nucleases

Another strategy to enhance the efficiency of gene targeting is to introduce site specific DSBs to target loci. DSBs are highly recombinogenic and can stimulate HR in hPSCs by three orders of magnitude (Zou et al. 2009). Cells are obligated to repair the introduced DSBs either by NHEJ, generating small deletions or insertions, or by the homology-directed repair pathway when a homologous donor template is provided. The endonucleases employed must then recognize DNA sequences that occur uniquely at target loci and have minimal off-target activity. Several endonucleases have been engineered to meet these requirements. Currently, the most used for site-specific gene targeting in hPSCs are: zinc finger nucleases (ZFNs), transcription activator-like (TAL) effector nucleases (TALENs), and more recently the clustered regulatory inter-spaced short palindromic repeats (CRISPRs)/Cas9 nucleases (Li et al. 2014). Importantly, owing to the high HR efficiency of genome editing achieved by such nucleases, the HR donor template can be supplied as a single-strand oligonucleotide (ssODN) as short as 80–150 bases, thus facilitating gene correction applications in hiPSCs (Soldner et al. 2011; Ding et al. 2013a, b).

ZFNs are hybrid nucleases that rely on a series of linked zinc finger motifs to recognize specific DNA sequences and the DNA-cleavage domain FokI restriction enzyme to sever DNA. Because FokI nuclease activity depends on dimerization, the ZFN system works as pairs of two monomers of ZFN in reverse orientation that can be designed to bind to a genomic sequence 18–36 nucleotides in length (Porteus and Carroll 2005; Carroll 2011a). Successful ZNF-mediated gene correction in hiPSCs was achieved for several disease-causing mutations (Sebastiano et al. 2011; Reinhardt et al. 2013b). However, due to the complexity of the required engineering steps, ZFNs have been largely supplanted by TALENs and more recently by the CRISPRs/Cas9 nuclease system.

TALENs have a similar structure to ZFNs, but the DNA-binding domain comes from TAL effector proteins and is a tandem array of amino acid repeats. Each of these units is able to bind to one of the four possible nucleotides. TALENs also cleave as dimers (Li et al. 2011) and display not only the unique advantage of easy modular assembly but also enhanced specificity as well as reduced off-target action compared to ZFNs (Li et al. 2011; Pattanayak et al. 2014). As demonstrated in a recent study, TALENs have greatly simplified genome editing in hiPSCs for generating disease models (Ding et al. 2013a). However, despite the initial enthusiasm, TALEN technology has several limitations for future clinical applications of gene-edited hiPSCs. TALEN target-site selection is restricted by the requirement of a preceding T base (Boch et al. 2009). Although this should not prohibit successful design of TALENs in most cases, it may be an issue when modifying a specific

mutation for future cell-based gene therapy. The reported sensitivity of TALENs to 5-methylcytosine could be a more serious drawback of the TALEN technology because of the prevalence of this DNA modification in the genome, though this problem may be overcome by engineering 5-methylcytosine- insensitive TALEN DNA-binding domains (Valton et al. 2012).

CRISPRs/Cas9 are RNA-guided engineered nucleases that have been developed from microbial adaptive immune systems named as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems (Ishino et al. 1987; Jansen et al. 2002). The system utilizes a protein component Cas9 (CRISPR-associated 9) and two small RNAs, crRNA (CRISPR RNA) and tracrRNA (trans crRNA), to mediate target sequence-specific cleavage of double-stranded DNA. To simplify expression in mammalian cells, crRNA and tracrRNA have been fused into one sgRNA (single guide RNA) by a tetranucleotide loop to generate a DSB at a target site. Remarkably, the RNA component of the CRISPR system determines the target sequence based on the Watson-Crick base pairing. Therefore, the design and construction of a target-specific sgRNA is versatile and straightforward and, because of the small size of the sgRNA (20 nucleotides), it is also possible to deliver multiple sgRNAs at the same time to achieve multiplex targeting (Cong et al. 2013; Mali et al. 2013). This makes CRISPRs/Cas9 system as the most accessible means to facilitate and optimize genetic engineering so far (Hsu et al. 2014) and since 2013, several groups have already demonstrated its usefulness for genome editing in hiPSCs (Ding et al. 2013b; Mali et al. 2013; Flynn et al. 2015; Song et al. 2015a). Despite its versatility, also the CRISPR/CAS9 system has several restraints. First, the targetable sites of Cas9 are constrained by the requirement of a $GN_{20}GG$ sequence motif (Jinek et al. 2012), which may cause a problem when targeting certain loci. Second, up to six mismatches between crRNA and target DNA are tolerated by Cas9, which may result in off-target cleavage (Jinek et al. 2012). Indeed, a recent study showed that CRISPR/CAS9 nucleases induce mutations at off-target sites with up to five mismatches (Fu et al. 2013). More importantly, frequencies of off-target mutations are equal to or higher than those of on-target mutations (Fu et al. 2013). Cas9 mutants with a more stringent requirement of crRNA-target DNA complementation may be engineered. For instance, Cas9 has been converted into a nickase, which reduces mutagenesis at off-target sites (Cong et al. 2013).

Thanks to the rapid development of engineered DNA nucleases, genome editing in hiPSCs has evolved from being a daunting task a few years ago to a routine procedure in most laboratories. However, the use of genome editing in the clinic requires very high levels of inspection to ensure safety. A systemic examination of off-target mutagenesis by whole-genome (Kiskinis et al. 2014; Smith et al. 2014; Suzuki et al. 2014; Veres et al. 2014; Yang et al. 2014) or exome sequencing (Yusa et al. 2011; Li et al. 2015) needs to be performed before any clinical translation of genome editing technologies and patient-specific, genome-corrected hiPSC derivatives will be possible.

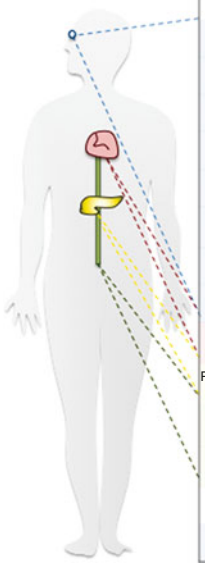
3.6 Clinical Applications of hiPSCs in Regenerative Medicine: Where Do We Stand?

Regenerative medicine aims to replace and/or regenerate damaged cells, organs, or tissues in order to restore normal function. Cell therapy is an important regenerative medicine approach. The inherent pluripotency of hiPSCs, along with their genetic identity to specific patients, raises the possibility of autologous transplantation to treat patients suffering from a myriad of disorders characterized by loss of a key cellular function, such as cardiomyocytes in myocardial infarct, dopaminergic neurons in Parkinson's disease, beta cells in type 1 diabetes, or hematopoietic stem cells in aplastic anemias. In the case of monogenic diseases, in which all the cells from the body initially carry the disease-causing mutation in their genomic DNA, a gene correction approach can be considered to generate disease-free autologous cells, as discussed in the previous paragraph.

However, compared to other cell-based therapies, the Investigational New Drug (IND) review process for hPSCs involves a higher level of scrutiny owing to their potential to form tumors and ectopic tissue. The lack of data on the potential untoward effects of hPSC-derived therapies in humans means that parameters surrounding efficacy, biodistribution, persistence, toxicity, presence of residual pluripotent cell contaminants and tumorigenicity potential all need to be thoroughly addressed before these therapies receive IND approval (Bailey 2012). Preclinical animal models need to be carefully designed to address these issues in a manner that satisfies the regulatory agencies (Frey-Vasconcells et al. 2012). Furthermore, the starting hPSC line itself needs to undergo extensive characterization for assurances of safety, such as analyses of genetic stability, virus and pathogen testing, derivation methods in the spirit of good manufacturing practices (GMPs), maintenance of the line under GMP conditions, and donor screening and eligibility (Carpenter et al. 2009). For hiPSCs, the reprogramming strategy is an additional consideration and those methods that do not involve integration of transgenes into the genome are definitely safer. Another major consideration for hPSC-based therapies is the route of administration. For the time being, therapies that are injected locally or contained within a device that limits their migration may have an easier time achieving IND status than those that are systemically injected, as these approaches help to limit the area in which potential adverse effects may occur. That being said, since 2010 several clinical trails using hPSC-based therapy have been initiated in various countries, as overviewed below.

3.6.1 Clinical Trials Involving hiPSCs

The current wave of clinical trials testing hPSC-based therapy predominantly focuses on hESC-derived cells (Fig. 3.4), including retinal pigment epithelium (for macula degeneration and related diseases) (Schwartz et al. 2012, 2015; Song et al. 2015b), pancreatic endoderm derivatives (for type 1 diabetes) (Schulz et al. 2012;



	Company	Trial location	Disease	Trial stage
hESC-derived cell type				
RPE (MA09-hRPE)	Ocata Therapeutics	USA	Dry AMD	Phase I/II
RPE (MA09-hRPE)	Ocata Therapeutics	USA	Stargardt	Phase I/II
RPE (MA09-hRPE)	Ocata Therapeutics	UK	Stargardt	Phase I/II
RPE (MA09-hRPE)	CHABiotech	Korea	Dry AMD	Phase I/II
RPE (MA09-hRPE)	CHABiotech	Korea	Stargardt	Phase I
RPE (MA09-hRPE)	University of California (with Ocata's cells)	USA	MMD	Phase I/II
RPE (PF-05206388)	Pfizer	UK	Wet AMD	Phase I
RPE (Opregen)	Cell Cure Neuroscience	Israel	Dry AMD	Phase I/II
CD15*ISL-1* cardiac progenitors	Assistance Publique, Hôpitaux de Paris	France	Severe heart failure	Phase I
Panacreatic endoderm (VC-01)	Viacyte	USA	Type I diabetes	Phase I/II
Oligodendrocyte progenitors (AST-OPC1)	Asterias Biotherapeutics	USA	Spinal cord injury	Phase I
Oligodendrocyte progenitors (AST-OPC1)	Asterias Biotherapeutics	USA	Spinal cord injury	Phase I/II
hiPSC-derived cell type				
RPE (autologous)	RIKEN Institute	Japan	Wet AMD	Phase I

Fig. 3.4 Clinical trials with hESC and hiPSC cell derivatives. Current clinical trials involving pluripotent stem cell derivatives target four organ systems: the eye, the heart, the pancreas, and the nervous system. Abbreviation: AMD, age-related macular degeneration; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; MMD, myopic macular degeneration; RPE, retinal pigment epithelium

Pagliuca et al. 2014), oligodendrocytes (for spinal cord injury) as well as cardiac progenitors (for severe heart failure) (Menasche et al. 2015). Yet, only one trial using autologous patient-specific hiPSC derivatives exists, which aims at curing the wet form of age-related macular degeneration using retinal pigment epithelium cells transplanted as sheets (Kamao et al. 2014) (Fig. 3.4). It started in September 2014 with the treatment of the first patient at the Riken Institute in Japan (Reardon and Cyranoski 2014), but was recently put on hold because hiPSCs from a second patient were found to carry genetic mutations.

Considering that hESCs took almost 12 years from the first establishment to the first transplantation into a spinal cord injury patient in October 2010, the transition of patient-specific hiPSCs from bench to bedside was relatively quick and, as time goes on, the number of hiPSC-based clinical trials will probably increase.

3.7 Concluding Remarks

Though hiPSC technology is not even a decade old, it has significantly revolutionized the world of stem cells, disease modeling, drug testing and regenerative medicine. The advent of improved reprogramming methods that do not involve integration

of transgenes into the genome and the rapid development of large-scale culture systems and efficient differentiation protocols as well as of advanced genome-editing technologies has begun to overcome the shortcomings of using hiPSCs in regenerative medicine.

The year 2014 marked the arrival of patient-specific hiPSCs onto the clinical stage, and this is just the beginning. Further efforts are needed to tap the full potential of hiPSC-mediated cell therapy to benefit human health. In addition, incorporating newly emerging genome-editing technologies might trigger a new era of gene therapy using hiPSCs.

Acknowledgments AM would like to acknowledge and thank the German Research Foundation and the German Ministry for Education and Research for their ongoing support of research in the laboratory. AM also acknowledge the Munich Heart Alliance, a member of the German Center for Cardiovascular Research.

References

Aasen T, Raya A, Barrero MJ et al (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 26:1276–1284

Bailey AM (2012) Balancing tissue and tumor formation in regenerative medicine. Sci Transl Med 4:147fs128

Ban H, Nishishita N, Fusaki N et al (2011) Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. Proc Natl Acad Sci U S A 108:14234–14239

Bao L, He L, Chen J et al (2011) Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors. Cell Res 21:600–608

Bar-Nur O, Russ HA, Efrat S et al (2011) Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell 9:17–23

Bellin M, Casini S, Davis RP et al (2013) Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome. EMBO J 32:3161–3175

Bett GC, Kaplan AD, Lis A et al (2013) Electronic "expression" of the inward rectifier in cardiocytes derived from human-induced pluripotent stem cells. Heart Rhythm 10:1903–1910

Boch J, Scholze H, Schornack S et al (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326:1509–1512

Brambrink T, Foreman R, Welstead GG et al (2008) Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell 2:151–159

Burridge PW, Keller G, Gold JD et al (2012) Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. Cell Stem Cell 10:16–28

Burridge PW, Matsa E, Shukla P et al (2014) Chemically defined generation of human cardiomyocytes. Nat Methods 11:855–860

Cao N, Liang H, Huang J et al (2013) Highly efficient induction and long-term maintenance of multipotent cardiovascular progenitors from human pluripotent stem cells under defined conditions. Cell Res 23:1119–1132

Carette JE, Pruszak J, Varadarajan M et al (2010) Generation of iPSCs from cultured human malignant cells. Blood 115:4039–4042

Carpenter MK, Frey-Vasconcells J, Rao MS (2009) Developing safe therapies from human pluripotent stem cells. Nat Biotechnol 27:606–613

Carroll D (2011a) Genome engineering with zinc-finger nucleases. Genetics 188:773–782

Carroll D (2011b) Zinc-finger nucleases: a panoramic view. Curr Gene Ther 11:2–10

Chang CW, Lai YS, Pawlik KM et al (2009) Polycistronic lentiviral vector for "hit and run" reprogramming of adult skin fibroblasts to induced pluripotent stem cells. Stem Cells 27:1042–1049

Chen G, Gulbranson DR, Hou Z et al (2011) Chemically defined conditions for human iPSC derivation and culture. Nat Methods 8:424–429

Cherry AB, Daley GQ (2013) Reprogrammed cells for disease modeling and regenerative medicine. Annu Rev Med 64:277–290

Chou BK, Mali P, Huang X et al (2011) Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Res 21:518–529

Cong L, Ran FA, Cox D et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823

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

Delacote F, Lopez BS (2008) Importance of the cell cycle phase for the choice of the appropriate DSB repair pathway, for genome stability maintenance: the trans-S double-strand break repair model. Cell Cycle 7:33–38

Devalla HD, Schwach V, Ford JW et al (2015) Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. EMBO Mol Med 7:394–410

Ding Q, Lee YK, Schaefer EA et al (2013a) A TALEN genome-editing system for generating human stem cell-based disease models. Cell Stem Cell 12:238–251

Ding Q, Regan SN, Xia Y et al (2013b) Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell 12:393–394

Emmert MY, Wolint P, Wickboldt N et al (2013) Human stem cell-based three-dimensional microtissues for advanced cardiac cell therapies. Biomaterials 34:6339–6354

Esteban MA, Xu J, Yang J et al (2009) Generation of induced pluripotent stem cell lines from Tibetan miniature pig. J Biol Chem 284:17634–17640

Evans SM, Yelon D, Conlon FL et al (2010) Myocardial lineage development. Circ Res 107:1428–1444

Ezashi T, Telugu BP, Alexenko AP et al (2009) Derivation of induced pluripotent stem cells from pig somatic cells. Proc Natl Acad Sci U S A 106:10993–10998

Feng B, Jiang J, Kraus P et al (2009) Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol 11:197–203

Flynn R, Grundmann A, Renz P et al (2015) CRISPR-mediated genotypic and phenotypic correction of a chronic granulomatous disease mutation in human iPS cells. Exp Hematol 43(10):838–848.e3

Frey-Vasconcells J, Whittlesey KJ, Baum E et al (2012) Translation of stem cell research: points to consider in designing preclinical animal studies. Stem Cells Transl Med 1:353–358

Fu JD, Rushing SN, Lieu DK et al (2011) Distinct roles of microRNA-1 and -499 in ventricular specification and functional maturation of human embryonic stem cell-derived cardiomyocytes. PLoS One 6:e27417

Fu Y, Foden JA, Khayter C et al (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31:822–826

Fusaki N, Ban H, Nishiyama A et al (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 85:348–362

Fussner E, Djuric U, Strauss M et al (2011) Constitutive heterochromatin reorganization during somatic cell reprogramming. EMBO J 30:1778–1789

Garry DJ, Olson EN (2006) A common progenitor at the heart of development. Cell 127:1101–1104

Gonzalez F, Barragan Monasterio M, Tiscornia G et al (2009) Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector. Proc Natl Acad Sci U S A 106:8918–8922

Gore A, Li Z, Fung HL et al (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471:63–67
Graichen R, Xu X, Braam SR et al (2008) Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation 76:357–370
Gramlich M, Pane LS, Zhou Q et al (2015) Antisense-mediated exon skipping: a therapeutic strategy for titin-based dilated cardiomyopathy. EMBO Mol Med 7:562–576
Guo L, Abrams RM, Babiarz JE et al (2011) Estimating the risk of drug-induced proarrhythmia using human induced pluripotent stem cell-derived cardiomyocytes. Toxicol Sci 123:281–289
Gupta MK, Illich DJ, Gaarz A et al (2010) Global transcriptional profiles of beating clusters derived from human induced pluripotent stem cells and embryonic stem cells are highly similar. BMC Dev Biol 10:98
Haase A, Olmer R, Schwanke K et al (2009) Generation of induced pluripotent stem cells from human cord blood. Cell Stem Cell 5:434–441
Han X, Han J, Ding F et al (2011) Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. Cell Res 21:1509–1512
Hanna J, Markoulaki S, Schorderet P et al (2008) Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell 133:250–264
Heng JC, Feng B, Han J et al (2010) The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell Stem Cell 6:167–174
Hirt MN, Boeddinghaus J, Mitchell A et al (2014a) Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation. J Mol Cell Cardiol 74:151–161
Hirt MN, Hansen A, Eschenhagen T (2014b) Cardiac tissue engineering: state of the art. Circ Res 114:354–367
Hou P, Li Y, Zhang X et al (2013) Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341:651–654
Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157:1262–1278
Huangfu D, Maehr R, Guo W et al (2008) Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol 26:795–797
Huch M, Koo BK (2015) Modeling mouse and human development using organoid cultures. Development 142:3113–3125
Humphreys BD (2014) Kidney structures differentiated from stem cells. Nat Cell Biol 16:19–21
Ichida JK, Blanchard J, Lam K et al (2009) A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell 5:491–503
Ishino Y, Shinagawa H, Makino K et al (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol 169:5429–5433
Jansen R, Embden JD, Gaastra W et al (2002) Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol 43:1565–1575
Jia F, Wilson KD, Sun N et al (2010) A nonviral minicircle vector for deriving human iPS cells. Nat Methods 7:197–199
Jiang J, Lv W, Ye X et al (2013) Zscan4 promotes genomic stability during reprogramming and dramatically improves the quality of iPS cells as demonstrated by tetraploid complementation. Cell Res 23:92–106
Jinek M, Chylinski K, Fonfara I et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821
Jung CB, Moretti A, Mederos Y, Schnitzler M et al (2012) Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. EMBO Mol Med 4:180–191
Kaji K, Norrby K, Paca A et al (2009) Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458:771–775
Kamao H, Mandai M, Okamoto S et al (2014) Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application. Stem Cell Rep 2:205–218

Kang X, Yu Q, Huang Y et al (2015) Effects of integrating and non-integrating reprogramming methods on copy number variation and genomic stability of human induced pluripotent stem cells. PLoS One 10:e0131128

Kawamura M, Miyagawa S, Miki K et al (2012) Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. Circulation 126:S29–S37

Kehat I, Kenyagin-Karsenti D, Snir M et al (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108:407–414

Kensah G, Roa Lara A, Dahlmann J et al (2013) Murine and human pluripotent stem cell-derived cardiac bodies form contractile myocardial tissue in vitro. Eur Heart J 34:1134–1146

Khan IF, Hirata RK, Wang PR et al (2010) Engineering of human pluripotent stem cells by AAV-mediated gene targeting. Mol Ther 18:1192–1199

Kim D, Kim CH, Moon JI et al (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4:472–476

Kim K, Doi A, Wen B et al (2010) Epigenetic memory in induced pluripotent stem cells. Nature 467:285–290

Kim J, Lengner CJ, Kirak O et al (2011a) Reprogramming of postnatal neurons into induced pluripotent stem cells by defined factors. Stem Cells 29:992–1000

Kim K, Zhao R, Doi A et al (2011b) Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. Nat Biotechnol 29:1117–1119

Kim HT, Lee KI, Kim DW et al (2013) An ECM-based culture system for the generation and maintenance of xeno-free human iPS cells. Biomaterials 34:1041–1050

Kiskinis E, Sandoe J, Williams LA et al (2014) Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. Cell Stem Cell 14:781–795

Laflamme MA, Chen KY, Naumova AV et al (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25:1015–1024

Lancaster MA, Knoblich JA (2014) Organogenesis in a dish: modeling development and disease using organoid technologies. Science 345:1247125

Lancaster MA, Renner M, Martin CA et al (2013) Cerebral organoids model human brain development and microcephaly. Nature 501:373–379

Laurent LC, Ulitsky I, Slavin I et al (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 8:106–118

Li W, Wei W, Zhu S et al (2009) Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. Cell Stem Cell 4:16–19

Li R, Liang J, Ni S et al (2010) A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. Cell Stem Cell 7:51–63

Li T, Huang S, Zhao X et al (2011) Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Res 39:6315–6325

Li M, Suzuki K, Kim NY et al (2014) A cut above the rest: targeted genome editing technologies in human pluripotent stem cells. J Biol Chem 289:4594–4599

Li HL, Fujimoto N, Sasakawa N et al (2015) Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. Stem Cell Rep 4:143–154

Lian X, Hsiao C, Wilson G et al (2012) Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci U S A 109:E1848–E1857

Lian X, Zhang J, Azarin SM et al (2013) Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. Nat Protoc 8:162–175

Liang P, Lan F, Lee AS et al (2013) Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. Circulation 127:1677–1691

Liao J, Cui C, Chen S et al (2009) Generation of induced pluripotent stem cell lines from adult rat cells. Cell Stem Cell 4:11–15

Lieu DK, Fu JD, Chiamvimonvat N et al (2013) Mechanism-based facilitated maturation of human pluripotent stem cell-derived cardiomyocytes. Circ Arrhythm Electrophysiol 6:191–201

Lister R, Pelizzola M, Kida YS et al (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471:68–73

Liu H, Zhu F, Yong J et al (2008) Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. Cell Stem Cell 3:587–590

Loh YH, Agarwal S, Park IH et al (2009) Generation of induced pluripotent stem cells from human blood. Blood 113:5476–5479

Lundy SD, Zhu WZ, Regnier M et al (2013) Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cells Dev 22:1991–2002

Luo J, Suhr ST, Chang EA et al (2011) Generation of leukemia inhibitory factor and basic fibroblast growth factor-dependent induced pluripotent stem cells from canine adult somatic cells. Stem Cells Dev 20:1669–1678

Ma Z, Wang J, Loskill P et al (2015) Self-organizing human cardiac microchambers mediated by geometric confinement. Nat Commun 6:7413

Maherali N, Hochedlinger K (2009) Tgfbeta signal inhibition cooperates in the induction of iPSCs and replaces Sox2 and cMyc. Curr Biol 19:1718–1723

Mali P, Chou BK, Yen J et al (2010) Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes. Stem Cells 28:713–720

Mali P, Yang L, Esvelt KM et al (2013) RNA-guided human genome engineering via Cas9. Science 339:823–826

Marson A, Foreman R, Chevalier B et al (2008) Wnt signaling promotes reprogramming of somatic cells to pluripotency. Cell Stem Cell 3:132–135

Mayshar Y, Ben-David U, Lavon N et al (2010) Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. Cell Stem Cell 7:521–531

Menasche P, Vanneaux V, Fabreguettes JR et al (2015) Towards a clinical use of human embryonic stem cell-derived cardiac progenitors: a translational experience. Eur Heart J 36:743–750

Mikkelsen TS, Hanna J, Zhang X et al (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454:49–55

Mitsui K, Suzuki K, Aizawa E et al (2009) Gene targeting in human pluripotent stem cells with adeno-associated virus vectors. Biochem Biophys Res Commun 388:711–717

Miyoshi N, Ishii H, Nagai K et al (2010) Defined factors induce reprogramming of gastrointestinal cancer cells. Proc Natl Acad Sci U S A 107:40–45

Miyoshi N, Ishii H, Nagano H et al (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8:633–638

Moon JH, Heo JS, Kim JS et al (2011) Reprogramming fibroblasts into induced pluripotent stem cells with Bmi1. Cell Res 21:1305–1315

Moretti A, Bellin M, Welling A et al (2010) Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N Engl J Med 363:1397–1409

Moretti A, Laugwitz KL, Dorn T et al (2013) Pluripotent stem cell models of human heart disease. Cold Spring Harb Perspect Med 3(11):a014027

Mummery C, Ward-Van Oostwaard D, Doevendans P et al (2003) Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107:2733–2740

Mummery C, Van Der Heyden MA, De Boer TP et al (2007) Cardiomyocytes from human and mouse embryonic stem cells. Methods Mol Med 140:249–272

Nagy K, Sung HK, Zhang P et al (2011) Induced pluripotent stem cell lines derived from equine fibroblasts. Stem Cell Rev 7:693–702
Nakagawa M, Koyanagi M, Tanabe K et al (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26:101–106
Nakagawa M, Takizawa N, Narita M et al (2010) Promotion of direct reprogramming by transformation-deficient Myc. Proc Natl Acad Sci U S A 107:14152–14157
Nakano T, Ando S, Takata N et al (2012) Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10:771–785
Narsinh KH, Jia F, Robbins RC et al (2011) Generation of adult human induced pluripotent stem cells using nonviral minicircle DNA vectors. Nat Protoc 6:78–88
Noseda M, Peterkin T, Simoes FC et al (2011) Cardiopoietic factors: extracellular signals for cardiac lineage commitment. Circ Res 108:129–152
Nunes SS, Miklas JW, Liu J et al (2013) Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. Nat Methods 10:781–787
Ohi Y, Qin H, Hong C et al (2011) Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. Nat Cell Biol 13:541–549
Okano H, Nakamura M, Yoshida K et al (2013) Steps toward safe cell therapy using induced pluripotent stem cells. Circ Res 112:523–533
Okita K, Nakagawa M, Hyenjong H et al (2008) Generation of mouse induced pluripotent stem cells without viral vectors. Science 322:949–953
Okita K, Matsumura Y, Sato Y et al (2011) A more efficient method to generate integration-free human iPS cells. Nat Methods 8:409–412
Ong SG, Lee WH, Kodo K et al (2015) MicroRNA-mediated regulation of differentiation and trans-differentiation in stem cells. Adv Drug Deliv Rev 88:3–15
Orban M, Goedel A, Haas J et al (2015) Functional comparison of induced pluripotent stem cell- and blood-derived GPIIbIIIa deficient platelets. PLoS One 10:e0115978
Otsuji TG, Minami I, Kurose Y et al (2010) Progressive maturation in contracting cardiomyocytes derived from human embryonic stem cells: qualitative effects on electrophysiological responses to drugs. Stem Cell Res 4:201–213
Ou DB, He Y, Chen R et al (2011) Three-dimensional co-culture facilitates the differentiation of embryonic stem cells into mature cardiomyocytes. J Cell Biochem 112:3555–3562
Pagliuca FW, Millman JR, Gurtler M et al (2014) Generation of functional human pancreatic beta cells in vitro. Cell 159:428–439
Paige SL, Osugi T, Afanasiev OK et al (2010) Endogenous Wnt/beta-catenin signaling is required for cardiac differentiation in human embryonic stem cells. PLoS One 5:e11134
Park IH, Zhao R, West JA et al (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451:141–146
Passier R, Oostwaard DW, Snapper J et al (2005) Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 23:772–780
Pattanayak V, Guilinger JP, Liu DR (2014) Determining the specificities of TALENs, Cas9, and other genome-editing enzymes. Methods Enzymol 546:47–78
Pera MF (2011) Stem cells: the dark side of induced pluripotency. Nature 471:46–47
Polo JM, Liu S, Figueroa ME et al (2010) Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat Biotechnol 28:848–855
Porteus MH, Carroll D (2005) Gene targeting using zinc finger nucleases. Nat Biotechnol 23:967–973
Rao C, Prodromakis T, Kolker L et al (2013) The effect of microgrooved culture substrates on calcium cycling of cardiac myocytes derived from human induced pluripotent stem cells. Biomaterials 34:2399–2411
Raya A, Rodriguez-Piza I, Guenechea G et al (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460:53–59
Reardon S, Cyranoski D (2014) Japan stem-cell trial stirs envy. Nature 513:287–288

Reinhardt P, Glatza M, Hemmer K et al (2013a) Derivation and expansion using only small molecules of human neural progenitors for neurodegenerative disease modeling. PLoS One 8:e59252

Reinhardt P, Schmid B, Burbulla LF et al (2013b) Genetic correction of a LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression. Cell Stem Cell 12:354–367

Robertson C, Tran DD, George SC (2013) Concise review: maturation phases of human pluripotent stem cell-derived cardiomyocytes. Stem Cells 31:829–837

Samavarchi-Tehrani P, Golipour A, David L et al (2010) Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell 7:64–77

Sanchez-Freire V, Lee AS, Hu S et al (2014) Effect of human donor cell source on differentiation and function of cardiac induced pluripotent stem cells. J Am Coll Cardiol 64:436–448

Schlaeger TM, Daheron L, Brickler TR et al (2015) A comparison of non-integrating reprogramming methods. Nat Biotechnol 33:58–63

Schulz TC, Young HY, Agulnick AD et al (2012) A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS One 7:e37004

Schwartz SD, Hubschman JP, Heilwell G et al (2012) Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 379:713–720

Schwartz SD, Regillo CD, Lam BL et al (2015) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 385:509–516

Sebastiano V, Maeder ML, Angstman JF et al (2011) In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. Stem Cells 29:1717–1726

Seki T, Yuasa S, Oda M et al (2010) Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell 7:11–14

Seki T, Yuasa S, Fukuda K (2012) Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus. Nat Protoc 7:718–728

Shi Y, Do JT, Desponts C et al (2008) A combined chemical and genetic approach for the generation of induced pluripotent stem cells. Cell Stem Cell 2:525–528

Shimada H, Nakada A, Hashimoto Y et al (2010) Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. Mol Reprod Dev 77:2

Sinnecker D, Goedel A, Laugwitz KL et al (2013) Induced pluripotent stem cell-derived cardiomyocytes: a versatile tool for arrhythmia research. Circ Res 112:961–968

Si-Tayeb K, Noto FK, Nagaoka M et al (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51:297–305

Smith C, Gore A, Yan W et al (2014) Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. Cell Stem Cell 15:12–13

Soldner F, Hockemeyer D, Beard C et al (2009) Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell 136:964–977

Soldner F, Laganiere J, Cheng AW et al (2011) Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. Cell 146:318–331

Somers A, Jean JC, Sommer CA et al (2010) Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette. Stem Cells 28:1728–1740

Song H, Chung SK, Xu Y (2010) Modeling disease in human ESCs using an efficient BAC-based homologous recombination system. Cell Stem Cell 6:80–89

Song B, Fan Y, He W et al (2015a) Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. Stem Cells Dev 24:1053–1065

Song WK, Park KM, Kim HJ et al (2015b) Treatment of macular degeneration using embryonic stem cell-derived retinal pigment epithelium: preliminary results in Asian patients. Stem Cell Rep 4:860–872

Spence JR, Mayhew CN, Rankin SA et al (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470:105–109

Stadtfeld M, Nagaya M, Utikal J et al (2008) Induced pluripotent stem cells generated without viral integration. Science 322:945–949

Stadtfeld M, Apostolou E, Akutsu H et al (2010) Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. Nature 465:175–181

Staerk J, Lyssiotis CA, Medeiro LA et al (2011) Pan-Src family kinase inhibitors replace Sox2 during the direct reprogramming of somatic cells. Angew Chem Int Ed Engl 50:5734–5736

Stevens KR, Kreutziger KL, Dupras SK et al (2009) Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. Proc Natl Acad Sci U S A 106:16568–16573

Suzuki K, Yu C, Qu J et al (2014) Targeted gene correction minimally impacts whole-genome mutational load in human-disease-specific induced pluripotent stem cell clones. Cell Stem Cell 15:31–36

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

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

Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Takebe T, Sekine K, Enomura M et al (2013) Vascularized and functional human liver from an iPSC-derived organ bud transplant. Nature 499:481–484

Thavandiran N, Dubois N, Mikryukov A et al (2013) Design and formulation of functional pluripotent stem cell-derived cardiac microtissues. Proc Natl Acad Sci U S A 110:E4698–E4707

Thomson JA, Itskovitz-Eldor J, Shapiro SS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Tomioka I, Maeda T, Shimada H et al (2010) Generating induced pluripotent stem cells from common marmoset (Callithrix jacchus) fetal liver cells using defined factors, including Lin28. Genes Cells 15:959–969

Tsai SY, Clavel C, Kim S et al (2010) Oct4 and klf4 reprogram dermal papilla cells into induced pluripotent stem cells. Stem Cells 28:221–228

Unternaehrer JJ, Daley GQ (2011) Induced pluripotent stem cells for modelling human diseases. Philos Trans R Soc Lond B Biol Sci 366:2274–2285

Utikal J, Maherali N, Kulalert W et al (2009) Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. J Cell Sci 122:3502–3510

Valton J, Dupuy A, Daboussi F et al (2012) Overcoming transcription activator-like effector (TALE) DNA binding domain sensitivity to cytosine methylation. J Biol Chem 287:38427–38432

Veres A, Gosis BS, Ding Q et al (2014) Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. Cell Stem Cell 15:27–30

Wang Y, Liang P, Lan F et al (2014) Genome editing of isogenic human induced pluripotent stem cells recapitulates long QT phenotype for drug testing. J Am Coll Cardiol 64:451–459

Warren L, Manos PD, Ahfeldt T et al (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7:618–630

Watanabe K, Ueno M, Kamiya D et al (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25:681–686

Wilmut I, Schnieke AE, Mcwhir J et al (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813

Woltjen K, Michael IP, Mohseni P et al (2009) piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458:766–770
Wu Z, Chen J, Ren J et al (2009) Generation of pig induced pluripotent stem cells with a drug-inducible system. J Mol Cell Biol 1:46–54
Xu H, Yi BA, Wu H et al (2012) Highly efficient derivation of ventricular cardiomyocytes from induced pluripotent stem cells with a distinct epigenetic signature. Cell Res 22:142–154
Yang L, Grishin D, Wang G et al (2014) Targeted and genome-wide sequencing reveal single nucleotide variations impacting specificity of Cas9 in human stem cells. Nat Commun 5:5507
Yu J, Vodyanik MA, Smuga-Otto K et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920
Yu J, Hu K, Smuga-Otto K et al (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science 324:797–801
Yusa K, Rad R, Takeda J et al (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. Nat Methods 6:363–369
Yusa K, Rashid ST, Strick-Marchand H et al (2011) Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. Nature 478:391–394
Zhang J, Klos M, Wilson GF et al (2012) Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. Circ Res 111:1125–1136
Zhao Y, Yin X, Qin H et al (2008) Two supporting factors greatly improve the efficiency of human iPSC generation. Cell Stem Cell 3:475–479
Zhou W, Freed CR (2009) Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27:2667–2674
Zhou H, Wu S, Joo JY et al (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4:381–384
Zhou T, Benda C, Duzinger S et al (2011) Generation of induced pluripotent stem cells from urine. J Am Soc Nephrol 22:1221–1228
Zhu S, Li W, Zhou H et al (2010) Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7:651–655
Zhu H, Lensch MW, Cahan P et al (2011) Investigating monogenic and complex diseases with pluripotent stem cells. Nat Rev Genet 12:266–275
Zou J, Maeder ML, Mali P et al (2009) Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell Stem Cell 5:97–110
Zwaka TP, Thomson JA (2003) Homologous recombination in human embryonic stem cells. Nat Biotechnol 21:319–321

Chapter 4
Spermatogonial Stem Cells

Ilya Chuykin, Michael Stauske, and Kaomei Guan

Abstract Spermatogonial stem cells (SSCs) constitute one of the most important stem cell systems in the adult body. SSCs are unipotent and respond for spermatogenesis in the male as they can differentiate only into sperms within the testicular niche. The long-term culture of SSCs without loss of their properties provides the opportunity to develop therapeutic strategies and re-initiate spermatogenesis for the patients who become infertile after cancer therapy. Moreover, SSCs can be spontaneously reprogrammed into pluripotent germline stem cells (GSCs) similar to embryonic stem cells (ESCs) when they are removed from their *in vivo* niche and cultured *in vitro* under specific conditions. The advantage of pluripotent GSCs over induced pluripotent stem cells is that the conversion of SSCs into pluripotent GSCs does not require addition of genes using the virus system, which may avoid unpredictable genetic dysfunction. In addition, this may also circumvent the ethical problems associated with human ESCs. The ability to generate patient-specific pluripotent GSCs for autologous transplantation provides the opportunity for cell replacement therapy without the need for immunosuppressant. In this review, we discuss the origin, properties and regenerative potential of SSCs. We summarize recent research findings regarding the mechanisms that regulate the self-renewal of SSCs. We believe that studying the biology of SSCs provides us important information to better understand male fertility. Furthermore, we address the contribution of SSCs and pluripotent GSCs to stem cell-based therapy for infertility treatment as well as for organ regeneration in the future.

Keywords Spermatogonial stem cells • Self-renewal • Differentiation capacity • Plasticity • Therapeutic applications

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_4

4.1 Introduction

In sexually reproducing animals the function of germ cells is to bring the genetic information from one generation to the next. In females oocytes provide one part of the genetic information, whereas in males spermatozoa own the other part. After fertilization both haploid genomes come together and a new offspring is able to grow up.

Spermatogenesis is a well-organized and complex process, which starts with a small number of spermatogonial stem cells (SSCs), the male germline stem cells (GSCs), and can generate 100 million spermatozoa each day in the adult male (de Rooij 1998). Spermatogenesis begins at 5–7 days after birth in rodents and 10–13 years after birth in men. The time from SSC differentiation to production of mature spermatozoa is about 35 days in the mouse and 64 days in the human (Brinster 2007).

In the adult mammalian testis, the seminiferous epithelium is mainly composed of somatic cells and spermatogenic cells (Fig. 4.1a, c). Sertoli cells, the somatic cells in the seminiferous epithelium are crucial for the coordination of spermatogenic

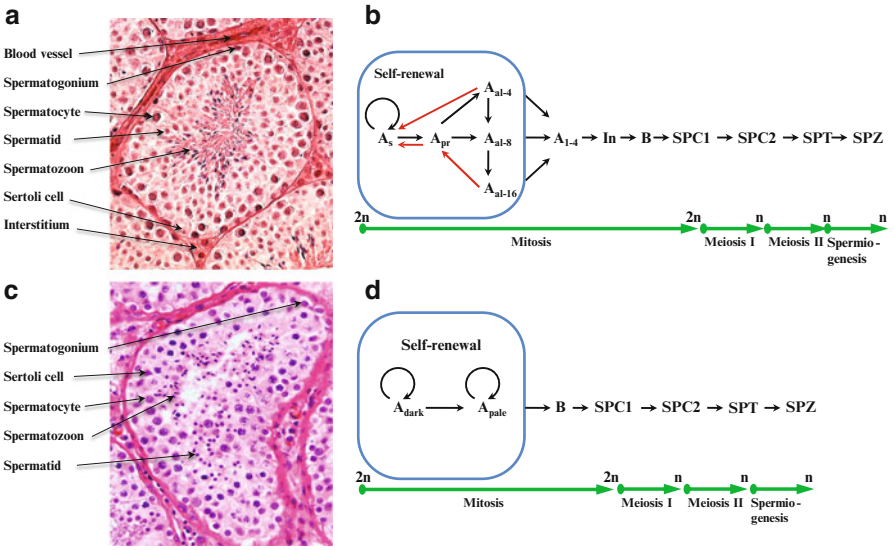

Fig. 4.1 Proposed models of spermatogonial subpopulations in mouse and human testes. (**a**) Cross section of mouse seminiferous tubule with spermatogonial subpopulations and somatic cells. (**b**) Model of self-renewal within the SSC compartment and spermatogenesis in the mouse suggested by Nakagawa et al. in 2010. According to this model spermatogonia type A_{pr} and A_{al} can dedifferentiate into SSCs (A_s spermatogonia). (**c**) Cross section of human seminiferous tubule with spermatogonial subpopulations and somatic cells. (**d**) Scheme for human SSC self-renewal and spermatogenesis proposed by Clermont in the 1960s. *SSC* spermatogonial stem cell, A_s single type A spermatogonium, A_{pr} paired type A spermatogonium, A_{al} aligned type A spermatogonium, *In* intermediate spermatogonium, *SPC1* primary spermatocyte, *SPC2* secondary spermatocyte, *SPT* spermatid, *SPZ* spermatozoon

events, thereby contributing to the well-organized structural and functional construction of the seminiferous epithelium (Jegou 1992). The spermatogenic cells (spermatogonia, spermatocytes, and spermatids) organized in layers are made of several generations of dividing and differentiating cells, which are involved in the production of spermatozoa. Spermatogonia reside at the basement membrane of seminiferous tubules. Spermatocytes are generally located in the middle of the seminiferous epithelium and spermatids (round and elongated) are located in the adluminal region. Spermatogenesis can be divided into three distinct phases: spermatogonial, spermatocyte and spermatid phases. In the spermatogonial phase (also known as mitotic phase or premeiosis), primary spermatocytes are generated as a result of proliferation and differentiation of spermatogonia. In the spermatocyte phase (meiosis I/II), each primary spermatocyte divides into two secondary spermatocytes during meiosis I, and each secondary spermatocyte into two spermatids during meiosis II. In the spermatid phase (also called as spermiogenesis), spermatozoa, also known as sperm cells are formed as a result of the metamorphosis of spermatids (Fig. 4.1b, d) (Clermont 1972). The timing of sequential steps in spermatogenesis is tightly regulated by genes of the germ cell, and Sertoli cells support the differentiation process.

SSCs, the undifferentiated spermatogonia constitute a small population of cells ($2–3 \times 10^4$ per adult mouse testis). In order for spermatogenesis to be maintained, it is essential that SSCs are able to self-renew in the testis. Indeed, SSCs have the capability to self-renew while remaining capable of generating numerous differentiated daughter cells. The small number of SSCs in the adult testis and the complexity of the microenvironment are the main difficulties in SSC research. Studies with SSC transplantation in the adult mouse demonstrate the potential of SSCs in clinical application for the treatment of male infertility (Brinster and Zimmermann 1994; Kanatsu-Shinohara et al. 2006; Kubota et al. 2004b; Kubota and Brinster 2006). Previous studies show that using cryopreservation and SSC transplantation, cancer patients undergoing chemotherapy or radiotherapy can retain their fertility to safeguard their germline (Brinster 2007; Ryu et al. 2006).

Furthermore, recent research shows that under appropriate culture conditions, both neonatal and adult SSCs in the mouse are able to convert into pluripotent embryonic stem cell (ESC)-like cells which can differentiate into derivatives of all three germ layers (Guan et al. 2006; Kanatsu-Shinohara et al. 2004; Seandel et al. 2007; Ko et al. 2009). The derivation of pluripotent stem cells from human testicular tissue may lead to a new source of autologous cells in regeneration of damaged organs. A thorough understanding of molecular mechanisms, especially growth factors and signaling pathways, regulating the fate determination of SSCs has important implications for basic research and for the potential therapeutic application of SSCs in patients.

In the present review, we focus on summarizing the development and characteristics of SSCs, on understanding growth factors and signaling pathways that regulate self-renewal, differentiation, and fate decisions of SSCs, as well as on discussing their implications for basic research and for therapeutic application, with special focus on organ regeneration and infertility.

4.2 Development of SSCs and Their Environment

4.2.1 Origin of SSCs

In many animal phyla, including insects, roundworms, and vertebrates, the distinction between somatic and germ cells occurs at the very early stage of embryonic development. All gametes arise from so-called primordial germ cells (PGCs), the embryonic precursors of the male and female germline. In insects and amphibians, there is a zone found in the cytoplasm of an egg cell, which contains determinants (RNA and protein components) that play an important role in PGC specification. This zone is called germ plasm or pole plasm. Only blastomeres incorporating the germ plasm develop into PGCs. The components of germ plasm prevent PGCs from differentiating into somatic cells by repression of the global transcriptional machinery (Strome and Lehmann 2007). Therefore, the germ plasm or pole plasm is often used as a convenient marker to trace the early ontogeny of germ cells in insects and amphibians (Eddy 1975; Eddy and Hahnel 1983; Mahowald and Hennen 1971).

In mammals such cytoplasmic determinants specifying PGCs are not detected. In early mammalian embryogenesis, the zygote divides three times resulting in a mass of eight cells having equal totipotency. At the 16-cell stage, the morula consists of a small group of internal cells, which remain pluripotent and give rise to the inner cell mass (ICM), and a larger group of external cells at the periphery, which become the trophectoderm cells, the first differentiated embryonic cell types. In the blastocyst, the ICM and the trophoblast cells become separate cell layers, neither of which contributes cells to the other group. Subsequently, a part of ICM differentiates into the primitive endoderm, and the remaining part of the ICM cells develops into the amniotic ectoderm and the embryonic epiblast. The embryonic epiblast is believed to be pluripotent and able to give rise to all cells of the three embryonic germ layers, as well as germ cells. PGCs are derived from the epiblast during gastrulation in rodents and humans (McLaren 2003).

In the mouse, a group of about 50–100 epiblast cells are first distinguishable at embryonic day 7.25–7.5 within the extraembryonic mesoderm in the distal portion of the primitive streak and at the base of allantoic buds. In humans, at about 21–22 days of gestation PGCs are first recognizable at the same region as that in the mouse, the wall of yolk sac near the developing allantois (De Felici et al. 2004). Both mouse and human PGCs are recognized by their alkaline phosphatase activity (McLaren 2003). The reason that PGCs evolve outside the actual embryo in extraembryonic tissues might be that here somatic paracrine factors cannot reach these cells and therefore they escape from a somatic cell fate. After collecting at the allantois, the PGCs propagate and at the same time move from the adjacent yolk sac through the hind gut and dorsal mesentery into the genital ridges.

At mouse embryonic day 12.5, the gonad of males becomes morphologically different from females. In humans, the first signs of sexual differentiation appear at the end of week seven. In the male genital ridge, PGCs become enclosed by the somatic supporting cells, the differentiating Sertoli cells, and seminiferous cords are

formed. The germ cells residing within seminiferous cords are called gonocytes and differ morphologically from PGCs. At embryonic day 13.5 in mice and at 18–20 weeks in humans, gonocytes arrest in the G0/G1 phase of the cell cycle and cease mitosis. The halt of proliferation is characteristic for the transition from PGCs to gonocytes, and at the same time the cell size increases in more than fourfold (Donovan and de Miguel 2004). In the fetus, gonocytes are located in the center of the tubules. Following birth in mammals, gonocytes migrate to the seminiferous tubule basement membrane and reenter the cell cycle. In the mouse testis, the ^{3}H Thymidine labeling index of gonocytes is 10.4 % at one day after birth, 20.1 % at day two, and 24.1 % at day three (Donovan and de Miguel 2004; Vergouwen et al. 1991), at which time the first A spermatogonia (also known as SSCs) are identified (de Rooij 1998). In mice, the SSC pool arises from gonocytes approximately 6 days after birth. From the puberty on the task of SSCs in the male is to provide an unlimited supply of progenitors for differentiation into mature spermatozoa.

4.2.2 Molecular Controls in SSC Development

In the mouse, bone morphogenetic proteins (BMPs) produced by the extraembryonic ectoderm induce a small number of epiblast cells to become PGCs (Lawson et al. 1999). Although the precise molecular mechanism to establish the germline in the early embryo is not yet completely clear, there are evidences pointing out an important role of transcription inhibitor B lymphocyte-induced maturation protein 1 (Blimp1, also Prdm1) in the establishment of the germ cell lineage in mice (Ohinata et al. 2005, 2006). Blimp1 expression is first detected in a few epiblast cells at embryonic day 6.25. Blimp1 together with Prdm14 plays a critical role during PGC specification from post-implantation epiblast cells. They together induce repression of the somatic program, and initiate epigenetic reprogramming in early germ cells toward an underlying pluripotent state, which is equivalent to ESCs (Ohinata et al. 2005, 2006). In addition, PGCs express germline-specific transcriptional factors and genes, such as Oct4, Stella (also known as PGC7 and Dppa3), Nanos3, Dead end, and Vasa (McLaren 2003; Saitou et al. 2002; Sato et al. 2002; Yabuta et al. 2006). The expression of homeotic genes (*Hox*-genes) commonly present in somatic cells is downregulated in developing PGCs (Saitou et al. 2002).

The process of division and migration of PGCs from the adjacent yolk sac into the genital ridges is strictly dependent on the c-Kit/stem cell factor (SCF) signal transduction pathway. Mouse embryos homozygous for mutation in *c-Kit* gene are deficient in germ cells (Buehr et al. 1993). In the absence of SCF, the ligand of c-Kit receptor, the motility of PGCs is dramatically decreased (Gu et al. 2009). The migration of PGCs is critically dependent on the interaction with extracellular matrix proteins, especially, with laminin (García-Castro et al. 1997). Cell surface receptor subunit β1-integrin plays an important role in colonization of the genital ridges, as in *β1-integrin* knockouts PGCs did not enter the embryonic gonads as efficiently as in wild-type mice (Anderson et al. 1999).

The transition from PGCs to gonocytes is marked by a decrease in germ cell proliferation and correlates with a decrease in the levels of c-Kit expression (Donovan and de Miguel 2004). At the same time, gonocytes lose expression of stage specific embryonic antigen 1 (SSEA-1), which is expressed by PGCs and begin to express the germ cell nuclear antigen 1 (GCNA-1), an antigen of unknown function. The gonocytes also lose adhesiveness to fibronectin and laminin (De Felici and Dolci 1989; Donovan and de Miguel 2004).

The gonocytes enter the mitotic arrest that persists until a few days after birth when mitotic activity resumes in these cells (McLaren 2003). In the adult testis, c-Kit is re-expressed in differentiating spermatogonia, but not in SSCs (Ohta et al. 2000; Schrans-Stassen et al. 1999).

Retinoic acid and other agents inducing spermatogonial differentiation, such as BMP4, stimulate c-Kit expression in undifferentiated spermatogonia (Pellegrini et al. 2003; Zhou et al. 2008a). The SCF, expressed by Sertoli cells, stimulates proliferation of type A spermatogonia (Dolci et al. 2001), but, together with retinoic acid it is also important for triggering meiotic entry of type B spermatogonia (Rossi et al. 2008). Indeed, a point mutation in the *Kit* gene does not cause any significant reduction in PGC number during embryonic development, or in SSC populations, but males are completely sterile for a block in the initial stages of spermatogenesis (Blume-Jensen et al. 2000; Kissel et al. 2000).

Furthermore, germ cell development involves epigenetic regulation of chromatin modifications and DNA methylation. The inheritance of the epigenetic modifications is reprogrammed in germ cells, but is relatively faithful in somatic cells. In mice, at embryonic day 7 when PGC specification occurs, levels of genome wide DNA methylation, histone H3 lysine-9 di-methylation (H3K9me2) and lysine-27 tri-methylation (H3K27me3) are similar to those in surrounding somatic cells (Seki et al. 2005). Immediately after their fate determination, the established PGCs undergo extensive erasure of genome-wide H3K9me2 and DNA methylation, two major repressive epigenetic modifications, and instead acquire high levels of H3K27me3 during their migration to genital ridges (Ohinata et al. 2006; Schaefer et al. 2007). Once arriving at the genital ridge at embryonic day 11.5 in mice and by the fifth week of human development, PGCs undergo erasure and re-establishment of parental imprints during male and female gametogenesis before being passed to the next generation. Re-establishment occurs only after sex determination has been initiated, for review see (Saitou et al. 2012). This is a critical point where the development of male and female germ cells goes differently. Under the influence of retinoic acid, produced by developing mesonephros, female germ cells enter meiosis. However, in the developing testis Cyp26b1, a retinoic acid-degrading enzyme expressed by Sertoli cells, prevents the meiosis in male germ cells. As found in *Cyp26b1* knockout mouse embryos, germ cells enter meiosis precociously (Bowles et al. 2006). Methylation of paternally imprinted genes is established in gonocytes up to the newborn. The newly established methylation imprints in gonocytes are then maintained through meiosis and passed to mature spermatozoa (Kato et al. 2007).

4.2.3 Classification of SSCs

In the mature mammalian testis, different germ cell types can be discriminated *in vivo*. Spermatogonia can be classified into different subtypes based on different morphologies. In the mouse, single type A spermatogonia are denoted as A_{single} (A_s), the most primitive cells located directly at the basal membrane of seminiferous tubules. Their percentage in the testis amounts to about 0.02–0.03 % of all germ cells (Tegelenbosch and de Rooij 1993). Through symmetrical division either two daughter A_s cells or two A_{paired} (A_{pr}, two cell cysts) spermatogonia arise out of one mother A_s cell (Dym and Fawcett 1971; Greenbaum et al. 2006). A_{pr} spermatogonia are connected through a cytoplasmic bridge. They can divide furthermore and generate up to 32 jointly connected spermatogonia of the subtype $A_{aligned}$ (A_{al}, 4, 8, or 16 cell clusters) (de Rooij 2001). Further differentiation is orientated from the basal membrane of the seminiferous tubule towards the lumen. A_{al} spermatogonia differentiate into A_1, which subsequently go through six synchronous mitoses generating A_2, A_3, A_4, intermediate (IN) and B spermatogonia and spermatocytes. The spermatocytes further undergo meiosis and continue differentiation into spermatids and mature spermatozoa (de Rooij 2001). This linear model proposed in 1971 by Huckins (1971) and Oakberg (1971) suggests that stem cell capacity resides within A_s cells, whereas other A-type spermatogonia represent transit-amplifying progenitors, which divide uni-directionally to generate longer cysts (Fig. 4.1b). The simplicity of the linear model has led it to be widely accepted, despite claims that early cysts could reverse their paths and even replenish stem cells after tissue damage (Dym and Clermont 1970). However, the transplant assay demonstrates that all A-type spermatogonia, including the A_s, A_{pr}, and A_{al} spermatogonia have stem cell potential (Orwig et al. 2008). Recently, Nakagawa et al. evaluated this straightforward linear model by applying the combination of lineage tracing and live imaging system and demonstrated that A_{pr} and A_{al} spermatogonia were not committed unidirectionally to differentiation but capable of reverting to A_s by fragmentation, and that the fate of individual spermatogonial populations was markedly altered during regeneration after damage (Fig. 4.1b) (Nakagawa et al. 2010).

In humans, there is still very little known about spermatogonial self-renewal. Clermont identified and characterized two spermatogonial subtypes of type A spermatogonia according to the staining pattern and the morphological characteristics of their nucleus nearly 50 years ago (Clermont 1963, 1966). They are referred to as dark type A (A_{dark}) and pale type A (A_{pale}) spermatogonia. The A_{dark} spermatogonia have a discoid nucleus containing a deeply stained dust-like chromatin and a cavity with a pale stained material in the central part of the nucleus. Very often one or more nucleoli closely to the nuclear membrane are visible. The A_{pale} spermatogonia have an ovoid or discoid nucleus containing a pale staining granulated chromatin and showing one or two nucleoli attached to the nuclear envelope. In the human testis, beginning at approximately 2 months of age, gonocytes are replaced by A_{dark} and A_{pale} spermatogonia. According to Clermont's model both A_{dark} and A_{pale} spermatogonia are the stem cells. Specifically, A_{dark} spermatogonia are the presumptive

reserve stem cells which represent a backup of SSCs, divide rarely but can be triggered to self-renew in case of injury or disease. In contrast, the A_{pale} spermatogonia represent the active stem cell pool which can self-renew and differentiate continuously to yield type B spermatogonia, which further differentiate into spermatocytes (Fig. 4.1d) (Clermont 1963, 1966, 1972). Therefore, in humans fewer mitotic steps are required to obtain spermatocytes and the efficiency of clonal expansion is much lower in comparison to rodents (Bustos-Obregon et al. 1975; Johnson 1994; Johnson et al. 1999, 2001). Although this classification for human spermatogonia has been adopted by most researchers, the described model is challenged recently by Ehmcke and Schlatt, who suggest that A_{pale} spermatogonia could undergo additional mitotic divisions (Ehmcke and Schlatt 2006). It has been demonstrated that in primates, a higher mitotic turnover is required from A_{pale} spermatogonia whose proliferation increases the total number of germ cells (Ehmcke et al. 2006). The role of stem cells is therefore limited to A_{dark} spermatogonia, which replenish the progenitor compartment (A_{pale} spermatogonia) in case of cytotoxic or natural depletion (Ehmcke et al. 2006). Nevertheless, up to now, very little new information is available on the true identity of human SSCs and on the process of their self-renewal and differentiation (Dym et al. 2009).

4.2.4 SSC Niche

The term "stem cell niche" is used to describe the microenvironment in which stem cells are found. It interacts with stem cells to regulate the stem cell fate. A stem cell niche comprises cells, extracellular matrix components, and local soluble factors. The maintenance and differentiation of SSCs in the adult mammalian testis take place in the seminiferous epithelium. Peritubular myoid cells and Sertoli cells are the structural basis for the SSC niche. Peritubular myoid cells form the outer layer of seminiferous tubules, whereas Sertoli cells encompass and nourish the germ cells forming the scaffolding structure of the seminiferous tubules. Peritubular myoid cells contribute to the contractile activity of testicular tubules and maintain mesenchymal-epithelial interactions with Sertoli cells both by cooperation in the deposition of extracellular matrix elements and by secretion of paracrine agonists, for example, PModS (Peritubular factor that Modulates Sertoli cell function) (Verhoeven et al. 2000). It has been reported that fibroblast growth factor (FGF) 2 and FGF9 can mediate mesenchymal-epithelial interactions of peritubular and Sertoli cells in the rat testis (El Ramy et al. 2005). However, peritubular myoid cells are present in all areas of the tubule basal membrane, and do not likely determine the location of the SSC niche (de Rooij 2009).

Sertoli cells play a key role in the formation of the niche for SSCs. Tight junctions formed between neighboring Sertoli cells constitute a protective blood-testis barrier, which separates seminiferous epithelium into basal and adluminal compartments. All the stages of spermatogonia before meiosis reside in the basal compartment (de Rooij and Russell 2000; Fijak et al. 2011). Advanced meiotic spermatocytes

and all post-meiotic germ cells are located in the immune privileged adluminal compartment (de Rooij and Russell 2000; Fijak et al. 2011). In the absence of Sertoli cells, successful and complete spermatogenesis resulting in mature sperm has not been demonstrated in mammals. Accumulating data have shown that Sertoli cells provide the necessary ligands to spermatogonia, such as glial cell-derived neurotrophic factor (GDNF) (Meng et al. 2000), SCF (Feng et al. 2000), FGF2 (Oatley and Brinster 2008), and the ligand of non-canonical Wnt pathway, Wnt5a (Yeh et al. 2011). These factors mediate external signals that define the proliferation rate and survival of spermatogonia and control spermatogenesis (see below).

The rate of spermatogenesis is controlled by sex hormones, but it appears that germ cells are affected by this regulation indirectly (Walker 2011). Interestingly, transplantation experiments revealed that rat SSCs supported by mouse Sertoli cells were differentiating with the timing characteristic of the rat, and generated the spermatogenic structural pattern of the rat, demonstrating that the timing of the cell differentiation process of spermatogenesis was regulated by germ cells alone (França et al. 1998; Griswold 2004). However, somatic cells of the seminiferous epithelium define the efficiency of spermatogenesis and connect the process to the hormonal regulation. It is known that peritubular and Sertoli cells, but not germ cells are affected by androgens produced by Leydig cells, which reside in the interstitial tissue of the testis (Fijak et al. 2011). The enhanced colonization of SSCs was observed in the testes of recipient mice treated with leuprolide which lowers testosterone levels (Ogawa et al. 1998).

Interestingly, small blood vessels running near the tubule wall or patches of Leydig cells determine the size of the niche (de Rooij 2009). Yoshida and colleagues found that undifferentiated spermatogonia (A_s, A_{pr}, and A_{al}) clustered along blood vessels and interstitium, whereas the later A_1 and A_2 divisions occurred away of this original position. Within the zone facing the interstitium and blood vessels, stem cells exhibited a further preference for the branch points of the blood vessels (Yoshida et al. 2007). It remains to be determined on what this SSC localization is dependent. It may be the components of the interstitial tissue that determine whether Sertoli cells produce factors to induce self-renewal and differentiation of SSCs. Furthermore, peritubular myoid cells, interstitial cells and blood-born factors may also have direct effects on maintenance and/or differentiation of SSCs.

4.3 Characteristics of SSCs

4.3.1 Marker Expression of Mouse SSCs

The surface phenotype of mouse undifferentiated spermatogonia, including SSCs is major histocompatibility complex (MHC) class 1 (MHC-1)$^-$ thymus cell antigen 1 (Thy-1)$^{low/+}$ c-Kit$^-$ β1-integrin$^+$ α6-integrin$^+$ αv-integrin$^{-/dim}$ at all postnatal ages (Kubota et al. 2003, 2004a; Shinohara et al. 1999). SSCs are also positive for cell

surface markers, such as Ep-CAM, CD9, GDNF receptor (GFR)α1, c-Ret receptor tyrosine kinase (RET), and an orphan adhesion-type G-protein-coupled receptor (GPR125) (Buageaw et al. 2005; Kubota et al. 2003; Naughton et al. 2006; Ryu et al. 2004; Seandel et al. 2007). Antibodies specific to the listed antigens allow enriching of SSCs by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). A well-known cell adhesion molecule E-cadherin is also a marker of mouse SSCs. E-cadherin appearance coincided with expression of promyelocytic leukemia zinc finger (PLZF), a crucial self-renewal factor of rodent SSCs, and its expression was decreased in c-Kit-positive differentiating spermatogonia (Tokuda et al. 2007; Tolkunova et al. 2009).

SSCs have a common feature with other adult stem cells, the ability to exclude DNA binding dye Hoechst. Therefore, SSCs can be identified as so-called side population after staining with Hoechst (Falciatori et al. 2004; Lassalle et al. 2004). Hoechst efflux can be prevented by a specific inhibitor Ko143 of ATP-binding cassette subfamily G member 2 (Abcg2 or Bcrp), suggesting that the 'side population' phenotype of SSCs is dependent on Abcg2 activity (Falciatori et al. 2004). RNA expression analysis demonstrated that the side population in testicular cells contains spermatogonial cells expressing germline stem cell markers *α6-integrin* and *Stra8* (Lassalle et al. 2004).

Recently, Nakagawa et al. propose that the marker expression appears to be the better indicator of the fate of individual spermatogonia over the morphological criteria. PLZF and E-cadherin have essentially identical expression patterns and are found in all A_s, A_{pr} and A_{al} spermatogonia (Nakagawa et al. 2010) whereas GFRα1 mostly marks A_s or A_{pr} spermatogonia and neurogenin (NGN)3-positive cells are mainly A_{al} (Nakagawa et al. 2010; Yoshida et al. 2007). A cytoplasmic protein encoded by the retinoic acid-responsive gene *Stra8*, is a specific marker for premeiotic spermatogonia and their progenitors (Giuili et al. 2002; Guan et al. 2006). Additionally, the pluripotency factor Lin28 marks also all A_s, A_{pr} and A_{al} spermatogonia, and Lin28-positive cells exist as two subpopulations: NGN3-negative (high stem cell potential) and NGN3-positive (high differentiation commitment) cells (Zheng et al. 2009). Another pluripotency factor Oct4 (POU5F1), a germline-specific transcriptional factor, is also expressed in mouse SSCs (Ko et al. 2009; Ohbo et al. 2003; Ohmura et al. 2004). Knockouts for several transcription factors, such as *TAF4b* (Falender et al. 2005), Ets variant gene 5 (*Etv5*) (Tyagi et al. 2009) and *PLZF* (Buaas et al. 2004; Costoya et al. 2004) resulted in impairment of spermatogonial compartment and therefore appear to be crucial for spermatogenesis. Mice with targeted disruption of *Etv5* showed total loss of undifferentiated spermatogonia resulting in a Sertoli cell-only phenotype and aspermia. Sertoli cells from *Etv5* knockout mice revealed a significant decrease in expression of several chemokines. Chemotaxis assays demonstrated that migration of SSCs towards Sertoli cells from *Etv5* knockout mice was significantly decreased in comparison to migration towards wild-type Sertoli cells. Rescue assays using recombinant chemokines indicated that C-C-motif ligand 9 (CCL9) facilitated Sertoli cell chemoattraction of SSCs, which express C-C-receptor type 1 (CCR1). This study also revealed that there was a protein-DNA interaction between *Etv5* and CCL9,

suggesting that *Etv5* might be a direct regulator of CCL9 expression (Simon et al. 2010).

During last 10 years many labs performed transcriptome profiling studies in order to identify gene signatures characteristic for germ cells at different stages of development. These studies include time course of testis development during embryogenesis (Shima et al. 2004; Small et al. 2005), SSCs derived from neonatal testis (Hofmann et al. 2005), and SSCs culture *in vitro* (Carlomagno et al. 2010; Hamra et al. 2004; Oatley et al. 2006). These studies describe new genes expressed in the SSCs as well as operating molecular pathways. For instance, Oatley et al. and Hoffmann et al. have discovered genes upregulated after stimulation of SSCs with GDNF (see below). Interestingly, genes with maximal change in expression are largely not coincident in these two studies. One reason could be the use of freshly isolated GFRα1$^+$ spermatogonia in one study (Hofmann et al. 2005) and long-term cultured SSCs in another (Oatley et al. 2006). Another reason could be different culture conditions used (Caires et al. 2010). Therefore, new high-throughput screenings, especially employing combination of enriched SSCs and long-term cultured germ cells from the same origin would help to identify new SSC genes and signaling pathways involved in propagation and differentiation of these cells.

4.3.2 Properties of Human SSCs

Studies related to the fundamental questions of SSC biology have been mostly performed with mice, to a less extent with rats and pigs and very few with primates including humans. Interesting and challenging question is to what extent the discovered mechanisms in animal models are relevant for human SSCs. Many studies showed that human and rodent spermatogonia share many but not all phenotypic markers (Dym et al. 2009). Similar to mouse SSCs, human SSCs are positive for CD49f (α6-integrin), GPR125, CD9, CD90 (Thy-1), GFRα1, MAGE-4, and VASA, and negative for CD117 (c-Kit) (He et al. 2010, 2012; Izadyar et al. 2011; Sadri-Ardekani et al. 2009). PLZF was found in monkey A_{dark}/A_{pale} (Hermann et al. 2007) and presumably human SSCs (Dym et al. 2009). It might be that the PLZF function is conserved between rodents and primates. Strategies developed for isolation of mouse SSCs including morphology-based selection, laminin selection and MACS using cell surface markers such as CD49f, GPR125, CD9 and SSEA4 have been applied for the enrichment of human SSCs (Golestaneh et al. 2009b; He et al. 2010, 2012; Izadyar et al. 2011; Kossack et al. 2009; Lim et al. 2010; Mizrak et al. 2010; Sadri-Ardekani et al. 2009).

In contrast to mouse SSCs, human SSCs do not express CD29 (β1-integrin) (Izadyar et al. 2011). Oct4 is not detected in adult human spermatogonia (Looijenga et al. 2003). In addition, other rodent markers, including NGN3, RET and Stra8 have not been studied in human spermatogonia. Further investigations to uncover the similarities and/or differences in spermatogonial phenotypes between humans and rodents are necessary.

4.3.3 Potential Plasticity of SSCs

It is well accepted that SSCs are unipotent when they are located within the testis. However, many studies demonstrate that mouse SSCs cultured *in vitro* acquire a remarkable potential plasticity once they are removed from their *in vivo* niche and can be reprogrammed by culture conditions alone into pluripotent stem cells (Guan et al. 2006; Kanatsu-Shinohara et al. 2004; Ko et al. 2009; Seandel et al. 2007), suggesting that pluripotency is kept in the adult male germline.

The first report on generation of germline cell-derived pluripotent stem cells can be traced back to 1992. When PGCs are cultured in the presence of SCF, leukemia inhibitory factors (LIF) and FGF2, they can become pluripotent stem cells, so called embryonic germ cells (EGCs) (Matsui et al. 1992; Resnick et al. 1992). They show similar characteristics as ESCs, which are derived from the inner cell mass of early blastocyst, can spontaneously differentiate into multiple cell phenotypes *in vitro*, and form teratomas in nude mice (Matsui et al. 1992). In 1998, human EGCs were obtained from human PGCs of 5- to 11-week embryos exposed to the same growth factors (Shamblott et al. 1998). In 2004, Shinohara and colleagues showed that SSCs isolated from mouse neonatal testis could be reprogrammed to pluripotent GSCs by culture conditions. These pluripotent GSCs show a morphological phenotype similar to ESCs, and can be maintained *in vitro* for a long time period and differentiated into various cell types both *in vivo* and *in vitro*. Two years later, we demonstrated that mouse adult spermatogonia were able to be reprogrammed into ESC-like pluripotent GSCs (Guan et al. 2006). Similar to mouse ESCs, the generated pluripotent GSCs express the cell surface marker SSEA-1, and transcription factors Oct4, Sox2, Nanog and Rex-1 and are also positive for alkaline phosphatase (Guan et al. 2006). It is worthy to mention that reprogramming of adult spermatogonia into pluripotent GSCs takes place spontaneously in culture. There is neither the addition of oncogenes nor the use of virus systems as described for generation of induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). This study was then confirmed by several groups (Izadyar et al. 2008; Ko et al. 2009; Seandel et al. 2007). Seandel et al. generated pluripotent GSCs from GPR125 positive spermatogonia (Seandel et al. 2007) whereas two other studies established pluripotent GSC cultures from Oct-EGFP positive SSCs (Izadyar et al. 2008; Ko et al. 2009). Although isolation methods of SSCs differ among these studies, the generated pluripotent GSCs exhibit similar characteristics as ESCs.

Following these mouse studies, many efforts have been put in the human SSC research. Several studies reported the generation of pluripotent GSCs from human testis (Conrad et al. 2008; Golestaneh et al. 2009b; Kossack et al. 2009; Mizrak et al. 2010). However, in comparison to human ESCs, these cells do not fulfill all criteria for pluripotency: (1) they show a limited potential for teratoma formation; (2) expression levels of pluripotency-specific genes such as *OCT4, NANOG* and *SOX2* in generated human pluripotent GSCs are much lower than that in human ESCs; and (3) the promoters of *OCT4* and *NANOG* genes are only partially demethylated compared to human ESCs. The possible explanation could be that the cells

have not been completely reprogrammed to the pluripotent state under the conditions used in those studies. Moreover, the cellular origin of these GSC cultures has been questioned. In all of these studies, the isolated cells were not analyzed for the expression of testicular somatic cell-specific genes to rule out the presence of other cell types, and the markers used for characterization of the isolated cells are not specific for human SSCs (Dym et al. 2009). It was demonstrated that the global gene expression profile in one of the reported human pluripotent GSC cultures was similar to that of human testicular fibroblasts but not to human SSCs (Ko et al. 2010; Tapia et al. 2011). The article published in Nature (Conrad et al. 2008) was retracted in 2014 due to that "the level of proof of pluripotency shown is not in line with regular criteria for such papers in Nature". Notably, GPR125 was also used for isolation of human SSCs (He et al. 2010). However, conversion of human GPR125-postive cells into pluripotent GSCs has not been reported.

Addition to SSCs giving rise to pluripotent GSCs spontaneously in culture, SSCs were reported to be able to directly transdifferentiate in *vivo* into somatic cell types including prostatic, uterine, and skin epithelium when they were recombined with the appropriate mesenchyme (Simon et al. 2009). Two other studies showed that SSCs were able to alter their cell fate *in vivo* upon interaction with the mammary gland microenvironment (Boulanger et al. 2007) or after injection into the bone marrow (Ning et al. 2010). Further studies are necessary to understand the molecular mechanism involved in the transdifferentiation process of SSCs (Cooke et al. 2015).

4.3.4 SSC Culture Conditions

Development of conditions for the long-term cultivation of SSCs *in vitro* is important to study the fundamental questions concerning the spermatogonial lineage and to make the use of these cells a valid clinical option. For the establishment of SSC culture *in vitro* the layer of feeder cells and appropriate medium containing all necessary supplements and growth factors are required. Initially, there were studies using Sertoli cell lines as feeder cells (Hamra et al. 2004). However, the maintenance of mouse SSCs was also achieved with mouse embryonic fibroblasts (MEFs) primarily derived from day 13.5 embryos (Guan et al. 2009). The concentration of feeder cells with $20–50 \times 10^3$ cells per cm^2 on the plate is an important point for successful establishment of SSC cultures. Whereas mouse SSCs can be cultured in the presence of fetal calf serum, the establishment of long-term culture of rat SSCs requires serum-free chemically defined culture medium (Wu et al. 2009b). Previous studies show that SSCs cultivated in this serum-free medium allows propagation of rat SSCs *in vitro* and ensures the maintenance of potential of cultured SSCs to restore spermatogenesis after transplantation (Wu et al. 2009b). Presumably this protocol with slight modifications might be used for cultivation of SSCs from other species.

Although intense interest and subsequent research surrounds the regenerative potential of human SSCs, only until recently two studies report the *in vitro* long-term propagation of human SSCs isolated from patients undergoing orchiectomy for treatment of prostate cancer for up to 15 weeks (Sadri-Ardekani et al. 2009), and from testicular tissues of patients with obstructive or non-obstructive azoospermia for more than 6 months (Lim et al. 2010). However, both studies demonstrate that human SSCs proliferate rather slowly, which may limit their clinical applications. Of note, human and rodent spermatogonia share many but not all phenotypic markers, and the cultivation of SSCs from both species needs application of GDNF and FGF2. This indicates that some common molecular mechanisms controlling self-renewal of SSCs may exist in both rodents and humans. Future research on optimization of culture conditions for human SSCs needs to test new factors known for regulating self-renewal of mouse SSCs (see below). However, one needs to keep in mind that characteristics of human SSCs also differ from rodent SSCs *in vivo* in some extent suggesting that the establishment of human SSC culture might also need additional factors, which are not essential for rodent SSCs.

4.4 Signaling Pathways Involved in Self-Renewal of SSCs

The continual production of mature spermatozoa throughout the whole lifespan of the organism requires the maintenance of stem cells capable of self-renewal and differentiation. In the next part of the review we focus on the most recent findings regarding signaling events controlling self-renewal of SSCs.

4.4.1 Regulation of Self-Renewal by GDNF and FGF2

GDNF is provided to SSCs by Sertoli cells and acts through a receptor heterodimer of RET and GFRα1 to promote SSC self-renewal and maintenance (Fig. 4.2) (Naughton et al. 2006). Mutant mice with one null *GDNF* allele underwent SSC depletion, whereas transgenic males overexpressing GDNF accumulated undifferentiated spermatogonia in the testis (Meng et al. 2000). Multiple signaling pathways are induced by GDNF via its interaction with RET (for interests, please also see review (Caires et al. 2010)). GDNF is shown to activate the Src family kinase (SFK) and PI3K/Akt pathways. Cultivation of SSCs with pharmacological inhibitors of these kinases followed by transplantation analysis shows impairment of SSC maintenance *in vitro* (Oatley et al. 2007). It has been shown that, through the SFK signaling pathway, stimulation of SSCs with GDNF results in upregulation of *bcl6b*, *etv5* and *lhx1* whereas the expression of these genes is decreased in the absence of GDNF (Oatley et al. 2007). The expression of *bcl6b*, *etv5* and *lhx1* is also identified in undifferentiated spermatogonia *in vivo*, and knockdown of these genes by small interfering RNAs reveals that all of them are essential for SSC maintenance *in vitro* (Oatley et al. 2007).

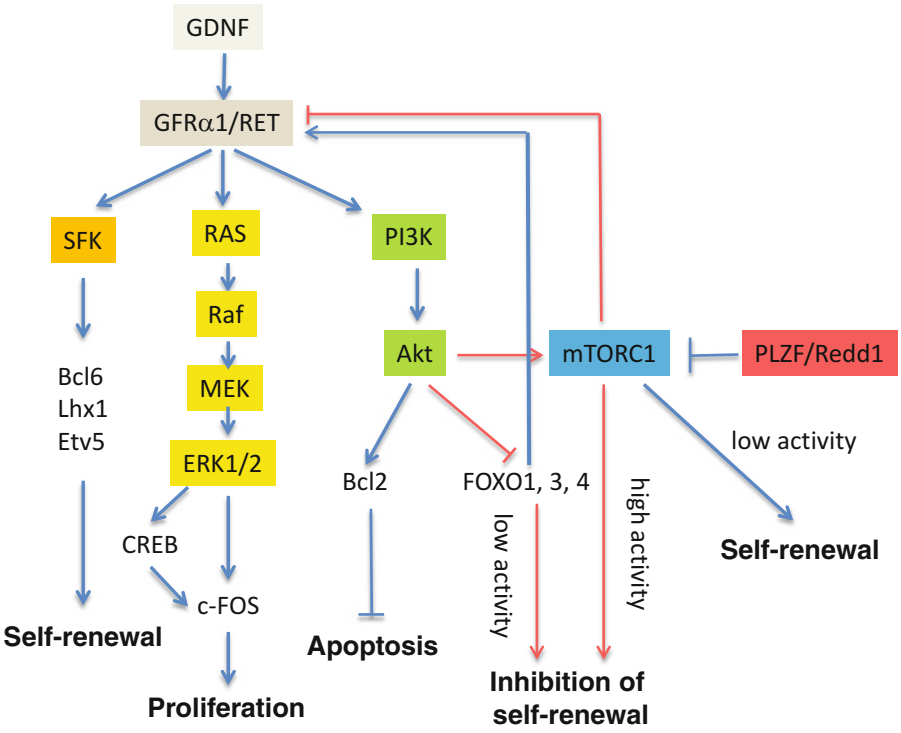

Fig. 4.2 GDNF mediated signaling pathways involved in self-renewal of SSCs. Briefly, GDNF is the most important factor for self-renewal of SSCs. It operates mainly through SFK and PI3K/Akt pathways controlling the expression of essential SSC genes (such as *Etv5*, *Bcl6b*, and *Lhx1*). In addition, GDNF can activate the canonical RAS/ERK1/2 pathway, which results in phosphorylation and activation of transcription factors such as CREB and c-FOS. Akt plays a controversial role in self-renewal of SSCs: on one hand it promotes self-renewal and inhibits apoptosis, on the other hand it phosphorylates and inactivates another important SSC factor, FOXO1. Additionally, PI3K/Akt might be also involved in activation of mTORC1 complex. PLZF counteracts excessive mTORC1 activity by controlling the expression of mTORC1 inhibitor Redd1. It is suggested that PI3K/Akt signaling must be carefully titrated *in vivo* to maintain SSC self-renewal and differentiation and that the Foxos and mTORC1 are pivotal intermediaries of this balance

In addition, GDNF can activate the canonical RAS/ERK1/2 pathway, which results in phosphorylation and activation of transcription factors such as CREB1, ATF1, CREM and c-FOS (He et al. 2008). He and colleagues also demonstrated that *ERK 1/2* (*MAPK 1/3*) was up-regulated in isolated human SSCs when cultured for 2 weeks in media containing GDNF, and phosphorylated ERK1/2 was increased in cultured cells compared to freshly isolated cells (Fig. 4.2) (He et al. 2010). It appears that the GDNF role is conserved between rodents and humans since GDNF allowed obtaining a short-term culture of presumable human SSCs (He et al. 2012).

The establishment of long-term culture of SSCs requires the application of not only GDNF but also FGF2 in culture medium. It has been discovered that *bcl6b*, *etv5* and *lhx1* are also induced by FGF2 via the MEK pathway (Ishii et al. 2012).

In another study, Akt kinase is rapidly phosphorylated in SSCs when GDNF is added to the medium, and the small-molecule inhibitor of PI3K prevents SSC self-renewal. Furthermore, conditional activation of the myristoylated form of Akt in SSCs promotes their proliferation in the absence of GDNF (Lee et al. 2007). Later studies point to a strictly controlled activity of PI3K/Akt pathway in spermatogonia, which is in part explained by the function of downstream targets, such as FOXO family of transcription factors (Goertz et al. 2011; Salih and Brunet 2008). FOXO proteins are well known to regulate cellular growth and organismal longevity inducing a variety of cellular responses including cell-cycle arrest and cell death. FOXO1, FOXO3 and FOXO4 may be regulated by Akt-dependent phosphorylation, leading to their functional inactivation by the export from the nucleus (Salih and Brunet 2008). It was found that FOXO1 was expressed in gonocytes and spermatogonia. During first week of postnatal development FOXO1 changed the subcellular localization from cytoplasmic in gonocytes to nuclear in spermatogonia. Conditional ablation of FOXO1 in germ cells did not affect the formation of gonocytes, whereas the amount of spermatogonia and more differentiated germ cells was diminished. The triple knockout of *FOXOs 1*, *3*, and *4* resulted in even fewer germ cells suggesting a partial functional redundancy in the FOXO family. Microarray analysis revealed that expression of *RET* gene was diminished after Cre-mediated ablation of *FOXO1* in germ cells, thereby explaining, in part, the role of FOXO1 in spermatogonia (Goertz et al. 2011). Taken together, the data suggest that PI3K-Akt signaling must be carefully titrated in vivo to maintain SSC self-renewal and differentiation and argue that the Foxos are pivotal intermediaries of this balance (Fig. 4.2).

It was found that functional inactivation of PI3K catalytic subunit in mice was detrimental for expansion of differentiating spermatogonia. Knock-in mice bearing a catalytically inactive subunit of p110β K805R demonstrated a decreased amount of differentiated germ cells in the male. These knock-in mice did not reveal any impairment in the development of SSCs during postnatal development, and SSCs were also unaffected in adult animals. Moreover, GDNF induced Akt phosphorylation and proliferation in cultured SSCs was not affected by TGX221, a specific pharmacological inhibitor of p110β PI3K subunit, but decreased after the treatment with a less selective PI3K inhibitor PIK75 (mainly inhibiting p110α subunit). Thus, presumably p110α is the main PI3K isoform activated by GDNF. Most strikingly, spermatogonia derived from testis of knockout mice failed to respond to SCF stimulation. Therefore, it appears that PI3K subunit p110β is necessary for c-Kit-mediated induction of proliferation and differentiation of spermatogonia (Ciraolo et al. 2010).

4.4.2 Self-Renewal of SSCs Regulated by the mTORC1-PLZF Interaction

As discussed above, the PLZF protein expression was observed in both human and mouse undifferentiated spermatogonia (Hermann et al. 2007; Nakagawa et al. 2010). A nonsense mutation in PLZF encoded by *zfp145* gene has been determined

in luxoid mutant mouse, which was previously found to be male infertile. Luxoid mice demonstrate a progressive loss of germ cells in the seminiferous tubules with the age. Phenotype similar to luxoid has been found in mice after targeting in the *zfp145* gene locus, the gonocyte numbers were not decreased but germ cells were eliminating with age and amount of sperm was decreased dramatically (Costoya et al. 2004). These suggest that although dispensable for germ cell development in embryogenesis and postnatal period, PLZF becomes important for the maintenance of SSC population in the testis. A recent work uncovered one of the functions of PLZF in SSCs. It turns out that SSCs lacking PLZF have enhanced activity of molecular target of rapamycin complex 1 (mTORC1), a key mediator of cell growth. PLZF opposes mTORC1 activity by inducing expression of the mTORC1 inhibitor Redd1 (Fig. 4.2). Increased mTORC1 activation in $PLZF^{-/-}$ SSCs inhibits their response to GDNF via negative feedback at the level of the GDNF receptors, GFRα1 and c-RET. The data also show that P$LZF^{-/-}$ SSCs have increased cell size compared to control, however, this change can be prevented by rapamycin, a small molecule inhibitor of mTORC1 complex. Furthermore, inhibition of mTORC1 via rapamycin attenuates $PLZF^{-/-}$ SSC defects and enhances wild-type SSC activity. The authors suggest that the mTORC1-PLZF functional interaction is a critical rheostat for maintenance of the spermatogonial pool and negative feedback from mTORC1 to the GDNF receptor balances SSC growth with self-renewal (Fig. 4.2) (Hobbs et al. 2010).

4.4.3 SSC Self-Renewal Controlled by Wnt Signaling

Wnts and their receptors Frizzleds (Fzds) are expressed in mouse spermatogonia (Golestaneh et al. 2009a). High expression of *Fzd3* was found in GFRα1-positive SSCs while only a very low expression of *Fzd* was found in the c-Kit-positive differentiating spermatogonial cells. Wnt3a and Wnt10b, activators of canonical Wnt signaling, increased cell proliferation in primary mouse SSC culture (Golestaneh et al. 2009a). However, study of Yeh and co-authors suggests that canonical Wnt signaling would rather promote differentiating spermatogonia than true SSCs. They showed that non-canonical Wnt5a supported self-renewal of mouse SSCs in a β-catenin-independent manner (Yeh et al. 2011). Wnt5a expression was restricted to Sertoli cells in the mouse testis and potential Wnt5a receptors Fzd5, Fzd7 and ROR2 were detected at the cell surface of SSCs. The inhibition of β-catenin signaling via application of Dickkopf-1 (Dkk1), which specifically blocks β-catenin signaling by binding to LRP5/6, did not affect SSC activity. However, secreted frizzled-related protein 1 (sFRP1), which inhibits both β-catenin-dependent and -independent signaling by binding Wnt ligands, led to a dose-dependent reduction of SSC activity. Wnt5a promoted SSC maintenance by supporting cell survival, and this pro-survival effect of Wnt5a was abolished by the inhibition of c-Jun N-terminal kinase (JNK) signaling. In addition, Wnt5a significantly increased JNK-*P* levels. Moreover, cells with activated β-catenin signaling had lost SSC function suggesting

that canonical Wnt pathway might be activated during early differentiation of SSCs. Therefore Wnt5a can be considered as a new member besides other SSC niche factors, such as GDNF and FGF2 (Yeh et al. 2011).

4.4.4 Integrins

Integrin-mediated cell adhesion to extracellular matrix plays an important role in regulating stem cell function and maintenance. In particular, integrins help define and shape the stem cell niche (Ellis and Tanentzapf 2010). SSCs reside at the basal membrane of seminiferous tubules, and are capable to bind to laminin. This feature is used for isolation of SSCs from testicular cells of mice (Guan et al. 2009) and rats (Hamra et al. 2008) suggesting that SSCs have a set of receptors for extracellular matrix. α6-integrin has been identified as cell surface marker of both rodent (Kubota et al. 2003; Ryu et al. 2004) and human SSCs (He et al. 2012). β1-integrin was discovered to be important for a proper homing of SSCs to the basal membrane of the seminiferous tubules after SSC transplantation (de Rooij et al. 2008; Kanatsu-Shinohara et al. 2008). Ablation of β1-integrin in SSCs influences their ability to colonize the recipient testis *in vivo* and to bind to laminin *in vitro* indicating that β1-integrin plays an important role in SSC function.

4.5 Differentiation Capacities and Their Progenitors

4.5.1 Differentiation Capacities of SSCs In Vivo and In Vitro

Previous studies showed that mouse SSCs were able to repopulate the seminiferous tubules 2 months after they were injected into the germ cell-depleted testis (Brinster and Zimmermann 1994). In this study, male mice were treated with the alkylating antineoplastic agent busulfan, which destroys SSCs and thus leads to a disruption of spermatogenesis. Testes of busulfan treated mice usually do not contain mature spermatozoa or even spermatogonia. After injection of SSCs from transgene *lacZ* (β-Galactosidase gene) donor mice into busulfan treated recipient mice functional spermatogenesis could be observed. Because the donor cells carried a transgene that produced β-Galactosidase in spermatids, these cells were identified by a blue staining after X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) treatment. The organization of the spermatogenic stages within the seminiferous tubules was normal and mature spermatozoa were produced. These data suggest that the SSCs are able to colonize the seminiferous tubules of infertile mice and subsequently undergo self-renewal division to support spermatogenesis. The repopulation capacity was also proved for SSCs derived from the rat (Hamra et al. 2002). Human SSCs were found as singlet or doublets on the seminiferous tubule basement membrane 3–6 months after transplantation to testes of immunodeficient mice. These results

indicate that human spermatogonia home to the basement membrane of the mouse recipient seminiferous tubule and are maintained as germ cells, but are unable to differentiate (Nagano et al. 2002; Wu et al. 2009a). Interestingly, when human spermatogonia were injected together with human testicular somatic cells, the greater number of singlet and doublets, the larger groups of human germ cells, and particularly the presence of dividing germ cells could be observed in mouse seminiferous tubules. These data indicate that human testicular somatic cells enhance the ability of human spermatogonia to colonize the mouse seminiferous tubule (Wu et al. 2009a). The ability of human SSCs to restore spermatogenesis would be the foundation for the treatment of men infertility.

In spite of the ability to differentiate *in vivo* in the testis, the complete process of spermatogenesis has not been achieved from SSCs under *in vitro* cell culture conditions. *In vitro* differentiation of SSCs and the use of *in vitro* derived male haploid gametes for intracytoplasmic sperm injection could be an option to restore fertility. The most advanced differentiation has been achieved using mouse SSCs. Treatment of mouse SSCs with SCF induced the formation of spermatocytes after 1 week. The latest differentiation stage observed was round spermatids, which appeared in culture after 3 weeks of SCF treatment (Feng et al. 2002). However, the study has not been repeated by another group or used to study signaling during differentiation of germ cells. One reason might be that cells used in this study were immortalized by overexpression of TERT (telomerase reverse transcriptase) and could be therefore different from primary SSCs (Feng et al. 2002).

It is worth to mention that there is only a minor percent of SSCs bearing self-renewal and capable of testis colonization under *in vitro* optimal culture conditions. Under the established culture conditions, SSCs consistently give rise to both new SSCs and differentiating progeny. The differentiating progeny have lost stemness and unlimited self-renewal capacity, but have developed functional intercellular bridges. Twin daughter cells of single SSCs often undergo self-renewal and differentiation side by side even though they have been exposed to virtually identical microenvironments. Moreover, quantitative experimental measurements and mathematical modeling indicate that fate decision is stochastic, with constant probability (Wu et al. 2009c). Therefore, SSCs seem to have an in-built program of self-renewal and differentiation, and probably the extent of survival of differentiating cells might be affected by external factors.

Previous studies showed that BMP4 induced the expression of early differentiation factor c-Kit in rat SSCs (Carlomagno et al. 2010). Vitamin A derivatives, such as retinoic acid are absolutely essential for the initiation of meiosis in germ cells in the testis. Mice and rats subjected to vitamin A deficient diet for several weeks become infertile and contain no mature germ cells in the testis. The injection of retinol induced the synchronous onset of spermatogenesis (van Pelt and de Rooij 1990a, b). *Stra8* gene is one of the targets of retinoic acid signaling, and is essential for meiosis in male and female germ cells (Anderson et al. 2008; Mark et al. 2008). Expression of Stra8 and c-Kit was induced in spermatogonia both *in vivo* and *in vitro* (Zhou et al. 2008a, b). Therefore, retinoic acid might be an essential component for *in vitro* differentiation of SSCs. Future works should test whether treatment

of SSCs with retinoic acid and other factors mentioned above can induce the initiation of meiosis *in vitro*, and should identify the specific factors required for a more efficient and complete *in vitro* spermatogenesis. Moreover, culture medium supporting the survival of spermatocytes and spermatids needs to be developed.

4.5.2 Differentiation of Pluripotent GSCs In Vitro

Pluripotent GSCs, similar to ESCs are able to spontaneously differentiate into derivatives of all three embryonic germ layers when they are removed from feeder cells (Cheng et al. 2012; Fagoonee et al. 2010; Guan et al. 2006, 2007; Streckfuss-Bomeke et al. 2009, 2014). We show that cardiomyocytes derived from pluripotent GSCs express cardiac-specific L-type Ca^{2+} channels and respond to Ca^{2+} channel-modulating drugs. Four different types of action potentials characteristic of pacemaker-, ventricle-, atrial- and Purkinje-like cardiomyocytes are observed. The cardiomyocytes derived from pluripotent GSCs also exhibit functional gap junctions as well as an intact calcium cycling (Guan et al. 2007). Pluripotent GSCs can also be differentiated into $Flk1^+$ cells, which are multipotent cardiovascular progenitors expressing Isl-1, Nkx2.5 and brachyury, and able to further differentiate into functional cardiomyocytes as well as functional endothelial cells (Cheng et al. 2012). These data indicate that GSC-derived cardiovascular progenitors as well as functional cardiomyocytes and endothelial cells may provide a useful source of cardiovascular cells for studying basic mechanisms of cardiogenesis and vasculogenesis and for cardiovascular regeneration.

Besides functional cardiomyocytes, mouse pluripotent GSCs can differentiate into neural progenitors under specific culture conditions, which can further differentiate into functional neurons (GABAergic, glutamatergic, serotonergic, and dopaminergic neurons) and glial cells (astrocytes and oligodendrocytes). Electrophysiological recordings of passive and active membrane properties and postsynaptic currents demonstrate the maturation of neural precursor cells into functional neurons and glial cells (Streckfuss-Bomeke et al. 2009). Therefore, pluripotent GSC-derived neural precursors and functional neurons and glial cells constitute a promising cell source for the treatment of many different nervous system disorders.

Several groups have reported that metabolically active hepatocytes can be derived from pluripotent GSCs *in vitro*, which are capable of albumin and haptoglobin secretion, urea synthesis, glycogen storage, and indocyanine green uptake (Fagoonee et al. 2010; Streckfuss-Bomeke et al. 2014). The pluripotent GSC-derived hepatocytes were found to be closer to fetal hepatocytes than adult hepatocytes (Fagoonee et al. 2010; Streckfuss-Bomeke et al. 2014). The functional hepatocytes may be a useful cell source for liver regeneration.

4.6 Potential Applications for Therapies

4.6.1 Therapeutic Application of SSCs in Male Infertility

Male infertility can be caused by genetic defects of the endocrine system, by defects in the development of the urogenital system or by defects in gametogenesis, cryptorchidism or erectile dysfunction. In addition, there are also secondary or acquired causes of infertility due to tubal disease or exposure to gonadotoxins from the environment (Matzuk and Lamb 2008). Among these causes, testicular cancer and the effect of radiotherapy or chemotherapy used in cancer treatment can also result in male infertility. In the testis, SSCs and differentiating spermatogonia divide most actively and are therefore extremely sensitive towards cytotoxic agents (Meistrich 1993). In contrast, Leydig and Sertoli cells can survive most cytotoxic therapies and may sustain a functional damage because of lower proliferation rate in adults. After cytotoxic therapies, seminiferous tubules contain only Sertoli cells, whereas germ cells appear to be absent (Shetty and Meistrich 2005). This could be the result of the deletion of SSCs and/or the loss of the ability of the remaining Sertoli cells to support the self-renewal and differentiation of a few surviving SSCs. At lower doses of cytotoxic agents recovery of spermatogenesis can be observed several months after termination of the treatment. At higher doses, however, azoospermia can be prolonged or even permanent. Adult male cancer patients have the ability of cryopreservation of their semen prior to chemo- or radiotherapy. This allows them to have children by artificial insemination after successful cancer treatment. However, prepubertal boys treated with high-dose chemotherapy, total body irradiation and/or irradiation involving the genital region cannot benefit from this approach since spermatogenesis at that age is not yet completed. At present, the only option for fertility preservation could be SSC preservation. Therefore, it is necessary to take a biopsy of testis tissue before chemo- or radiotherapy (Fig. 4.3). The establishment of isolation and long-term cultivation of human SSCs *in vitro*, and cryopreservation of these cells provides the opportunity for clinical applications with regard to the treatment of male infertility. After successful cancer treatment the cultivated SSCs could be transplanted back into the seminiferous tubules of the patient (Geens et al. 2008). Autologous intra-testicular transplantation of SSCs is a hypothetical option that is currently thoroughly studied by a few research groups, mainly in rodent models. Already in 1994, Brinster and Zimmermann (Brinster and Zimmermann 1994) could show that successful restoration of spermatogenesis occurs in infertile mice after injection of SSCs into seminiferous tubules. The transplantation of SSCs has been shown to produce live offspring in mice (Goossens et al. 2003, 2006). The success of these transplantation experiments in rodents suggests therapeutic potential for the patients. However, some safety concerns related to this technique should be taken into consideration, the risk of transmitting tumor cells back to the patient and other aspects of the procedure, like germ cell retrieval, cell sorting and preservation, still need to be optimized prior to clinical applications in men.

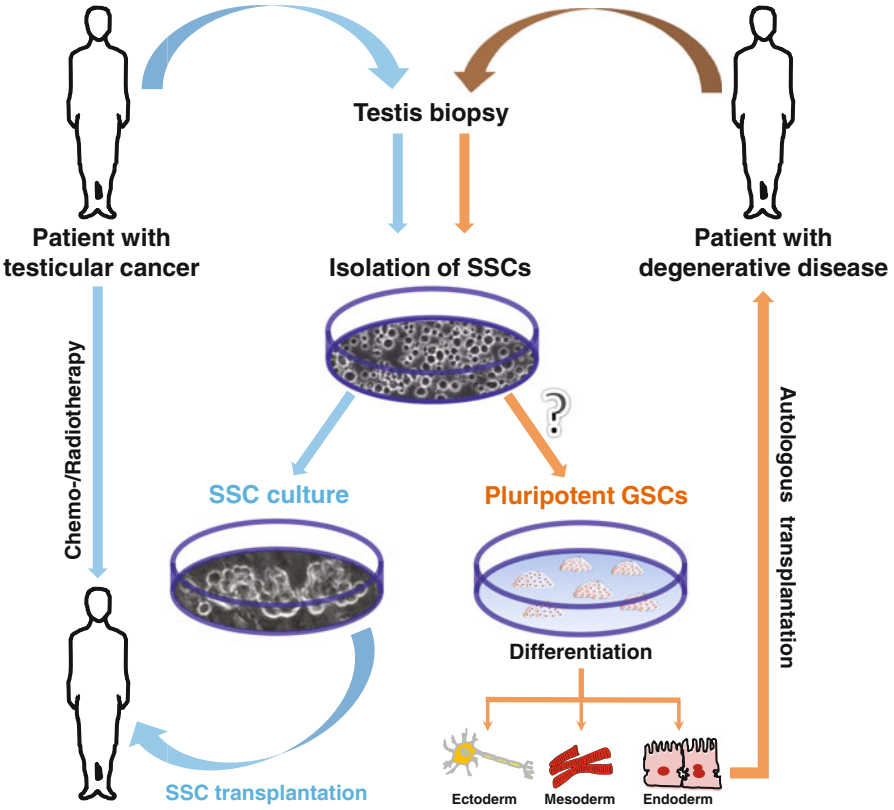

Fig. 4.3 Potential application of SSCs in treatment of infertility after chemo-/radiotherapy in patients with testicular cancer and potential application of SSC-derived pluripotent GSCs in regenerative medicine. *SSCs* spermatogonial stem cells, *GSCs* germline stem cells

4.6.2 Therapeutic Applications of Pluripotent GSCs

As mentioned above, mouse SSCs can be converted into pluripotent GSCs *in vitro* under defined culture conditions. Two characteristics of pluripotent stem cells make them interesting for regenerative medicine: their high proliferation rate and their ability to differentiate into all different cell types of the body. The establishment of protocols for a directed differentiation of pluripotent stem cells allows the production of any somatic cell type which is needed. The main focus of stem cell research has been on cell therapy for pathological conditions with no current methods of treatment, such as neurodegenerative diseases, heart attacks, retinal dysfunction and lung and liver diseases. The idea is to replace diseased or damaged tissue by using the desired cells derived from pluripotent stem cells. The overall aim is to develop methods of application either of pure cell populations or of whole tissue parts to the diseased organ. Transplantation of Flk1^{+} cardiovascular progenitors derived from

mouse pluripotent GSCs directly into the ischemic heart of mouse resulted in the improvement of cardiac function by promoting angiogenesis and postponing host cell death (Iwasa et al. 2010). Although mouse pluripotent GSCs can differentiate into functional neurons, glial cells as well as hepatocytes, the *in vivo* colonization capacity of hepatocytes in mouse models of liver diseases, or regeneration potential of functional neurons in the damaged brain remains to be demonstrated. These mouse *in vitro* and *in vivo* studies pave the way for therapeutic application of pluripotent GSCs; however, up to now there are no evidences showing that so far generated human pluripotent GSCs are able to differentiate into functional cardiomyocytes, neurons, or hepatocytes. The drawback of so far generated human pluripotent GSCs is that they may be not truly pluripotent and thus their differentiation potential is limited. Therefore, development of a culture system for establishing truly pluripotent human GSCs is of paramount importance. Optimizing culture conditions by application of small molecules may facilitate the generation of human pluripotent GSCs (Zhu et al. 2011).

With regard to clinical applications, the use of patient-specific stem cells, such as human pluripotent GSCs, for autologous stem cell-based therapies avoids immunological and ethical problems related to human ESCs. SSCs could be obtained from testis biopsies, cultivated *in vitro* and converted into pluripotent stem cells (Fig. 4.3). These cells can then be differentiated into somatic cells and transplanted back into the patient. Thus, the risk of immunological rejection is reduced onto a minimum and the patient may not be dependent on immune suppressant drugs.

So far, many studies using human pluripotent ESCs in organ regeneration have been performed and discussed, for interests and more details, please see reviews (Kung and Forbes 2009; Ronaghi et al. 2010; Shiba et al. 2009; Varanou et al. 2008). The first FDA approval (http://www.fda.gov) for the preclinical usage of differentiated human ESCs for the treatment of spinal cord injury makes human ESCs a very attractive source for clinical applications. However, in August 2009, the FDA put a clinical hold on human ESC clinical trials because further characterization of differentiated cells and more nonclinical trials/applications of human ESC-derived neural cells into animal models are requested (Ronaghi et al. 2010).

4.7 Conclusions and Future Development in Research

SSCs constitute one of the most important stem cell systems in the adult body. SSCs are unipotent and respond for spermatogenesis in the male as they can only differentiate into sperms within the testicular niche. Methods for isolation and cultivation of SSCs from the rodent testis have been well established. Rodent SSCs can be expanded *in vitro* for a long term without loss of their properties. Only recently some studies report that human SSCs can be isolated from adult testis biopsies and propagated *in vitro*. This provides the opportunity to develop therapeutic strategies and re-initiate spermatogenesis for the patients who become infertile after cancer therapy.

Since pluripotent stem cells can be generated from PGCs, neonatal and adult SSCs, we believe that the pluripotency is maintained in the male germline. SSCs can be spontaneously reprogrammed into pluripotent GSCs when they are removed from their *in vivo* niche and cultured *in vitro* under specific conditions. The advantage of pluripotent GSCs over iPSCs is that the conversion of SSCs into pluripotent GSCs does not require addition of genes using the virus system, which may avoid unpredictable genetic dysfunction. This makes them a safer cell source for autologous transplantation than iPSCs. In addition, this may also circumvent the ethical and immunological problems associated with human ESCs. Future works should focus on optimizing protocols for isolation and long-term culture of human SSCs as well as establishing culture conditions for generation of pluripotent GSCs fulfilling all criteria for pluripotency. In addition, it is of paramount importance to identify the cell type in the testis that is capable of the conversion into the pluripotent stem cells, presumably it is the human SSCs, but this needs to be determined. Xenotransplantation studies might be used to confirm the function of human SSCs (Hermann et al. 2010). This will bring the realization of personalized regenerative medicines closer. However, this approach is suitable only for men, the half of the world's population. For women, derivation of parthenogenetic stem cells from the woman whose unfertilized eggs are artificially activated may provide another potential source for cell-based autologous transplantation therapy (Turovets et al. 2011).

Finally, successful development of pluripotent stem cell-based replacement strategies for various diseases needs to address three important questions: (1) how to generate an adequate number of cells sufficient for active improvement of organ function? More intensive work to obtain a better understanding of stem cell differentiation pathways and to improve differentiation protocols of pluripotent stem cells may help to find out a solution for it. (2) How to improve the survival of transplanted cells in the damaged organ upon transplantation? (3) How to eliminate residual undifferentiated cells from differentiated cells which are destined for *in vivo* transplantation as these undifferentiated cells may form tumors? Moreover, the ideal source of stem cells for efficient and safe cell replacement has remained a challenging issue that requires more investigation.

Acknowledgments The authors studies were funded by Bundesministerium für Bildung und Forschung (01GN0822 and 01GN0957) and Deutsche Forschungsgemeinschaft (GU 595/2-1, SFB1002/A04 to K.G.). The authors would like to thank Dr. Felix Bremmer (Dept. Pathology, University of Göttingen) for providing us the histochemical sections of human testicular tissue.

References

Anderson R, Fässler R, Georges-Labouesse E, Hynes RO, Bader BL, Kreidberg JA, Schaible K, Heasman J, Wylie C (1999) Mouse primordial germ cells lacking beta1 integrins enter the germline but fail to migrate normally to the gonads. Development 126(8):1655–1664

Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, Page DC (2008) Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci U S A 105(39):14976–14980

Blume-Jensen P, Jiang G, Hyman R, Lee KF, O'Gorman S, Hunter T (2000) Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3′-kinase is essential for male fertility. Nat Genet 24(2):157–162. doi:10.1038/72814

Boulanger CA, Mack DL, Booth BW, Smith GH (2007) Interaction with the mammary microenvironment redirects spermatogenic cell fate in vivo. Proc Natl Acad Sci U S A 104(10):3871–3876. doi:10.1073/pnas.0611637104

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, Koopman P (2006) Retinoid signaling determines germ cell fate in mice. Science 312(5773):596–600

Brinster RL (2007) Male germline stem cells: from mice to men. Science 316(5823):404–405

Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91(24):11298–11302

Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE (2004) Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 36(6):647–652

Buageaw A, Sukhwani M, Ben-Yehudah A, Ehmcke J, Rawe VY, Pholpramool C, Orwig KE, Schlatt S (2005) GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod 73(5):1011–1016

Buehr M, McLaren A, Bartley A, Darling S (1993) Proliferation and migration of primordial germ cells in We/We mouse embryos. Dev Dyn 198(3):182–189

Bustos-Obregon E, Courot M, Flechon JE, Hochereau-de-Reviers MT, Holstein AF (1975) Morphological appraisal of gametogenesis. Spermatogenetic process in mammals with particular reference to man. Andrologia 7(2):141–163

Caires K, Broady J, McLean D (2010) Maintaining the male germline: regulation of spermatogonial stem cells. J Endocrinol 205(2):133–145

Carlomagno G, van Bragt MPA, Korver CM, Repping S, de Rooij DG, van Pelt AMM (2010) BMP4-induced differentiation of a rat spermatogonial stem cell line causes changes in its cell adhesion properties. Biol Reprod 83(5):742–749

Cheng I-F, Kaiser D, Huebscher D, Hasenfuss G, Guan K, Schaefer K (2012) Differentiation of multipotent adult germline stem cells derived from mouse testis into functional endothelial cells. J Vasc Res 49(3):207–220

Ciraolo E, Morello F, Hobbs RM, Wolf F, Marone R, Iezzi M, Lu X, Mengozzi G, Altruda F, Sorba G, Guan K, Pandolfi PP, Wymann MP, Hirsch E (2010) Essential role of the p110beta subunit of phosphoinositide 3-OH kinase in male fertility. Mol Biol Cell 21(5):704–711

Clermont Y (1963) The cycle of the seminiferous epithelium in man. Am J Anat 112:35–51

Clermont Y (1966) Renewal of spermatogonia in man. Am J Anat 118(2):509–524

Clermont Y (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52(1):198–236

Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, Aicher W, Buhring HJ, Mattheus U, Mack A, Wagner HJ, Minger S, Matzkies M, Reppel M, Hescheler J, Sievert KD, Stenzl A, Skutella T (2008) Generation of pluripotent stem cells from adult human testis. Nature 456(7220):344–349

Cooke PS, Simon L, Nanjappa MK, Medrano TI, Berry SE (2015) Plasticity of spermatogonial stem cells. Asian J Androl. doi:10.4103/1008-682X.148072

Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36(6):653–659

De Felici M, Dolci S (1989) In vitro adhesion of mouse fetal germ cells to extracellular matrix components. Cell Differ Dev 26(2):87–96

De Felici M, Scaldaferri ML, Lobascio M, Iona S, Nazzicone V, Klinger FG, Farini D (2004) Experimental approaches to the study of primordial germ cell lineage and proliferation. Hum Reprod Update 10(3):197–206
de Rooij DG (1998) Stem cells in the testis. Int J Exp Pathol 79(2):67–80
de Rooij DG (2001) Proliferation and differentiation of spermatogonial stem cells. Reproduction 121(3):347–354
de Rooij DG (2009) The spermatogonial stem cell niche. Microsc Res Tech 72(8):580–585
de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21(6):776–798
de Rooij DG, Repping S, van Pelt AM (2008) Role for adhesion molecules in the spermatogonial stem cell niche. Cell Stem Cell 3(5):467–468. doi:10.1016/j.stem.2008.10.010
Dolci S, Pellegrini M, Di Agostino S, Geremia R, Rossi P (2001) Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor. J Biol Chem 276(43):40225–40233. doi:10.1074/jbc.M105143200
Donovan P, de Miguel M (2004) Gonocyte development and differentiation. In: Skinner MK, Griswold MD (eds) Sertoli cell biology, Elsevier LTD, Oxford , pp 417–435
Dym M, Clermont Y (1970) Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. Am J Anat 128(3):265–282
Dym M, Fawcett DW (1971) Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 4(2):195–215
Dym M, Kokkinaki M, He Z (2009) Spermatogonial stem cells: mouse and human comparisons. Birth Defects Res C Embryo Today 87(1):27–34
Eddy EM (1975) Germ plasm and the differentiation of the germ cell line. Int Rev Cytol 43:229–280
Eddy EM, Hahnel AC (1983) Establishment of the germ cell line in mammals. In: McLaren A, Wylie C (eds) Current problems in germ cell differentiation. British Society for development biology symposium, Cambridge University Press, Cambridge, pp 41–69
Ehmcke J, Schlatt S (2006) A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction 132(5):673–680
Ehmcke J, Wistuba J, Schlatt S (2006) Spermatogonial stem cells: questions, models and perspectives. Hum Reprod Update 12(3):275–282
El Ramy R, Verot A, Mazaud S, Odet F, Magre S, Le Magueresse-Battistoni B (2005) Fibroblast growth factor (FGF) 2 and FGF9 mediate mesenchymal-epithelial interactions of peritubular and Sertoli cells in the rat testis. J Endocrinol 187(1):135–147
Ellis SJ, Tanentzapf G (2010) Integrin-mediated adhesion and stem-cell-niche interactions. Cell Tissue Res 339(1):121–130
Fagoonee S, Hobbs RM, De Chiara L, Cantarella D, Piro RM, Tolosano E, Medico E, Provero P, Pandolfi PP, Silengo L, Altruda F (2010) Generation of functional hepatocytes from mouse germ line cell-derived pluripotent stem cells in vitro. Stem Cells Dev 19(8):1183–1194
Falciatori I, Borsellino G, Haliassos N, Boitani C, Corallini S, Battistini L, Bernardi G, Stefanini M, Vicini E (2004) Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB J 18(2):376–378
Falender AE, Freiman RN, Geles KG, Lo KC, Hwang K, Lamb DJ, Morris PL, Tjian R, Richards JS (2005) Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 19(7):794–803
Feng LX, Ravindranath N, Dym M (2000) Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. J Biol Chem 275(33):25572–25576
Feng LX, Chen Y, Dettin L, Pera RA, Herr JC, Goldberg E, Dym M (2002) Generation and in vitro differentiation of a spermatogonial cell line. Science 297(5580):392–395
Fijak M, Bhushan S, Meinhardt A (2011) Immunoprivileged sites: the testis. Methods Mol Biol 677:459–470

França LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD (1998) Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59(6):1371–1377

García-Castro MI, Anderson R, Heasman J, Wylie C (1997) Interactions between germ cells and extracellular matrix glycoproteins during migration and gonad assembly in the mouse embryo. J Cell Biol 138(2):471–480

Geens M, Goossens E, De Block G, Ning L, Van Saen D, Tournaye H (2008) Autologous spermatogonial stem cell transplantation in man: current obstacles for a future clinical application. Hum Reprod Update 14(2):121–130

Giuili G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M, Cuzin F (2002) Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. EMBO Rep 3(8):753–759

Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH (2011) Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest 121(9):3456–3466

Golestaneh N, Beauchamp E, Fallen S, Kokkinaki M, Uren A, Dym M (2009a) Wnt signaling promotes proliferation and stemness regulation of spermatogonial stem/progenitor cells. Reproduction 138(1):151–162

Golestaneh N, Kokkinaki M, Pant D, Jiang J, DeStefano D, Fernandez-Bueno C, Rone JD, Haddad BR, Gallicano GI, Dym M (2009b) Pluripotent stem cells derived from adult human testes. Stem Cells Dev 18(8):1115–1126

Goossens E, Frederickx V, De Block G, Van Steirteghem AC, Tournaye H (2003) Reproductive capacity of sperm obtained after germ cell transplantation in a mouse model. Hum Reprod 18(9):1874–1880

Goossens E, Frederickx V, de Block G, van Steirteghem A, Tournaye H (2006) Evaluation of in vivo conception after testicular stem cell transplantation in a mouse model shows altered post-implantation development. Hum Reprod 21(8):2057–2060

Greenbaum MP, Yan W, Wu MH, Lin YN, Agno JE, Sharma M, Braun RE, Rajkovic A, Matzuk MM (2006) TEX14 is essential for intercellular bridges and fertility in male mice. Proc Natl Acad Sci U S A 103(13):4982–4987

Griswold MD (2004) Perspective on the function of Sertoli cells. In: Skinner MK, Griswold MD (eds) Sertoli cell biology, Elsevier LTD, Oxford, pp 15–18

Gu Y, Runyan C, Shoemaker A, Surani A, Wylie C (2009) Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. Development 136(8):1295–1303

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440(7088):1199–1203

Guan K, Wagner S, Unsold B, Maier LS, Kaiser D, Hemmerlein B, Nayernia K, Engel W, Hasenfuss G (2007) Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. Circ Res 100(11):1615–1625

Guan K, Wolf F, Becker A, Engel W, Nayernia K, Hasenfuss G (2009) Isolation and cultivation of stem cells from adult mouse testes. Nat Protoc 4(2):143–154

Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL (2002) Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 99(23):14931–14936

Hamra FK, Schultz N, Chapman KM, Grellhesl DM, Cronkhite JT, Hammer RE, Garbers DL (2004) Defining the spermatogonial stem cell. Dev Biol 269(2):393–410

Hamra FK, Chapman KM, Wu Z, Garbers DL (2008) Isolating highly pure rat spermatogonial stem cells in culture. Methods Mol Biol 450:163–179

He Z, Jiang J, Kokkinaki M, Golestaneh N, Hofmann MC, Dym M (2008) Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. Stem Cells 26(1):266–278

He Z, Kokkinaki M, Jiang J, Dobrinski I, Dym M (2010) Isolation, characterization, and culture of human spermatogonia. Biol Reprod 82(2):363–372

He Z, Kokkinaki M, Jiang J, Zeng W, Dobrinski I, Dym M (2012) Isolation of human male germline stem cells using enzymatic digestion and magnetic-activated cell sorting. Methods Mol Biol 825:45–57

Hermann BP, Sukhwani M, Lin C-C, Sheng Y, Tomko J, Rodriguez M, Shuttleworth JJ, McFarland D, Hobbs RM, Pandolfi PP, Schatten GP, Orwig KE (2007) Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. Stem Cells 25(9):2330–2338

Hermann BP, Sukhwani M, Hansel MC, Orwig KE (2010) Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction 139(3):479–493

Hobbs RM, Seandel M, Falciatori I, Rafii S, Pandolfi PP (2010) Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell 142(3):468–479

Hofmann M-C, Braydich-Stolle L, Dym M (2005) Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 279(1):114–124

Huckins C (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 169(3):533–557

Ishii K, Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2012) FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. Development 139(10):1734–1743. doi:10.1242/dev.076539

Iwasa T, Baba S, Doi H, Kaichi S, Yokoo N, Mima T, Kanatsu-Shinohara M, Shinohara T, Nakahata T, Heike T (2010) Neonatal mouse testis-derived multipotent germline stem cells improve the cardiac function of acute ischemic heart mouse model. Biochem Biophys Res Commun 400(1):27–33. doi:10.1016/j.bbrc.2010.07.131

Izadyar F, Pau F, Marh J, Slepko N, Wang T, Gonzalez R, Ramos T, Howerton K, Sayre C, Silva F (2008) Generation of multipotent cell lines from a distinct population of male germ line stem cells. Reproduction 135(6):771–784

Izadyar F, Wong J, Maki C, Pacchiarotti J, Ramos T, Howerton K, Yuen C, Greilach S, Zhao HH, Chow M, Chow YC, Rao J, Barritt J, Bar-Chama N, Copperman A (2011) Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 26(6):1296–1306

Jegou B (1992) The Sertoli cell. Baillieres Clin Endocrinol Metab 6(2):273–311

Johnson L (1994) A new approach to study the architectural arrangement of spermatogenic stages revealed little evidence of a partial wave along the length of human seminiferous tubules. J Androl 15(5):435–441

Johnson L, Neaves WB, Barnard JJ, Keillor GE, Brown SW, Yanagimachi R (1999) A comparative morphological study of human germ cells in vitro or in situ within seminiferous tubules. Biol Reprod 61(4):927–934

Johnson L, Staub C, Neaves WB, Yanagimachi R (2001) Live human germ cells in the context of their spermatogenic stages. Hum Reprod 16(8):1575–1582

Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A, Shinohara T (2004) Generation of pluripotent stem cells from neonatal mouse testis. Cell 119(7):1001–1012

Kanatsu-Shinohara M, Ikawa M, Takehashi M, Ogonuki N, Miki H, Inoue K, Kazuki Y, Lee J, Toyokuni S, Oshimura M, Ogura A, Shinohara T (2006) Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. Proc Natl Acad Sci U S A 103(21):8018–8023

Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fässler R, Shinohara T (2008) Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. Cell Stem Cell 3(5):533–542

Kato Y, Kaneda M, Hata K, Kumaki K, Hisano M, Kohara Y, Okano M, Li E, Nozaki M, Sasaki H (2007) Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum Mol Genet 16(19):2272–2280

Kissel H, Timokhina I, Hardy MP, Rothschild G, Tajima Y, Soares V, Angeles M, Whitlow SR, Manova K, Besmer P (2000) Point mutation in kit receptor tyrosine kinase reveals essential roles for kit signaling in spermatogenesis and oogenesis without affecting other kit responses. EMBO J 19(6):1312–1326. doi:10.1093/emboj/19.6.1312

Ko K, Tapia N, Wu G, Kim JB, Bravo MJ, Sasse P, Glaser T, Ruau D, Han DW, Greber B, Hausdorfer K, Sebastiano V, Stehling M, Fleischmann BK, Brustle O, Zenke M, Scholer HR (2009) Induction of pluripotency in adult unipotent germline stem cells. Cell Stem Cell 5(1):87–96

Ko K, Arauzo-Bravo MJ, Tapia N, Kim J, Lin Q, Bernemann C, Han DW, Gentile L, Reinhardt P, Greber B, Schneider RK, Kliesch S, Zenke M, Scholer HR (2010) Human adult germline stem cells in question. Nature 465(7301):E1; discussion E3

Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, Gromoll J, Turek PJ, Reijo-Pera RA (2009) Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells 27(1):138–149

Kubota H, Brinster RL (2006) Technology insight: In vitro culture of spermatogonial stem cells and their potential therapeutic uses. Nat Clin Pract Endocrinol Metab 2(2):99–108

Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 100(11):6487–6492

Kubota H, Avarbock MR, Brinster RL (2004a) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731

Kubota H, Avarbock MR, Brinster RL (2004b) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101(47):16489–16494

Kung JW, Forbes SJ (2009) Stem cells and liver repair. Curr Opin Biotechnol 20(5):568–574

Lassalle B, Bastos H, Louis JP, Riou L, Testart J, Dutrillaux B, Fouchet P, Allemand I (2004) 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 131(2):479–487

Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL (1999) Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13(4):424–436

Lee J, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, Kimura T, Nakano T, Ogura A, Shinohara T (2007) Akt mediates self-renewal division of mouse spermatogonial stem cells. Development 134(10):1853–1859

Lim JJ, Sung SY, Kim HJ, Song SH, Hong JY, Yoon TK, Kim JK, Kim KS, Lee DR (2010) Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture conditions. Cell Prolif 43(4):405–417

Looijenga LH, Stoop H, de Leeuw HP, de Gouveia Brazao CA, Gillis AJ, van Roozendaal KE, van Zoelen EJ, Weber RF, Wolffenbuttel KP, van Dekken H, Honecker F, Bokemeyer C, Perlman EJ, Schneider DT, Kononen J, Sauter G, Oosterhuis JW (2003) POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. Cancer Res 63(9):2244–2250

Mahowald AP, Hennen S (1971) Ultrastructure of the "germ plasm" in eggs and embryos of Rana pipiens. Dev Biol 24(1):37–53

Mark M, Jacobs H, Oulad-Abdelghani M, Dennefeld C, Feret B, Vernet N, Codreanu CA, Chambon P, Ghyselinck NB (2008) STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation. J Cell Sci 121(Pt 19):3233–3242

Matsui Y, Zsebo K, Hogan BL (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70(5):841–847

Matzuk MM, Lamb DJ (2008) The biology of infertility: research advances and clinical challenges. Nat Med 14(11):1197–1213

McLaren A (2003) Primordial germ cells in the mouse. Dev Biol 262(1):1–15

Meistrich ML (1993) Effects of chemotherapy and radiotherapy on spermatogenesis. Eur Urol 23(1):136–141; discussion 142

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493

Mizrak SC, Chikhovskaya JV, Sadri-Ardekani H, van Daalen S, Korver CM, Hovingh SE, Roepers-Gajadien HL, Raya A, Fluiter K, de Reijke TM, de la Rosette JJ, Knegt AC, Belmonte JC, van der Veen F, de Rooij DG, Repping S, van Pelt AM (2010) Embryonic stem cell-like cells derived from adult human testis. Hum Reprod 25(1):158–167

Nagano M, Patrizio P, Brinster RL (2002) Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril 78(6):1225–1233

Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S (2010) Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328(5974):62–67

Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod 74(2):314–321

Ning L, Goossens E, Geens M, Van Saen D, Van Riet I, He D, Tournaye H (2010) Mouse spermatogonial stem cells obtain morphologic and functional characteristics of hematopoietic cells in vivo. Hum Reprod 25(12):3101–3109. doi:10.1093/humrep/deq269

Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169(3):515–531

Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263–286

Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL (2006) Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A 103(25):9524–9529

Oatley JM, Avarbock MR, Brinster RL (2007) Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem 282(35):25842–25851

Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (1998) Leuprolide, a gonadotropin-releasing hormone agonist, enhances colonization after spermatogonial transplantation into mouse testes. Tissue Cell 30(5):583–588

Ohbo K, Yoshida S, Ohmura M, Ohneda O, Ogawa T, Tsuchiya H, Kuwana T, Kehler J, Abe K, Schöler HR, Suda T (2003) Identification and characterization of stem cells in prepubertal spermatogenesis in mice. Dev Biol 258(1):209–225

Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton SC, Obukhanych T, Nussenzweig M, Tarakhovsky A, Saitou M, Surani MA (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 436(7048):207–213

Ohinata Y, Seki Y, Payer B, O'Carroll D, Surani MA, Saitou M (2006) Germline recruitment in mice: a genetic program for epigenetic reprogramming. Ernst Schering Res Found Workshop 60:143–174

Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T, Ohbo K (2004) Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol 67(4):285–296

Ohta H, Yomogida K, Dohmae K, Nishimune Y (2000) Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development 127(10):2125–2131

Orwig KE, Ryu BY, Master SR, Phillips BT, Mack M, Avarbock MR, Chodosh L, Brinster RL (2008) Genes involved in post-transcriptional regulation are overrepresented in stem/progenitor spermatogonia of cryptorchid mouse testes. Stem Cells 26(4):927–938

Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S (2003) Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. J Cell Sci 116(Pt 16):3363–3372. doi:10.1242/jcs.00650

Resnick JL, Bixler LS, Cheng L, Donovan PJ (1992) Long-term proliferation of mouse primordial germ cells in culture. Nature 359(6395):550–551

Ronaghi M, Erceg S, Moreno-Manzano V, Stojkovic M (2010) Challenges of stem cell therapy for spinal cord injury: human embryonic stem cells, endogenous neural stem cells, or induced pluripotent stem cells? Stem Cells 28(1):93–99

Rossi P, Lolicato F, Grimaldi P, Dolci S, Di Sauro A, Filipponi D, Geremia R (2008) Transcriptome analysis of differentiating spermatogonia stimulated with kit ligand. Gene Expr Patterns 8(2):58–70. doi:10.1016/j.modgep.2007.10.007

Ryu B-Y, Orwig KE, Kubota H, Avarbock MR, Brinster RL (2004) Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol 274(1):158–170

Ryu BY, Orwig KE, Oatley JM, Avarbock MR, Brinster RL (2006) Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells 24(6):1505–1511

Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, Hovingh S, de Reijke TM, de la Rosette JJ, van der Veen F, de Rooij DG, Repping S, van Pelt AM (2009) Propagation of human spermatogonial stem cells in vitro. JAMA 302(19):2127–2134

Saitou M, Barton SC, Surani MA (2002) A molecular programme for the specification of germ cell fate in mice. Nature 418(6895):293–300

Saitou M, Kagiwada S, Kurimoto K (2012) Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development 139(1):15–31

Salih DAM, Brunet A (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr Opin Cell Biol 20(2):126–136

Sato M, Kimura T, Kurokawa K, Fujita Y, Abe K, Masuhara M, Yasunaga T, Ryo A, Yamamoto M, Nakano T (2002) Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. Mech Dev 113(1):91–94

Schaefer CB, Ooi SK, Bestor TH, Bourc'his D (2007) Epigenetic decisions in mammalian germ cells. Science 316(5823):398–399

Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM (1999) Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 140(12):5894–5900. doi:10.1210/endo.140.12.7172

Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW, Yancopoulos GD, Murphy A, Valenzuela DM, Hobbs RM, Pandolfi PP, Rafii S (2007) Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature 449(7160):346–350

Seki Y, Hayashi K, Itoh K, Mizugaki M, Saitou M, Matsui Y (2005) Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. Dev Biol 278(2):440–458

Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A 95(23):13726–13731

Shetty G, Meistrich ML (2005) Hormonal approaches to preservation and restoration of male fertility after cancer treatment. J Natl Cancer Inst Monogr 34:36–39

Shiba Y, Hauch KD, Laflamme MA (2009) Cardiac applications for human pluripotent stem cells. Curr Pharm Des 15(24):2791–2806

Shima JE, McLean DJ, McCarrey JR, Griswold MD (2004) The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 71(1):319–330

Shinohara T, Avarbock MR, Brinster RL (1999) beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96(10):5504–5509

Simon L, Ekman GC, Kostereva N, Zhang Z, Hess RA, Hofmann MC, Cooke PS (2009) Direct transdifferentiation of stem/progenitor spermatogonia into reproductive and nonreproductive tissues of all germ layers. Stem Cells 27(7):1666–1675. doi:10.1002/stem.93

Simon L, Ekman GC, Garcia T, Carnes K, Zhang Z, Murphy T, Murphy KM, Hess RA, Cooke PS, Hofmann M-C (2010) ETV5 regulates sertoli cell chemokines involved in mouse stem/progenitor spermatogonia maintenance. Stem Cells 28(10):1882–1892

Small CL, Shima JE, Uzumcu M, Skinner MK, Griswold MD (2005) Profiling gene expression during the differentiation and development of the murine embryonic gonad. Biol Reprod 72(2):492–501

Streckfuss-Bomeke K, Vlasov A, Hulsmann S, Yin D, Nayernia K, Engel W, Hasenfuss G, Guan K (2009) Generation of functional neurons and glia from multipotent adult mouse germ-line stem cells. Stem Cell Res 2(2):139–154

Streckfuss-Bomeke K, Jende J, Cheng IF, Hasenfuss G, Guan K (2014) Efficient generation of hepatic cells from multipotent adult mouse germ-line stem cells using an OP9 co-culture system. Cell Reprogram 16(1):65–76. doi:10.1089/cell.2013.0057

Strome S, Lehmann R (2007) Germ versus soma decisions: lessons from flies and worms. Science 316(5823):392–393

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

Tapia N, Arauzo-Bravo MJ, Ko K, Scholer HR (2011) Concise review: challenging the pluripotency of human testis-derived ESC-like cells. Stem Cells 29(8):1165–1169

Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290(2):193–200

Tokuda M, Kadokawa Y, Kurahashi H, Marunouchi T (2007) CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 76(1):130–141

Tolkunova EN, Malashicheva AB, Chikhirzhina EV, Kostyleva EI, Zeng W, Luo J, Dobrinskiĭ I, Hierholzer A, Kemler R, Tomilin AN (2009) E-cadherin as a novel surface marker of spermatogonial stem cells. Tsitologiia 51(3):212–218

Turovets N, Semechkin A, Kuzmichev L, Janus J, Agapova L, Revazova E (2011) Derivation of human parthenogenetic stem cell lines. Methods Mol Biol 767:37–54

Tyagi G, Carnes K, Morrow C, Kostereva NV, Ekman GC, Meling DD, Hostetler C, Griswold M, Murphy KM, Hess RA, Hofmann M-C, Cooke PS (2009) Loss of Etv5 decreases proliferation and RET levels in neonatal mouse testicular germ cells and causes an abnormal first wave of spermatogenesis. Biol Reprod 81(2):258–266

Van Pelt AM, De Rooij DG (1990a) The origin of the synchronization of the seminiferous epithelium in vitamin A-deficient rats after vitamin A replacement. Biol Reprod 42(4):677–682

van Pelt AM, de Rooij DG (1990b) Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. Biol Reprod 43(3):363–367

Varanou A, Page CP, Minger SL (2008) Human embryonic stem cells and lung regeneration. Br J Pharmacol 155(3):316–325

Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA, de Rooij DG (1991) Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 93(1):233–243

Verhoeven G, Hoeben E, De Gendt K (2000) Peritubular cell-Sertoli cell interactions: factors involved in PmodS activity. Andrologia 32(1):42–45

Walker WH (2011) Testosterone signaling and the regulation of spermatogenesis. Spermatogenesis 1(2):116–120. doi:10.4161/spmg.1.2.16956

Wu X, Schmidt JA, Avarbock MR, Tobias JW, Carlson CA, Kolon TF, Ginsberg JP, Brinster RL (2009a) Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci U S A 106(51):21672–21677

Wu Z, Falciatori I, Molyneux LA, Richardson TE, Chapman KM, Hamra FK (2009b) Spermatogonial culture medium: an effective and efficient nutrient mixture for culturing rat spermatogonial stem cells. Biol Reprod 81(1):77–86

Wu Z, Luby-Phelps K, Bugde A, Molyneux LA, Denard B, Li WH, Suel GM, Garbers DL (2009c) Capacity for stochastic self-renewal and differentiation in mammalian spermatogonial stem cells. J Cell Biol 187(4):513–524

Yabuta Y, Kurimoto K, Ohinata Y, Seki Y, Saitou M (2006) Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. Biol Reprod 75(5):705–716

Yeh JR, Zhang X, Nagano MC (2011) Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. J Cell Sci 124(Pt 14):2357–2366

Yoshida S, Sukeno M, Nabeshima Y-I (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317(5845):1722–1726

Zheng K, Wu X, Kaestner KH, Wang PJ (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9:38. doi:10.1186/1471-213X-9-38

Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, Pouchnik D, Banasik B, McCarrey JR, Small C, Griswold MD (2008a) Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. Biol Reprod 78(3):537–545

Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess RA, Small C, Griswold MD (2008b) Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. Biol Reprod 79(1):35–42

Zhu S, Wei W, Ding S (2011) Chemical strategies for stem cell biology and regenerative medicine. Annu Rev Biomed Eng 13:73–90

Chapter 5
Hematopoietic Stem Cells

Mary Clarke and Jonathan Frampton

Abstract Hematopoietic stem cells represent the most studied and understood adult stem cell, and have consequently set the trends for the investigation of a wide array of stem cells, while their clinical use for over half a century and ever improving efficacy encourages the view that stem cell therapy will one day be useful in the treatment of a whole host of diseases that involve cell loss. In this chapter we describe how hematopoietic stem cells can be identified, isolated and characterized, and how important it is to be able to conduct experiments on animal models as well as humans, especially as studies in animals can provide the best, sometimes only, way to test stem cell potential and new protocols for their therapeutic use. The increasing possibilities for bone marrow regenerative medicine raised by the rapid developments in our ability to derive pluripotent stem cells from any individual are discussed, in particular because these are likely to be a very effective source of hematopoietic stem cells for all people requiring them to be replaced, as well as the exciting prospect that they can provide a route for the correction of inherited diseases affecting the blood system.

Keywords Hematopoietic stem cells • Bone marrow • Lymphoid cells • Myeloid cells • Classification • Colony-forming unit • Differentiation • Antigenic phenotype • Myeloproliferation • Anemia

5.1 Introduction

The hematopoietic stem cell (HSC) represents a paradigm for much of present day stem cell biology and regenerative medicine. The first therapeutic application of HSC, predating any characterization or even knowledge of its actual existence, was the pioneering development by E. Donnall Thomas in 1957 of bone marrow transplantation (BMT) as a therapy to alleviate the consequences of radiation and

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_5

chemotherapy (Thomas et al. 1957). This ground breaking bone marrow therapy gave rise to the belief that tissue stem cells hold the future promise for regenerative medicine for numerous diseases. The strategies for characterization, purification and bioassay of HSC have therefore been adapted for many other tissue-specific stem cells, while the drive to understand the cellular and molecular properties of HSC has provided a framework for studies of various embryonic and adult stem cell types. Comparison of the behaviour of HSC and leukemia cells was also highly influential in the origin of the concept of the cancer stem cell as the underlying component of many tumors, highlighting an exciting potential target for novel therapies that may succeed in achieving life-long remission where traditional anti-cancer treatments often fail.

Given the extensive history in the use of HSC in therapeutic practice, it would be easy to assume that they are well understood, however research on these stem cells is a dynamic area, continually leading to re-evaluation of their nature and potential. Once believed to be a homogenous population, it has more recently emerged that HSC are actually not a single entity, but rather a collection of cell subtypes with pre-programmed differentiation and self-renewal behaviours. The nature of HSC varies throughout development, distinct cell types arising to provide a transient source of hematopoietic cells. Since it is beyond the scope of this chapter, the reader is referred to one of the many excellent reviews that discuss the developmental aspects of HSC biology (Dzierzak et al. 1998; Mikkola and Orkin 2006; Cumano and Godin 2007; Dzierzak and Speck 2008), and here we will concentrate on those HSC that have the most relevance to regenerative medicine, namely adult bone marrow derived cells and those HSC that can be isolated from umbilical cord blood.

The necessity for continual regeneration of the various lymphoid (B-cells, T-cells, natural killer cells, dendritic cells) and myeloid (red cells, platelets, monocytes and macrophage, dendritic cells, granulocytes) cells that constitute the hematopoietic system is emphasized when we consider the vast number of cells, approximately 10^{12}, arising in human bone marrow on a daily basis (Doulatov et al. 2012). These mature adult hematopoietic cells are generated through a succession of hierarchical steps initiating at the apex of the hematopoietic system with the HSC. The HSC gives rise to a series of more committed progenitors with the potential to proliferate ('transit amplifying' cells). Increasing cell specialization is accompanied by a gradual decrease in proliferation potential, resulting ultimately in the supply of terminally differentiated functional blood cell types that make up the lymphoid and myeloid compartments. Although the hematopoietic system has been extensively studied for several decades, it is only in the last few years that we have begun to understand some of the molecular mechanisms that dictate HSC function. These developments have been made with the help of technology enabling complex cell sorting strategies for the isolation of HSC at high purity and viability combined with sophisticated assays of stem cell function both in vitro and in vivo. The ultimate extension of cell sorting to provide single cells for analysis has allowed detailed descriptions of the cell and molecular characteristics of individual HSC, which has revealed a somewhat different picture of their properties than is apparent simply from studying the averaged results obtained from a purified population of cells.

Historically, the majority of studies defining HSC and their differentiation potentials have been conducted using the mouse as a model, while investigations of human HSC have trailed somewhat behind so that some of our understanding of them is fairly rudimentary. Apart from the fact that there is a relative dearth of useful surface markers for the characterization of human HSC, the fact that the most meaningful indicator of stem cell activity is their ability to function in vivo as revealed through repopulation assays poses a particular difficulty in human stem cell research.

In addition to increasingly refined definition of HSC, especially those that have the greatest potential for application in a therapeutic context, key areas of investigation that will impinge heavily on the success or otherwise of advances in regenerative medicine include finding ways to expand HSC in vitro without loss of any aspect of their functional potential, and improving upon the efficiency with which transplanted cells integrate into the hematopoietic system. Perhaps the most exciting challenge, which could one day lead to an unlimited ability to provide replacement HSC personalized for the patient, is their derivation from pluripotent stem cells, and this will be discussed with respect to advances that have been made using embryonic stem (ES) cells and more recently with the discovery of methods to produce so called induced pluripotent stem (iPS) cells from any cell in the body.

5.2 Derivation and Classification

A major obstacle in HSC research is that the cells are incredibly rare, with only 1 in 10^6 cells in human bone marrow being a functional transplantable stem cell (Wang et al. 1997). Although the first application of bone marrow derived stem cells in a therapeutic context was over five decades ago, the vast majority of our understanding of the nature of HSC has come from studies on mouse bone marrow. Modern day laboratories utilize two main methods for isolating HSC from bone marrow, which depending on the precise criteria applied, represent only 0.05 % or less of the nucleated cells in the mouse, meaning that a mere 5000 HSC can be isolated from an individual animal. The first method (MACS) involves enrichment employing magnetic beads conjugated to antibodies against a specific surface marker. The second, and notably more precise separation technique, utilizes fluorescence activated cell sorting (FACS), which is based on immunofluorescent labeling of surface antigens as a means to mark cells for separation from the bulk population (Challen et al. 2009). State-of-the-art cell sorters are now equipped with up to 7 powerful lasers and have the capability simultaneously to detect fluorescence from up to 18 fluorochrome-labeled antibodies directed against multiple markers (usually designated as ‘Cluster of Differentiation’ or CD markers) enabling prospective isolation of more infrequent cells. Many current cell sorters are able to separate single HSC into individual wells in multi-well plates permitting molecular characterization of gene expression and assessment of their self-renewal and differentiation potentials.

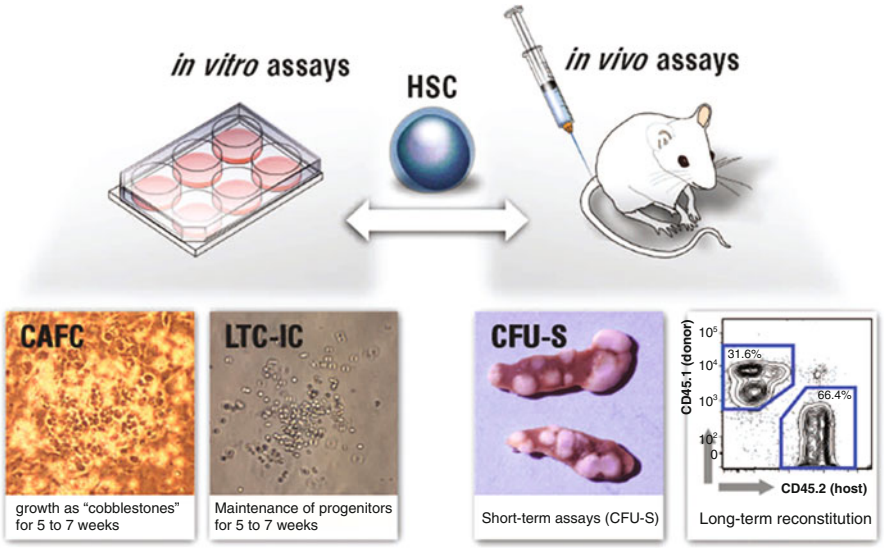


Fig. 5.1 Assays HSC. In vitro culture assays of HSC include the ability of the cells to grow for 5–7 weeks as 'cobblestones' (the *dark cells* in the picture) leading to the term 'cobblestone area forming cell' (CAFC). The Long Term Culture-Initiating Cell (LTC-IC) assay measures whether hematopoietic progenitor cells (capable of forming colonies in secondary assays, as shown in the picture) are still present after 5–7 weeks of culture. In vivo assays in mice include the CFU-S assay, the original stem cell assay developed by Till and McCulloch. The picture shows spleens isolated from mice injected with CFU-S that exhibit several hematopoietic colonies developed from the stem cells. The most stringent HSC assay involves looking for the long-term presence of donor-derived cells in a reconstituted mouse host. The example shows host-donor recognition by antibodies that recognize two different mouse alleles of CD45 (Adapted from Bone Marrow (Hematopoietic) Stem Cells by Domen et al. http://stemcells.nih.gov/info/Regenerative_Medicine)

5.2.1 Bioassays of HSC

In the strictest sense, the HSC is defined by its functional capacity to reconstitute the entire hematopoietic system for the lifetime of the individual or animal; however, a number of less stringent bioassays are also widely used, often as a preliminary guide because the definitive in vivo bone marrow transplantation assay is both time-consuming and costly (Fig. 5.1).

The first assays of hematopoietic progenitor cell potential in vivo can be attributed to James Till and Ernest McCulloch, who famously demonstrated that colonies of myeloid cells developing in the spleen following transplantation of bone marrow into lethally irradiated mice were clonal (Till and McCulloch 1961). However, the existence of long-lived stem cells in the bone marrow was deduced from subsequent experiments involving clonal tracking of serial transplantations (Dick et al. 1985; Lemischka et al. 1986). Arising out of these studies, the current gold standard assay of mouse HSC is generally accepted to be long-term repopulation of lethally irradiated mice in a situation in which the cells being tested are compared to a reference

wild type population (Harrison et al. 1993). Most often, test and reference strains are used that are congenic (i.e. genetically identical save at a specific genetic locus) for allelic variants of CD45 (previously known as Ly5), which can easily be distinguished by immunofluorescent flow cytometry. 'Long-term' is taken to mean a sustained output from the graft of at least 1 % of all circulating white blood cells for at least 4 months (Purton and Scadden 2007). The most rigorous test of HSC potential involves assessment of their ability to be serially transplanted from the primary reconstituted recipient to a secondary irradiated host, thereby demonstrating that engrafting cells are undergoing self-renewal. Competitive repopulation assays performed this way are at best semi-quantitative, and a more refined method, involving limiting dilution, allows determination of the frequency of HSC. In this assay, a series of dilutions of the test population are competed against reference wild type bone marrow cells. The number of mice negative for reconstitution in each cell dose is measured and the frequency of HSC ('competitive repopulating units' or CRU) is estimated using Poisson statistics (Szilvassy et al. 1990). Purton and Scadden (2007) discuss the finer details of repopulation assays, how they are best interpreted, and their possible limitations.

Although in vivo assays are essential in order fully to define and quantify stem cell potential, they have some limitations that can be complemented by a range of assays that can be performed in vitro. First, and rather obviously, in vivo assays can take many months to complete and require extensive and costly facilities, so it is often useful to have a more simple assay that can be used to make an initial assessment of the likely HSC content, for example while developing a strategy for prospective cell sorting or following some experimental manipulation that is expected to have a significant effect on HSC function. Second, the output from an in vivo assay is the consequence of many biological events following transplantation, including homing, self-renewal, HSC commitment and the behaviour of downstream progenitors and differentiated cells, and it is often important to be able to determine cellular properties at a single cell level immediately following isolation of putative HSC. Several distinct in vitro assays are used that measure the frequency of progenitors (colony-forming unit in culture; CFU-C), stem cells (long-term culture-initiating cell; LTC-IC), or both (cobblestone area-forming cell assay; CAFC), the latter two correlating at least to some extent with in vivo activity (van Os et al. 2004).

CFU-C assays, pioneered by the work of Don Metcalf and colleagues (Bradley and Metcalf 1966; Moore et al. 1973), allow detection and quantification of myeloid progenitors present in the population of cells being analyzed or that could have arisen in vitro from more immature cells, including the HSC. The culture conditions rely on the presence of growth factors and nutrients that will permit complete differentiation along one or more of the pathways of differentiation that a particular cell is expected to be capable of adopting. CFU-C assays have been essential for determining the specific growth factors necessary for HSC maintenance, proliferation and differentiation. These assays have also been crucial in the characterization of leukemic stem cells (LSC). The assessment of lymphoid CFU potential in vitro has in the past been more difficult, requiring co-culture systems such as that of

OP9-DL1 cells, a mouse stromal cell line that expresses the Notch ligand Delta-like 1 (DL1), for establishing T-cell differentiation (Whitlock and Witte 1982; Schmitt and Zuñiga-Pflucker 2002). However, recent demand for improved mouse B-cell differentiation has led to the development of media capable of supporting such specification, similar to that already used for myeloid lineages. The progress for human lymphoid cell differentiation is, however, still somewhat marred due to the insufficient knowledge of the cytokines responsible for this (Doulatov et al. 2012). Human B-cell differentiation is feasible when HSC are co-cultured for 2–4 weeks upon the stromal cell lines MS-5 or S17 in the presence of SCF, TPO, IL-7 and IL-2.

The basic principle of in vitro assays in the presence of specific growth factors and cytokines is to determine what a stem cell or progenitor is capable of giving rise to and their proliferative abilities following gene manipulation, as can be recognized after a number of days by the specific features of the differentiated cells (eg surface marker expression, cell morphology and the presence of characteristic cytoplasmic enzyme activities, etc). In the right conditions, a single HSC can give rise to multiple cell lineages, whereas a more mature hematopoietic progenitor cell will have a more restricted capability. Since it would not be possible to discriminate from whence the individual differentiated cells originated if such assays were performed in a liquid culture of the whole sorted population, these assays are generally carried out in one of two ways so that the potential of individual cells can be observed. Most commonly, a cell population is seeded into the appropriate growth conditions in media that also contain a substance that is like a soft gel (usually methycellulose). This prevents the cells from moving around extensively, and if seeded at the correct density means that the differentiated derivatives from each cell are clearly separated and can eventually be collected for phenotypic analysis. Alternatively, sorted stem cells can be deposited as single cells into tiny individual wells in a plastic dish where they then can be allowed to grow and differentiate in liquid conditions (Ema et al. 2000; Takano et al. 2004).

The CAFC and LTC-IC assays, based on the original studies by Dexter and colleagues (1977), involve culture of stem cell populations with adherent cells that mimic the normal HSC microenvironment. The CAFC assay measures the frequency of cells that are capable of growing under the stromal layer, and by enumerating so-called ‘cobblestone’ areas at various times it is possible to assess mature progenitors back to repopulating HSC (Ploemacher et al. 1989; Ploemacher et al. 1991). The LTC-IC assay is similar to the CAFC assay except that the readout is the presence of progenitors that can themselves be assayed for CFU-C capability (Sutherland et al. 1989; Lemieux et al. 1995).

Just as many of the in vitro assays are adaptable for the measurement of both murine and human stem cells and progenitors, there is an equal and ever growing need in the context both of regenerative medicine and for therapeutic targeting of diseased cells to be able to test in vivo potential of human HSC. Since this is clearly not feasible in humans a number of approaches have been developed over the last 30 years that rely on the generation of in vivo chimeras of human cells, or ‘xenografts’, in animals. Compared to assays of mouse HSC in congenic recipients, the testing of

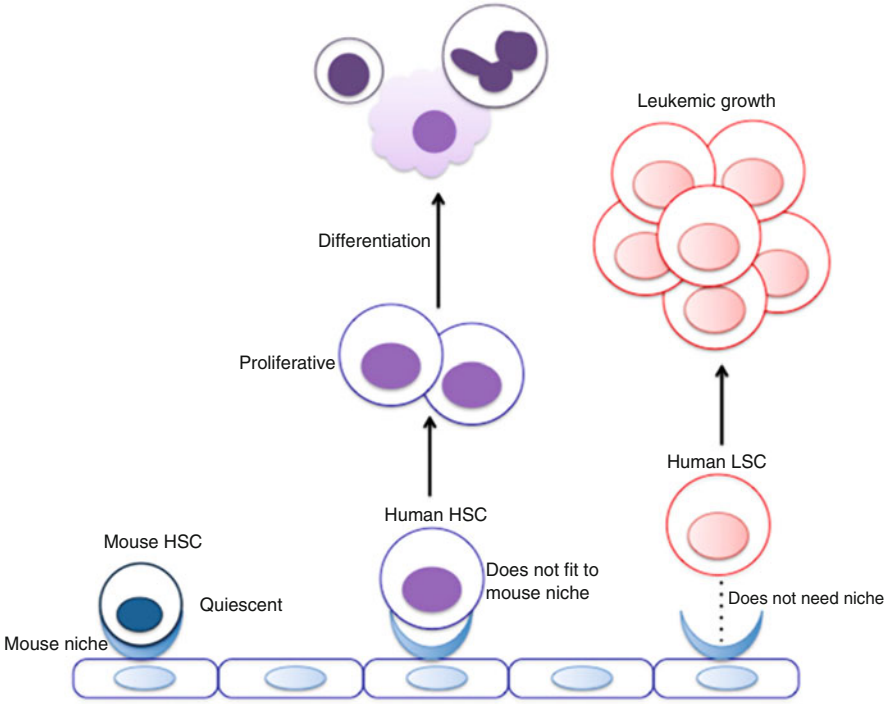


Fig. 5.2 Mouse and human HSC may not be equally supported by the mouse bone marrow niche. The schematic illustrates how mouse HSC engage with elements of the niche and can thereby persist in a quiescent state. In contrast, human HSC engage less well and as a consequence tend towards proliferation and differentiation. Human LSC from aggressive types of leukemia appear to be less dependent on the support from the mouse niche to maintain leukemic growth (Taken from Goyama et al. 2015)

human HSC in animal models is far less definitive and quantitative, especially because of the diversity of the model systems that have been employed, and there is no agreed standard to which different investigators can adhere. The usual host animal for xenograft assays of human HSC is the mouse, although larger animals have been utilized in some circumstances. There are several limitations associated with the xenograft approach, not least of which is the effect of the host immune system in the face of 'foreign' cells. Additionally, the importance of the 'niche' (discussed in Chap. 3) as a determinant of stem cell engraftment is a major limitation of xenograft efficiency since species differences mean that the host bone marrow environment does not necessarily represent an appropriate niche for stem cell maintenance (Fig. 5.2), although some niche elements such as the SCF/Kit axis are able to operate across the species barrier (Cosgun et al. 2014). It is likely that at least some of the apparent differences between mouse and human HSC in terms of the surface antigens used to characterize them can be attributed to the shortcomings of the xenograft assays.

Although not ideal from the perspective of logistics, cost, and time, several investigators have shown that human HSC can be engrafted in sheep by direct introduction of cells into the early gestational age fetus. By in utero injection of human fetal liver HSC, Esmail Zanjani and colleagues were able to demonstrate long-term (greater than 2 years) engraftment in sheep (Zanjani et al. 1992). Subsequently, this approach was used to prove for the first time that human adult bone marrow cells could elicit long-term chimerism and sustain human hematopoiesis (Srour et al. 1993), and has been further developed (Zanjani et al. 1995, 1998), enabling most recently the demonstration that engrafted human cells can be mobilized in the sheep model in the same way that they would normally be in human donors (Almeida-Porada et al. 2007).

By far the majority of xenograft experiments of human HSC have been performed in mice (reviewed in Goyama et al. 2015), but unlike the experiments involving sheep, the human cells are injected into adults, thereby requiring that the hosts are immunologically deficient so that they will not bring about rejection of the xenogenic cells. Two strategies for human-into-mouse engraftment were developed originally by the work of McCune and Dick. In the first approach, human cells within a fragment of hematopoietic tissue are grafted under the kidney capsule, which provides a permissive environment for the donor cells (McCune et al. 1988; Namikawa et al. 1990; Chen et al. 1994). The much more widely utilized method involves adaptation of the usual protocol for transplantation of mouse HSC into host animals, the main difference being that the mice are subjected to sub-lethal irradiation to pre-condition the bone marrow by increasing the opportunity for HSC to occupy vacant niches (Kamel-Reid and Dick 1988).

To minimize the possibility of the human cells being rejected, several immunocompromised mouse models have been developed, in particular relying upon the Severe Combined Immunodeficiency (SCID) mutant strain. As for mouse-into-mouse repopulation assays, xenografts of human cells can be made quantitative through a limiting dilution approach, the term SCID Repopulating Unit (SRU) usually being adopted to define the numbers of functional long-term HSC. SCID mice are deficient in both B- and T-cell mediated immunity, and their usefulness has been enhanced in various ways through combination with other spontaneous or engineered mutations (Fig. 5.3).

The strain that has been most commonly used for xenografting brings together the Non-Obese Diabetic (NOD) mutation with SCID, usually referred to as NOD/SCID, which lacks not only functional B and T lymphocytes, but also has low levels of natural killer (NK) cell activity (Shultz et al. 1995). A reduced overall cellularity of the bone marrow in NOD/SCID mice may also facilitate engraftment of HSC because of the availability of suitable niches for stem cells. The NOD/SCID mouse exhibits some features that partially limit its usefulness, especially for long-term xenograft models, such as shortened lifespan due to high incidence of thymic lymphoma, some spontaneous production of functional lymphocytes with aging, and residual innate immunity. Further incorporation of the β2 microglobulin knockout into the NOD/SCID background increased the efficiency of repopulation by umbilical cord blood cells by over tenfold (Kollet et al. 2000).

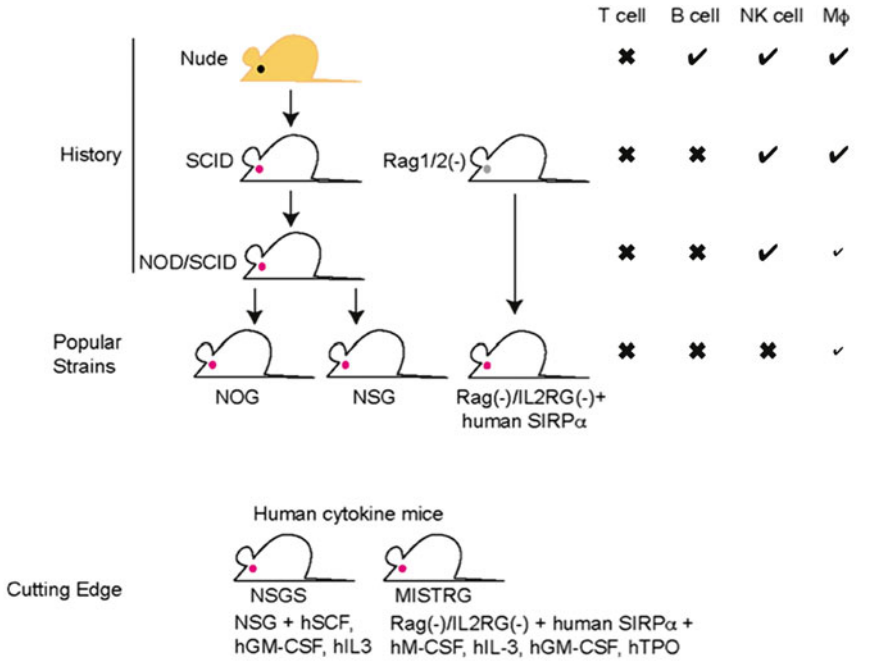


Fig. 5.3 Mouse models for engraftment of human cells. The first strains of mice (Nude, SCID and Rag knockout) that were used as hosts for human cells are compromised (indicated by a cross) in their lymphoid compartment but retain (indicated by a tick) NK and myeloid cell aspects of immunity. Subsequent crossing of strains, deletion of the IL2 receptor γ chain, and introduction of specific transgenes such as SIRPα and cytokine genes, has created strains that are both more immune deficient and support HSC engraftment (Adapted from Goyama et al. 2015)

To circumvent the problems associated with NOD/SCID mice as hosts for xenografts, NOD/SCID mice with a truncation or a deletion of the IL-2R common γ chain, which is a critical component for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signaling, were developed (the so-called NOG and NSG strains: Ito et al. 2002; Shultz et al. 2005). The deletion of the IL-2R common γ chain gene results in a complete loss of B, T and NK cells. The NSG strain was shown to support a fivefold higher $CD34^+$ cell engraftment compared with NOD/SCID mice. A second benefit of these mice is that the deficiency in cytokine signaling prevented the formation of lymphomas, permitting long-term studies (Ito et al. 2002; Ishikawa et al. 2005; reviewed by Ito et al. 2008).

Mouse lines lacking the Rag2 recombinase activity involved in the rearrangement of immunoglobulin and T-cell receptor genes have also been much applied as immunodeficient models, especially in combination with deletion of the IL-2R common γ chain (Goldman, 1998). More recently this model has been improved through transgenic expression of the human signal-regulatory protein alpha (SIRPα), which is a receptor-type transmembrane glycoprotein known to be involved in the negative regulation of receptor tyrosine kinase-coupled signaling processes (Strowig et al. 2011).

As hematopoietic research progresses and the importance of specific cytokines are determined, many investigators have found that the efficiency of engraftment of human HSC can be enhanced by treatment of the host with cytokines or co-transplantation of accessory cells. For example, Bonnet et al. (1999) demonstrated that low numbers of purified cord blood-derived immature cells would engraft NOD/SCID mice effectively if co-transplanted with more mature cell populations that had been irradiated to prevent cell division or by short-term in vivo treatment with the growth factors and cytokines stem cell factor (SCF), interleukin 3 (IL3) and granulocyte macrophage colony stimulating factor (GM-CSF). Similarly, bone marrow chimerism in the SCID or Rag2/IL2Rγ double knockout models could be facilitated by administration of IL3, GM-CSF and erythropoietin (Lapidot et al. 1992) or IL3, GM-CSF and erythropoietin (Mazurier et al. 1999), respectively. Co-transplantation of stromal cells has also been shown to have some benefit in establishing HSC xenografts in mice. Hence, primary bone marrow stroma modified to express IL3 was able to enhance HSC engraftment (Nolta et al. 1994), while unmodified mesenchymal stem cells (MSC) derived from the fetal lung or bone marrow increased HSC engraftment, but in the latter case it appeared that the effect might not to require homing of MSC to the bone marrow (Noort et al. 2002; in 't Anker et al. 2003; Bensidhoum et al. 2004). More recent advances in strains for xenografting have seen the generation of mice with cytokine-expressing human transgene knock-ins, encoding for example thrombopoietin (TPO), SCF, IL-3, and GM-CSF, all of which have exhibited augmented engraftment of the human cells following transplantation (Wunderlich et al. 2010; Rongvaux et al. 2011, 2014; Willinger et al. 2011).

Despite the improvements achieved in the short-term engraftment of human HSC through the use of sophisticated immunodeficient mice, the degree to which the cells can self-renew and undergo effective serial transplantation is limited. As mentioned already, differences in the bone marrow niche environment certainly play a part, hindering the ability of transplanted HSC from entering a quiescent state. Such an explanation could account for the high percentage (10–20 %) of human CD34+ cells observed in the bone marrow of a xenograft compared to nearer 1 % in adult humans.

The protocols for engraftment of HSC have undergone a number of modifications over the years, including additional preconditioning of mice by treatment with clodronate-containing liposomes in order to delete macrophage (Fraser et al. 1995; van Rijn et al. 2003) or with an antibody against the surface antigen CD122 in order to target NK cells and macrophage that act as a barrier to stem cell engraftment (McKenzie et al. 2005). Furthermore, to overcome the limitations of homing and cellular loss in the lungs that is inherent in intravenous injection of cells via the tail vein, a number of investigators have achieved much improved rates of engraftment by direct injection of HSC into the bone marrow cavities of either the femur or tibia (Kushida et al. 2001; Wang et al. 2003; Yahata et al. 2003; McKenzie et al. 2006).

5.2.2 HSC Antigenic Phenotype and Purification Schemes

Over the last quarter of a century the combined power of flow cytometry and the availability of monoclonal antibodies raised against a vast array of hematopoietic cell surface molecules, together with the various bioassays described above, has enabled an incredibly detailed definition of the heterogenous population of cells with stem cell activity in the hematopoietic hierarchy of the mouse. No single surface molecule has yet been found that enables identification of HSC; however, a number of markers have been described that can be used in combination to very precisely define stem cells, the particular drive being to isolate the rare cells with the highest potential for long-term reconstitution. The advances in this area have been most successful in the case of mouse bone marrow, and in comparison, the definition of human HSC is by no means as refined, partly due to of an absence of sufficient definitive surface markers but also, as already discussed, because the in vivo assays of stem cell activity are not ideal.

5.2.2.1 Mouse HSC

Most sorting strategies rely upon negative selection for markers of the differentiated hematopoietic lineages (Lin), which usually include B220, CD4 (sometimes CD3 or CD5 instead), CD8, Gr1, CD11b (Mac-1) and Ter119, in combination with positive selection for c-Kit (the receptor for SCF) and Sca-1 (stem cell antigen-1) (Okada et al. 1992), giving rise to the acronym LSK (or KSL, depending on laboratory preference). Historically, the Weissman group has been the driving force for the purification of HSC and their favoured protocol incorporates staining for the Thy1.1 antigen and selection of cells that express only low levels together with and absence of lineage markers and the presence of Sca-1 ($Thy1.1^{lo}$ Lin^- $Sca\text{-}1^+$ or TLS cells; Spangrude et al. 1988), although this precise strategy has not been widely adopted because the Thy1.1 antigen is not expressed on many of the most commonly used laboratory strains of mice. Both LSK and TLS populations contain long-term repopulating cells (LT-HSC), but these represent less than 10 % of the LSK cells, the remains of which have only short-term activity (ST-HSC) or are multipotent progenitors (MPP) with no capacity for self-renewal. Following these early studies, there has been an ever-driving urge to discover a unique marker of the mouse LT-HSC that truly distinguishes it from the heterogeneous population of stem cells. A number of investigators have identified additional markers that can be used to resolve the stem cell and progenitor components within the LSK population, and to this day improvements are still being published on a fairly regular basis. The Nakauchi laboratory were first to show that the expression of CD34 could be used to discriminate LT-HSC, in that single LSK $CD34^-$ cells were able to bring about long-term reconstitution (Osawa et al. 1996). The subsequent addition of Flt-3 (also known as Flk-2 and CD135) into the mix allowed prospective purification of not only LT-HSC (LSK $CD34^-$ $Flt3^-$), but also ST-HSC (LSK $CD34^+$ $Flt3^-$) and MPP

(LSK CD34$^+$ Flt3$^+$) (Christensen and Weissman 2001; Adolfsson et al. 2001; Yang et al. 2005). More recently, due to the adoption of more precise cell sorting strategies, it has become possible to fractionate these stem cell populations further into more discrete fractions with more specific properties. Hence, data suggests that the MPP initially differentiates into lymphoid-primed multipotential progenitors (LMPP), which retain the potential to give rise to lymphoid and granulocyte-macrophage cells but which lack megakaryocyte-erythroid potential (Adolfsson et al. 2005; Lai and Kondo 2006), while more committed myeloid and lymphoid progenitors lie downstream of these LMPP cells (Akashi et al. 2000; Pronk et al. 2007). These latter publications led to a redrawing of the accepted hematopoietic hierarchy model, but as further work elucidates more and more discrete subpopulations within the HSC compartment, our view of the hematopoietic hierarchy will continue to be modified.

In addition to surface marker expression, there are other characteristics of HSC that can be employed for their identification and isolation using flow cytometry, often most effective when used in combination with strategies such as those employing LSK or related staining protocols. The most widely used characteristic relies on the ability of HSC to actively expel small molecules from their cytoplasm, a mechanism of cytotoxic evasion. A family of transmembrane proteins known as ABC transporters are involved in a wide variety of normal cells and stem cells with the purpose of removing diverse chemicals. One family member, ABC-G2, is often expressed by stem cells and has the ability to export certain chemical dyes that have entered the cytoplasm by passive diffusion. Empirically, it was found that one such DNA binding supravital dye, Hoechst 33342, is removed by ABC-G2 and that this can be visualized with a flow cytometer by measuring red and blue fluorescent light emissions upon stimulating with a UV laser. In the complex pattern of light emitted by a mixture of cells treated with Hoechst 33342, many stem cells appear as a population, usually called the 'side population', which exhibits low red and blue fluorescence because the dye has been largely removed by the transporter (Goodell et al. 1996). The drawbacks of the Hoechst 33342 exclusion method are that the staining method is highly sensitive to slight changes in protocol, producing inconsistencies between HSC isolations. Unfortunately, side population characteristics are not restricted to stem cells, with approximately 15 % of whole bone marrow side population being negative for the stem cell markers c-Kit and Sca-1 (Challen et al. 2009). Also, not all stem cells exhibit the property, and therefore the technique is best utilized in combination with other methods, especially surface marker staining, as a means to refine stem cell identification and isolation (Challen et al. 2009). The other major flow cytometry method not involving specific antibodies makes use of the fluorescent vital dye rhodamine 123 (Rh-123), which preferentially accumulates in mitochondrial membranes and acts as an indicator of mitochondrial, and hence cellular, activity. Since the more immature HSC tend to be quiescent, sorting for cells exhibiting a low degree of fluorescence in the presence of Rh-123 enriches for long-term repopulating cells (Spangrude and Johnson 1990).

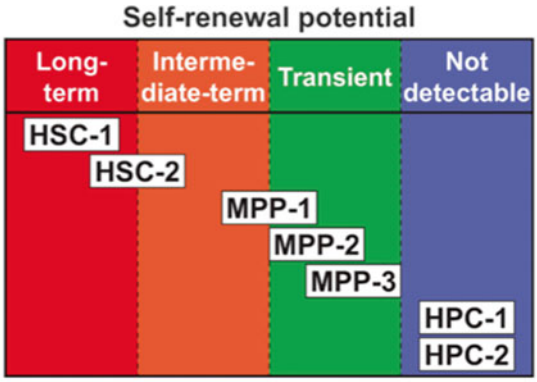
Subsequent, highly resolved strategies for the isolation of long-term repopulating HSC have largely built upon the basis of one or more of the LSK, side popula-

tion and Rh-123 staining methods. Chen et al. (2003) found that immunofluorescent staining for the ancillary TGFβ receptor endoglin (CD105) in combination with Sca-1 expression and low staining for Rh-123 defines a nearly homogenous population of LT-HSC without the use of CD34, c-Kit or Lin markers. As discussed later in this chapter, another feature of immature HSC is their tendency to be niche-associated and a marker linked to this property, namely the angiopoietin-2 receptor Tie2, has been used to select a subpopulation of LSK cells that are enriched in LT-HSC (Arai et al. 2004). Two advances based on RNA microarray screening for genes expressed exclusively in subfractions of HSC have probably made the most significant contribution to the robust isolation of highly enriched long-term repopulating cells. First, following initial identification from expression screening, antibodies against cell surface receptors of the SLAM family, including CD48, CD150 and CD244, were shown to discriminate HSC ($CD48^-$ $CD150^+$ $CD244^-$), MPP ($CD48^-$ $CD150^-$ $CD244^+$) and the most restricted progenitors ($CD48^+$ $CD150^-$ $CD244^+$) (Kiel et al. 2005). This is the first family of receptors whose combinatorial expression can be used to precisely distinguish stem and progenitor cells in the mouse. Similarly, microarray technology led to the identification of murine endothelial protein C receptor (EPCR, CD201) as a marker to sort cells, especially when used in combination with positivity for the antigen Sca-1, as it is expressed at high levels in HSC with a high reconstitution activity, and probably represents the first known marker that 'explicitly' identifies HSC within murine bone marrow (Balazs et al. 2006). The group of Conny Eaves combined these two latter approaches and have shown that LT-HSC with the most durable self-renewal potential, as demonstrated following serial transplantation, are selectively and highly enriched in the $CD150^+$ subset of the $EPCR^+$ $CD48^-$ $CD45^-$ fraction of bone marrow cells (Kent et al. 2009).

Most recently, the group of Sean Morrison has refined their use of the SLAM markers CD48, CD150, CD229 and CD244 to be able to discriminate seven subpopulations within the HSC LSK population in mice (Oguro et al. 2013). CD229 expression distinguishes functionally distinct subsets of HSC and MPP, $CD229^-$ HSC being mainly myeloid biased and more quiescent than $CD229^+$ HSC (Fig. 5.4).

In an interesting and distinctive strategy, the group of Bertrand Göttgens have used an iterative bioinformatics approach in which single sorted HSC, 'indexed' by their precise surface antigen profile, were analysed for expression of known regulatory transcription factors (Wilson et al. 2015; Schulte et al. 2015). Linking this data to the efficiency of single cell engraftment, they were then able to define a new and smaller set of surface antigens that uniquely characterize the most highly potent stem cells, including high expression of Sca-1 and EPCR.

The theme of heterogeneity and differing potentialities has also been pursued using transgenic lineage reporters. Notably, the groups of Nerlov and Jacobsen have used a fluorescent reporter driven by the von Willebrand Factor gene to demonstrate the presence of HSC in the mouse that have a preferential capacity to differentiate towards megakaryocytes (Sanjuan-Pla et al. 2013). Although the jury is still out to some extent, these latter sorts of observations, together with other definitions of HSC heterogeneity and differentiation potential based on single cell transplantations,



Fraction	Surface marker expression	% of WBM
HSC-1	$CD150^{+}CD48^{-/low}CD229^{-/low}CD244^{-}Lineage^{-/low}Sca\text{-}1^{+}c\text{-}Kit^{+}$	0.0020±0.0005
HSC-2	$CD150^{+}CD48^{-/low}CD229^{+}CD244^{-}Lineage^{-/low}Sca\text{-}1^{+}c\text{-}Kit^{+}$	0.0065±0.0021
MPP-1	$CD150^{-}CD48^{-/low}CD229^{-/low}CD244^{-}Lineage^{-/low}Sca\text{-}1^{+}c\text{-}Kit^{+}$	0.0006±0.0001
MPP-2	$CD150^{-}CD48^{-/low}CD229^{+}CD244^{-}Lineage^{-/low}Sca\text{-}1^{+}c\text{-}Kit^{+}$	0.0159±0.0038
MPP-3	$CD150^{-}CD48^{-/low}CD229^{+}CD244^{+}Lineage^{-/low}Sca\text{-}1^{+}c\text{-}Kit^{+}$	0.0237±0.0056
HPC-1	$CD150^{-}CD48^{+}Lineage^{-/low}Sca\text{-}1^{+}c\text{-}Kit^{+}$	0.1132±0.0271
HPC-2	$CD150^{+}CD48^{+}Lineage^{-/low}Sca\text{-}1^{+}c\text{-}Kit^{+}$	0.0032±0.0014

Fig. 5.4 Schematic and table summarizing the self-renewal potentials and surface antigen characteristics of each fraction of LSK mouse hematopoietic stem cells (HSC), multipotent progenitors (MPP) and hematopoietic progenitor cells (HPC) subdivided according to SLAM marker (CD48, CD150, CD229 and CD244) expression. The numbers in the table represent the proportion of whole bone marrow (WBM) that each fraction represents (Taken from Oguro et al. 2013)

point to there being distinct subtypes of HSC that have predetermined lineage bias. Moreover, the long-appreciated age-associated change in HSC potential that is reflected in increased myelopoiesis at the expense of lymphopoiesis appears to be underpinned by alterations in the relative contribution of these different subtypes to the overall pool of HSC (reviewed in Eaves 2015).

5.2.2.2 Human HSC

The abilities to identify and purify long-term reconstituting human HSC are at present somewhat less sophisticated compared to the situation with the mouse due to the lack of adequate methods to segregate HSC from MPP. Similar to the mouse, purification of human HSC requires simultaneous detection of several cell surface markers, and although informative, the specific strategies for isolating mouse HSC cannot be duplicated for human HSC (Fig. 5.5). This is due to differences in characteristic marker expression between the two species, the most prominent difference residing in their expression of CD34. The two principal sources of human HSC for therapeutic application, namely bone marrow and umbilical cord blood, also

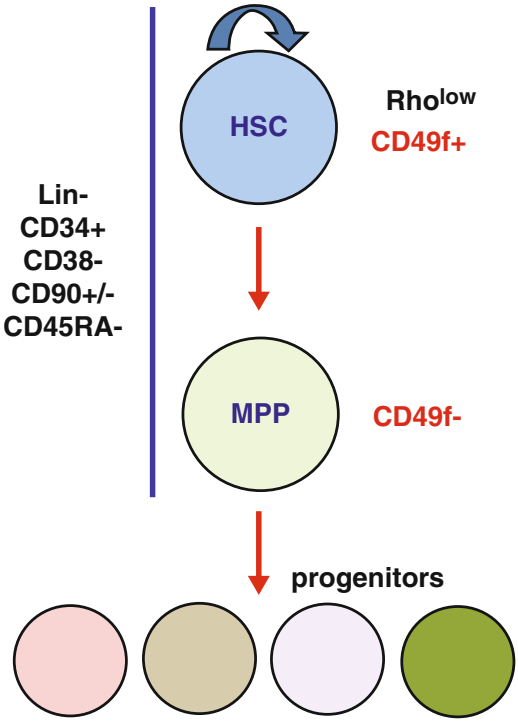


Fig. 5.5 Schematic of the surface marker differences that distinguish the most immature human HSC, which has the ability to repopulate all blood cell types, from the multipotent progenitor (MPP). The CD49f (α5 integrin) marker was key in discriminating the two cell types (Adapted from Notta et al. 2011)

demonstrate some differences in the precise pattern of markers, raising extra difficulties in determining the best strategies for cell purification in the clinic. Nevertheless, human HSC capable of multilineage engraftment in animal models can now be resolved with a reasonably high degree of enrichment.

The majority of human HSC are $CD34^+$ in contrast to mouse, as was first demonstrated during the Nineties when human Lin^-CD34^+ fetal bone marrow cells were shown to be able to engraft in SCID mice (Baum et al. 1992). However, although capable of engrafting in SCID mice, most $CD34^+$ cells were subsequently shown to be lineage-restricted progenitors and the true HSC remained elusive. Enrichment of human HSC can be achieved further on the basis of expression of CD45RA (Mayani et al. 1993), Thy-1 (Baum et al. 1992; Craig et al. 1993; Majeti et al. 2007) and CD38 (Hao et al. 1995; Bhatia et al. 1997). The recognized pattern of expression that segregates human HSC from MPP is that of $CD34^+$ $CD38^-CD45RA^-$ and loss of Thy1 expression (Majeti et al. 2007).

In contrast to $CD34^+$ cells, Lin^- $CD34^-$ $CD38^-$ cells have low clonogenicity in short-and long-term in vitro assays. However, the number of $CD34^-$ SRUs increased in short-term suspension cultures in conditions that did not maintain SRU derived from $CD34^+$ populations (Bhatia et al. 1998). Based on its association with colony-forming potential and repopulation capacity, CD34 expression has, until recently, remained as a convenient marker for human HSC. However, it has since been postulated that there is in fact a human $CD34^-$ HSC that is analogous to that of the

mouse (Bhatia et al. 1998; Ando 2002; Engelhardt et al. 2002; Guo et al. 2003; Ishii et al. 2011).

Chimeras generated in either sheep or immunocompromised mice have shown that $CD34^-$ cells from cord blood, bone marrow, and granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood do have in vivo HSC activity in spite of failing to exhibit significant clonogenic activity in vitro. Further defining the phenotype of cord blood-derived $CD34^-$ SRUs, Kimura et al. (2007) proposed that the immunophenotype of very primitive long-term repopulating human HSC is Lin^- $CD34^-$ c-Kit^- $Flt3^-$. Paralleling studies on mouse HSC, Goodell et al. (1997) showed that human bone marrow contains side population cells present at a very low frequency, which interestingly are also $CD34^-$. It has been hypothesized that the $CD34^-$ HSC may represent the most immature and quiescent population of stem cells in humans at the apex of the hematopoietic hierarchy (Anjos-Afonso et al. 2013). As in the mouse, Rh-123 staining can be employed in defining human HSC, low dye retention being associated closely with the Lin^- $CD34^+$ $CD38^-$ population (McKenzie et al. 2007). However, taking all of this knowledge into account and using simple calculations of reported HSC frequencies, it can be established that more than 99 % of human HSC must be $CD34^+$.

The differences between antigen expression on mouse and human HSC is not unique to CD34, and other distinctive variations can be seen in the expression of the Flt-3 receptor, which is expressed on the surface of human HSC but not on the mouse (Sitnicka et al. 2003), and the SLAM markers CD150 and CD48, which unlike in the mouse are absent on human HSC (Sintes et al. 2008; Larochelle et al. 2011). Due to the discrepancies in the expression of CD34 and its relationship to stem cell activity, research continues to define better markers of human HSC. A recent publication from the laboratory of John Dick revealed a novel human HSC marker, namely CD49f (α6 integrin). Single $CD49f^+$ cells were shown to be capable of generating highly efficient long-term multilineage grafts and that loss of CD49f expression coincided with transient engrafting MPPs (Notta et al. 2011). Such markers could pave the way for the isolation of pure populations of human HSC for therapeutic use and further research into HSC properties.

A number of additional discriminators of human HSC subpopulations have been investigated. Two features of human HSC that have proven useful for the isolation of the most immature cells are worthy of mention. First is the relative high expression of aldehyde dehydrogenase (ALDH) in hematopoietic progenitor cells (Kastan et al. 1990). Cord blood cells stained for ALDH activity using the substrate BODIPY-aminoacetaldehyde ('Aldefluor') and depleted for Lin^+ cells are enriched for $CD34^+$ $CD38^-$ cells (Storms et al. 1999). Second, and perhaps the more useful property of long-term repopulating human HSC, is the expression of CD133, an antigen that characterizes several types of adult stem cells. For example, a rare population of cord blood cells expressing CD133 and negative for CD7 were found to be highly enriched for progenitor activity at a frequency equivalent to purified fractions of $CD34^+$ stem cells, and they were the only subset among the Lin^- $CD34^-$ $CD38^-$ population capable of giving rise to $CD34^+$ cells in defined liquid cultures and of engrafting in NOD/SCID mice (Yin et al. 1997; Gallacher et al. 2000). Cell

selection combining Lin antigen depletion together with staining for ALDH activity and CD133 expression provides a purification of HSC with long-term repopulating function that has been considered to be an alternative to CD34 cell selection for stem cell therapies. Hence, limiting dilution analysis demonstrates a tenfold increase in the frequency of repopulating cells compared with Lin^- $CD133^+$ cells, with maintenance of immature hematopoietic phenotypes ($CD34^+$ $CD38^-$) and enhanced repopulating function in recipients of serial, secondary transplants (Hess et al. 2006).

5.3 Characteristics and Properties

Like other adult stem cells, HSC are regulated and supported by the surrounding tissue microenvironment, generally referred to as the stem cell 'niche'. The niche includes all cellular and non-cellular components that interact in order to control the adult stem cell, and the reader is also referred to a number of excellent recent reviews that specifically discuss the nature of these in relation to the HSC in the bone marrow (Wilson and Trumpp 2006; Kiel and Morrison 2008; Morrison and Scadden 2014; Schepers et al. 2015; Boulais and Frenette 2015).

In brief, the current perception of HSC in the bone marrow is that they reside at the interface of bone and the bone marrow (the endosteum), but it remains uncertain whether this interface itself is a niche, or whether endosteal cells secrete factors that diffuse to nearby niches. Indeed, recent work has shown that HSC can reside in a niche that appears to involve a very close juxtaposition of both osteoblasts and microvessel endothelial cells (Lo Celso et al. 2009), while close association with both $Nestin^+$ MSC and megakaryocytes also have important consequences for HSC maintenance (Méndez-Ferrer et al. 2010; Bruns et al. 2014).

It is important that the bone marrow niche should not be viewed as a static environment, since both the hematopoietic and immune systems are required to respond rapidly and adapt to the needs of the individual, it should therefore be regarded as a fluid system that continually processes information from the organism as a whole (Mercier et al. 2011). Much of the knowledge that we have obtained from studies of the normal bone marrow microenvironment is leading us to a better understanding of the ways in which LSC can manipulate the niche to enhance their survival and proliferation. Due to this adaptable nature of the bone marrow niche it is becoming clear that it represents a novel therapeutic target, its manipulation, for example by pharmacological enhancement of the number and function of osteoblasts, being a potential way to augment the effectiveness of stem cell therapies (Adams and Scadden 2008).

Much of the life of a HSC within its niche is one of inactivity in which it replicates only relatively infrequently. This state of quiescence is thought to be an indispensable property for the maintenance of HSC, protecting them from stress and hence the accumulation of DNA mutations and enabling them to sustain life-long hematopoiesis. The molecular mechanisms through which the niche controls the

HSC cell cycle to establish quiescence are beginning to be elucidated. For example, it has been shown that the interaction of the Tie2 receptor tyrosine kinase with its ligand Angiopoietin-1 leads to tight adhesion of HSC to stromal cells, and maintenance of their long-term repopulating activity (Arai et al. 2004). In spite of their generally quiescent state, the normal homeostatic balance in the hematopoietic system requires HSC to be able to exit the niche and then achieve several transits through vascular endothelium to be able to migrate through the blood, enter different organs and then return back to the bone marrow. These processes of migration and specific homing need to be amplified during stress-induced recruitment of leukocytes from the bone marrow reservoir and during stem cell mobilization as part of defense and repair. Both HSC mobilization (reviewed by Pelus and Fukuda 2008) and homing (reviewed by Lapidot et al. 2005; Chute 2006) are also crucially important in the context of clinical stem cell transplantation.

HSC induced to exit the bone marrow and mobilize to the peripheral blood following treatment with granulocyte-colony stimulating factor (G-CSF) have become the most widely used source of HSC for engraftment and show significant superiority to cells obtained directly from the bone marrow. In addition to G-CSF, the growth factor SCF, adhesion molecules such as VLA-4 and P- and E-selectins, chemokines, proteolytic enzymes such as elastase and cathepsin G, and various matrix metalloproteinases (MMP) have all been shown to have a role in stem cell mobilization. The chemokine stromal-derived factor 1 (SDF-1 or CXCL12) and its receptor CXCR4 are major players involved in the regulation of HSC mobilization and homing. During steady-state homeostasis, CXCR4 is expressed by HSC and also by stromal cells, which are the main source for SDF-1 in the bone marrow. Stress-induced modulations in SDF-1 and CXCR4 levels participate in recruitment of immature and maturing leukocytes from the bone marrow reservoir to damaged organs as part of host defense and repair mechanisms. The recent finding that murine HSC rapidly mobilized by the CXCR2 receptor agonist GROβ show superior repopulation kinetics and more competitive engraftment than the equivalent cells mobilized by G-CSF demonstrates that the chemokine/chemokine receptor axis has potentially superior therapeutic potential compared to the use of G-SCF (Fukuda et al. 2007).

In addition to the complex interplay with niche cells and diffusible mediators, it has emerged recently that there is an element of dynamic regulation via neurotransmitter signaling. Hence, ablation by genetic or chemical means of adrenergic neurotransmission or administration of a β2 adrenergic agonist results, respectively, in decreased or enhanced HSC mobilization indicating the involvement of norepinephrine signaling in the process (Katayama et al. 2006; Spiegel et al. 2008).

HSC homing involves rolling and firm adhesion to endothelial cells in small marrow sinusoids under blood flow, followed by trans-endothelial migration across the physical endothelium/extracellular matrix barrier, ultimately leading to access and anchorage to their specialized niches. Like mobilization, this coordinated, multistep process also involves signaling by SDF-1 and SCF, and activation of LFA-1, VLA-4/5 and CD44 and a role for MMP.

Although HSC and their niche clearly have to persist throughout life, a number of studies have shown that there are age-related changes in HSC that have functional consequences for the hematopoietic system and are likely the result of a combination of cell-intrinsic and microenvironmental influences (for a review see Geiger et al. 2013). Studies of X-chromosome inactivation in elderly females have suggested that the pool of HSC normally diminishes with age, resulting in oligoclonal or even monoclonal hematopoiesis; however, by analyzing the pattern of allele-specific gene expression, Swierczek et al. (2008) have provided convincing evidence against this hypothesis and suggest that clonal hematopoiesis is not a normal consequence of aging. Nevertheless, consideration of HSC differentiation potential suggests that a degree of selection can operate. HSC isolated from young and aged donors have been reported to differ in functional capacity, the complement of proteins on the cell surface, transcriptional activity, and genome integrity (reviewed by Woolthius et al. 2011). In the mouse, several hallmark age-dependent changes in the HSC compartment have been identified, including an increase in HSC numbers and a decrease in homing efficiency. Increased proliferation and decreased function with age can be correlated with dramatic alterations in gene expression; one analysis of HSC from mice aged 2 to 21 months identified approximately 1500 genes that were age-induced and 1600 that were age-repressed (Chambers and Goodell 2007). Genes associated with the stress response, inflammation, and protein aggregation dominated the up-regulated expression profile, while the down-regulated profile was marked by genes involved in the preservation of genomic integrity and chromatin remodeling. One gene in particular that has attracted attention in this context, and may have implications for treatment strategies, is the cyclin-dependent kinase inhibitor p16INK4a, the level of which accumulates and modulates specific age-associated HSC functions (Janzen et al. 2006). Notably, in the absence of p16INK4a, HSC repopulating defects and apoptosis were mitigated, improving the stress tolerance of cells and the survival of animals in successive transplants, suggesting that therapeutic inhibition of genes such as p16INK4a may ameliorate the physiological impact of ageing on stem cells. The differences in 'aged' behavior of HSC were later explained by an accumulation of myeloid-biased HSC with age in both mice (Challen et al. 2010) and humans (Pang et al. 2011) at the expense of lymphoid-biased cells (Cho et al. 2008). The fact that myeloid-biased HSC from young and aged sources behave similarly in all aspects tested might suggest that aging does not change individual HSC. Indeed, as discussed above, this shift in the balance of HSC potential likely reflects a change in the relative proportions of cells predetermined for a myeloid/lymphoid fate or a more restricted myeloid differentiation preference.

There has been a growing appreciation over the last 15 years of the role that reactive oxygen species (ROS) play in a variety of cellular processes. ROS are formed by the partial reduction of oxygen and include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) (Turrens 2003). ROS have been shown to regulate cell cycle progression, cell motility and growth factor signaling in a variety of normal cell types (Valko et al. 2007). ROS production and consequent oxidative stress has been linked to aging and degenerative disease (Sardina et al. 2012),

although beneficial effects of moderate levels of ROS have been noted (Goldstone et al. 1996; Tatla et al. 1999). The importance of ROS in HSC was made evident from studies on mouse models in which genes involved in the regulation of ROS levels were genetically reduced. For example, reduction of FOXO transcription factor function leads to loss of HSC quiescence and self-renewal capacity (Jang and Sharkis 2007). In the absence of external stimuli, FOXO proteins normally reside in the nucleus in an active state, promoting cell cycle arrest, resistance to stress, apoptosis and ROS detoxification (Coffer and Burgering 2007). Although it is evident that ROS levels are crucial to the function of HSC, the precise mechanisms affected are not clear. There are actually distinct HSC niches in the bone marrow depending on oxygen availability and the consequent levels of ROS. Hence, ROS^{low} and ROS^{high} HSC exhibit the same surface phenotype, but differ in that the population with lower ROS levels displays higher self-renewal (Jang and Sharkis 2007). The association between the oxidative state and HSC self-renewal capacity has led to interest in the manipulation of ROS levels as a way to enhance bone marrow transplantation and to delay the aging of HSC. Such manipulations have been achieved in the mouse by application of the lipid sphingosine-1-phosphate, which induces the production of ROS in HSC and consequently causes an increase in their motility.

5.4 Differentiation Capacity and Their Precursors

What happens downstream of the HSC in the hematopoietic hierarchy is important in a therapeutic context when considering the specific requirements for progenitors and differentiated progeny to regenerate the normal hematopoietic status. Work largely emanating from the laboratory of Irving Weissman has defined committed progenitors in the mouse that are immediately downstream of the most mature component of the LSK compartment. These cells mark the first distinction between the lymphoid and myeloid lineages. The existence of a common lymphoid progenitor (CLP) that can only give rise to T cells, B cells, and NK cells was first reported by Kondo et al. (1997), who described a bone marrow Lin^{-} IL-$7R^{+}$ Thy-1^{-} Sca-1^{lo} c-Kit^{lo} population with these characteristics. A complementary clonogenic common myeloid progenitor (CMP) that gives rise to all myeloid lineages was similarly defined by Akashi et al. (2000) who also demonstrated that this cell can give rise to either megakaryocyte/erythrocyte progenitors (MEP) or granulocyte/macrophage progenitors (GMP). The resulting model, which proposes that the first lineage commitment step of HSC results in a strict separation into CLP and CMP, was challenged, as discussed above, by the identification of LMPP, a population of cells with lympho-myeloid differentiation potential that have lost the ability to adopt erythroid and megakaryocyte lineage fates (Adolfsson et al. 2005). Hence, LSK HSC that co-express high levels of the tyrosine kinase receptor Flt3 were shown to sustain granulocyte, monocyte, and B and T cell potentials, but in contrast to LSK $Flt3^{-}$ HSC failed to produce significant erythroid and megakaryocytic progeny. The equivalent details of the hierarchy downstream of the HSC are yet to be fully elucidated in

humans, and it cannot be assumed that these will be comparable between species. Using similar strategies for the identification of down-stream human progenitors, populations corresponding to the mouse LMPP have also been defined in humans (MLP) that sustain both lymphoid and myeloid lineages but excluding megakaryocytic/erythroid potential (Hoebeke et al. 2007; Six et al. 2007; Goardon et al. 2011).

Such details of the pathways of commitment and differentiation of HSC as described above, and how these may differ during development, are becoming ever more important in attempts to optimize the production of replacement hematopoietic cells from ES cells. As for the definition of the HSC phenotype and functional testing of HSC in vivo, attempts to elucidate protocols for the induction of hematopoietic differentiation from ES cells have been led by work in the mouse. Although conditions have been worked out to enable the derivation of most mature hematopoietic cell types from both mouse and human ES cells, it is important to be aware of the developmental stage that these cells represent and the extent to which it is possible to generate adult HSC with repopulation potential (for a review on the derivation of hematopoietic cells from ES cells see Vo and Daley, 2015). Following on from extensive studies on the differentiation of hematopoietic cells from murine ES cells (reviewed in Olsen et al. 2006), production from human ES cells was first described by Kaufman et al. (2001) who employed co-culture with murine bone marrow stromal or yolk sac endothelial cell lines. Amongst a number of subsequent modifications to this strategy, Vodyanik et al. (2005) were able to obtain large numbers of $CD34^+$ cells at greater than 95 % purity using co-culture with the mouse stromal line OP9. Although the latter ES cell-derived $CD34^+$ cells contained $ALDH^+$ $Rh\text{-}123^{lo}$ cells and were highly enriched in colony-forming cells, even after in vitro expansion, they displayed a phenotype of primitive hematopoietic progenitors. A potential solution to the problem of the stage of developmental maturity was found in the case of mouse ES cell differentiation in that expression of HoxB4 in primitive progenitors combined with culture on hematopoietic stroma induced a switch to the definitive HSC phenotype capable of engrafting primary and secondary recipients (Kyba et al. 2002), however a similar approach with human ES cells did not generate engraftable cells.

Encouragingly, since the first successes with human ES cell differentiation into HSC-like cells, conditions have been improved considerably leading ultimately to the in vitro generation of HSC with repopulation activity. For example, Narayan et al. (2006) were able to engraft sheep using Lin^- $CD34^+$ or $CD34^+$ $CD38^-$ obtained by culturing human ES cells on stromal feeders, their long-term engrafting potential being confirmed by successful transplantation into secondary recipients. Similarly, using co-culture with stromal cells, this time derived from mouse aorta-gonad-mesonephros (AGM) and fetal liver, Ledran et al. (2008) obtained cells expressing CD34 at day 18–21 of differentiation that were capable of primary and secondary hematopoietic engraftment into immunocompromised mice at substantially higher levels than described previously.

The inability to produce genuine HSC from human ES cells (and pluripotent cells in general) remains a challenge, but one that is likely to be resolved by detailed understanding of the cell and molecular biology of development and the paths that

distinguish primitive and definitive hematopoiesis. An alternative route to fully functional HSC that can engraft and produce adult hematopoietic cell types has been suggested by recent findings of cell type conversion using enforced expression of specific combinations of transcription factors. Success in transcription factor-mediated reprogramming to a pluripotent state or of conversion between hematopoietic lineages has encouraged the testing of strategies for the direct generation of HSC from somatic cells (Szabo et al. 2010). Some of the most convincing and significant recent attempts have yielded human HSC with engraftment potential. Hence, starting with primary vascular endothelial cells, Sandler et al. (2014) used introduction of FOSB, GFI1, RUNX1 and SPI1 to produce cells that could be serially engrafted, although their lymphoid contribution was limited. Doulatov et al. (2013) were also successful in producing HSC with engraftment potential, but in their case the cells used were human pluripotent cells (ES and iPS cells) into which they introduced a combination of HOXA9, ERG, RORA, SOX4 and MYB. Again, lymphoid capacity of the engrafted cells was minimal but significantly there was evidence of definitive erythroid maturation.

5.5 Potential Applications for Therapies

The utilization of stem cells in the clinic has already met with great success and remains one of the most appealing prospects in regenerative medicine today. The therapeutic use of HSC pioneered in the 1950s through the development of BMT, initially used matched siblings as donors but has subsequently come to involve the use of partially matched or mismatched donors, which although deemed necessary in most situations can result in problems arising from immunogenic matching, resulting in rejection or graft-versus host disease (reviewed by Copelan 2006). A range of diseases have been successfully treated by BMT, including principally cancers of blood cells, but also other hematological disorders such as myeloproliferation, anemia and genetic defects that cause immunodeficiency. BMT is also an option for treatment of some inherited metabolic disorders that result from an enzyme deficiency affecting cells in addition to, or other than, blood cells, but which can be ameliorated through the production of the deficient protein from engrafted donor blood cells.

Possible improvements in HSC therapies can essentially be broken down into those that increase the availability of suitable, preferably autologous, cells in large numbers, and those that maximize the efficiency of engraftment of the transplanted cells. The latter prospect relates to understanding of the mechanisms of homing and the factors that control niche occupancy as discussed above, and it is likely that this knowledge will have a significant impact in the years to come. To date, means to improve the availability of HSC for BMT have received far more attention. Roughly 30 % of patients requiring BMT have a matched sibling, while another 50 % potentially have a good match to an individual amongst the nine million registered donors worldwide, although less than half of these will actually receive a donation.

Although cord blood is a viable alternative source of HSC it is not ideal because it only contains a limited number of HSC, so that ex vivo expansion is almost certainly necessary. Ex vivo expansion of HSC in combinations of cytokines and other soluble factors, designed to mimic the signals provided within the niche, has met with mixed success, although more recently quite significant degrees of amplification in the numbers of cells retaining engraftment potential have been achieved. For example, using a combination of SCF, Flt3 ligand (FL), TPO and IL6, two independent groups achieved significant expansion of $CD34^+$ cord blood cells that retained the capacity to repopulate NOD/SCID mice (Kusadasi et al. 2000; Ueda et al. 2000). Direct manipulation of molecular mechanisms that are linked to proliferation and self-renewal is another potential way to expand stem cells and ectopic overexpression of the transcriptional regulator HoxB4 has proved to be effective at inducing rapid, extensive, and highly polyclonal expansions of murine HSC that retained full lympho-myeloid repopulating potential and enhanced in vivo regenerative potential (Antonchuk et al. 2002). Other approaches that have been investigated include the use of fibroblast growth factors (FGF), in particular FGF 1 and 2, which can maintain long-term repopulating activity of mouse bone marrow HSC in vitro (de Haan et al. 2003; Yeoh et al. 2006), while the Notch ligand Delta 1 has a moderate effect in enhancing the expansion of SRUs in cultures of cord blood $CD133^+$ cells employing the SCF, FL, TPO, IL-6 cocktail of factors described above (Suzuki et al. 2006). Perhaps the greatest success has come from the laboratory of Harvey Lodish who identified angiopoietin-like 2 and angiopoietin-like 3 proteins as factors produced by HSC-supportive mouse fetal liver $CD3^+$ cells (Zhang et al. 2006). These produced a roughly 30-fold expansion of long-term HSC in culture, which has subsequently been applied to human cord blood cells by developing a serum-free culture containing SCF, TPO, FGF-1, angiopoietin-like 5, and IGFBP2 (Zhang et al. 2008).

Many believe that the solution to producing more cells for transplantation lies in the derivation of HSC from alternative sources, and a number of options have been considered in order to achieve this goal. The prospect of in vitro production of HSC as a futuristic potential supply for BMT derived from ES cells is an exciting opportunity for regenerative medicine as they represent a theoretically unlimited source of HSC. Nevertheless as for cord blood-derived HSC there are at present significant limitations to the number of appropriate cells that may be obtained.

As discussed already, ES cells can be differentiated into HSC with repopulating capability and it may soon be possible to produce these in quantities that are sufficient for clinical application. However, the use of ES cell-derived HSC is ultimately limited because they are unlikely to be perfectly matched to the donor and ethical consequences of the generation of human embryos for therapeutic applications must be considered. Alternatively, what if ES cells could be made that match every individual so that truly personalized stem cell transplantations would become a reality? Efforts have been made to do just this with ES-like cells being generated through processes such as nuclear transfer, involving either the fusion of ES cells with somatic cell or the transfer of somatic nuclear contents into an oocyte (Wilmut et al. 1997).

The real breakthrough came in 2006 when the Japanese researcher Shinya Yamanaka showed that it is possible to convert normal differentiated adult cells, firstly from the mouse (Takahashi and Yamanaka 2006; Okita et al. 2007) and then from humans (Takahashi et al. 2007), to become like ES cells by forced expression of specific pluripotent genes; namely OCT4, SOX2, KLF4 and c-MYC. These cells, which are discussed in detail in Chapter 3, are usually referred to as induced pluripotent stem (iPS) cells and have the additional advantage that their generation does not involve the use of an embryo bypassing many ethical concerns. The subsequent demonstration that iPS cells can be differentiated, like ES cells, into HSC (Hanna et al. 2007; Schenke-Layland et al. 2008; Niwa et al. 2009) means that they offer the real prospect of limitless autologous HSC for all. Of course there are many details yet to be ironed out, but the progress in this area of stem cell science is nothing if not meteoric (Robinton and Daley 2012; Inoue et al. 2014). Interestingly, a recent study has shown that immature hematopoietic cells derived from human iPS cells are more permissive to engraft the bone marrow of xenotransplantation recipients compared to phenotypically identical cells obtained from ES cells, although these HSC failed to demonstrate multilineage differentiation unless they were removed from the animal, a phenomenon that could be attributed to their inability to down regulate key micro RNAs involved in hematopoiesis (Risueño et al. 2012).

The considerable recent effort in reprogramming cell phenotype towards a pluripotent state has also led to renewed interest in trans-differentiation directly from one cell type to another, without progressing through an iPS cell stage. This has been achieved, with varying degrees of success, for a number of cell types, including neural cells, cardiomyocytes and hepatocytes. As discussed already, one potentially exciting advantage of a trans-differentiation approach is that it may be possible more easily to produce mature cells with an adult rather than embryonic or fetal phenotype.

The ability to produce iPS cells, and consequently patient-specific HSC, also offers the exciting prospect that inherited blood-related disorders might be corrected. Proof-of-principle for this concept was first provided by the laboratory of Rudi Jaenisch who created a humanized sickle cell anemia mouse model, which was then rescued after transplantation with HSC obtained in vitro from autologous iPS cells in which the mutant hemoglobin allele had been reverted to the normal sequence by gene-specific targeting (Hanna et al. 2007). A second important proof-of-principle, this time with human cells, has been the demonstration that somatic cells from Fanconi anemia patients can be used to generate iPS cells in which the Fanconi-anemia defect could be corrected by over expression of the normal version of the affected protein and then used to give rise to hematopoietic progenitors of the myeloid and erythroid lineages that are disease-free (Raya et al. 2009). The opportunities for genetic correction have also recently received a technological boost through the development of a simple, precise and efficient genome editing system based on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and a CRISPR-associated (Cas) nuclease that are part of a bacterial defence mechanism for the elimination of invading genetic material. The protein Cas9 has been

adapted to utilize a single 'guide' RNA that leads to double-strand cleavage at specific DNA sequences, which then enables mutation or modification of the genome by harnessing cell repair mechanisms (see review by Sternberg and Doudna (2015) for a detailed description of the technology).

Although these exciting advances in iPS cell generation raise the prospect of patient-specific stem cell therapies, the transition from the bench to the patient bedside is still some distance into the future. Numerous obstacles must be overcome before these therapies are put into routine practice. Firstly, the original methods of producing iPS cells utilized retroviruses as the means to introduce pluripotency genes. This process would be entirely unacceptable in the clinic since retroviruses are known cancer-causing agents (Okita et al. 2007). New methods of generation of iPS cells have evolved with the means to remove the oncogenes after the induction of pluripotency reducing the risk of tumorigenesis (Yu et al. 2009).

Although it is some distance into the future before these techniques are put into clinical practice, the generation of HSC from iPS cells from patients can be used in the present for phenotypic based drug screens in complex diseases for which the under-lying genetic mechanism is unknown.

5.6 Conclusions and Future Development in Research

Research into the nature and application of HSC has come a long way since the earliest forays into transplantation of bone marrow into patients. Apart from the highly detailed understanding that we now have of the molecular and cellular characteristics of HSC and ways in which they can be manipulated and used for clinical benefit, research in this area has provided the guiding light for the whole field of stem cell biology. The means of identifying and purifying HSC and the sophisticated in vitro and in vivo tests that have been developed to assay their potential have been adopted and modified for the investigation of the now burgeoning array of stem cells that play roles in both development and the maintenance of adult tissues. The study of HSC illustrates so well how investigations in animal models, in particular the mouse, can inform studies in man and how they can provide important pre-clinical information on the potency and behaviour of stem cells following ex vivo expansion or on ways in which to improve the efficiency of engraftment once cells are introduced into the recipient.

The improvements in the ability of clinicians to treat more patients more effectively with transplantations as a result of the increasing knowledge of HSC are likely to be given an even greater boost as a result of the astonishing developments in the ability to generate pluripotent stem cells and to then use these to produce HSC with long-term engraftment potential. Apart from the chance to treat more people successfully, there is now opened up the real prospect that individuals born with genetic defects that affect blood cell production or function, as well as some other inherited disorders such as those affecting aspects of metabolism, can expect to

have their deficiencies corrected by gene targeting in iPS cells generated from nothing more than a few skins cells.

For sure there are many hurdles yet to be overcome, but the future looks very exciting.

References

Adams GB, Scadden DT (2008) A niche opportunity for stem cell therapeutics. Gene Ther 15:96–99

Adolfsson J, Borge OJ, Bryder D et al (2001) Upregulation of Flt3 expression within the bone marrow Lin(−)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15:659–669

Adolfsson J, Månsson R, Buza-Vidas N et al (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121:295–306

Akashi K, Traver D, Miyamoto T et al (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404:193–197

Almeida-Porada G, Porada C, Gupta N et al (2007) The human-sheep chimeras as a model for human stem cell mobilization and evaluation of hematopoietic grafts' potential. Exp Hematol 35:1594–1600

Ando K (2002) Human CD34- hematopoietic stem cells: basic features and clinical relevance. Int J Hematol 75:370–375

Anjos-Afonso F, Currie E, Palmer HG et al (2013) CD34(−) cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. Cell Stem Cell 13:161–174

Antonchuk J, Sauvageau G, Humphries RK (2002) HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 109:39–45

Arai F, Hirao A, Ohmura M et al (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149–161

Balazs AB, Fabian AJ, Esmon CT et al (2006) Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. Blood 107:2317–2321

Baum CM, Weissman IL, Tsukamoto AS et al (1992) Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci U S A 89:2804–2808

Bensidhoum M, Chapel A, Francois S et al (2004) Homing of in vitro expanded Stro-1- or Stro-1+ mesenchymal stem cells into the NOD/SCID mouse and their role in supporting human CD34 cell engraftment. Blood 103:3313–3319

Bhatia M, Wang JC, Kapp U et al (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A 94:5320–5325

Bhatia M, Bonnet D, Murdoch B et al (1998) A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med 4:1038–1045

Bonnet D, Bhatia M, Wang JC et al (1999) Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive hematopoietic cells transplanted at limiting doses into NOD/SCID mice. Bone Marrow Transplant 23:203–209

Boulais PE, Frenette PS (2015) Making sense of hematopoietic stem cell niches. Blood 125:2621–2629

Bradley TR, Metcalf D (1966) The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 44:287–299

Bruns I, Lucas D, Pinho S et al (2014) Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. Nat Med 20:1315–1320

Challen GA, Boles NC, Lin KK et al (2009) Mouse hematopoietic stem cell identification and analysis. Cytometry A 75:14–24
Challen GA, Boles NC, Chambers SM et al (2010) Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell 6:265–278
Chambers SM, Goodell MA (2007) Hematopoietic stem cell aging: wrinkles in stem cell potential. Stem Cell Rev 3:201–211
Chen BP, Galy A, Kyoizumi S et al (1994) Engraftment of human hematopoietic precursor cells with secondary transfer potential in SCID-hu mice. Blood 84:2497–2505
Chen CZ, Li L, Li M et al (2003) The endoglin(positive) sca-1(positive) rhodamine(low) phenotype defines a near-homogeneous population of long-term repopulating hematopoietic stem cells. Immunity 19:525–533
Cho RH, Sieburg HB, Muller-Sieburg CE (2008) A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. Blood 111:5553–5561
Christensen JL, Weissman IL (2001) Flk-2 is a marker in hematopoietic stem cell differentiation: a simple model to isolate long-term stem cells. Proc Natl Acad Sci U S A 98:14541–14546
Chute JP (2006) Stem cell homing. Curr Opin Hematol 13:399–406
Coffer PJ, Burgering BM (2007) Stressed marrow: FoxOs stem tumour growth. Nat Cell Biol 9:251–253
Copelan EA (2006) Hematopoietic stem-cell transplantation. N Engl J Med 354:1813–1826
Cosgun KN, Rahmig S, Mende N et al (2014) Kit regulates HSC engraftment across the human-mouse species barrier. Cell Stem Cell 15:227–238
Craig W, Kay R, Cutler RL et al (1993) Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med 177:1331–1342
Cumano A, Godin I (2007) Ontogeny of the hematopoietic system. Annu Rev Immunol 25:745–785
de Haan G, Weersing E, Dontje B et al (2003) In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. Dev Cell 4:241–251
Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of hematopoietic stem cells in vitro. J Cell Physiol 91:335–344
Dick JE, Magli MC, Huszar D et al (1985) Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. Cell 42:71–79
Doulatov S, Notta F, Laurenti E et al (2012) Hematopoiesis: a human perspective. Cell Stem Cell 10:120–136
Doulatov S, Vo LT, Chou SS et al (2013) Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. Cell Stem Cell 13:459–470
Dzierzak E, Speck NA (2008) Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat Immunol 9:129–136
Dzierzak E, Medvinsky A, de Bruijn M (1998) Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. Immunol Today 19:228–236
Eaves CJ (2015) Hematopoietic stem cells: concepts, definitions, and the new reality. Blood 125:2605–2613
Ema H, Takano H, Sudo K et al (2000) In vitro self-renewal division of hematopoietic stem cells. J Exp Med 192:1281–1288
Engelhardt M, Lübbert M, Guo Y (2002) CD34(+) or CD34(−): which is the more primitive? Leukemia 16:1603–1608
Fraser CC, Chen BP, Webb S et al (1995) Circulation of human hematopoietic cells in severe combined immunodeficient mice after Cl2MDP-liposome-mediated macrophage depletion. Blood 86:183–192
Fukuda S, Bian H, King AG et al (2007) The chemokine GRObeta mobilizes early hematopoietic stem cells characterized by enhanced homing and engraftment. Blood 110:860–869

Gallacher L, Murdoch B, Wu DM et al (2000) Isolation and characterization of human CD34(−) Lin(−) and CD34(+)Lin(−) hematopoietic stem cells using cell surface markers AC133 and CD7. Blood 95:2813–2820

Geiger H, de Haan G, Florian MC (2013) The ageing haematopoietic stem cell compartment. Nat Rev Immunol 13:376–389

Goardon N, Marchi E, Atzberger A et al (2011) Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. Cancer Cell 19:138–152

Goldman JP, Blundell MP, Lopes L et al (1998) Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. Br J Haematol 103:335–342

Goldstone SD, Milligan AD, Hunt NH (1996) Oxidative signalling and gene expression during lymphocyte activation. Biochim Biophys Acta 1314:175–182

Goodell MA, Brose K, Paradis G et al (1996) Isolation and functional properties of murine hemopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806

Goodell MA, Rosenzweig M, Kim H et al (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3:1337–1345

Goyama S, Wunderlich M, Mulloy JC (2015) Xenograft models for normal and malignant stem cells. Blood 125:2630–2640

Guo Y, Lübbert M, Engelhardt M (2003) CD34- hematopoietic stem cells: current concepts and controversies. Stem Cells 21:15–20

Hanna J, Wernig M, Markoulaki S et al (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318:1920–1923

Hao QL, Shah AJ, Thiemann FT et al (1995) A functional comparison of CD34+CD38- cells in cord blood and bone marrow. Blood 86:3745–3753

Harrison DE, Jordan CT, Zhong RK et al (1993) Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple bionomial, correlation and covariance calculations. Exp Hematol 21:206–219

Hess DA, Wirthlin L, Craft TP et al (2006) Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. Blood 107:2162–2169

Hoebeke I, De Smedt M, Stolz F et al (2007) T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34(+)CD38(−)CD7(+) common lymphoid progenitors expressing lymphoid-specific genes. Leukemia 21:311–319

in't Anker PS, Noort WA, Kruisselbrink AB et al (2003) Nonexpanded primary lung and bone marrow-derived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol 31:881–889

Inoue H, Nagata N, Kurokawa H et al (2014) iPS cells: a game changer for future medicine. EMBO J 33:409–417

Ishii M, Matsuoka Y, Sasaki Y et al (2011) Development of a high-resolution purification method for precise functional characterization of primitive human cord blood-derived CD34-negative SCID-repopulating cells. Exp Hematol 39:203–213

Ishikawa F, Yasukawa M, Lyons B et al (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. Blood 106:1565–1573

Ito M, Hiramatsu H, Kobayashi K et al (2002) NOD/SCIDgamma(c) (null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 100:3175–3182

Ito M, Kobayashi K, Nakahata T (2008) NOD/Shi-scid IL2rgamma(null) (NOG) mice are more appropriate for humanized mouse models. Curr Top Microbiol Immunol 324:53–76

Jang YY, Sharkis SJ (2007) A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood 110:3056–3063

Janzen V, Forkert R, Fleming HE et al (2006) Stem cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443:42142–42146

Kamel-Reid S, Dick JE (1988) Engraftment of immune-deficient mice with human hematopoietic stem cells. Science 242:1706–1709
Kastan MB, Schlaffer E, Russo JE et al (1990) Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. Blood 75:1947–1950
Katayama Y, Battista M, Kao WM et al (2006) Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell 124:407–421
Kaufman DS, Hanson ET, Lewis RL et al (2001) Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 98:10716–10721
Kent DG, Copley MR, Benz C et al (2009) Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. Blood 113:6342–63450
Kiel MJ, Morrison SJ (2008) Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol 8:290–301
Kiel MJ, Yilmaz OH, Iwashita T et al (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121:1109–1121
Kimura T, Asada R, Wang J et al (2007) Identification of long-term repopulating potential of human cord blood-derived CD34-flt3- severe combined immunodeficiency-repopulating cells by intra-bone marrow injection. Stem Cells 25:1348–1355
Kollet O, Peled A, Byk T et al (2000) Beta2 microglobulin-deficient (B2m(null)) NOD/SCID mice are excellent recipients for studying human stem cell function. Blood 95:3102–3105
Kondo M, Weissman IL, Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91:661–672
Kusadasi N, van Soest PL, Mayen AE et al (2000) Successful short-term ex vivo expansion of NOD/SCID repopulating ability and CAFC week 6 from umbilical cord blood. Leukemia 14:1944–1953
Kushida T, Inaba M, Hisha H et al (2001) Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 97:3292–3299
Kyba M, Perlingeiro RC, Daley GQ (2002) HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 109:29–37
Lai AY, Kondo M (2006) Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. J Exp Med 203:1867–1873
Lapidot T, Pflumio F, Doedens M et al (1992) Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. Science 255:1137–1141
Lapidot T, Dar A, Kollet O (2005) How do stem cells find their way home? Blood 106:1901–1910
Larochelle A, Savora M, Wiggins M et al (2011) Human and rhesus macaque hematopoietic stem cells cannot be purified based only on SLAM family markers. Blood 117:1550–1554
Ledran MH, Krassowska A, Armstrong L et al (2008) Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches. Cell Stem Cell 3:85–98
Lemieux ME, Rebel VI, Lansdorp PM et al (1995) Characterization and purification of a primitive hematopoietic cell type in adult mouse marrow capable of lymphomyeloid differentiation in long-term marrow "switch" cultures. Blood 86:1339–1347
Lemischka IR, Raulet DH, Mulligan RC (1986) Developmental potential and dynamic behavior of hematopoietic stem cells. Cell 45:917–927
Lo Celso C, Fleming HE, Wu JW et al (2009) Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 457:92–96
Majeti R, Park CY, Weissman IL (2007) Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 1:635–645
Mayani H, Dragowska W, Lansdorp PM (1993) Cytokine-induced selective expansion and maturation of erythroid versus myeloid progenitors from purified cord blood precursor cells. Blood 81:3252–3258

Mazurier F, Fontanellas A, Salesse S et al (1999) A novel immunodeficient mouse model RAG2 x common cytokine receptor gamma chain double mutants requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. J Interferon Cytokine Res 19:533–541

McCune JM, Namikawa R, Kaneshima H et al (1988) The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science 241:1632–1639

McKenzie JL, Gan OI, Doedens M et al (2005) Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of CD122+ cells. Blood 106:1259–1261

McKenzie JL, Gan OI, Doedens M et al (2006) Individual stem cells with highly variable proliferation and self-renewal properties comprise the human hematopoietic stem cell compartment. Nat Immunol 7:1225–1233

McKenzie JL, Takenaka K, Gan OI et al (2007) Low rhodamine 123 retention identifies long-term human hematopoietic stem cells within the Lin-CD34+CD38- population. Blood 109:543–545

Méndez-Ferrer S, Michurina TV, Ferraro F et al (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829–834

Mercier FE, Ragu C, Scadden DT (2011) The bone marrow at the crossroads of blood and immunity. Nat Rev Immunol 12:49–60

Mikkola HK, Orkin SH (2006) The journey of developing hematopoietic stem cells. Development 133:3733–3744

Moore MA, Williams N, Metcalf D (1973) In vitro colony formation by normal and leukemic human hematopoietic cells: characterization of the colony-forming cells. J Natl Cancer Inst 50:603–623

Morrison SJ, Scadden DT (2014) The bone marrow niche for haematopoietic stem cells. Nature 505:327–334

Namikawa R, Weilbaecher KN, Kaneshima H et al (1990) Long-term human hematopoiesis in the SCID-hu mouse. J Exp Med 172:1055–1063

Narayan AD, Chase JL, Lewis RL et al (2006) Human embryonic stem cell-derived hematopoietic cells are capable of engrafting primary as well as secondary fetal sheep recipients. Blood 107:2180–2183

Niwa A, Umeda K, Chang H et al (2009) Orderly hematopoietic development of induced pluripotent stem cells via Flk-1(+) hemangiogenic progenitors. J Cell Physiol 221:367–377

Nolta JA, Hanley MB, Kohn DB (1994) Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: analysis of gene transduction of long-lived progenitors. Blood 83:3041–3051

Noort WA, Kruisselbrink AB, in't Anker PS et al (2002) Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol 30:870–878

Notta F, Doulatov S, Laurenti E et al (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 333:218–221

Oguro H, Ding L, Morrison SJ (2013) SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell 13:102–116

Okada S, Nakauchi H, Nagayoshi K et al (1992) In vivo and in vitro stem cell function of c-kit and Sca-1-positive murine hematopoietic cells. Blood 80:3044–3050

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317

Olsen AL, Stachura DL, Weiss MJ (2006) Designer blood: creating hematopoietic lineages from embryonic stem cells. Blood 107:1265–1275

Osawa M, Hanada K, Hamada H et al (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245

Pang WW, Price EA, Sahoo D et al (2011) Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci U S A 108:20012–20017

Pelus LM, Fukuda S (2008) Chemokine mobilized adult stem cells: defining a better hematopoietic graft. Leukemia 22:466–473

Ploemacher RE, van der Sluijs JP, Voerman JS et al (1989) An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood 74:2755–2763

Ploemacher RE, van der Sluijs JP, van Beurden CA et al (1991) Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulation and spleen colony-forming hematopoietic stem cells in the mouse. Blood 78:2527–2533

Pronk CJ, Rossi DJ, Mansson R et al (2007) Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor hierarchy. Cell Stem Cell 1:428–442

Purton LE, Scadden DT (2007) Limiting factors in murine hematopoietic stem cell assays. Cell Stem Cell 1:263–270

Raya A, Rodríguez-Pizà I, Guenechea G et al (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460:53–59

Risueño RM, Sachlos E, Lee JH et al (2012) Inability of human induced pluripotent stem cell-hematopoietic derivatives to downregulate micro RNAs in vivo reveals a block in xenograft hematopoietic regeneration. Stem Cells 30:131–139

Robinton DA, Daley GQ (2012) The promise of induced pluripotent stem cells in research and therapy. Nature 481:295–305

Rongvaux A, Willinger T, Takizawa H et al (2011) Human thrombopoietin knockin mice efficiently support human hematopoiesis in vivo. Proc Natl Acad Sci U S A 108:2378–2383

Rongvaux A, Willinger T, Martinek J et al (2014) Development and function of human innate immune cells in a humanized mouse model. Nat Biotechnol 32:364–372

Sandler VM, Lis R, Liu Y et al (2014) Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. Nature 511:312–318

Sanjuan-Pla A, Macaulay IC, Jensen CT et al (2013) Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature 502:232–236

Sardina JL, López-Ruano G, Sánchez-Sánchez B et al (2012) Reactive oxygen species: are they important for hematopoiesis? Crit Rev Oncol Hematol 81:257–274

Schenke-Layland K, Rhodes KE, Angelis E et al (2008) Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. Stem Cells 26:1537–1546

Schepers K, Campbell TB, Passegué E (2015) Normal and leukemic stem cell niches: insights and therapeutic opportunities. Cell Stem Cell 16:254–267

Schmitt TM, Zúñiga-Pflücker JC (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity 17:749–756

Schulte R, Wilson NK, Prick JC et al (2015) Index sorting resolves heterogeneous murine hematopoietic stem cell populations. Exp Hematol 43(9):803–811

Shultz LD, Schweitzer PA, Christianson SW et al (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol 154:180–191

Shultz LD, Lyons BL, Burzenski LM et al (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 174:6477–6489

Sintes J, Romero X, Marin P et al (2008) Differential expression of CD150 (SLAM) family receptors by human hematopoietic stem and progenitor cells. Exp Hematol 36:1199–1204

Sitnicka E, Buza-Vidas N, Larsson S et al (2003) Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. Blood 102:881–886

Six EM, Bonhomme D, Monteiro M et al (2007) A human postnatal lymphoid progenitor capable of circulating and seeding the thymus. J Exp Med 204:3085–3093

Spangrude GJ, Johnson GR (1990) Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc Natl Acad Sci U S A 87:7433–7437
Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241:58–62
Spiegel A, Kalinkovich A, Shivtiel S et al (2008) Stem cell regulation via dynamic interactions of the nervous and immune systems with the microenvironment. Cell Stem Cell 3:484–492
Srour EF, Zanjani ED, Cornetta K et al (1993) Persistence of human multilineage, self-renewing lymphohematopoietic stem cells in chimeric sheep. Blood 82:3333–3342
Sternberg SH, Doudna JA (2015) Expanding the biologist's toolkit with CRISPR-Cas9. Mol Cell 58:568–574
Storms RW, Trujillo AP, Springer JB et al (1999) Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci U S A 96:9118–9123
Strowig T, Rongvaux A, Rathinam C et al (2011) Transgenic expression of human signal regulatory protein alpha in Rag2−/−gamma(c)−/− mice improves engraftment of human hematopoietic cells in humanized mice. Proc Natl Acad Sci U S A 108:13218–13223
Sutherland HJ, Eaves CJ, Eaves AC et al (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 74:1563–1570
Suzuki T, Yokoyama Y, Kumano K et al (2006) Highly efficient ex vivo expansion of human hematopoietic stem cells using Delta1-Fc chimeric protein. Stem Cells 24:245624–245665
Swierczek SI, Agarwal N, Nussenzveig RH et al (2008) Hematopoiesis is not clonal in healthy elderly women. Blood 112:3186–3193
Szabo E, Rampalli S, Risueño RM et al (2010) Direct conversion of human fibroblasts to multilineage blood progenitors. Nature 468:521–526
Szilvassy SJ, Humphries RK, Lansdorp PM et al (1990) Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proc Natl Acad Sci U S A 87:8736–8740
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
Takano H, Ema H, Sudo K et al (2004) Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med 199:295–302
Tatla S, Woodhead F et al (1999) The role of reactive oxygen species in triggering proliferation and IL-2 secretion in T cells. Free Radic Biol Med 26:14–24
Thomas ED, Lochte HL Jr, Lu WC et al (1957) Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med 257:491–496
Till JE, McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222
Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344
Ueda T, Tsuji K, Yoshino H et al (2000) Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. J Clin Invest 105:1013–1021
Valko M, Leibfritz D, Moncol J et al (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84
van Os R, Kamminga LM, de Haan G (2004) Stem cell assays: something old, something new, something borrowed. Stem Cells 22:118111–118190
van Rijn RS, Simonetti ER, Hagenbeek A et al (2003) A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG−/− gammac−/− double-mutant mice. Blood 102:2522–2531
Vo LT, Daley GQ (2015) Denovo generation of HSCs from somatic and pluripotent stemcell sources. Blood 125:2641–2648

Vodyanik MA, Bork JA, Thomson JA et al (2005) Human embryonic stem cell-derived CD34+ cells: efficient production in coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood 105:617–626

Wang JC, Doedens M, Dick JE (1997) Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating assay. Blood 89:3919–3924

Wang J, Kimura T, Asada R et al (2003) SCID-repopulating cell activity of human cord blood-derived CD34- cells assured by intra-bone marrow injection. Blood 101:2924–2931

Whitlock CA, Witte ON (1982) Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci U S A 79:3608–3612

Willinger T, Rongvaux A, Stowig T et al (2011) Improving human hemato-lymphoid-system mice by cytokine knock-in gene replacement. Trends Immunol 32:321–327

Wilmut I, Schnieke AF, McWhir J et al (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813

Wilson A, Trumpp A (2006) Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol 6:93–106

Wilson NK, Kent DG, Buettner F et al (2015) Combined single-cell functional and gene expression analysis resolves heterogeneity within stem cell populations. Cell Stem Cell 16:712–724

Woolthuis CM, de Haan G, Huls G (2011) Aging of hematopoietic stem cells: intrinsic changes or micro-environmental effects? Curr Opin Immunol 23:512–517

Wunderlich M, Chou FS, Link KA et al (2010) AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia 24:1785–1788

Yahata T, Ando K, Sato T et al (2003) A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood 101:2905–2913

Yang L, Bryder D, Adolfsson J et al (2005) Identification of Lin(−)Sca1(+)kit(+)CD34(+)Flt3-short-term hematopoietic stem cells capable of rapidly reconstituting and rescueing myeloablated transplant recipients. Blood 105:2717–2723

Yeoh JS, van Os R, Weersing E et al (2006) Fibroblast growth factor-1 and -2 preserve long-term repopulating ability of hematopoietic stem cells in serum-free cultures. Stem Cells 24:1564–1572

Yin AH, Miraglia S, Zanjani ED et al (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90:5002–5012

Yu J, Hu K, Smugga-Otto K et al (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science 324:797–801

Zanjani ED, Pallavicini MG, Ascensao JL et al (1992) Engraftment and long-term expression of human fetal hematopoietic stem cells in sheep following transplantation in utero. J Clin Invest 89:1178–1188

Zanjani ED, Srour EF, Hoffman R (1995) Retention of long-term repopulating ability of xenogeneic transplanted purified adult human bone marrow hematopoietic stem cells in sheep. J Lab Clin Med 126:24–28

Zanjani ED, Almeida-Porada G, Livingston AG et al (1998) Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Exp Hematol 26:353–360

Zhang CC, Kaba M, Ge G et al (2006) Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat Med 12:240–245

Zhang CC, Kaba M, Iizuka S et al (2008) Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. Blood 111:3415–3423

Chapter 6
Stem Cells for Cardiovascular Regeneration

Christoph Brenner, Robert David, and Wolfgang-Michael Franz

Abstract Ischemic disorders are the main cause of death in the Western world. With more patients surviving their acute myocardial infarction and an aging population, congestive heart failure is the rising health problem. At present, heart transplantation remains the only curative treatment for end stage heart failure. The discrepancy between demand and supply of donor organs does not fill the clinical need. This explains the huge efforts made in the field of stem cell research trying to establish alternative methods for endogenous tissue regeneration and to find sources for tissue replacement. In contrast to adult stem cells mainly acting in a paracrine fashion pluripotent stem cells have the potential to generate transplantable myocardial and vascular tissue.

Due to the low percentage of cardiovascular progenitor cells in pluripotent stem cell cultures, various approaches using exogenous factors aim for their amplification and purification in vitro. However, one future key technology may be genetic forward programming based on profound understanding of differentiation pathways in order to direct stem cell differentiation towards cardiovascular fates. In this regard, subtype specific programming has already been achieved by overexpression of distinct early cardiovascular transcription factors leading to populations of either predominantly early/intermediate type cardiomyocytes or differentiated ventricular myocardial cells, respectively. In addition, techniques for gentle purification of myocardial and vascular progenitor cells will have to be further refined in order to enable the generation of highly specific, pure and safe cell populations for transplantations and for tissue engineering.

In contrast, circulating bone marrow-derived progenitor cells have the potential not to replace diseased cardiovascular tissue but to stimulate its endogenous regeneration. Various approaches have recently been introduced to mobilise these cells from their physiological niche and to facilitate a sufficient recruitment into the diseased tissue. While the therapeutic impact of these cells is already being investigated

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_6

in clinical trials at present, further methodological refinements may once allow their use in clinical routine.

Keywords Pluripotent stem cell-derived cardiovascular progenitor cells • Tissue engineering • Bone marrow-derived progenitor cells • Paracrine regeneration

6.1 Introduction

Cardiovascular diseases are the leading cause of death in the Western world. For instance, in the year 2012 a 40.2 % mortality was related to diseases of the heart or circulatory system in Germany. And this trend is even likely to climb in the foreseeable future (DESTATIS 2013). The loss of cardiomyocytes irreversibly leads to adverse remodeling of the myocardium and chamber dilatation associated with a decreased ejection fraction and heart insufficiency. And with more patients surviving their acute myocardial infarction and a senescent population, congestive heart failure has already become a major health concern. Today, one of the main obstacles responsible for the high mortality of cardiovascular diseases are the limited therapeutical options available reflected by a 1-year-mortality of 50 % for patients suffering from end-stage heart failure (NYHA stage III or IV) (Kessler and Byrne 1999). Furthermore, the lack of donor organs for heart transplantations, which is the only causal therapy available, aggravates this shortage of therapeutical options. And in case of successful transplantation allograft vasculopathy and chronic heart transplant rejection are the causes for a poor clinical outcome (Neumayer 2005; Boyle and Colvin-Adams 2004; Hunt 1998). The direct medical costs for treatment of cardiovascular diseases within the EU are estimated at more than 100 billion Euros per year (Leal et al. 2006).

Thus, scientists are encouraged to seek for new therapeutic options. Currently, the main objective is to find a way increasing the regeneration capacity of a diseased organ instead of "simply" replacing it. Therefore, several types of stem or progenitor cells have been introduced, characterized and tested in animal models. The transplantation of skeletal myoblasts in ischemic cardiomyopathy was a promising approach as these autologous cells are easily accessible and available in a sufficient quantity (Menasche et al. 2003). Unfortunately, this non-cardiac muscular tissue did not electrically couple to the working myocardium (Menasche 2005). After transplantation of fetal cardiomyocytes in a mouse model the formation of intercalated discs and long term survival postengraftment could be documented (Soonpaa et al. 1994) so that this cell type could be regarded as a possible source for cell therapy. Merely the clinical use of these cells is hampered by the lacking availability, at least for ethical reasons. Only the use of autologous bone-marrow-derived stem cells is not impaired by the problems mentioned above. Both the transendocardial injection of these cells as well as the mobilization in combination with improved homing or the intracoronary cell-application via heart catheter into the ischemic myocardium appeared to be feasible, save and ethically inoffensive. Furthermore, encouraging results could be generated in animal models and initial clinical trials (Schachinger et al. 2006a; Zaruba et al. 2009; Engelmann et al. 2009; Theiss et al. 2010; Assmus

et al. 2014; Wollert et al. 2004). However, the underlying mechanisms still remain unclear. At least, it appears to be more than unlikely that bone-marrow-derived stem cells can transdifferentiate into a myocardial cell line even though first observations claimed to demonstrate that (Orlic et al. 2001; Balsam et al. 2004). It rather seems that potential beneficial effects caused by these adult stem cells are based on paracrine influence on the surrounding tissue resulting in neo-angiogenesis, anti-apoptosis and probably stimulated proliferation of resident progenitor cells (Deindl et al. 2006; Murry et al. 2004). In summary, bone marrow-derived stem cells may have positive effects on the endogenous healing process of the damaged myocardium but depict no direct source for cardiovascular tissue engineering. Besides, recently published clinical trials on cell-based cardiac therapies spark suspicion that currently used routes and techniques of progenitor cell administration may not be sufficient to significantly improve myocardial recovery (Gyongyosi et al. 2015). Applied methods will need further refinement in future clinical trials.

Even though far away from curative clinical application the better source for the generation of viable myocardial tissue at present are pluripotent stem cells. These cells, depending on their derivation, have the ability to form all different cell types required to form myocardial (atrial, ventricular and stromal cells) and vascular tissue (endothelial and smooth muscle cells) or specialized cardiomyocytes subtypes (e.g. pacemaker, conduction-system) and can be generated as autologous, i.e. genetically compatible to the recipient, stem cells in a theoretically infinite amount (Maltsev et al. 1993). Since the technique of tissue engineering, i.e. the formation of transplantable three-dimensional constructions from beating cardiomyocytes, e.g. by *biological assembly* (Akins et al. 1999) or the *cell sheet approach* (Okano et al. 1995), is already well established and has been refined over the past 15 years (see Chaps. 9 and 10), the scientific community is focusing on the identification of the appropriate progenitor cell types from the pluripotent stem cell culture. Possible applications among others are infarct repair (Bel et al. 2010; Kraehenbuehl et al. 2011; Singla et al. 2011) or the generation of biological pacemakers (Shiba et al. 2009; Jung et al. 2014). While the use of autologous and patient-specific inducible pluripotent stem cells (iPS-cells, see chapter 8) will most likely solve the problem of immunological tolerance of the transplanted tissue there still exist some more hurdles that have to be overcome. The generation of sufficient amounts of transplantable cells is one main goal as cardiomyocytes make up less than 10 % of all cells in the murine embryonic stem cell culture (Yuasa et al. 2005) and even less in the human ES cell system (Xu et al. 2008). Furthermore, the identification of the appropriate subtypes within the vast diversity of developing cardiovascular cells and their developmental stages within the pluripotent stem cell culture is crucial to achieve the best functional results after transplantation. And last but not least the isolation methods have to be further improved to guarantee the generation of pure and distinct graft cells for transplantation to minimize the risk for cardiac arrhythmias or even teratoma formation (see chapter 7) (Liao et al. 2010; Lin et al. 2010).

With the identification of stage- and lineage-specific markers and progressing decipherment of the molecular cardiovascular development various opportunities for meeting the challenges mentioned above will appear.

6.2 Pluripotent Stem Cell-Derived Progenitor Cells for Tissue Replacement

6.2.1 Characteristics and Classification

The development of the cardiovascular system, i.e. the first organ system to develop in vertebrate embryos, begins with the gastrulation in the third week of human embryonic development. Due to its considerable size at this point in time the embryo is not able to nourish itself by diffusion alone any more but needs circulating blood for the supply of the highly proliferative tissues (Eisenberg and Eisenberg 2006). While the heart itself represents the first functioning organ in the mammalian body its development begins at day 19 in the cardiac neural crest and the bilateral cardiogenic zones in the anterior visceral mesoderm with the induction of angioblasts by the endoderm (Sadler 1998; Buckingham et al. 2005). At the beginning of the fourth week of embryonic development forming endothelial cell clusters connect to endocardial tubes, translocate into the thoracic region and form the primitive heart tube with an inner endocardial layer and a surrounding myocard (Gerecht-Nir et al. 2003; Sadler 1998). During weeks five to seven the four chambered heart develops by folding and septum formation and starts beating after connection to the first vascular loops (Larsen 1998). The myocardial derivation from angioblasts and endothelial cell clusters thereby clearly shows that heart and vessels arise from the same original cardiovascular progenitor cells.

However, regarding the cardiovascular development from a molecular point of view is even more complicated. It is well-known that the common cardiovascular stem cells can be derived in various developmental stages from all available pluripotent cell lines like embryonic (see chapter 7), induced pluripotent (see chapter 8), spermatogonial (see chapter 9) or parthenogenetic stem cells (Yamanaka 2007). These pluripotent stem cells show an in vitro differentiation roughly comparable even though not identical to the embryonic development in vivo. And as these cells in the native state express the pluripotency markers Oct3/4, Nanog, Sox etc. (Srivastava and Ivey 2006; Takahashi and Yamanaka 2006) they are all able to form early cardiac mesoderm which is the prerequisite for the development of cardiovascular organs and the production of cardiovascular stem cells (see Fig. 6.1).

Fig. 6.1 (continued) determined to form myocardium or blood vessels. (**d**) Whereas vascular progenitors are further characterized by the expression of Islet1 (*Isl1*), *HOXB5* and the VEGF-receptor 2 (*Flk-1*), myocardial precursors follow a genetic program determined by the cardiac transcriptional key factor *Nkx2.5*. The further development of the heart can be distinguished between the first and the second heart field, which are characterized by the expression of *Tbx20, Tbx5, Nkx2.5, Hand1* and *Isl1, FGF8, Mef2c* and *Tbx1*, respectively. Fully differentiated vascular structures express the structural proteins SM-actin and SM-MHC in the smooth muscle cells and CD31 and VE-Cadherin in endothelial cells. Typical cardiac structural proteins are myosin heavy chain (*αMHC*), ventricular myosin light chain (*MLC2v*), Troponin and Connexin40 for electrical coupling

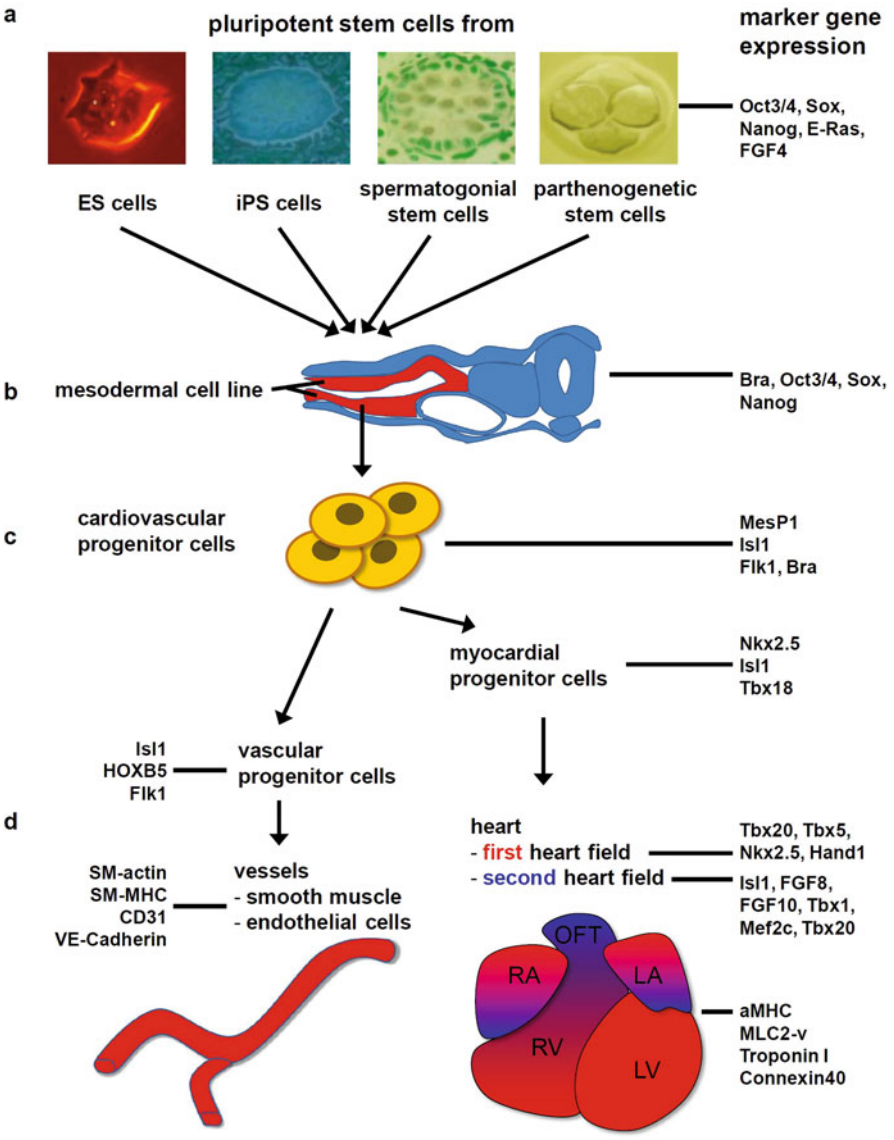

Fig. 6.1 Cardiovascular progenitors: Expression of marker genes during development from pluripotent stem cells to complete organs

(**a**) Pluripotent stem cells express the pluripotency markers Oct3/4, Sox, Nanog, E-Ras and FGF4 and can be derived from embryonic stem cells, induced pluripotent stem cells, spermatogonial stem cells or parthenogenetic stem cells. (**b**) All of the cell lines mentioned above are able to form a mesodermal cell lineage during stem cell differentiation. During this stage the pluripotency markers are downregulated and the first mesodermal transcription factor Brachyury (*Bra*) is expressed and again downregulated during further specification into the cardiovascular lineage. (**c**) The earliest marker of cardiovascular progenitor cells is the basic helix-loop-helix transcription factor *mesoderm posterior 1* (*MesP1*), which is expressed in early cardiovascular progenitor cells

While forming the cardiogenic regions in the lateral-plate mesoderm the cardiovascular precursors downregulate the earliest pan-mesodermal marker Brachyury (Bra) and start expressing the transcription factor mesoderm posterior 1 (MesP1) which is highly specific for all cardiogenic and several vasculogenic regions giving rise to the dorsal aorta, intersomitic and cranial vessels (Kitajima et al. 2000; Saga et al. 2000; Saga et al. 1999). Interestingly, Brachyury directly mediates the expression of MesP1 in the cardiovascular progenitor cells by binding to the MesP1 promotor region (David et al. 2011). This explains why MesP1 can serve as a first target gene for reprogramming or purification assays next to the markers described in the following sentences. MesP1 expressing cells therefore represent the first largely specific population of multipotent cardiovascular progenitor cells during embryogenesis that is able to form all components required to build up the heart. After creation of the cardiac crescent the cardiovascular stem cells get further specialized. Heart progenitors get committed irreversibly to a cardiac fate expressing the homeodomain transcription factors Islet1 (Isl1) and Nkx2.5 whereas the vascular progenitor cells are marked mainly by the expression of the homeobox transcription factor HOXB5 and the VEGF-receptor 2 (Flk1) (see Fig. 6.1) (Srivastava and Ivey 2006). Flk1 as well as the cell surface markers CD31 and VE-Cadherin expressed by fully differentiated endothelial cells can easily be used for antibody-based purification of vascular cells for further use in the means of therapeutical utilization by the methods described below. Moreover, an ES-cell derived cell population expressing Flk1 in a second wave and Brachyury was shown to have the ability not only to form endothelial cells but to work also as multipotent cardiovascular progenitors comparable to the MesP1 expressing cell line described above. This cell population showed cardiomyocytic, endothelial and vascular smooth muscular potential under cardiac cytokine stimulation (Kattman et al. 2006) and therefore could also be a suitable cell-source for transplantation.

Further cardiac development is based on two myocardial cell lineages that form the various regions of the heart. In particular, the cell lines can be roughly distinguished by their contribution to the formation of the left ventricle *(first heart field)* and the outflow tract *(second heart field)*, respectively (Kelly et al. 2001; Zaffran et al. 2004). The progenitor cells of the first heart field are mainly characterized by the expression of Nkx2.5, Tbx5 and Hand1 whereas this list seems not to be exhaustive at present. First heart field progenitors form both ventricles, atria and the atrioventricular canal. Cells expressing Isl1, FGF8, FGF10, Tbx1 and Mef2c originate from the mesodermal core of the pharyngeal arches and are attributed to the second heart field. They colonize the outflow tract and all other heart regions except of the left ventricle (see Fig. 6.1) (Kelly et al. 2001; Buckingham et al. 2005). The markers of the second heart field are explored quite well in contrast to those specific for the first heart field, but further investigation is required. For instance, Isl1 expressing cells can be used to generate smooth muscle, endothelial cells and cardiomyocytes but its knockout leads to a deficient development which is restricted to the outflow tract and right ventricle (Meilhac et al. 2004). In contrast, Nkx2.5 is expressed in both the first and second heart field but the Nkx2.5 knockout model lacks only the formation of Hand1 expressing cells which corresponds merely to the first heart

field structures (Lyons et al. 1995). The Tbx5-knockout mouse shows quite similar defects even though not as pronounced.

It becomes clear that many overlapping expression patterns exist in parallel to each other and depending on the stage of development. And, without a doubt, not all (especially transient) expressions of certain cardiovascular markers have been detected, yet. Furthermore, till today it was not possible to isolate a specific progenitor cell for neither the first nor the second heart field as the markers mentioned above are preferably but not exclusively expressed in the respective precursor field (Lam et al. 2009). The knowledge about the presently known specific markers expressed by the respective multipotent cardiovascular progenitor cells during the distinctive differentiation stages (in particular the $Flk1^{+}Bra^{+}$ (Kattman et al. 2006), the $MesP1^{+}$ (David et al. 2008a) and the $Isl1^{+}$ (Laugwitz et al. 2008) cell population) and the information about their developmental relevance is far from being complete today but nevertheless can already be used to identify, amplify and isolate the desired cell types in vitro from a pluripotent stem cell culture for generation of new cardiac tissue (David et al. 2009; Kattman et al. 2006; Müller et al. 2000; Wobus et al. 1997). To support this challenge various techniques have been introduced in recent years. These will be described in the following passages.

6.2.2 Derivation

The isolation of multipotent cardiovascular progenitor cells from a pluripotent stem cell culture has to face several obstacles. As described above, all conceivable types of precursor cells develop during the differentiation in vitro and can be identified by their specific gene expression program. Before being able to use these cells, e.g. for tissue engineering, they have to be cultured in a sufficient quantity and purified to avoid adverse reactions like teratoma formation (see chapter 7) or malicious cardiac arrhythmias (Lee and Makkar 2004) after transplantation.

The differentiating ES cell culture only contains a few percent of beating cardiomyocytes (Xu et al. 2008) and therefore various attempts for increasing this number have been performed. The first approaches succeeded in stimulating the cardiogenesis by using exogenous stimulation methods (see Fig. 6.2 and Table 6.1). The addition of retinoic acid to the murine embryonic stem cell culture enhances the development of ventricular cardiomyocytes shown by increased expression of the αMHC and MLC2v genes (Wobus et al. 1997). A comparable effect can be reached by stimulation with ascorbic acid (Takahashi et al. 2003) or cultivation of ES cells under influence of a low frequency magnetic field (Ventura et al. 2005). A guided differentiation via pacemaker-like cardiomyocytes with expression of Cx40, Cx45 and typical transmembranous action potential can be attained by exposure of murine ES cells to endothelin-1. Regarding the human embryonic stem cell culture the increase of beating cardiomyocytes can be facilitated by coculturing of ES cells with endodermal END2 cells (Mummery et al. 2007) via yet undefined excreted serum factors or the addition of SB203580, a specific p38 MAP kinase inhibitor

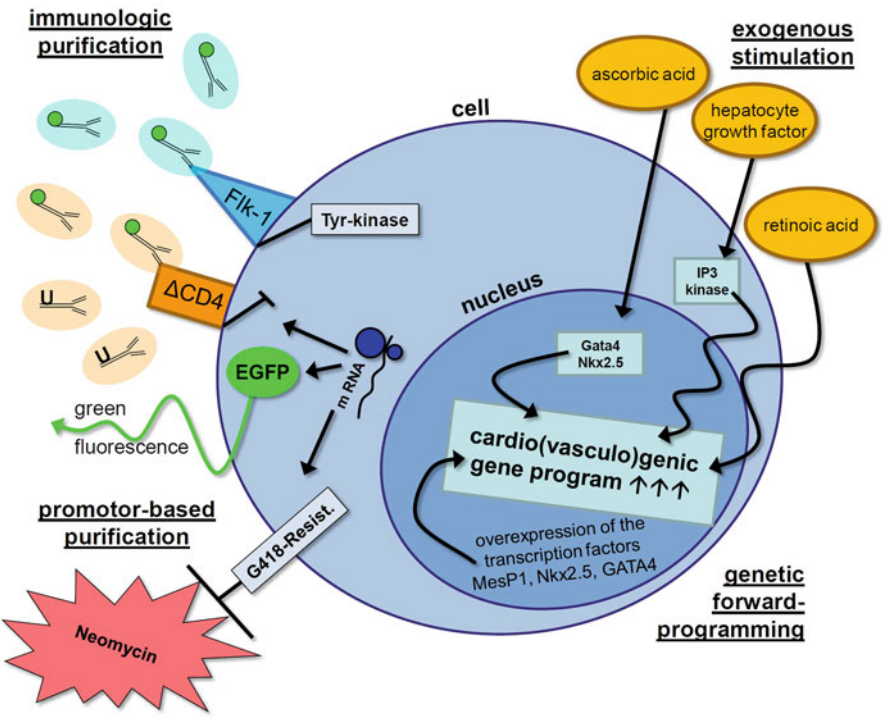

Fig. 6.2 Amplification and isolation of cardiovascular progenitor cells
Exogenous stimulation: The cardiovasculogenic program can be induced by addition of various agents (ascorbic and reinoic acid or hepatocyte growth factor) or the cultivation of the pluripotent stem cell culture in a low-frequency magnetic field. The underlying mechanisms are largely unknown here
Genetic forward programming: The differentiation process of pluripotent stem cells can be driven towards a cardiovascular fate via overexpression of selected cardiac (*Nkx2.5*) and cardiovascular (*MesP1*) transcription factors under control of the cytomegalovirus promotor
Immunologic purification: Cardiovascular progenitor cells can be purified from the differentiating stem cell culture via antibody-based fluorescence-activated or magnetic cell sorting. Antibodies can either bind to cardiovascular-specific endogenously expressed (*Flk1*) or transgenetically (promotor-based) expressed cell surface markers (ΔCD4)
Promotor-based purification: The expression of structural proteins under control of specific cardiac and cardiovascular gene promotors facilitates the purification of cardiac or cardiovascular (progenitor) cells via magnetic cell sorting (ΔCD4), fluorescence activated cell sorting (EGFP) or antiobiotic selection (G418-resistance)

(Graichen et al. 2008). A complete review over the latest approaches for stimulating cardiogenesis with the help of external factors can be found in Table 6.1. These attempts all provided promising results but lacked the detailed analysis of an underlying signaling pathway and therefore await further investigation (see Fig. 6.2).

A more elegant way for generating increased numbers of cardiovascular progenitors in the pluripotent stem cell culture is *genetic forward programming* (see Fig. 6.2). Recently, David et al. have shown proof of principle for cardiovascular

Table 6.1 Exogenous induction of cardiovascular development in embryonic stem cells

Authors	Agent/method	Cell type	Effects
Wobus et al. (1997)	Retinoic acid	Mouse ES cells	Enhanced development of ventricular cardiomyocytes, up regulated MLC2v- and myosin heavy chain expression
Paquin et al. (2002)	Oxytocin	P19 embryonic stem cells	Increased number of beating cell colonies, up regulated ANP- and myosin heavy chain expression
Takahashi et al. (2003)	Ascorbic acid	Mouse ES cells	Increased number of alpha myosin heavy chain-positive cells, up regulated expression of GATA4, beta-MHC
Gassanov et al. (2004)	Endothelin	Mouse ES cells	Increased number of pacemaker-like cardiomyocytes, confirmed by electrophysiology (If) and immunostaining (Cx40, Cx45)
Ventura et al. (2004)	Butyric and retinoic mixed ester of hyaluron	Mouse ES cells	Increased number of spontaneously beating cardiomyocytes, up regulated expression of GATA4 and Nkx2.5
Ventura et al. (2005)	Extremely low frequency magnetic field	Mouse ES cells	Increased number of spontaneously beating cardiomyocytes, up regulated expression of GATA4 and Nkx2.5
Yuasa et al. (2005)	Noggin, inhibitor of BMP-signaling	Mouse ES cells	Increased beating EB incidence
Roggia et al. (2006)	Hepatocyte growth factor	Mouse ES cells	Increased number of beating embryoid bodies via PI3 kinase/Akt pathway, up regulated expression of GATA4 and Nkx2.5
Mummery et al. (2007)	Coculture with endodermal END2 cells	Human ES cells	Increased number of beating cardiomyocytes
Graichen et al. (2008)	SB203580, specific p38 MAP kinase inhibitor	Human ES cells	Increased cardiomyocyte yield via enhanced early mesoderm formation
Xu et al. (2008)	Prostaglandin I2	Human ES cells	Enhanced cardiogenic activity
Wiese et al. (2011)	Suramin	Mouse ES cells	Induction of differentiation into sinus node-like cells
Sharma et al. (2015)	Small molecule-modulation and glucose starvation	Human iPS cells	Induction of cardiomyocytes differentiation

subtype specific programming of pluripotent stem cells. Via overexpression of early cardiovascular transcription factors like Nkx2.5 (under control of the cytomegalovirus (CMV) promoter) the process of cardiogenesis could be induced in murine ES cells (David et al. 2009). Forced expression of this cardiac key gene leads to increased numbers of differentiated beating ventricular cardiomyocytes without affecting vascular progenitors in vitro whereas MesP1 overexpression causes an enhancement of

vascular development shown by an increased number of electrophysiologically detectable early/intermediate type cardiomyocytes and spontaneously sprouting endothelial structures in the culture dish (David et al. 2008a; David et al. 2009). This is a strong sign for MesP1 lying upstream of Nkx2.5 in the molecular hierarchy for cardiovascular specification as the first-mentioned is acting on the level of cardiovascular progenitor cells whereas the influence of Nkx2.5 is restricted to the more specified ventricular cardiomyocyte progenitors (see Fig. 6.1). David et al. could detect a stimulation of cardiovascular development via MesP1-overexpression not only in early vertebrates but also in murine embryonic stem cells (David et al. 2008a). Subsequently, this key finding was confirmed by several other groups (Bondue et al. 2008; Lindsley et al. 2008). In the work of David et al. the underlying signaling pathway of MesP1 has been deciphered in detail. MesP1-overexpression and -knockdown experiments revealed a prominent function of MesP1 within a gene regulatory cascade causing Dkk1 mediated blockade of canonical Wnt-signalling. Independent evidence from chromatin immunoprecipitation, in vitro DNA binding studies, expression analysis in wild-type and MesP1/2 double knockout mice and reporter gene assays confirmed the Dkk1 promoter as a direct target, activated by MesP1 protein (David et al. 2008a). This mechanism is supported by findings published by Lindsley et al. and Bondue et al. that detected a 50-fold and 1.5-fold upregulation of Dkk1-expression, respectively, caused by overexpression of MesP1 (Bondue et al. 2008; Lindsley et al. 2008).

Thus, it is evident that MesP1 is located at the top of the transcriptional network that controls cardiovascular differentiation by directly regulating the spatial and temporal expression of key cardiac transcription factors such as Nkx2.5, Tbx20, Hand2, Mef2c and indirectly by enhancing the transcription of Dkk1 (Bondue et al. 2008; Wu 2008; David et al. 2008a; Lindsley et al. 2008). Further attempts for genetic forward programming even though without a detailed related signaling pathway are summarized in Table 6.2.

Despite the progressing decryption of the molecular development of the heart and vessel formation, methods are not yet refined enough for scientists being able to direct pluripotent stem cell differentiation exclusively in the direction of cardiovascular development not to mention to generate pure cultures of specific differentiated myocardial, endothelial or smooth muscle cells with the help of gene technology methods. Therefore, to minimize the hazard of undifferentiated stem cells or improper cell types within the transplantable cell mass various purification methods have been introduced. The best established ways of isolating specific cells from a differentiating pluripotent stem cell culture are using the fluorescence activated or the magnetic cell sorting (FACS, MACS) (Kanno et al. 2004; Kattman et al. 2006; Müller et al. 2000; David et al. 2005). After labeling of the desired cells by antibodies binding to stage and cell type specific surface antigens the antibody-coupled cells can be detected and isolated by their fluorescence and magnetic properties, respectively (see Fig. 6.2). A suitable cell population for this approach are the $Flk1^{+}Bra^{+}$ cardiovascular progenitors described by Kattman et al. (Kattman et al. 2006) as Flk1 is endogenously expressed on the surface of these cells and therefore can be used for the methods described above. Merely the parallel expres-

Table 6.2 Induction of cardiovascular differentiation via manipulations on gene activity

Authors	Genetic modification	Main affected signaling	Effect
Grepin et al. (1997)	GATA-4 overexpression	Nuclear target of inductive factors for precardiac cells	Accelerated cardiogenesis, increased number of terminally differentiated beating cardiomyocytes
Kanno et al. (2004)	NO overexpression	Not known	Accelerated cardiomyocyte differentiation, apoptosis of cells not committed to Cardiomyocyte-differentiation
Singh et al. (2007)	Chibby overexpression	Wnt/β-Catenin-pathway	Increased cardiac differentiation
David et al. (2009)	Nkx2.5 overexpression	Not known	Increased number of ventricular cardiomyocytes
David et al. (2009)	MesP1 overexpression	Wnt/β-Catenin-pathway	Increased number of early/intermediate type cardiomyocytes
Izarra et al. (2014)	miR1, miR133a	Mesodermal development, cardiac differentiation	Overexpression of miR1 and miR133 simultaneously leads to mesodermal commitment of P19.CL6 cells, miR1 overexpression enhances cardiac differentiation

Table 6.3 Purification of cardiovascular and cardiac progenitors by specific cellular markers

Authors	Endogenous cellular marker	Method	Cell type
Kattman et al. (2006)	Flk-1	FACS	Cardiovascular progenitor cells from murine ES cell culture
	Cell surface		
Nelson et al. (2008)	Flk-1, CXCR-4	FACS	Cardiopoietic lineage from murine ES cell culture
	Cell surface		
Ban et al. (2013)	MHC1-MB	FACS	Cardiac troponin expressing cardiomyocytes
	Intracellular molecular beacons		

sion of Flk1 in specified vascular progenitor cells (see Fig. 6.1) makes this marker less specific and thus can decrease the purity of the desired cardiovascular progenitor cell population. Possibly, the co-staining with Flk1- and CXCR4-antibodies may reduce this contamination (see Table 6.3) (Nelson et al. 2008). Unlike early cardiovascular or especially hematopoietic stem cells specified cardiac progenitors and differentiated cardiomyocytes do not express any known exclusive surface proteins that are accessible for antibody-based purification methods in their native state (Müller et al. 2000). For this reason the *promotor-based labeling* of cardiomyocytes was introduced (David et al. 2005; Kolossov et al. 2005). The transfection of a gene vector including a reporter gene (eGFP, ΔCD4 or

Table 6.4 Promotor-based purification methods of cardiac and cardiovascular cells from the embryonic stem cell culture

Authors	Promoter-based construct	Method	Cell type
Müller et al. (2000)	MLC2v-eGFP, CMV-enhancer	Percoll-gradient, FACS	Ventricular-like cardiomyocytes from murine ES cell culture
Zweigerdt et al. (2003) and Zandstra et al. (2003)	αMHC-Neomycin-Resistance	Antibiotic selection	Beating cardiomyocytes
Gassanov et al. (2004)	ANP-eGFP	Suitable for FACS	Pacemaker-like cardiomyocytes from murine ES cell culture
David et al. (2005)	PGK-ΔCD4	MACS	Suitable for all cardiac-specific ΔCD4 expression constructs
Kolossov et al. (2005)	αMHC-eGFP	FACS	Atrial and pacemaker cardiomyocytes from murin ES cell culture
David et al. (2008b)	Cx40-eGFP	FACS	Cardiovascular progenitor cells from murine ES cell culture
Potta et al. (2010)	Acta3-PuroIRES2-EGFP	Antibiotic selection	Pacemaker-, atrial- and ventricular-like cardiomyocytes from murine ES cell culture

Neomycin(G418)-Resistance) expressed under the control of a cardiac specific genetic promotor like MLC2v, αMHC (both specific for ventricular cardiomyocytes, (Kolossov et al. 2005; Müller et al. 2000; Zweigerdt et al. 2003)), Cx40 (cardiovascular progenitors, (David et al. 2008b)), or ANP (pacemaker cells, (Gassanov et al. 2004)) or the targeted knock-in of a reporter gene into the respective gene locus thereby facilitates the stable labeling of the desired cell type during a specific differentiation stage (see Fig. 6.2 and Table 6.4). The purification method is dependent on the used reporter gene. The intracellular expression of enhanced green fluorescent protein (EGFP) is only suitable for fluorescence activated cell sorting (FACS) whereas the intracellularly deleted (i.e. lacking any intracellular signal transduction) cluster of differentiation 4 (ΔCD4) can be stained by FITC- (for FACS) or magnetic beat-coupled (for MACS) antibodies (see Fig. 6.2). Another recently introduced technique for FACS-dependent purification of cardiomyocytes is based on the nucleofection of fluorescing molecular beacons (MBs) that specifically target cardiomyocytes-specific mRNA. After intracellular delivery the MBs can bind e.g. to myosin heavy chain 6/7 mRNA. Using this technique, purities of up to 97 % could be reached (Ban et al. 2013).

Another method, not less elegant but contrarian to fluorescence-activated cell sorting, is the negative selection with help of the neomycin-resistance gene. While the desired cells survive the treatment with the antibiotic G418 all needless cells are eradicated by this toxin (Zandstra et al. 2003; Zweigerdt et al. 2003).

Yet not all of the purification methods mentioned above are potentially suitable for the isolation of cells destined for the use in human beings. Using the antibiotic

selection for example, it is not possible to gain stage-specific cardiovascular progenitor cells from the pluripotent stem cell culture as cardiovascular development is fast and the selection period in general takes several weeks (with the hazard of resistance and possible harmful effects of the antibiotic on terminally differentiated cells themselves) to guarantee a reliable purity of the desired cells (Klug et al. 1996; Zandstra et al. 2003; Zweigerdt et al. 2003). But also cytometry (FACS) cannot be seen as the gold standard of cell sorting as the cells get highly accelerated and irradiated by laser light as a side effect of the underlying method, which is likely to harm the purified cardiovascular cell types. Moreover, fluorescent proteins used for cell labeling has been reported to bear pro-apoptotic properties (Liu et al. 1999) which would be a major hindrance for transplantation of such fluorescent cells into damaged myocardium. However, with less manipulating methods being developed, with high purity grades and a minor risk for tumor formation, generation of transplantable cardiac tissue appears feasible in the future.

6.2.3 Potential Therapeutic Applications (Tissue Engineering)

Various applications for cardiac repair via pluripotent stem cell-derived cardiovascular progenitors have been proposed within recent years. With ischemic and dilative cardiomyopathy being on the list of the top cardiovascular diseases the generation of contractile myocardium presently is a main goal of current tissue engineering approaches (Zimmermann and Cesnjevar 2009; Tulloch et al. 2011). The transplantation of genetically and physiologically compatible myocardial tissue with an included and appropriate blood vessel supply will allow compensation of lost myocardial tissue – largely independent from its underlying disease (Masumoto et al. 2014).

Another important application of cardiovascular tissue engineering is the generation of biological pacemaker and conducting cells (Gassanov et al. 2004; Wiese et al. 2011; Jung et al. 2014) for patients suffering from sick-sinus-syndrome or disturbances of the conduction system like atrioventricular or bundle branch block. The implementation of those specified cells could be an alternative to electronic pacemaker implantations.

Besides generation of myocardium cardiovascular stem cells could also be utilized for the generation of large vessels that might be used as vascular bypass grafts or arterial prostheses (Srivastava and Ivey 2006). However, as today acceptable autologous alternatives exist (e.g. internal mammarian or radial arteries as bypass grafts) the generation of macrovascular tissues currently is only a secondary goal.

However, comparable to other organ systems the ultimate objective while working with pluripotent stem cell-derived progenitor cells will be the generation of a whole working organ system, i.e. the artificial engineering of a working transplantable heart.

A first step towards clinical implementation of ES cell derived progenitors has just been initiated by the group of Philippe Menasché who is planning to transplant

human embryonic stem cell-derived CD15+ Isl1+ progenitor cells into the epicardium of diseased hearts to improve their viability (ESCORT trial, NCT02057900).

6.2.4 Future Development in Pluripotent Stem Cell Research

Until today, a decent part of cardiovascular development has already been investigated. It is possible to obtain, multiply, purify and maintain cardiovascular progenitors and their derivatives from pluripotent stem cells in vitro for generation of clinically applicable cardiac and cardiovascular replacement tissue. The knowledge and technology available today thereby provide the prerequisites necessary to let the vision of cardiac tissue engineering appear feasible within the next decades. For the remaining obstacles still lying in the way various approaches are already at least being investigated now. Thus, the problem of immunologic rejection of graft tissue is most likely to be solved by the use of induced pluripotent stem cells. Furthermore, the ongoing decryption of the cellular signal transduction system in developing human pluripotent stem cells will finally enable us to exclusively direct stem cell differentiation into the desired cardiovascular fate. Ideally, the control of the molecular development should then be feasible by exogenous manipulation without using transgenic cells any more. This will then facilitate the generation of highly specific, pure and riskless transplantable cell populations.

A very different approach for using pluripotent stem cells for cardiac tissue regeneration lies in the generation of differentiated epicardial cells in vitro. While classical approaches for tissue replacement focus on engineering of contractile transplantable tissue, epicardial cells might be used as a kind of specified progenitor cell for myocardial regeneration. Witty et al. showed that an epicardial lineage differentiation can be induced in pluripotent stem cells. During heart development epicardial cells directly contribute to the formation of the myocardium via the epithelial-to-mesenchymal transition (EMeT) or the epicardium-to-myocardium transition (EMyT) (see Fig. 6.3) (Brenner and Franz 2014; Witty et al. 2014). Transplanting pluripotent stem cell-derived epicardial cells into the pericardium of a diseased heart may thus depict an elegant and easy method to regenerate myocardial tissue in the future.

6.3 Circulating Progenitor Cells for Endogenous Tissue Regeneration

6.3.1 Characteristics and Therapeutic Applications

Another therapeutic approach for the regeneration of the heart and blood vessels instead of complete tissue replacement is based on circulating progenitor cells (ciPCs). Certain ischemic or inflammatory stimuli as well as pharmacological

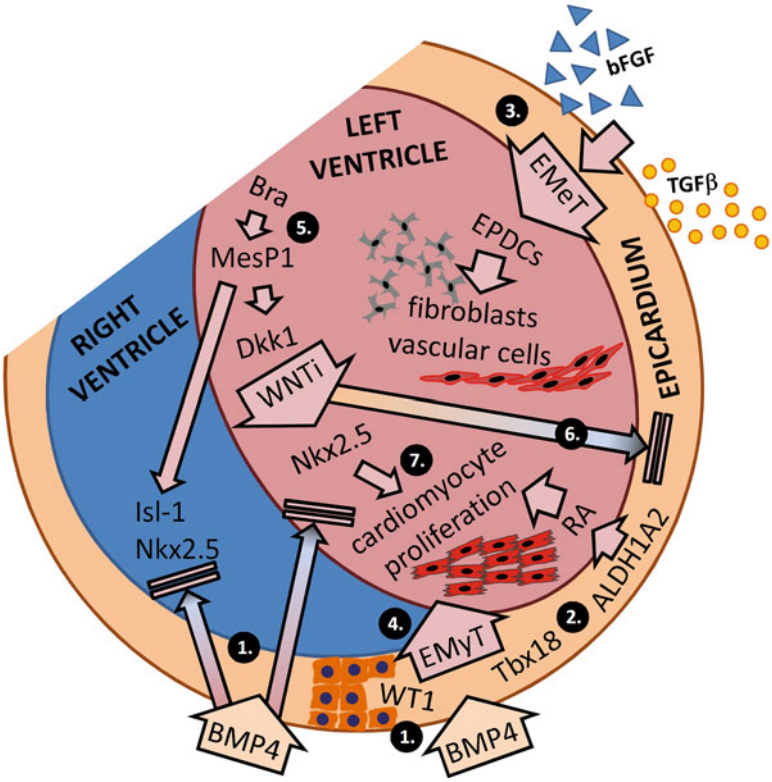

Fig. 6.3 Schematic Description of Epicardial involvement in Cardiac Development
(*1*) Alterations in BMP signaling lead to proliferation of the epicardial lineage and inhibit cardiomyocyte development. (*2*) In contrast to endocardial and myocardial cells, epicardial cells typically express *WT1, Tbx18*, and *ALDH1A2*, which is necessary for production of retinoic acid (*RA*). (*3*) Epicardial-derived cells (*EPDCs*) directly contribute to the formation of the myocardium as noncardiomyocyte cells via the epithelial-to-mesenchymal transition (*EMeT*). (*4*) Epicardial cells may directly differentiate into myocardial cells (epicardium-to-myocardium transition, *EMyT*) and contribute to the ventricular septum and atrial and ventricular walls. (*5*) Brachyury induces the expression of *MesP1*, which inhibits canonical WNT signaling (*WNTi*). This leads to an accelerated cardiomyogenesis in the primary heart field (which gives rise to the *left* ventricle and atria) and the secondary heart field (which gives rise to the *right* ventricle and outflow tract). (*6*) Inhibition of WNT signaling (*WNTi*) by *Dkk1* reduces the formation of epicardial tissue. (*7*) *Nkx2.5* expression is induced by *MesP1* and enhances the formation of ventricular cardiomyocytes
Reprinted from Brenner and Franz (2014), with kind permission from Elsevier

induction using e.g. the granulocyte-colony-stimulating factor GCSF can mobilize these cells from their bone marrow niche (Deindl et al. 2006). Circulating progenitor cells are recruited from the blood into injured tissues that express adhesion molecules on their cell surfaces and secret specific homing factors. These progenitor cells generally do not appear to have the ability to transdifferentiate and thus cannot directly replace diseased cardiovascular tissue. However, ciPCs work in a paracrine fashion to promote proliferation of local differentiated and resident progenitor cells,

inhibit apoptosis and induce neovascularization (Gnecchi et al. 2008; Zaruba et al. 2009; Brenner et al. 2014).

Different approaches for using the regenerative potential of circulating progenitor cells have been introduced within the recent years. Besides direct intravenous or intracoronary injection of in vitro cultivated progenitors also non-invasive techniques for enhancing tissue recruitment have successfully been investigated in preclinical models and clinical trials. After endogenous or pharmacological mobilization of bone marrow progenitor cells, inhibition of the cleaving enzyme dipeptidyl peptidase 4 (DPP4) could enhance their recruitment into diseased cardiac and vascular tissue. In ischemic myocardium as well as in injured arterial blood vessels local expression of the homing factor SDF1 (stromal cell-derived factor 1) is highly upregulated as compared to healthy tissue. SDF1 binds to the CXCR4 receptor on the surface of ciPCs and thus facilitates their homing into the tissue. SDF1 itself is inactivated by the DPP4, which is ubiquitously available and cleaves two amino acids at the N-terminus of SDF1. Administration of a DPP4 inhibitor blocks SDF1 inactivation which boosts the invasion of progenitor cells into the diseased tissue (Christopherson et al. 2004).

Various recently published animal studies could demonstrate that pharmacological stabilization of SDF1 indeed improves myocardial recovery and survival after acute myocardial infarction (Theiss et al. 2013; Theiss et al. 2011; Zaruba et al. 2009). Likewise, inhibition of DPP4 could also accelerate reendothelialization of denuded arterial blood vessels in mice (see Fig. 6.4) (Brenner et al. 2014).

6.3.2 Future Prospects

Due to the promising preclinical studies, various therapeutic approaches have already been translated into clinical trials investigating the regenerative potential of bone marrow-derived progenitor cells. While the REPAIR-AMI trial demonstrated beneficial effects of intracoronary progenitor cell delivery after myocardial infarction various other studies regarding this route of application remained negative (Schachinger et al. 2006b; Abbasi et al. 2011). Future large-scale randomized clinical studies like the ongoing BAMI trial (NCT01569178) will help to further determine the therapeutic impact of intracoronary injected progenitor cells. Besides, also another trial investigating the regenerative effect of circulating progenitor cells using the pharmacological approach (GCSF and the DPP4 inhibitor Sitagliptin, SITAGRAMI trial) has recently been finished (Theiss et al. 2010). We expect publication of the results in 2015.

6.4 Conclusion

Both, pluripotent and circulating stem and progenitor cells provide the potential for a therapeutic application in humans. While pluripotent stem cells are able to form beating myocardial cells and vascular tissue several hurdles still have to be

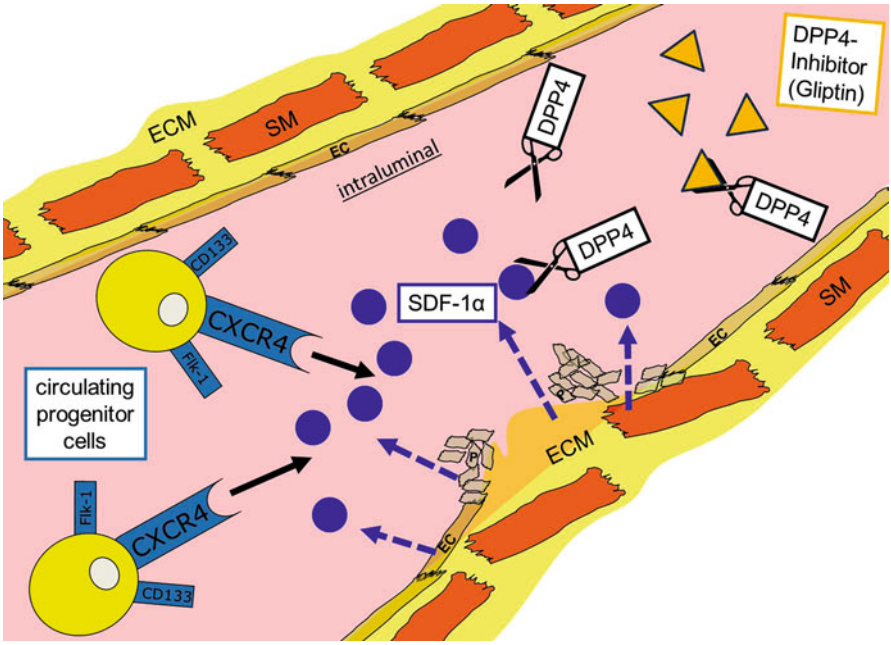

Fig. 6.4 Essential molecular mechanism for the recruitment of circulating progenitor cells into the injured vascular wall
After deendothelialization of the arterial blood vessel, attached platelets and activated smooth muscle and endothelial cells secret the cytokine *SDF1*. This binds to the *CXCR4* receptor on the surface of circulating progenitor cells and thus facilitates their recruitment into the injured tissue. Recruited ciPCs can improve tissue regeneration by paracrine stimulation of local differentiated and progenitor cells. *SDF1* is inactivated by the dipeptidyl peptidase 4 (*DPP4*). Thus, local active *SDF1* concentration can be increased by the pharmacological inhibition of *DPP4* which enhances the therapeutic effect of *SDF1*. (*EC* endothelial cell, *ECM* extracellular matrix, *P* attached platelet, *SM* smooth muscle cell)

overcome before a therapeutic administration. In contrast, the use of adult circulating progenitor cells appears to be closer to clinical application. However, also this type of progenitor cell still has to prove its efficacy in diseased patients.

References

Abbasi M, Javan H, Alizadeh B, Afzalnia S (2011) Can intracoronary stem cell injection permanently improve cardiac function after myocardial infarction? Interact Cardiovasc Thorac Surg 12(2):229–231. doi:10.1510/icvts.2010.252106

Akins RE, Boyce RA, Madonna ML, Schroedl NA, Gonda SR, McLaughlin TA, Hartzell CR (1999) Cardiac organogenesis in vitro: reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. Tissue Eng 5(2):103–118

Assmus B, Leistner DM, Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Sedding D, Yu J, Corti R, Mathey DG, Barth C, Mayer-Wehrstein C, Burck I, Sueselbeck T, Dill T, Hamm CW, Tonn T, Dimmeler S, Zeiher AM, Group R-AS (2014) Long-term clinical outcome

after intracoronary application of bone marrow-derived mononuclear cells for acute myocardial infarction: migratory capacity of administered cells determines event-free survival. Eur Heart J 35(19):1275–1283. doi:10.1093/eurheartj/ehu062

Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428(6983):668–673. doi:10.1038/nature02460, nature02460 [pii]

Ban K, Wile B, Kim S, Park HJ, Byun J, Cho KW, Saafir T, Song MK, Yu SP, Wagner M, Bao G, Yoon YS (2013) Purification of cardiomyocytes from differentiating pluripotent stem cells using molecular beacons that target cardiomyocyte-specific mRNA. Circulation 128(17):1897–1909. doi:10.1161/CIRCULATIONAHA.113.004228

Bel A, Planat-Bernard V, Saito A, Bonnevie L, Bellamy V, Sabbah L, Bellabas L, Brinon B, Vanneaux V, Pradeau P, Peyrard S, Larghero J, Pouly J, Binder P, Garcia S, Shimizu T, Sawa Y, Okano T, Bruneval P, Desnos M, Hagege AA, Casteilla L, Puceat M, Menasche P (2010) Composite cell sheets: a further step toward safe and effective myocardial regeneration by cardiac progenitors derived from embryonic stem cells. Circulation 122(11):S118–S123. doi:122/11_suppl_1/S118 [pii], 10.1161/CIRCULATIONAHA.109.927293

Bondue A, Lapouge G, Paulissen C, Semeraro C, Iacovino M, Kyba M, Blanpain C (2008) Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. Cell Stem Cell 3(1):69–84

Boyle A, Colvin-Adams M (2004) Recipient selection and management. Semin Thorac Cardiovasc Surg 16(4):358–363

Brenner C, Franz WM (2014) Pluripotent-stem-cell-derived epicardial cells: a step toward artificial cardiac tissue. Cell Stem Cell 15(5):533–534. doi:10.1016/j.stem.2014.10.007

Brenner C, Krankel N, Kuhlenthal S, Israel L, Remm F, Fischer C, Herbach N, Speer T, Grabmaier U, Laskowski A, Gross L, Theiss H, Wanke R, Landmesser U, Franz WM (2014) Short-term inhibition of DPP-4 enhances endothelial regeneration after acute arterial injury via enhanced recruitment of circulating progenitor cells. Int J Cardiol 177(1):266–275. doi:10.1016/j.ijcard.2014.09.016

Buckingham M, Meilhac S, Zaffran S (2005) Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet 6(11):826–835

Christopherson KW 2nd, Hangoc G, Mantel CR, Broxmeyer HE (2004) Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 305(5686):1000–1003. doi:10.1126/science.1097071

David R, Groebner M, Franz WM (2005) Magnetic cell sorting purification of differentiated embryonic stem cells stably expressing truncated human CD4 as surface marker. Stem Cells 23(4):477–482

David R, Brenner C, Stieber J, Schwarz F, Brunner S, Vollmer M, Mentele E, Muller-Hocker J, Kitajima S, Lickert H, Rupp R, Franz WM (2008a) MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. Nat Cell Biol 10(3):338–345

David R, Theiss H, Franz WM (2008b) Connexin 40 promoter-based enrichment of embryonic stem cell-derived cardiovascular progenitor cells. Cells Tissues Organs 188(1–2):62–69

David R, Stieber J, Fischer E, Brunner S, Brenner C, Pfeiler S, Schwarz F, Franz WM (2009) Forward programming of pluripotent stem cells towards distinct cardiovascular cell types. Cardiovasc Res 84(2):263–272

David R, Jarsch V, Schwarz F, Nathan P, Gegg M, Lickert H, Franz WM (2011) Induction of MesP1 by Brachyury(T) generates the common multipotent cardiovascular stem cell. Cardiovasc Res 92(1):115–122. doi:cvr158 [pii], 10.1093/cvr/cvr158

Deindl E, Zaruba MM, Brunner S, Huber B, Mehl U, Assmann G, Hoefer IE, Mueller-Hoecker J, Franz WM (2006) G-CSF administration after myocardial infarction in mice attenuates late ischemic cardiomyopathy by enhanced arteriogenesis. FASEB J 20(7):956–958

DESTATIS (2013) Number of deaths up 2 % on a year earlier. Press release from 12 December 2013, German federal statistical office (422)

Eisenberg LM, Eisenberg CA (2006) Wnt signal transduction and the formation of the myocardium. Dev Biol 293(2):305–315

Engelmann MG, Theiss HD, Theiss C, Henschel V, Huber A, Wintersperger BJ, Schoenberg SO, Steinbeck G, Franz WM (2009) G-CSF in patients suffering from late revascularised ST elevation myocardial infarction: final 1-year-results of the G-CSF-STEMI trial. Int J Cardiol 144(3):399–404

Gassanov N, Er F, Zagidullin N, Hoppe UC (2004) Endothelin induces differentiation of ANP-EGFP expressing embryonic stem cells towards a pacemaker phenotype. FASEB J 18(14):1710–1712

Gerecht-Nir S, David R, Zaruba M, Franz WM, Itskovitz-Eldor J (2003) Human embryonic stem cells for cardiovascular repair. Cardiovasc Res 58(2):313–323

Gnecchi M, Zhang Z, Ni A, Dzau VJ (2008) Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 103(11):1204–1219. doi:10.1161/CIRCRESAHA.108.176826

Graichen R, Xu X, Braam SR, Balakrishnan T, Norfiza S, Sieh S, Soo SY, Tham SC, Mummery C, Colman A, Zweigerdt R, Davidson BP (2008) Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation 76(4):357–370

Grepin C, Nemer G, Nemer M (1997) Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. Development 124(12):2387–2395

Gyongyosi M, Wojakowski W, Lemarchand P, Lunde K, Tendera M, Bartunek J, Marban E, Assmus B, Henry TD, Traverse JH, Moye LA, Surder D, Corti R, Huikuri H, Miettinen J, Wohrle J, Obradovic S, Roncalli J, Malliaras K, Pokushalov E, Romanov A, Kastrup J, Bergmann MW, Atsma DE, Diederichsen A, Edes I, Benedek I, Benedek T, Pejkov H, Nyolczas N, Pavo N, Bergler-Klein J, Pavo IJ, Sylven C, Berti S, Navarese EP, Maurer G, Investigators* A (2015) Meta-Analysis of Cell-based CaRdiac stUdiEs (ACCRUE) in patients with acute myocardial infarction based on individual patient data. Circ Res 116(8):1346–1360. doi:10.1161/CIRCRESAHA.116.304346

Hunt SA (1998) Current status of cardiac transplantation. JAMA 280(19):1692–1698

Izarra A, Moscoso I, Canon S, Carreiro C, Fondevila D, Martin-Caballero J, Blanca V, Valiente I, Diez-Juan A, Bernad A (2014) miRNA-1 and miRNA-133a are involved in early commitment of pluripotent stem cells and demonstrate antagonistic roles in the regulation of cardiac differentiation. J Tissue Eng Regen Med. doi:10.1002/term.1977

Jung JJ, Husse B, Rimmbach C, Krebs S, Stieber J, Steinhoff G, Dendorfer A, Franz WM, David R (2014) Programming and isolation of highly pure physiologically and pharmacologically functional sinus-nodal bodies from pluripotent stem cells. Stem Cell Rep 2(5):592–605. doi:10.1016/j.stemcr.2014.03.006

Kanno S, Kim PK, Sallam K, Lei J, Billiar TR, Shears LL 2nd (2004) Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. Proc Natl Acad Sci U S A 101(33):12277–12281

Kattman SJ, Huber TL, Keller GM (2006) Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell 11(5):723–732

Kelly RG, Brown NA, Buckingham ME (2001) The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. Dev Cell 1(3):435–440

Kessler PD, Byrne BJ (1999) Myoblast cell grafting into heart muscle: cellular biology and potential applications. Annu Rev Physiol 61:219–242

Kitajima S, Takagi A, Inoue T, Saga Y (2000) MesP1 and MesP2 are essential for the development of cardiac mesoderm. Development 127(15):3215–3226

Klug MG, Soonpaa MH, Koh GY, Field LJ (1996) Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J Clin Invest 98(1):216–224

Kolossov E, Lu Z, Drobinskaya I, Gassanov N, Duan Y, Sauer H, Manzke O, Bloch W, Bohlen H, Hescheler J, Fleischmann BK (2005) Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes. FASEB J 19(6):577–579

Kraehenbuehl TP, Ferreira LS, Hayward AM, Nahrendorf M, van der Vlies AJ, Vasile E, Weissleder R, Langer R, Hubbell JA (2011) Human embryonic stem cell-derived microvascular grafts for cardiac tissue preservation after myocardial infarction. Biomaterials 32(4):1102–1109. doi:S0142-9612(10)01290-1 [pii], 10.1016/j.biomaterials.2010.10.005

Lam JT, Moretti A, Laugwitz KL (2009) Multipotent progenitor cells in regenerative cardiovascular medicine. Pediatr Cardiol 30(5):690–698

Larsen W (1998) Essentials of human embryology. Library of congress cataloging-in-publication data, vol 2. Churchill Livingstone, New York

Laugwitz KL, Moretti A, Caron L, Nakano A, Chien KR (2008) Islet1 cardiovascular progenitors: a single source for heart lineages? Development 135(2):193–205

Leal J, Luengo-Fernandez R, Gray A, Petersen S, Rayner M (2006) Economic burden of cardiovascular diseases in the enlarged European Union. Eur Heart J 27(13):1610–1619. doi:ehi733 [pii], 10.1093/eurheartj/ehi733

Lee MS, Makkar RR (2004) Stem-cell transplantation in myocardial infarction: a status report. Ann Intern Med 140(9):729–737

Liao SY, Liu Y, Siu CW, Zhang Y, Lai WH, Au KW, Lee YK, Chan YC, Yip PM, Wu EX, Wu Y, Lau CP, Li RA, Tse HF (2010) Proarrhythmic risk of embryonic stem cell-derived cardiomyocyte transplantation in infarcted myocardium. Heart Rhythm 7(12):1852–1859. doi:S1547-5271(10)00904-5 [pii], 10.1016/j.hrthm.2010.09.006

Lin Q, Fu Q, Zhang Y, Wang H, Liu Z, Zhou J, Duan C, Wang Y, Wu K, Wang C (2010) Tumourigenesis in the infarcted rat heart is eliminated through differentiation and enrichment of the transplanted embryonic stem cells. Eur J Heart Fail 12(11):1179–1185. doi:hfq144 [pii], 10.1093/eurjhf/hfq144

Lindsley RC, Gill JG, Murphy TL, Langer EM, Cai M, Mashayekhi M, Wang W, Niwa N, Nerbonne JM, Kyba M, Murphy KM (2008) Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs. Cell Stem Cell 3(1):55–68

Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ (1999) Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 260(3):712–717

Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP (1995) Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev 9(13):1654–1666

Maltsev VA, Rohwedel J, Hescheler J, Wobus AM (1993) Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. Mech Dev 44(1):41–50

Masumoto H, Ikuno T, Takeda M, Fukushima H, Marui A, Katayama S, Shimizu T, Ikeda T, Okano T, Sakata R, Yamashita JK (2014) Human iPS cell-engineered cardiac tissue sheets with cardiomyocytes and vascular cells for cardiac regeneration. Sci Rep 4:6716. doi:10.1038/srep06716

Meilhac SM, Esner M, Kelly RG, Nicolas JF, Buckingham ME (2004) The clonal origin of myocardial cells in different regions of the embryonic mouse heart. Dev Cell 6(5):685–698

Menasche P (2005) Skeletal myoblast for cell therapy. Coron Artery Dis 16(2):105–110

Menasche P, Hagege AA, Vilquin JT, Desnos M, Abergel E, Pouzet B, Bel A, Sarateanu S, Scorsin M, Schwartz K, Bruneval P, Benbunan M, Marolleau JP, Duboc D (2003) Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol 41(7):1078–1083

Müller M, Fleischmann BK, Selbert S, Ji GJ, Endl E, Middeler G, Muller OJ, Schlenke P, Frese S, Wobus AM, Hescheler J, Katus HA, Franz WM (2000) Selection of ventricular-like cardiomyocytes from ES cells in vitro. FASEB J 14(15):2540–2548

Mummery CL, Ward D, Passier R (2007) Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum-free medium. Curr Protoc Stem Cell Biol Chapter 1:Unit 1F 2

Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428(6983):664–668

Nelson TJ, Faustino RS, Chiriac A, Crespo-Diaz R, Behfar A, Terzic A (2008) CXCR4+/FLK-1+ biomarkers select a cardiopoietic lineage from embryonic stem cells. Stem Cells 26(6):1464–1473

Neumayer HH (2005) Introducing everolimus (Certican) in organ transplantation: an overview of preclinical and early clinical developments. Transplantation 79(9 Suppl):S72–S75

Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y (1995) Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. Biomaterials 16(4):297–303

Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P (2001) Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 938:221–229; discussion 229–230

Paquin J, Danalache BA, Jankowski M, McCann SM, Gutkowska J (2002) Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. Proc Natl Acad Sci U S A 99(14):9550–9555

Potta SP, Liang H, Winkler J, Doss MX, Chen S, Wagh V, Pfannkuche K, Hescheler J, Sachinidis A (2010) Isolation and functional characterization of alpha-smooth muscle actin expressing cardiomyocytes from embryonic stem cells. Cell Physiol Biochem 25(6):595–604. doi:000315078 [pii], 10.1159/000315078

Roggia C, Ukena C, Bohm M, Kilter H (2006) Hepatocyte growth factor (HGF) enhances cardiac commitment of differentiating embryonic stem cells by activating PI3 kinase. Exp Cell Res 313(5):921–930

Sadler T (1998) Medizinische Embryologie – Die normale menschliche Entwicklung und ihre Fehlbildungen (trans: Drews U), 9th edn

Saga Y, Miyagawa-Tomita S, Takagi A, Kitajima S, Miyazaki J, Inoue T (1999) MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development 126(15):3437–3447

Saga Y, Kitajima S, Miyagawa-Tomita S (2000) Mesp1 expression is the earliest sign of cardiovascular development. Trends Cardiovasc Med 10(8):345–352

Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Suselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM (2006a) Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355(12):1210–1221

Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Suselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM, Investigators R-A (2006b) Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 355(12):1210–1221. doi:10.1056/NEJMoa060186

Sharma A, Li G, Rajarajan K, Hamaguchi R, Burridge PW, Wu SM (2015) Derivation of highly purified cardiomyocytes from human induced pluripotent stem cells using small molecule-modulated differentiation and subsequent glucose starvation. J Vis Exp 97: e52628. doi:10.3791/52628

Shiba Y, Hauch KD, Laflamme MA (2009) Cardiac applications for human pluripotent stem cells. Curr Pharm Des 15(24):2791–2806

Singh AM, Li FQ, Hamazaki T, Kasahara H, Takemaru KI, Terada N (2007) Chibby, an antagonist of the Wnt/{beta}-catenin pathway, facilitates cardiomyocyte differentiation of murine embryonic stem cells. Circulation 115(5):617–626

Singla DK, Long X, Glass C, Singla RD, Yan B (2011) iPS cells repair and regenerate infarcted myocardium. Mol Pharm. doi:10.1021/mp2001704

Soonpaa MH, Koh GY, Klug MG, Field LJ (1994) Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 264(5155):98–101

Srivastava D, Ivey KN (2006) Potential of stem-cell-based therapies for heart disease. Nature 441(7097):1097–1099

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

Takahashi T, Lord B, Schulze PC, Fryer RM, Sarang SS, Gullans SR, Lee RT (2003) Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 107(14):1912–1916

Theiss HD, Brenner C, Engelmann MG, Zaruba MM, Huber B, Henschel V, Mansmann U, Wintersperger B, Reiser M, Steinbeck G, Franz WM (2010) Safety and efficacy of SITAgliptin plus GRanulocyte-colony-stimulating factor in patients suffering from Acute Myocardial Infarction (SITAGRAMI-Trial)-rationale, design and first interim analysis. Int J Cardiol 145(2):282–284. doi:S0167-5273(09)01501-0 [pii], 10.1016/j.ijcard.2009.09.555

Theiss HD, Vallaster M, Rischpler C, Krieg L, Zaruba MM, Brunner S, Vanchev Y, Fischer R, Grobner M, Huber B, Wollenweber T, Assmann G, Mueller-Hoecker J, Hacker M, Franz WM (2011) Dual stem cell therapy after myocardial infarction acts specifically by enhanced homing via the SDF-1/CXCR4 axis. Stem Cell Res 7(3):244–255. doi:10.1016/j.scr.2011.05.003

Theiss HD, Gross L, Vallaster M, David R, Brunner S, Brenner C, Nathan P, Assmann G, Mueller-Hoecker J, Vogeser M, Steinbeck G, Franz WM (2013) Antidiabetic gliptins in combination with G-CSF enhances myocardial function and survival after acute myocardial infarction. Int J Cardiol 168(4):3359–3369. doi:10.1016/j.ijcard.2013.04.121

Tulloch NL, Muskheli V, Razumova MV, Korte FS, Regnier M, Hauch KD, Pabon L, Reinecke H, Murry CE (2011) Growth of engineered human myocardium with mechanical loading and vascular coculture. Circ Res 109(1):47–59. doi:CIRCRESAHA.110.237206 [pii], 10.1161/CIRCRESAHA.110.237206

Ventura C, Maioli M, Asara Y, Santoni D, Scarlata I, Cantoni S, Perbellini A (2004) Butyric and retinoic mixed ester of hyaluronan. A novel differentiating glycoconjugate affording a high throughput of cardiogenesis in embryonic stem cells. J Biol Chem 279(22):23574–23579

Ventura C, Maioli M, Asara Y, Santoni D, Mesirca P, Remondini D, Bersani F (2005) Turning on stem cell cardiogenesis with extremely low frequency magnetic fields. FASEB J 19(1):155–157

Wiese C, Nikolova T, Zahanich I, Sulzbacher S, Fuchs J, Yamanaka S, Graf E, Ravens U, Boheler KR, Wobus AM (2011) Differentiation induction of mouse embryonic stem cells into sinus node-like cells by suramin. Int J Cardiol 147(1):95–111. doi:S0167-5273(09)00871-7 [pii], 10.1016/j.ijcard.2009.08.021

Witty AD, Mihic A, Tam RY, Fisher SA, Mikryukov A, Shoichet MS, Li RK, Kattman SJ, Keller G (2014) Generation of the epicardial lineage from human pluripotent stem cells. Nat Biotechnol 32(10):1026–1035. doi:10.1038/nbt.3002

Wobus AM, Kaomei G, Shan J, Wellner MC, Rohwedel J, Ji G, Fleischmann B, Katus HA, Hescheler J, Franz WM (1997) Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. J Mol Cell Cardiol 29(6):1525–1539

Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H (2004) Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 364(9429):141–148

Wu SM (2008) Mesp1 at the heart of mesoderm lineage specification. Cell Stem Cell 3(1):1–2

Xu XQ, Graichen R, Soo SY, Balakrishnan T, Rahmat SN, Sieh S, Tham SC, Freund C, Moore J, Mummery C, Colman A, Zweigerdt R, Davidson BP (2008) Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. Differentiation 76(9):958–970

Yamanaka S (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1(1):39–49

Yuasa S, Itabashi Y, Koshimizu U, Tanaka T, Sugimura K, Kinoshita M, Hattori F, Fukami SI, Shimazaki T, Okano H, Ogawa S, Fukuda K (2005) Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. Nat Biotechnol 23(5):607–611

Zaffran S, Kelly RG, Meilhac SM, Buckingham ME, Brown NA (2004) Right ventricular myocardium derives from the anterior heart field. Circ Res 95(3):261–268

Zandstra PW, Bauwens C, Yin T, Liu Q, Schiller H, Zweigerdt R, Pasumarthi KB, Field LJ (2003) Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng 9(4):767–778

Zaruba MM, Theiss HD, Vallaster M, Mehl U, Brunner S, David R, Fischer R, Krieg L, Hirsch E, Huber B, Nathan P, Israel L, Imhof A, Herbach N, Assmann G, Wanke R, Mueller-Hoecker J, Steinbeck G, Franz WM (2009) Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction. Cell Stem Cell 4(4):313–323

Zimmermann WH, Cesnjevar R (2009) Cardiac tissue engineering: implications for pediatric heart surgery. Pediatr Cardiol 30(5):716–723

Zweigerdt R, Burg M, Willbold E, Abts H, Ruediger M (2003) Generation of confluent cardiomyocyte monolayers derived from embryonic stem cells in suspension: a cell source for new therapies and screening strategies. Cytotherapy 5(5):399–413

Chapter 7
Neural Stem Cells

Yoko Arai, Wieland B. Huttner, and Federico Calegari

Abstract Neural stem cells are the source of all neurons, astrocytes and oligodendrocytes of the central nervous system. While the vast majority of neural stem cells are consumed during embryonic development, a subpopulation persists in specialized regions of the adult mammalian brain where addition of cells, notably neurons, continues throughout life. The significance and physiological role of adult neurogenesis are still debated but it is generally believed that neural stem cells may be used to establish novel therapies for certain neural pathologies. In this chapter we describe the main features of neural stem cells during embryonic development and adulthood as well as the key mechanisms known to influence their proliferation versus differentiation. We then discuss the current views on the function of adult neurogenesis and the first attempts to use neural stem cells in therapy. Since the focus of this book is on regenerative medicine, we will mainly describe neural stem cells of mammalian organisms and briefly mention studies on other phyla only if particularly relevant.

Keywords Neural stem cells • Nervous system • Basal lamina • Neurogenesis miRNA • DNA methylation • Chromatin remodeling • Subgranular zone • Subependymal zone • Neurodegenerative diseases • Stroke • Alzheimer • Parkinson • Spinal cord lesions

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_7

Abbreviations

AP	anterior-posterior
AraC	arabinosyde-C
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BrdU	bromodeoxyuridine
CSL	CBF1/RBPJk/*Supplessor of hairless*/Lag1
CNS	central nervous system
CDK	cyclin-dependent kinase
Dnmts	DNA methyltransferases
DV	dorso-ventral
EGF	epidermal growth factor
FGF2	fibroblast growth factor 2
GABA	γ-aminobutyric acid
HATs	histone acetylases
HDACs	histone deacetylases
HIF-Ia	hypoxia-inducible factor Iα
INM	interkinetic nuclear migration
miRNAs	microRNAs
NSC	Neural stem cells
Ngn	neurogenin
NICD	notch receptor
RA	retinoic acid
Shh	sonic hedgehog
SGZ	subgranular zone
SVZ	sub-ventricular zone
VZ	ventricular zone

7.1 Embryonic Neural Stem Cells, Their Lineage and Characteristics

Regenerative medicine for intractable brain disease benefits from the remarkable progress of neuroscience research. The aim and hope of regenerative medicine is the recovery or replacement of diseased cells and tissue in patients by application of *in vitro* developed cells or tissue recapitulating *in vivo* brain development (Ringe et al. 2002; Shastri 2006). Advantages of therapies using neural stem cells (NSC) are to regenerate organs without rejection by implanting regenerated cells into damaged organs and to supply nerve nutrition factors to support unhealthy brain cells (Okano 2002). These therapies are actively progressing together with the accumulation of results on the properties of NSC obtained by more fundamental neuroscience research (Okano and Sawamoto 2008). Therefore, it is important to understand the basic process of brain development. In this chapter, as a foundation for regenerative

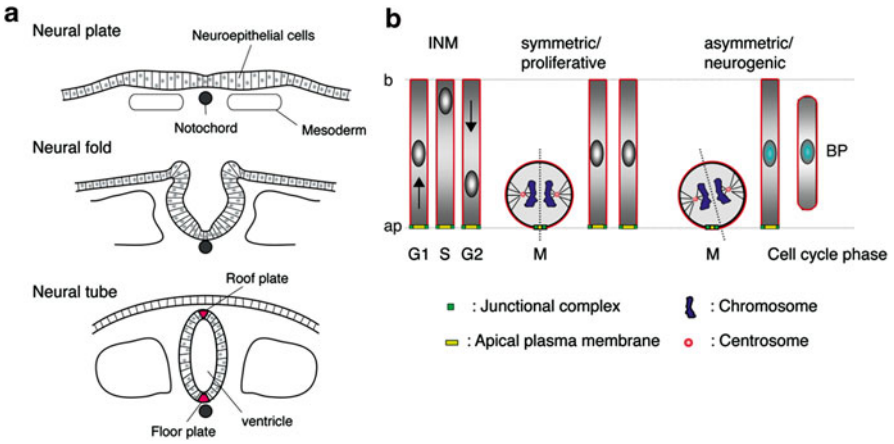

Fig. 7.1 Neural tube formation (a) and cell division of neuroepithelial progenitors (radial glial cells) (b). (**a**) At the neural plate stage, ectoderm overlying the notochord at the midline thickens to form the neural plate. At the neural fold stage, the neural plate invaginates to form the neural tube. At the neural tube stage, floor plate and roof plate cells become evident (*red*). (**b**) Interkinetic nuclear migration (INM) during the different phases of the cell cycle and symmetric *versus* asymmetric division of neuroepithelial cells. During M phase, the cleavage plane (*dotted line*) of apical progenitors bisects the apical domain for symmetric/proliferative division, whereas it bypasses this domain for asymmetric/neurogenic division, which generates a neuron (not illustrated) or a neuronally committed basal progenitor (BP). *ap* apical surface, *b* basal lamina

therapy, the development of the mammalian central nervous system (CNS) is described, particularly how multipotency of NSC is maintained and specification of differentiated cells is acquired. From a developmental point of view, embryonic NSC will form all neural cell types of the adult brain including the adult NSC.

7.1.1 Stem Cell Niche

NSC, collectively, can be regarded as multipotent progenitor cells and have (i) self-renewal capacity and (ii) the potential to give rise to all neural cell types: neurons, astrocytes and oligodendrocytes. NSC are highly polarized epithelial cells, that is, the neuroepithelial cells that form the neural tube upon invagination of the neural plate during the process of neurulation (Fig. 7.1a). Neuroepithelial cells are arranged in a single layer of cells that forms the ventricular zone (VZ) (BoulderComm 1970). The VZ, whose apical side faces the ventricles and whose basal side faces the basal lamina, is colonized by blood vessels (Bautch and James 2009; Götz and Huttner 2005). This environment provides "stem cell niche"–like features to the neuroepithelial cells during development. Specifically, the ventricles are filled with lipoprotein- and membrane particle-rich cerebrospinal fluid, and the basal lamina is a rich source of extracellular molecules including morphogens and growth factors (Raballo et al. 2000; Vaccarino et al. 1999a) (for more details, see Sect. 7.3). NSC are exposed to this environment through receptor interactions and endocytosis (Logan and Nusse

2004). In addition, the neurovascular communication is an important factor for the stem cell niche (Bautch and James 2009). Many molecules are supplied to NSC *via* blood vessels. At early stages of embryogenesis, endothelial cells, surrounding perivascular cells and neural cells interact and form "neurovascular units", which will be a basic unit of the future blood-brain barrier (Lok et al. 2007). Oxygen (O_2) is one of the important chemicals in the regulation of the NSC fate (Panchision 2009). In the mammalian CNS, the O_2 partial pressure (pO_2) is much lower than in air (20.8 %) (Erecińska and Silver 2001; Panchision 2009). At such low pO_2, hypoxia-inducible factor Iα (HIF-Ia) facilitates signal transduction pathways stimulating self-renewal of NSC, whereas high pO_2 degrades HIF-Ia to promote neurogenesis (Gustafsson et al. 2005) and gliogenesis (Pistollato et al. 2007). Taken together, the ventricular fluid–, basal lamina– and blood vessel–based microenvironment of NSC provides important signals for the proliferation *versus* differentiation of these cells, and thus in the development of the CNS.

7.1.2 Characteristics of NSC In Vivo and In Vitro

In vivo, hallmarks of neuroepithelial cells in the CNS are (i) interkinetic nuclear migration (INM), the movement of cell nuclei from the apical luminal side (apical surface) to the basal side of the VZ (basal lamina) in concert with the progression of the cell cycle, and (ii) cell polarity, with the plasma membrane of neuroepithelial cells being divided into two principal domains, an apical domain (apical plasma membrane) facing the ventricles and a basolateral domain which are separated from each other by junctional complexes (Fig. 7.1b). These characteristics have been well studied in the developing vertebrate CNS (Farkas and Huttner 2008; Götz and Huttner 2005; Kriegstein and Alvarez-Buylla 2009). At the beginning of brain development, neuroepithelial cells proliferate, generating two equivalent daughter cells in the VZ and increasing exponentially in number (Kageyama et al. 2008). At the onset of neurogenesis, intrinsic and extrinsic factors acting in concert control the production of neurons from neuroepithelial cells in a stepwise manner (Kriegstein and Götz 2003). Neuroepithelial cells transform into radial glial cells, collectively referred to as apical progenitors, that will progressively engage in differentiation. Apical progenitors change their division mode from a proliferative to a differentiative mode that leads to the production of neurons through the formation of so-called basal (or intermediate) neural progenitors (Götz and Huttner 2005; Haubensak et al. 2004; Hevner 2006; Noctor et al. 2004). In the developing cerebral cortex, basal progenitors form a second progenitor layer located basal to the VZ, the subventricular zone (SVZ). In rodents, basal progenitors typically undergo one cell cycle and then generate two post-mitotic neurons which migrate out from the SVZ to appropriate neuronal layers (Hevner 2006). The sequential production of various types of neurons (neurogenesis) is then followed by gliogenesis (the birth of astrocytes and oligodendrocytes).

Region–specific *in vitro* NSC lines have been derived from fetal and adult CNS (Reynolds et al. 1992; Reynolds and Weiss 1992). Cells isolated from adult germinal regions are cultured in the presence of two growth factors, FGF2 (Fibroblast growth factor 2 or basic FGF) and EGF (epidermal growth factor) (see more detail in Sect. 7.3), and propagated in suspension culture as multicellular spherical aggregates termed "neurospheres" (Pollard et al. 2008). EGF, a known mitogen, and its receptors (EGFRs) are expressed in the CNS (Seroogy et al. 1995). EGF induces the proliferation of stem cells, which gives rise to neurospheres of undifferentiated cells that can differentiate into neurons, astrocytes and oligodendrocytes (Reynolds and Weiss 1996). Indeed, one NSC can expand more than 10^7 fold in the presence of EGF (Reynolds and Weiss 1996), as determined from clonal population analyses of embryonic and adult NSC *in vitro*. The simplified environment used in *in vitro* systems, though distinct from the physiological stimulation that NSC face in the niche, nonetheless provides an excellent tool to manipulate and dissect how cells undergo self-renewal and differentiation. In the following sections, mainly *in vivo* mechanisms will be reviewed, as the insight gained here provides an important basis to the development of regenerative applications.

7.1.3 Cell Cycle and Division

Fundamental biological mechanisms such as cell cycle and division are tightly linked to cell fate changes of NSC. Cell cycle kinetics of NSC are controlled by signaling pathways and cell-intrinsic determinants in order to obtain the appropriate balance between the growth of progenitors and their differentiation into neurons (Dehay and Kennedy 2007; Lukaszewicz et al. 2002; Ochiai et al. 2009; Ohnuma and Harris 2003; Ohnuma et al. 2001; Shimogori et al. 2004). The transition from one cell cycle phase to the next is controlled by the activation, via phosphorylation, of CDKs (cyclin-dependent kinases) that ensure that all cell cycle phases are executed in the correct order. Each CDK is dependent on a partner cyclin, which oscillates during the cell cycle to control its progression. Cyclin-CDK complexes form a driving force for the cell cycle (Ekholm and Reed 2000), which is inhibited by CKI (CDK inhibitor), a braking system in the cycle (Ohnuma et al. 2001) regulated by protein degradation (Nakayama and Nakayama 2006).

During neurogenesis, cell cycle progression of NSC is linked to INM (Fig. 7.1b) (Hayes and Nowakowski 2000). For M phase, the nucleus migrates to the apical side of the VZ where mitosis occurs, then the nucleus is translocated to the basal side during the G1 phase. DNA synthesis (S phase) occurs in the basal region of the VZ and is followed by the return of the nucleus to the apical side during the G2 phase (Kriegstein and Alvarez-Buylla 2009). Before overt neurogenesis in the cerebral cortex, NSC progression through the cell cycle is relatively fast, allowing self-amplification of NSC (Takahashi et al. 1995). During the progression of neurogenesis, the average cell cycle length increases, with lengthening notably in the G1 phase, as determined by cumulative BrdU labeling (Caviness et al. 1995; Takahashi et al.

1995), suggesting that the control of the G1 phase is a key step for the neurogenesis. Activation of cyclin D/cdk4 and cyclin E/cdk2 complexes is an important step for the transition from G1 to S phase (Ekholm and Reed 2000), and accordingly the manipulation of G1/S transition by overexpression of cyclin D/cdk4 or cyclin E1 changes the cell fate of neural progenitors by shortening the G1 phase and total cell cycle length (Lange et al. 2009; Pilaz et al. 2009).

Before neurogenesis, NSC amplify their pool exponentially by symmetric proliferative division to generate two equivalent, proliferating daughter cells. At the beginning of neurogenesis, neurons and basal progenitors arise from asymmetric division of apical progenitors (radial glial cells), which allows their self-renewal as the other daughter cell remains an apical progenitor (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004, 2008). Symmetric divisions of basal progenitors generate neurons (Götz and Huttner 2005; Kriegstein and Alvarez-Buylla 2009). In the *Drosophila* CNS, asymmetric division is governed by various determinants, with notably the cleavage plane orientation during division being a critical fate determinant for the daughter cells (Doe and Skeath 1996). Specifically, a cleavage plane parallel to the apico-basal axis gives rise to symmetric division, while a cleavage plane perpendicular to this axis gives rise to asymmetric division (Doe and Skeath 1996). This orientation of cleavage planes results in the equal or unequal distribution of polarized cell fate determinants. In the developing mammalian CNS (Chenn and McConnell 1995), subtle variations in cleavage plane orientation are typically observed and the vast majority of cleavage planes are oriented parallel to the apico-basal axis, with an important feature being whether or not the tiny apical plasma membrane and the apical junctional complexes are bisected (Kosodo et al. 2004). Symmetric proliferative divisions bisect, and asymmetric neurogenic divisions bypass, this apical domain and thus have differential effects on its inheritance (Huttner and Brand 1997; Kosodo et al. 2004) (Fig. 7.1b). Lack of a precise orientation of the mitotic spindle perpendicular to, and of the cleavage plane parallel to, the apico-basal axis upon knock-down of Aspm (abnormal spindle-like microcephaly-associated) causes precocious neurogenesis (Fish et al. 2006, 2008). In contrast, deletion of the spindle regulator LGN results in the delamination and somal translocation of neural progenitors without significant consequences for neurogenesis (Konno et al. 2008; Morin et al. 2007). These findings indicate that precise control of spindle/cleavage orientation appears to be important for cell fate determination in a context-dependent manner.

Neuronal differentiation involves the specification of neural progenitors and neurons, and hence the appropriate alterations in gene expression controlled by cell-intrinsic and -extrinsic cues (Kriegstein and Götz 2003). After production of neurons, radial glial cells finally differentiate into glial cells such as astrocytes and oligodendrocytes. Therefore, in the rodent CNS, the following three steps occur sequentially; (i) expansion and self-renewal: symmetric proliferative and asymmetric divisions, respectively, of apical progenitors, (ii) neurogenesis: differentiative divisions of apical and basal progenitors, (iii) gliogenesis: differentiative divisions of neural progenitors (Qian et al. 2000). These aspects will be discussed in the following sections. First, the major intracellular players, complex intrinsic regulations

involved in the determination of neural progenitors, will be discussed. Then, important extracellular factors that influence neurogenesis and their respective signaling pathways will be reviewed. In the adult neurogenesis section, the generic aspects of gliogenesis will be addressed and regenerative applications will be discussed.

7.2 Cell-Intrinsic Factors in Neural Stem Cell Differentiation

To generate the variety of brain cells, NSC collectively have to be multipotent and provide the proper cell type at the proper time. The processes by which NSC generate a variety of functionally integrated neural progenitors and then neurons during embryonic development have been intensively studied, and the variety of neural progenitors is established by combinations of various cell-intrinsic factors: several types of transcription factors, receptors, ligands, cell cycle modulators and polarity proteins, etc (Kriegstein and Alvarez-Buylla 2009). A platform for neurogenesis is established by the coordinated functions of these players through signal transduction pathways.

7.2.1 Transcriptional Regulators

The major cell-intrinsic regulators are the basic helix-loop-helix (bHLH) transcription factors, which contribute to changing the characteristics of NSC over time during brain development: the self-renewal capacity, neurogenesis and gliogenesis (Kageyama and Nakanishi 1997). There are two types of bHLH genes: (i) the repressor-type bHLH genes, *Hes* genes, are mammalian homologues of *Drosophila hairy* and *Enhancer of split*, and (ii) the activator-type bHLH genes, *Neurogenin (Ngn)*, *Mash1* and *Math* genes, are mammalian homologues of *Drosophila* proneural genes *achaete–scute* complex and *atonal* (Kageyama et al. 2008). *Hes* genes not only regulate the maintenance of NSC but also promote gliogenesis in cooperation with Notch signaling (Lathia et al. 2008) (discussed below), while proneural genes including *Neurogenin (Ngn)*, *Mash1* and *Math* are responsible for promoting neurogenesis (Ross et al. 2003).

7.2.1.1 Regulators of Maintenance and Proliferation

There are seven members in the *Hes* family (Kageyama et al. 2008). Among them, *Hes1*, *Hes3* and *Hes5* genes are highly expressed in the early stage of embryonic brain development to maintain NSC in an undifferentiated state and to inhibit their differentiation. Functional studies of *Hes* genes have been done using gene knock-out strategies. In *Hes1:Hes3:Hes5* triple knock-out mice, neuroepithelial cells prematurely differentiate into neurons as early as embryonic day (E) 8.5 when neuroepithelial cells in the wild-type are extensively self-renewing (Hatakeyama

et al. 2004). Importantly, in the triple knock-out mice, premature neurogenesis is already completed by E10 without generation of later-born cells, glia and ependymal cells, demonstrating that *Hes1*, *Hes3* and *Hes5* are also essential for fate determination of neural progenitors (Hatakeyama et al. 2004). Furthermore, *Hes1:Hes5* double knock-out neurospheres do not expand properly in contrast to wild-type (Ohtsuka et al. 1999). Hes genes are therefore important to regulate not only self-renewal and differentiation but also the multipotency of NSC for formation of neural tissue.

Interestingly, *Hes* genes also seem to have a role in tissue architecture, an important aspect with regard to tissue engineering. In *Hes1:Hes3:Hes5* triple knock-out embryos at E8.5, intracellular apical junctional complexes (adherens and tight junctions) and the basal lamina are disrupted and premature neurons scattered into the lumen and surrounding tissues (Hatakeyama et al. 2004). Therefore, *Hes* genes are essential for the structural integrity of the CNS.

7.2.1.2 Regulators of Differentiation

The activator-type bHLH genes, *Neurogenin 2 (Ngn2)*, *Mash1* and *Math* are expressed in neural progenitors that have a limited potential for proliferation (Fode et al. 2000). Upon expression of these bHLH genes, differentiation into neurons after repeated asymmetric cell division occurs as represented in Fig. 7.1b. Overexpression of the activator-type bHLH genes in NSC induces neuron-specific genes, and therefore these genes are known as proneural genes (Parras et al. 2002). Proneural genes also specify the neuronal subtypes. For example, in the dorsal part of cerebral cortex, *Ngn2* promotes generation of neurons and specifies glutamatergic pyramidal identity. In the ventral telencephalon, *Mash1* specifies γ-aminobutyric acid (GABA) – ergic inhibitory interneurons. Upon a change in progenitor identity as observed in *Ngn2* knock-out embryos, dorsal progenitors generate GABAergic instead of glutamatergic neurons, demonstrating the role of *Ngn2* in neuronal subtype specification (Parras et al. 2002; Perez et al. 1999). Thus, proneural genes are not only crucial for the generation of neurons but also for the acquisition of their proper identity.

7.2.1.3 Notch Signaling: Control Between Proliferation and Differentiation

Notch is a transmembrane receptor expressed by neural progenitors. Notch binds to its ligands Delta and Jagged, which are also transmembrane proteins and are also expressed by neural progenitors (Lathia et al. 2008). Once the Notch signaling pathway is activated, NSC maintain the proliferative and undifferentiated properties by induction of *Hes1* and *Hes5* genes which inhibit proneural gene expression. After receptor-ligand binding, the intracellular domain of the Notch receptor (NICD) is enzymatically cleaved by presenilin-1/γ-secretase and translocates to the nucleus

where the transcriptional repressor complex CSL (CBF1/RBPJk/*Suppressor of hairless*/Lag1) downregulates the transcription of *Hes* genes (Lathia et al. 2008). Nuclear NICD binds to CSL, which turns it into a transcription activator complex acting on chromatin remodeling factors to transcribe *Hes1* and *Hes5* genes (Artavanis-Tsakonas et al. 1999; Honjo 1996). Hes proteins in turn repress the transcription of proneural genes (*Ngn2, Mash1* and *Math3*), and their targets Delta and neuron-specific genes are not transcribed (Artavanis-Tsakonas et al. 1999). Conversely, in neuronally committed progenitors, expression of bHLH proneural genes promotes the transcription of neuron-specific genes and of *Delta* (Castro et al. 2006). Delta expressed from the committed progenitor activates Notch in the neighbouring cells to keep them in the undifferentiated state (Castro et al. 2006). This process is called "lateral inhibition" (Artavanis-Tsakonas et al. 1999). Therefore, Notch-Delta binding is the initial event for neuronal differentiation and a paradigmatic example of the crosstalk between neighboring cells.

7.2.1.4 Regulators of Regional Specificity

Higher brain functions rely on complex neuronal circuit formation between functionally specified regions. The fundamental regional organization of the brain is specified during early embryonic development by region-specific expression of transcription factors (Kriegstein and Götz 2003; Osumi et al. 2008). The transcription factor Pax6 is a key molecule to define the regional specificity of the CNS (Götz et al. 1998). Pax6 is specifically expressed in the dorsal telencephalon that gives rise to the neocortex (glutamatergic pyramidal neurons), the dorsal diencephalon that gives rise to the thalamus (thalamic neurons), the hindbrain which gives rise to the cerebellum (Purkinje neurons), and the dorsal brainstem (motor and sensory neurons) and spinal cord (motor and somatic neurons) (Simpson and Price 2002). In *Pax6* mutant animals, *Dlx1* and *Gsh2*, two genes identifying the ventral part of the telencephalon giving rise to basal ganglia (GABAergic neurons), are misexpressed in the dorsal cortex leading to a ventralization of the dorsal cortex (Stoykova et al. 1996; Toresson et al. 2000).

Pax6 is also important to control self-renewal (Arai et al. 2005) and differentiation (Götz et al. 1998) to generate neural progenitors (Estivill-Torrus et al. 2002; Warren et al. 1999). In the dorsal cortex, gain- and loss-of-function studies of Pax6 identified regulatory networks that control these processes. Pax6 acts by changing the combination of co-binding transcription factors in a dose-dependent manner (Sansom et al. 2009), which allows the regulation of expression of various downstream genes. Examples include (Sansom et al. 2009) (1) neural stem and progenitor maintainance of self-renewal capacity – Emx2, Sox9 and Hmga2, (2) cell cycle progression – G1 cyclin-dependent kinase (Cdk4) and Pten1, (3) neurogenesis – Ngn2 (bHLH) and Eomes/Tbr2, (4) chromatin binding – Cbx1 and Rnf2. Thus, Pax6 allows the integration of many biological pathways together with regional specificity cues.

7.2.2 *Epigenetic Control in the Course of Differentiation*

Other mechanisms that also contribute to cellular differentiation are epigenetic modifications. While the DNA sequence itself is generally conserved in somatic cells throughout the life of an organism, specific transcriptional regulation can be maintained by epigenetic mechanisms in individual progenitor cells and inherited by their differentiating progeny. Epigenetic modifications of cell type-specific genes contribute to cell-autonomous changes in NSC that regulate both neurogenic and gliogenic differentiation processes. Neurons, astrocytes and oligodendrocytes differentiate sequentially from NSC, and these programs can be recapitulated *in vitro*. NSC isolated form early embryonic stages generate, in terms of differentiated cells, predominantly neurons, while seemingly identical NSC isolated form later developmental stages generate predominantly astrocytes under the same culture conditions (Qian et al. 2000), suggesting that epigenetic mechanisms contribute to this change in the potential of NSC.

7.2.2.1 Histone Modifications and DNA Methylation

NSC fate is profoundly controlled by the spatiotemporal pattern of expression of transcription factors in concert with epigenetic modifications of their genome, including (1) histone modifications (acetylation, methylation, phosphorylation, ubiquitination and sumoylation) and (2) DNA methylation (Vincent and Van Seuningen 2009). When NSC self-renew, expression of lineage-specific genes is turned off and their chromatin is found in a "repressed" status as indicated by chemical modifications (deacetylation and methylation) of histones (Shi et al. 2008). These histone modifications occur most commonly on amino-terminal histone tails and provide a "histone code" that can be read by nuclear proteins to influence a multitude of cellular activities (Turner 2002).

The level of histone acetylation is regulated by the activity of histone acetylases (HATs) and histone deacetylases (HDACs) (Shi et al. 2008). It has been reported that HDAC-mediated transcriptional repression is essential for the maintenance and self-renewal of NSC (Sun et al. 2007). HDACs deacetylate lysine residues of histones resulting in chromatin condensation, which was shown to block access of transcription factors involved in neuronal differentiation (Shi et al. 2008). Tlx, a transcription factor essential for NSC proliferation, recruits HDACs onto *p21* (a CDK inhibitor) and *Pten* (a phosphatase and tumor suppressor gene) promoter regions to repress their expression, resulting in inhibition of neuronal differentiation (Sun et al. 2007), indicating that histone deacetylation by HDACs is a key step for gene silencing in NSC.

More recently, the potential role of histone methylation in CNS development has gained attention. Methylation of lysine residues of histone H3 and histone H4 has been observed in neuroepithelial cells, and variations in the degree of histone methylation (mono-, di- or trimethyl histones) has been implicated in neuronal differentiation (Biron et al. 2004). Trimethyl histone H3 and monomethyl histone H4 have been found to be elevated in proliferating neural progenitors, while

trimethyl histone H4 is enriched in differentiating neurons (Biron et al. 2004). Thus, an epigenetic program based on a highly dynamic regulation of histone lysine methylation seems to participate in the neural differentiation process.

The epigenetic status is also regulated by DNA methylation. The level of DNA methylation of the promoter region of a gene often reflects its state of repression. DNA methylation is a post-replicative modification of cytosine (C) that occurs predominantly within CpG dinucleotides (Rottach et al. 2009) and is catalyzed by DNA methyltransferases (Dnmts) (Robertson and Wolffe 2000). Dnmt1 null embryos show embryonic lethality (Li et al. 1992), and conditional Dnmt1 depletion in neural progenitors results in DNA hypomethylation and precocious astroglial differentiation (Fan et al. 2005), suggesting that the maintenance of DNA methylation is important for normal development and controls the timing of gliogenesis. Another two independently encoded DNA methyltransferase genes, *Dnmt3a* and *Dnmt3b*, are expressed in the CNS (Okano et al. 1999; Watanabe et al. 2002, 2006). While *Dnmt3b* is specifically expressed in neural progenitors, *Dnmt3a* is expressed in postmitotic neurons (Watanabe et al. 2006). *Dnmt3b* null embryos have multiple developmental defects, indicating an important role of DNA methylation in the initial steps of differentiation. *Dnmt3a* null embryos develop until 4 weeks after birth (Okano et al. 1999), and it has been suggested that Dnmt3a is required for the establishment of proper tissue-specific DNA methylation patterns.

Recently, a link between DNA methylation and cell type-specific gene expression was reported. DNA methylation itself is involved in the repression of *GFAP*, which is expressed in astrocytes (Takizawa et al. 2001). Interestingly, DNA methylation coupled to chromatin remodeling also plays a crucial role in regulating neuronal activity-dependent genes like *BDNF* (brain derived neurotrophic factor) (Martinowich et al. 2003). Demethylation of the *BDNF* promoter region was observed upon depolarization, releasing its repression and therefore allowing its expression in active neuronal networks. This again puts emphasis on the crucial role of epigenetic mechanisms for the regulation of factors involved in neuronal function, in this case plasticity (Martinowich et al. 2003).

7.2.2.2 Chromatin Remodeling

A role of chromatin-based epigenetic mechanisms in early neural development has been reported (Aigner et al. 2007; Lessard et al. 2007). Chromatin remodeling involves the effective shifting of nucleosome cores along the length of the DNA molecule, a process known as “nucleosome sliding”. Chromatin remodeling is accomplished, at least in part, by ATPase-containing complexes, referred to as ATP-dependent SWI/SNF-like chromatin remodeling complexes (Cheng et al. 2005; Strahl and Allis 2000). Mammalian NSC and proliferating progenitor cells express the complexes SWI2/SNF2-like ATPases together with BAF45a, a Krüppel/PHD domain protein, and BAF53a (Lessard et al. 2007). Conversely, when NSC exit the cell cycle, the homologues BAF45b, BAF45c and BAF53b replace the respective subunits specific of proliferating NSC (Lessard et al. 2007). The combination of chromatin remodeling

factors therefore seems to add another degree of complexity to the regulation of factors involved in the proliferation/differentiation fate choice of NSC.

7.2.3 MicroRNAs

MicroRNAs (miRNAs) are 20–25 nucleotide-long non-coding RNAs that negatively regulate the stability and translation of target mRNA (Ambros 2004; Bartel 2009). Approximately 70 % of the known miRNAs are found in the brain (Du and Zamore 2005). miRNAs are expressed in a tissue-specific and developmentally regulated manner (Ambros 2004; Bartel 2009). A large fraction of miRNA genes are found within introns of transcripts generated by RNA polymerase II (Kim 2005). These primary transcripts of miRNAs (pri-miRNA) are first processed into 60–75 nucleotide-long hairpin-like precursors (pre-miRNAs) by the RNase III endonuclease. They are then exported to the cytoplasm where they are cleaved into mature miRNAs by Dicer, a cytoplasmic RNase III-type endonuclease. miRNA recognition of a target mRNA results in its decreased stability and translation and hence in reduced expression of the respective gene (Klein et al. 2005).

The deletion of Dicer1 causes embryonic lethality and loss of stem cell pools (Bernstein et al. 2003). However, conditional Dicer knock-out in the developing cerebral cortex does not impair the early expansion of NSC and the generation of basal progenitors but does result in a dramatic size reduction of the cerebral cortex and in a disruption of its neuronal layering (De Pietri Tonelli et al. 2008), indicating that miRNAs control, in particular, neuronal differentiation.

miRNAs are key regulators of stem cell biology in general, and of neural development in particular, and have been implicated in cell fate decisions based on their expression patterns, computationally predicted targets and overexpression analyses (Cao et al. 2006; Houbaviy et al. 2003; Smirnova et al. 2005; Suh et al. 2004). During neurogenesis, several neuronal miRNAs show linage-specific expression (Smirnova et al. 2005). miR9 and miR125 are expressed in the neural tube and found in both the germinal and the neuronal layers, whereas miR124 expression is predominantly observed in neurons, miR23 in astrocytes and miR26 and miR29 in both neurons and astrocytes (Cao et al. 2006; Smirnova et al. 2005). Overexpression of miR9 and miR124 in neuronal progenitors decreases astrocyte differentiation, while inhibition of miR9 alone or together with miR124 reduces the number of neurons (Smirnova et al. 2005), consistent with a role of neural miRNAs (miR9 and miR124) in neurogenesis (Cheng et al. 2009). Recently, *laminin γ1* and *integrin β1*, which are highly expressed in neural progenitors and repressed during neuronal differentiation, have been reported to be target genes of miR124 (Cao et al. 2007), thereby providing a possible mechanism for the miR124-induced alteration in neural progenitor proliferation. In addition, miR124 binds to the 3′UTR of SCP1 to antagonize its anti-neural function (Visvanathan et al. 2007), thus acting on broader signal transduction pathways which may affect various cellular functions. Clearly, a comprehensive identification of miRNAs and their targets genes will provide important insight into the regulation of NSC proliferation *versus* differentiation.

7.3 Cell-Extrinsic Factors in Neural Stem Cell Differentiation

Neurogenesis is highly dependent on the proper environment, which affects cell behavior and identity. In other words, NSC proliferation and differentiation will be influenced by extracellular signals that convey information about growth conditions as well as positional information (Cayuso and Martí 2005). Extracellular signaling molecules, notably growth factors and morphogens, are key factors of the microenvironment in which NSC reside, the stem cell niche, and are crucial for coordinating CNS development. "Pattern formation is the mechanism by which initially equipotent embryonic cells proliferate and organize into an intricate spatial arrangement of diverse cell types" (Wolpert 1969).

7.3.1 Morphogens and Identity

In the developing brain, positional information along the anterior-posterior (AP) and dorso-ventral (DV) body axes is encoded by morphogens. Morphogens are secreted molecules that influence gene expression in a concentration-dependent manner. Morphogens are produced from sources called signal-organizing centers and diffuse to form a concentration gradient, which is then integrated by the receiving cells and will affect various cellular aspects including cell migration, organization and identity.

Shh (sonic hedgehog), a major morphogen, is a member of the Hh (hedgehog) family and the best studied ligand of this signaling pathway. Shh is produced in two ventral midline signaling centers, the floor plate of the neural tube and the underlying notochord, an axial mesodermal structure (Martí et al. 1995). To travel far (long-range activity) from its source along the DV axis, Shh requires an auto-processing event that releases an active, cholesterol-modified, N-terminal fragment (N-Shh) (Ingham and McMahon 2001). Graded Shh concentration along the DV axis in a ventral-high, dorsal-low profile allows the initial patterning of the progenitor domains within the ventral neural tube (Briscoe et al. 2000; Jessell 2000; Pierani et al. 1999). This gradient is converted into the intracellular expression of various homeodomain transcription factors that define progenitor domain identity (Briscoe et al. 2000). In the developing ventral spinal cord, five different types of post-mitotic neurons (four interneurons and one motor neuron) are generated from these progenitor domains (Briscoe et al. 2000; Pierani et al. 1999, 2001). Thus, Shh controls the generation of distinct post-mitotic neurons along the DV axis. With regard to the field of regenerative medicine, for example spinal cord injury which typically is localized to a specific area and hence to neuronal subgroups, these findings provide crucial basic knowledge for designing appropriate therapeutic approaches.

BMPs (bone morphogenetic proteins), belong to the transforming growth factor β (TGF-β) family, and multiple BMPs are secreted from the roof plate and the dorsal

neural tube (Liem et al. 1997). Several studies indicate that BMPs function in dorsal patterning of the spinal cord to antagonize the ventral patterning effect by Shh (Chesnutt et al. 2004; Liem et al. 1997; McMahon et al. 1998; Wine-Lee et al. 2004). BMPs bind to two families of receptor serine/threonine kinases, type I and II, and propagate the signal by phosphorylation of Smad proteins (Shi and Massagué 2003). Disrupting BMP signaling with the BMP antagonist Noggin affects particularly the dorsal neuron identity in the spinal cord where BMP acts as morphogen, and can be rescued by BMP4 exposure (Liem et al. 1997). The double knock-out of BMP receptors (BMPRIa and Ib) and knock-down of Smad4 by RNA interference result in loss of the dorsal-most phenotype (Chesnutt et al. 2004; Wine-Lee et al. 2004), thus adding further evidence for the role of BMPs in the establishment of the dorsal progenitor domain identity in the spinal cord.

The proper development of the CNS requires differentiation to proceed not only along the DV, but also the AP, body axis. FGFs (fibroblast growth factors), which have various functions in the cell biology of the NSCs (Mason 2007), are also involved in the AP "body plan" formation process. FGFs are monomeric ligands and activate FGF receptor (FGFR) tyrosine kinase (Mason 2007). FGF8 from the presomitic mesoderm is known to be important for the caudal body axis extension by controlling the proliferation in a "stem cell zone", composed of self-renewing progenitors, in the ridge of the caudal neural tube (Diez del Corral et al. 2002). FGF8-exposed progenitors differentiate into neurons only after neural tube closure and following exposure to retinoic acid (RA), produced from the somitic mesoderm surrounding the neural tube (Diez del Corral et al. 2003). Thus, FGF signaling is involved the maintenance of self-renewal and an undifferentiated state of progenitors, whereas RA promotes neurogenesis, with the interplay between these two factors governing the progression of neurogenesis along the AP axis.

7.3.2 Morphogens and Growth

Morphogens such as Shh and BMPs are not only involved in patterning but also influence the proliferation and survival of progenitors. Shh has a known mitogenic function (Dahmane et al. 2001; Ulloa and Briscoe 2007) as shown by gain- and loss-of-function studies analyzing the proliferation of neural progenitors in the CNS (Cayuso et al. 2006; Chiang et al. 1996; Ishibashi and McMahon 2002) including the cerebral cortex (Komada et al. 2008). In Shh signal-receiving cells, binding to Patched (Ptc) receptors (Ingham and McMahon 2001) releases the inhibition of the receptor Smoothened and activates downstream target genes involved in cell proliferation like Cyclin D and N-Myc through Gli activation (Jacob and Briscoe 2003; Kenney et al. 2003; Kenney and Rowitch 2000; Ulloa and Briscoe 2007). Proliferation and cell cycle kinetics are affected in *Shh* conditional knock-out mice, which show an increase in cell cycle length (Komada et al. 2008). BMPs also control proliferation of NSC and progenitors either through their downstream targets (cyclin D1 and cdk4) (Hu et al. 2001) or Wnt canonical signaling pathways (Nusse

et al. 2008). Transgenic embryos with a constitutively active BMPRIa (caBMPR1a) (see also Sect. 7.2.1) show a robust proliferation of neural progenitors at early stages, and the morphology of the neuroepithelium is severely altered, with the appearance of gyrus-like structures (Panchision et al. 2001), whereas constitutively active BMPRIb (caBMPR1b) promotes neurogenesis (Panchision et al. 2001). Furthermore, caBMPR1a induces expression of *Wnt1* and *Wnt3*, two mitogenic *Wnt* genes, indicating that the mitogenic effects of BMPs may depend on the mitogenic activity of Wnt. Induction by the BMP pathway of *Msx1*, a known inhibitor of proneural genes (see Sect. 7.2.1.2), could also contribute to the mitogenic activity of BMPs (Liu et al. 2004).

Wnt ligands form a family of secreted glycoproteins related to *Drosophila* Wingless and participate in multiple developmental events during embryogenesis (Logan and Nusse 2004). Wnt effects are pleiotropic and include mitogenic stimulation, cell fate specification and differentiation. Wnt signaling through its receptors (Frizzled) leads to the translocation of β-catenin to the nucleus to form a transcriptional complex with TCFs (T-cell factor), a pathway called canonical Wnt pathway (Logan and Nusse 2004). Evidence for the control of proliferation by the canonical Wnt pathway has been obtained by gain- and loss-of-function approaches for Wnts (Lange et al. 2006) and β-catenin (Chenn and Walsh 2003; Machon et al. 2003; Megason and McMahon 2002). In the developing CNS, the most prominent member of the Wnt family, Wnt1, is expressed at the dorsal midline along the entire AP axis (Gavin et al. 1990; Parr et al. 1993), whereas β-catenin is expressed ubiquitously in the VZ, with relatively strong immunoreactivity at the apical, luminal side of the VZ (Chenn and Walsh 2003; Megason and McMahon 2002). Ectopic expression of Wnt1 and Wnt3a in transgenic mice causes overgrowth of the neural tube at the dorsal midline (Dickinson et al. 1994; Megason and McMahon 2002). Wnt7a and 7b are also important for cell proliferation in the VZ and SVZ of the cerebral cortex (Viti et al. 2003). Consistent with the role of Wnts in stimulating proliferation, expression of constitutively active β-catenin increases progenitor proliferation and decreases neurogenesis (Megason and McMahon 2002; Zechner et al. 2003) (Chenn and Walsh 2003). In contrast, ablation of β-catenin causes a reduction of tissue mass (Zechner et al. 2003). These Wnt/β-catenin-mediated mitogenic effects occur through downstream target genes (cyclin D, c-Myc and connexin43) which regulate G1/S transition of the cell cycle (He et al. 1998; Lange et al. 2006; Logan and Nusse 2004; Shtutman et al. 1999; Tetsu and McCormick 1999).

FGF2 (basic FGF) is also known to stimulate the proliferation of progenitors in primary cultures isolated from embryonic cerebral cortex by shortening cell cycle length (specifically G1) (Lukaszewicz et al. 2002). FGF2 is highly expressed in the developing VZ and SVZ, and knock-out mice show a reduction in proliferation, VZ volume and total cell number during development. Furthermore, in adult knock-out mice, the numbers of pyramidal neurons and glial cells are equally reduced (Korada et al. 2002; Raballo et al. 2000; Vaccarino et al. 1999b), consistent with FGF2 being a potent mitogen *in vivo*. Interactions between FGFs and the Notch signaling pathway have been described (Yoon et al. 2004). Progenitors isolated from the VZ in the presence of FGF2 increase *Notch1* and decrease *Delta1* expression (Yoon et al.

2004), suggesting that at least part of the FGF activity is mediated through the Notch pathway.

Some FGFs are produced locally by signal-organizing centers, in particular in the anterior medial part of the cerebral cortex. Conditional inactivation of FGF8 in the cortex results in a significant reduction of cortical size and mitotic index, and in robust apoptosis (Storm et al. 2006). In contrast to FGF2, FGF8 promotes differentiation and cell cycle exit (Borello et al. 2008). The various FGFs and their receptors play pivotal roles in the regulation of proliferation and the genesis of cortical neurons and glia. EGF, often used as a supplement in cell culture to sustain proliferation, behaves like a mitogen for late embryonic and adult NSC (Reynolds et al. 1992; Reynolds and Weiss 1992).

To conclude, many extrinsic factors are required, in appropriate spatio-temporal patterns, for proper CNS development. Most of them act as either mitogens or morphogens involved in proliferation control and providing positional cues to neural progenitors, which will affect the timing of neurogenesis and neuronal identity.

Many factors, some extrinsic other intrinsic, orchestrate the proliferation *versus* differentiation fate choice of NSC (Fig. 7.2a, b). The underlying integration of information is amazingly complex and occurs at various regulatory levels. Understanding normal development and neural stem cell biology will bring us closer to designing the future tools for regenerative medicine.

7.4 Adult Neural Stem Cells

7.4.1 Introduction: A Historical Perspective

It was 1906 when for the first time two scientists shared the Nobel award. These were Bartolomeo Camillo Golgi for developing the silver impregnation reaction (*la reazione nera*) and Santiago Ramon y Cajal for the demonstration, using Golgi's method, that the brain is made of contiguous, individual nerve cells (*the neuron theory*) (Lopez-Munoz et al. 2006).

The works of Golgi and Cajal represented a fundamental leap to start revealing the extraordinary complex cytoarchitecture of the adult brain, which, on the other hand, made it difficult to consider that this organ may undergo remodeling during adult life. The vision of the adult brain as a static and unmodifiable structure was also corroborated by clinical and functional observations, at least those that could be made with the tools available at the time. For example, it was known that patients with injuries to the central nervous system (CNS) or neurological pathologies had very little, if any, possibility of recovery and that even a minor lesion to the CNS may lead to major deficits in its function. Therefore, the scientific community was confident that CNS plasticity must be terminated after development, as summarized by Cajal himself in an often-cited statement: *"Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres, the nerve paths are something fixed and immutable: everything may*

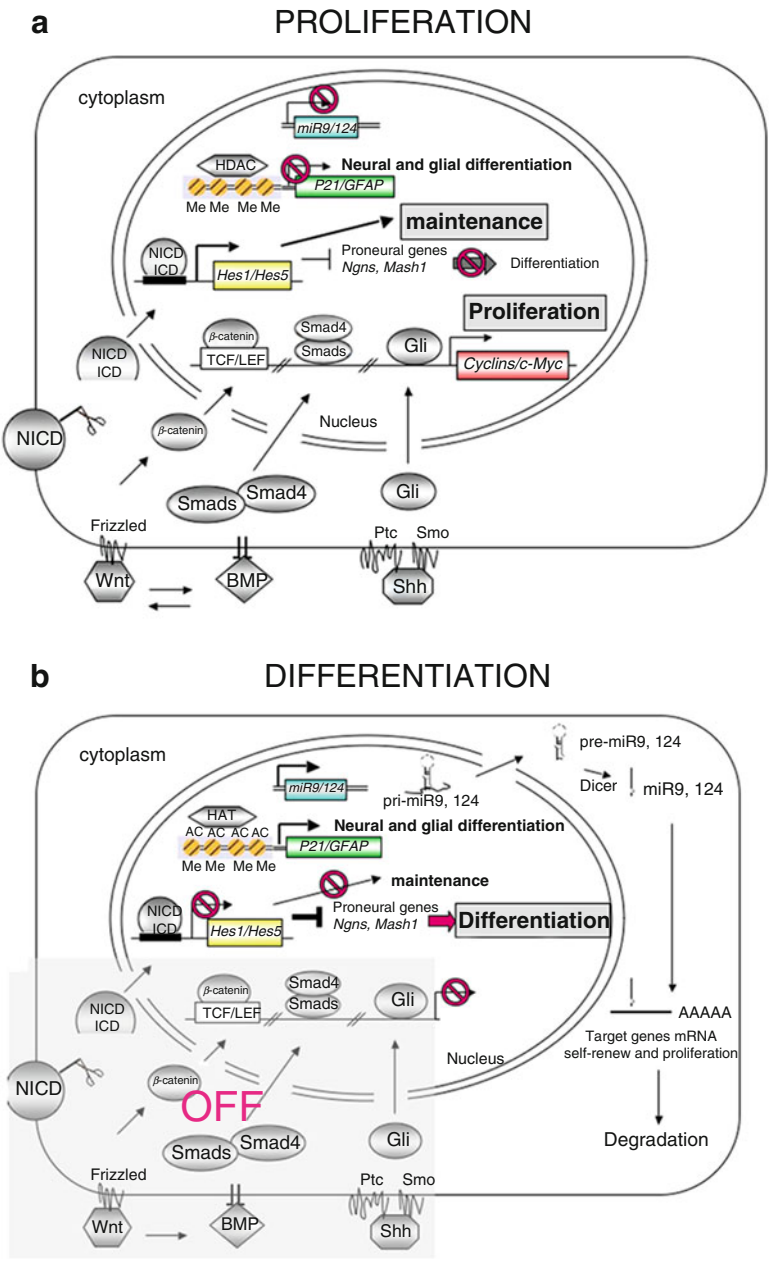

Fig. 7.2 Cross-talk between cell-intrinsic and -extrinsic factors in neural progenitors. Schematic representation of the various signaling pathways that affect the fate of NSC; (**a**) maintenance of NSC proliferation and an undifferentiated state, (**b**) differentiation of progenitors

die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree" (Ramon y Cajal 1913).

And indeed, science has changed this view. The first evidence of adult neurogenesis came nearly 50 years later, after the characterization of tritiated thymidine and autoradiography as a system to identify newly synthesized DNA and, thus, cells undergoing S phase in living tissues (Firket and Verly 1958). To appreciate the impact of this revolutionary approach, it should be considered that this was the first time that scientists had the opportunity to retrospectively analyze the time of origin, lineage and migration of cells in living organisms. This was due to the fact that the radiolabeled compound (i) is rapidly metabolized after administration, which allows pulse-chase labeling of cells, and (ii) is irreversibly incorporated in the DNA allowing the identification of daughter cells long after their mother had concluded S phase.

It was Joseph Altman to first use this approach to investigate the possibility of adult neurogenesis by administering the radiolabeled compound to the injured brain followed, 1 month later, by its detection in cells that were morphologically classified as neurons (Altman 1962). Interestingly for stem cell biologists today, Altman did not interpret this finding as to indicate that neurons may be activated to enter S phase and, eventually, divide. Rather, he correctly concluded that *"…new neurons may arise from non differentiated precursors, such as ependymal cells. After multiplication, such embryonic cells could differentiate and thus add new neurons to the existing population."* (Altman 1962). Notably, the identity of ependymal cells as true neural stem cells is still debated at the time of writing (Chojnacki et al. 2009).

Altman also made similar observations in other brain regions. However, since the neurons observed were, admittedly, very few and no technique was yet available to ascertain their identity, the scientific community tended to consider adult neurogenesis unconvincing or, in the most benevolent cases, negligible and unimportant. This view did not change even after the advent of electron microscopy and a more reliable identification of newborn adult neurons by ultrastructural analysis, as first established by Michael Kaplan (Kaplan and Hinds 1977).

Only the combination of S phase radiolabeling, ultrastructural analyses and electrophysiology undertaken by Fernando Nottebohm in the 1980s could finally provide proof of adult neurogenesis (Nottebohm 1985). This is not to say that adult neurogenesis was accepted as a reality for Nottebohm's studies were limited to canaries, a species of songbirds known to undergo seasonal brain remodeling, which suggested that adult neurogenesis was, if at all, limited to few and rare species. Nevertheless, Nottebohm's work acted as a catalyzer for new investigations in mammals, which were gaining momentum also due to the use of the thymidine-analogue bromodeoxyuridine (BrdU), which, in contrast to radio-labeling, allowed immuno-detection together with established molecular markers of neurons.

This lead in the 1980s and 1990s to a series of studies that (re-) discovered adult neurogenesis in a variety of species including rat, mouse, rabbit, macaque and human and showed that newborn functional neurons can integrate into preexisting neuronal networks (Doetsch et al. 1997, 1999a; Eriksson et al. 1998; Gould et al. 1999a; Gueneau et al. 1982; Kempermann et al. 1997; Kornack and Rakic 1999;

Kuhn et al. 1996; Stanfield and Trice 1988; van Praag et al. 2002). In addition, and equally important, systems started to be established to obtain cells from the adult brain that could generate neurons and glia in vitro (Lois and Alvarez-Buylla 1993; Reynolds and Weiss 1992).

These works, and several others, finally led to the acceptance of adult mammalian neurogenesis and to the following boost in neural stem cell research. It should be said, however, that this period of great discoveries was, and somehow still is, a period of confusion too. For example, while adult neurogenesis in the hippocampus and subventricular zone is firmly established, adult neurogenesis in the cortex has been *proven* (Dayer et al. 2005; Gould et al. 1999b, 2001) and *disproven* (Ackman et al. 2006; Frielingsdorf et al. 2004; Koketsu et al. 2003; Kornack and Rakic 2001) various times and certain observations on adult neurogenesis in humans (Curtis et al. 2007) have been openly challenged (Sanai et al. 2007). These conflicting reports, in part explained by an inappropriate use of recent technologies (Breunig et al. 2007a), reflect the novelty and dynamism of the field and should be kept in mind while studying adult neurogenesis.

Nevertheless, it took science nearly a century to change the *"harsh decree"* and view the adult brain as a dynamic and plastic organ where newborn neurons are integrated into existing circuits each day (Gross 2000). Adapting Cajal's statement one century later, we may now conclude that *it is for the science of the future to manipulate, if possible, brain plasticity for therapeutic intervention.*

7.4.2 Derivation/Classification

7.4.2.1 Origin

At the end of mammalian embryonic development, radial glial cells are believed to undergo a series of morphological and molecular changes that progressively transform them into astrocytes of the adult brain (Barry and McDermott 2005; Kriegstein and Alvarez-Buylla 2009; Mission et al. 1991; Voigt 1989). In particular, bipolar radial glia cells were recently observed by time-lapse microscopy on embryonic brain cultures to lose apical contact, migrate to the cortical plate and assume a stellate morphology characteristic of mature astrocytes (Noctor et al. 2008). This process is accompanied at neonatal stages by a reduction in the proliferative potential of newborn astrocytes as they progressively slow their rate of division to become quiescent (Ichikawa et al. 1983). Alternatively, some radial glial cells of specific brain regions escape this fate and, while keeping astrocytic features, become adult neural stem cells (Kriegstein and Alvarez-Buylla 2009).

Unfortunately, the terminology in the field may lead to confusion as radial glial cells of the developing CNS are neural stem cells but it would be inappropriate to call adult neural stem cells radial glia. Moreover, while adult neural stem cells have astrocytic features, not all astrocytes are neural stem cells. Nevertheless, the two regions of the adult brain in which neural stem cells have been more consistently

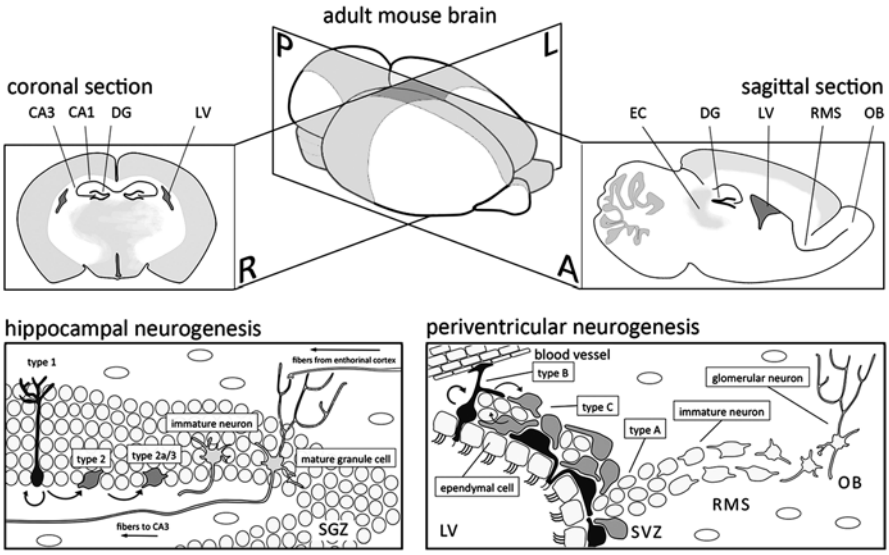

Fig. 7.3 The adult neurogenic niche. (*top*) Coronal (*left*) and sagittal (*right*) sections of the adult mouse brain (*center*; P-A-L-R = posterior-anterior-left-right, respectively) showing the sites of adult neurogeneis. (*bottom*) Cytoarchitecture of the SGZ (*left*) and SVZ (*right*). Type 1/B, 2/C, 3/A cells and neurons are depicted. *Arrows* indicate their lineage. *DG* dentate gyrus, *LV* lateral ventricle, *EC* entorhinal cortex, *RMS* rostral migratory stream, *OB* olfactory bulb, SGZ subgranular zone

reported and rigorously studied are the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Fig. 7.3) (Doetsch et al. 1997, 1999a; Eriksson et al. 1998; Gould et al. 1999a; Gueneau et al. 1982; Kempermann et al. 1997; Kornack and Rakic 1999; Kuhn et al. 1996; Stanfield and Trice 1988; van Praag et al. 2002). In these two neurogenic niches different stem and progenitor cells coexist that are reminiscent of embryonic precursors, as we shall see later.

7.4.2.2 Identification and Nomenclature

The terminology used to identify the various precursors of the SGZ differs from that of the SVZ because the two neurogenic niches have been independently characterized by different groups. This may seem unfortunate because neural stem and progenitor cells in these two areas are similar with regard to their origin, morphology and function, which would call for a consistent terminology. However, important differences exist, in particular with regard to the expression of certain molecular markers and the neuronal subtypes generated, making it convenient to keep a different nomenclature for a more rigorous classification.

The main approach currently used to identify precursor subtypes in vivo is by performing immunohistochemistry with antibodies against specific molecular

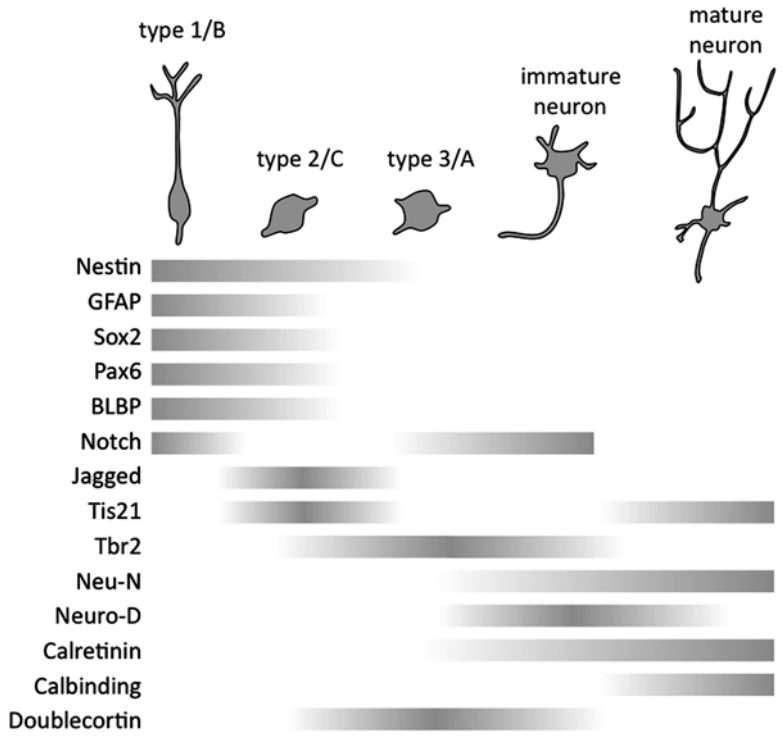

Fig. 7.4 Molecular markers commonly used to identify neural precursors in adult neurogenesis (Adapted from Attardo et al. (2008), Breunig et al. (2007b) and Zhao et al. (2008))

markers (Fig. 7.4). In essence, the detection of immunolabeling, or lack thereof, is taken as an evidence of cell identity. Though technically very practicable, the problematics inherent in such an approach are several such as that (i) no single individual marker has yet been described to selectively label an entire precursor subpopulation, (ii) certain cell types are identified based on quantitative assessment of labeling intensity, which is often problematic, (iii) immunolabeling does not necessarily indicate gene expression as synthesized proteins may be inherited from mother to daughter cell, and lastly, (iv) cells may change expression levels of certain genes while progressing through the cell cycle and, thus, a different expression level of a marker may not necessarily indicate different cell identity. Due to these limitations, immunolabeling for molecular markers, or the use of transgenic reporter mouse lines for that particular marker, is typically combined with BrdU labeling and estimation of cell cycle parameters, which provides further evidence of cell identity because of the different cell cycle kinetics characteristic of precursor subtypes. Finally, but certainly more difficult, cell identity could be ascertained by electron microscopy and ultrastructural analyses, which, together with advanced light microscopy, has been critical to 3D-reconstruct the cytoarchitecture of the neurogenic niches (Mirzadeh et al. 2008; Shen et al. 2008; Tavazoie et al. 2008).

The combination of these techniques has led to the identification of three main precursors types in the SGZ and SVZ.

In the SGZ, neural precursors have been defined to as type 1, 2 and 3 with an additional subdivision of type 2 cells in 2a and 2b (Kempermann et al. 2004). Similarly, in the SVZ neural precursors have been defined to as type B, C and A cells, with the subdivision of B cells in type B1 and B2 as original proposed (Doetsch et al. 1997) being later abandoned (Doetsch et al. 1999a). The morphological and functional features of these precursor subtypes and their lineage will be more thoroughly described later; it suffices here to say that the type 1/B cells are thought to generate type 2/C cells, and these then generate type 3/A cells, also referred to as neuroblasts of newborn neurons.

Finally, a fourth cell type is present specifically in the periventricular area. These are ependymal cells forming a single-cell layer that delimits the boundary between the SVZ (also called subependymal zone) and the lumen of the ventricle. Ependymal cells were also proposed to be neural stem cells (Johansson et al. 1999) but this view was later disputed (Capela and Temple 2002; Chiasson et al. 1999; Doetsch et al. 1999a) leading to a long controversy in the field (Chojnacki et al. 2009). Recently, the neurogenic capacity of ependymal cells was shown to occur only during certain neurological disorders (Carlen et al. 2009) and, thus, the consensus at the time of writing is that ependymal cells are not neural stem cells under physiological conditions.

7.4.3 Characteristics/Properties

7.4.3.1 Anatomy and Cytoarchitecture of the Neurogenic Niches

The hippocampus lies within the temporal lobes of the telencephalic hemispheres and is more generally subdivided into CA1, CA3 and dentate gyrus, which form the trisynaptic circuit of this brain area. A high nuclear density in the three regions makes the hippocampus easy to identify on cross-sections. Specifically, CA1 (dorsally) and CA3 (ventrally) fuse to form the characteristic shape of a C (with its concavity oriented medially) while the dentate gyrus, resembling a V (with its concavity oriented laterally), is adjacent to CA3 (Fig. 7.3) (see the Allan Brain Atlas for 3D-reconstructions of brain anatomy: http://www.brain-map.org).

Simplifying a complex neuronal circuit, inputs to the hippocampus are transmitted from the entorhinal cortex to the dentate gyrus, which is connected to CA3 through the mossy fibers. Signals from pyramidal neurons in CA3 are then sent via the Schaffer collateral to CA1, which finally project outside the hippocampus back to the entorhinal cortex.

The only region of the hippocampus where neural stem cells are known to reside is the SGZ of the dentate gyrus, a particularly vascularized region with a diverse population of highly packed cells, including type 1, 2 and 3 precursors. Advanced light microscopy has shown that bipolar, type 1 cells are oriented perpendicularly to the SGZ, have their nuclei towards the concavity of the V and span with their pro-

cess the entire thickness of the SGZ until branching into several smaller processes at the level of the inner molecular layer, where they contact blood vessels. In contrast, type 2 and 3 cells lose radial morphology and assume a more rounded shape. Newborn neurons remain in the SGZ and extend axon and dendrites to integrate into the circuitry of the hippocampus (Fig. 7.3) (Alvarez-Buylla and Lim 2004; Kempermann et al. 2004; Kriegstein and Alvarez-Buylla 2009).

The anatomy and cytoarchitecture of the SVZ is very different from the hippocampus. With the end of embryonic development, the cavity of the neural tube collapses and is reduced to minimal proportions. Delimiting and in direct contact with the cerebrospinal fluid lies a single cell layer of ciliated ependymal cells, supposedly derived from radial glial cells (Kriegstein and Alvarez-Buylla 2009). In contrast to the hippocampus, which is both a neuronal network in itself and a neurogenic niche, the SVZ serves only the latter function. In fact, neurons derived from the SVZ migrate through the rostral migratory stream toward the rostral-most region of the brain, the olfactory bulbs, where they integrate into the networks mediating olfaction.

The olfactory bulbs receive information from the adjacent olfactory epithelium, laying the nasal cavity. In this context, it should be mentioned that the olfactory epithelium is yet another site of adult neurogenesis, though in this case neurons are part of the peripheral, rather than central, nervous system (Martinez-Marcos et al. 2000), which makes them somehow less attractive for therapy.

Advanced light microscopy and 3D-reconstruction by electron microscopy allowed to define the cytoarchitecture of the adult SVZ (Mirzadeh et al. 2008; Shen et al. 2008; Tavazoie et al. 2008). Relatively flat ependymal cells surround type B cells in a pin-wheel arrangement, which allows B cells to extend their primary cilium into the lumenal fluid. Nuclei of B cells are located underneath ependymal cells (Mirzadeh et al. 2008) and extend a basal process to surround C cells, which lack lumenal contact and themselves surround A cells. Finally, in the center of this B-C-A layering, type A cells form chains of migrating neuroblasts/newborn neurons forming the rostral migratory stream connecting the neurogenic niche with the final destination of the newborn neurons, the olfactory bulb.

7.4.3.2 Cell Biological Features of Adult Neural Stem Cells

It is interesting to observe that, despite many differences, adult neural stem and progenitor cells retain certain features of their functionally equivalent embryonic counterparts, i.e. radial glial cells and basal progenitors, respectively.

For example, adult type 1/B cells, like radial glia, are bipolar and highly elongated, and are characterized by the expression of astrocytic markers and the contact of their terminal process with blood vessels (Filippov et al. 2003; Fukuda et al. 2003; Shen et al. 2008; Tavazoie et al. 2008). In addition, they retain a primary cilium, an organelle long neglected through which important signaling pathways act (Doetsch et al. 1999b; Mirzadeh et al. 2008). In contrast to type 1/B cells, and similar to basal progenitors, type 2/C progenitors assume a rounded morphology and, in the SVZ, lose contact with the lumen of the ventricle (Doetsch et al. 1999b;

Mirzadeh et al. 2008). An important feature distinguishing adult neural stem cells from radial glia, besides the lack of interkinetic nuclear migration, concerns their cell cycle kinetics.

As already described, the developing cortex is a highly proliferative tissue with a cell cycle length of 10–20 h (Calegari et al. 2005; Takahashi et al. 1995), which is similar to type 2/C progenitors (Cameron and McKay 2001; Hayes and Nowakowski 2002; Morshead and van der Kooy 1992; Zhang et al. 2006). In contrast, the rate of division of type 1/B cells is extremely slow, which has earned them the epithet of slowly-dividing or label-retaining cells as S-phase tracers are less frequently diluted by cell division and, thus, can be detected in daughter stem cells a long time after incorporation into their mothers. The slow division rate of type 1/B cells has been demonstrated after administration of cytostatic agents that kill cells undergoing mitosis (in particular, arabinosyde-C (AraC)) (Doetsch et al. 1999b; Morshead et al. 1994; Seri et al. 2001). First, AraC treatment depletes type 2/C and 3/A cells, but not type 1/B cells. Second, removal of AraC allows the repopulation of all cell types. Thus, type 1/B cells are mostly quiescent and are true stem cells as they alone can regenerate the entire neurogenic niche (Doetsch et al. 1999b; Morshead et al. 1994; Seri et al. 2001). Calculation upon different times of AraC treatment allowed to estimate cell cycle length of adult type 1/B neural stem cells that divide, on average every 2–4 weeks (Doetsch et al. 1999b; Morshead et al. 1994). This, however, should not be interpreted as to indicate that the cell cycle of neural stem cells requires several weeks to be completed; rather it suggests quiescence for most of this time, with the cell cycle length proper being a small fraction of it.

Finally, type 3/A cells, the third precursor type with no counterpart in the developing cortex, are also considered proliferating precursors, hence their name neuroblasts (Doetsch et al. 1999a), which implies that they undergo cell division. In fact, AraC treatment also depletes type 3/A cells, and a relatively short BrdU exposure is sufficient to label a proportion of them. The view that type 3/A cells progress through the cell cycle is supported by the expression, in a subpopulation of them, of markers of proliferation (Doetsch et al. 1999a). However, considering (i) the limitations in assessing cell identity by immunohistochemistry, (ii) that depletion of type 2/C cells would alone be sufficient to deplete also their progeny, and (iii) that BrdU is inherited from a proliferating mother to a postmitotic daughter, these experiments cannot exclude the possibility that a proportion of type 3/A cells are postmitotic neurons rather than proliferating precursors.

7.4.4 Differentiation Capacity and Their Precursors

7.4.4.1 Lineage and Mode of Division

As already mentioned, the exact lineage and mode of division of adult neural precursors is much less defined than those during embryonic development, which is due to intrinsic difficulties in performing lineage tracing experiments in the adult

brain. For example, contrary to the developing cortex, adult neural stem and progenitor cells constitute a particularly small population distributed within postmitotic neurons and glia. In addition, no individual marker for specific progenitor subtypes has yet been found and, thus, transgenic reporter mouse lines cannot be effectively used to identify the relevant cell type to image, which is very important for lineage tracing experiments in tissue. Finally, neural stem cells are mostly quiescent requiring very long culture experiments, which may be unphysiological or harmful to the tissue. Nevertheless, various morphological, functional and molecular studies suggest that type 1/B cells generate type 2/C cells while the latter generate type 3/A cells. No direct evidence is available to suggest that any given precursor undergoes symmetric or asymmetric cell division.

7.4.4.2 Factors Influencing Differentiation

For many decades, morphogens, transcription and trophic factors that influence neural differentiation have been almost exclusively studied during embryonic development. Not surprisingly, however, the same factors are now shown to have similar effects on adult neural stem cells. We will here only briefly summarize the most important examples of these signaling pathways and refer the reader to comprehensive reviews for more information (Ever and Gaiano 2005; Guillemot 2007; Ninkovic and Gotz 2007; Suh et al. 2009).

The vascular niche Adult neurogenesis occurs is a highly vascularized environment, which suggests that signals may be transmitted from the blood to neural precursors in order to control their activity. The concept of a vascular niche (Palmer et al. 2000) was somehow present three decades ago from studies on seasonal songbirds as, in fact, adult neurogenesis was known to be triggered by hormones released into the blood (Nottebohm 1985). More recently, a direct link between testosterone, angiogenesis and adult neurogenesis has been established in birds (Louissaint et al. 2002) while neural precursors in mammals have been found to cluster around blood vessels (Shen et al. 2008; Tavazoie et al. 2008). The cross-talk between angiogenesis and neurogenesis is attracting now more attention (Alvarez-Buylla and Lim 2004; Suh et al. 2009), and it is presumably not a coincidence that factors promoting angiogenesis, most notably VEGF, also promote neurogenesis (Cao et al. 2004; Jin et al. 2002) while their inhibition has the opposite effect (Cao et al. 2004; Fabel et al. 2003). Finally, it is worth noting that increased angiogenesis is the primary response in many models of neurodegenerative disease and that treatments that improve angiogenesis also improve brain recovery after injury (Zhang and Chopp 2009).

Notch Both Notch and its ligands Jagged1 and Dll1 are expressed in the adult neurogenic niche (Givogri et al. 2006; Nyfeler et al. 2005), and activation of the Notch pathway in adult neural stem cells promotes their expansion and inhibits differentiation (Androutsellis-Theotokis et al. 2006; Nyfeler et al. 2005). Interestingly, Notch activity, and thus neural precursor self-renewal, seems to be mediated by an autoregulatory loop between Notch and Shh signaling (Androutsellis-Theotokis

et al. 2006), which is also implicated in controlling quiescence of ependymal cells (Carlen et al. 2009). In addition, certain neurodegenerative diseases, such as stroke, induce Notch signaling (Carlen et al. 2009), and administration of Notch-ligands can be used to better restore brain function upon stroke (Androutsellis-Theotokis et al. 2006).

Shh Similar to the effects observed during corticogenesis, activation of Shh signaling in adult neural precursors induces a strong, dose-dependent increase in proliferation while its inhibition has the opposite effect (Breunig et al. 2008; Lai et al. 2003; Machold et al. 2003). Interestingly, key mediators of the Shh pathway in adult neural stem cells are localized on the primary cilium (Breunig et al. 2008; Han et al. 2008), an organelle recognized to play important functions during development (Gerdes et al. 2009). Finally, transgenic mouse lines to genetically monitor Shh activity have allowed to fate-map neural precursors, providing the first evidence that quiescent neural stem cells can self-renew for over 1 year (Ahn and Joyner 2005).

Wnt Again similar to the effects during corticogenesis, increasing Wnt signaling in the adult hippocampus promotes cell cycle progression and increases neuronal output while, conversely, inhibiting Wnt has the opposite effects (Lie et al. 2005). Wnt function in adult neural stem cells has recently been proposed to act through a sophisticated crosstalk between the transcription factor Sox2 and the proneural gene NeuroD1 (Kuwabara et al. 2009).

7.4.5 Function and Potential Application for Therapies

7.4.5.1 Role of Adult Neurogenesis

One of the most challenging goals in neuroscience is to understand the molecular and cellular mechanisms underlying elaborate cognitive functions. It is a fact, however, that this is one of the fields in life science where we still know relatively little. The physiological processes that allow learning, memory, feeling emotions and elaborate functions that are emphasized in humans, such as self-consciousness, are even difficult to conceptualize, but the recognition of adult neurogenesis has led scientists to investigate whether neural stem cells may be involved in any of these functions (Abrous et al. 2005; Imayoshi et al. 2009; Kempermann 2008; Lledo et al. 2006; Zhao et al. 2008).

A striking finding in this context is that external physiological stimuli have an effect on neurogenesis (Kempermann et al. 1997; Leuner et al. 2004). In the SGZ, genetic differences in various mouse strains have been correlated to the extent of precursors proliferation, neurogenesis and mouse performance in learning and memory tasks (Kempermann and Gage 2002). Moreover, voluntary exercise, such as allowing mice to train on a running wheel, or an enriched environment, such as larger housing conditions with toys and other mice for stimulating social behavior, increases neurogenesis and neuronal survival, respectively (Olson et al. 2006; Zhao et al. 2008). Interestingly, these stimuli also increase the mouse performance in learning and memory tasks (Olson et al. 2006; Zhao et al. 2008).

While these experiments could show a direct correlation between adult neurogenesis and learning and memory, other experiments aimed to manipulate adult neurogenesis failed to report consistent effects. For example, inhibiting adult hippocampal neurogenesis by depletion of precursors by means of X-ray irradiation did not prevent the beneficial effects on learning and memory that was induced by the subsequent exposure to an enriched environment. This suggests that factors other than neurogenesis, such as an increased angiogenesis or hormone activity, may be responsible for the improved behavioral response (Meshi et al. 2006).

In addition to radiation, cytostatic drugs or genetic manipulations are also used to investigate the behavioral effects upon inhibition of neurogenesis. Yet, alternative approaches have shown opposite outcomes (Olson et al. 2006; Saxe et al. 2006; Zhang et al. 2008a; Zhao et al. 2008). Thus, although adult hippocampal neurogenesis has been suggested to play a role in learning, memory, fear conditioning, depression and other elaborate brain functions, none of these effects are firmly established (Abrous et al. 2005; Imayoshi et al. 2009; Kempermann 2008; Lledo et al. 2006; Zhao et al. 2008).

The role of adult neurogenesis in olfaction is certainly less controversial. In this context, neurogenesis in the SVZ is essential to preserve cell homeostasis of the olfactory bulb as newborn neurons are needed to replace old, dying ones (Imayoshi et al. 2008). This is in contrast to the SGZ in which addition of newborn neurons is cumulative, leading to an increase, though extremely modest, in neuron number over time (Imayoshi et al. 2008). It is important to mention, however, that in both cases the vast majority of newborn neurons will not integrate into preexisting circuits but will undergo cell death. Nevertheless, the fact that neurogenesis is needed to preserve cell number in the olfactory bulb has corroborated the previous hypothesis that SVZ neurogenesis is essential for acquisition and memory of olfactory stimuli, which in rodents is of paramount importance for interacting with the environment and for social behavior (Alonso et al. 2006; Lledo et al. 2006).

7.4.5.2 Use of Neural Stem Cells in Therapy

A justification for the great efforts and huge investments in neural stem cell research is its possible application in therapies for neurodegenerative diseases or CNS injuries (Okano et al. 2007; Steiner et al. 2006; Zhang and Chopp 2009; Zuccato and Cattaneo 2009). As we shall see in this book, however, cell-based treatments of neural pathologies are at the moment the least developed and effective when compared to similar treatments in other tissues, such as in the bone marrow, skin, pancreas or bone (part IV of this book).

This gap is easily explained by the many difficulties inherent in the study and manipulation of the CNS in human patients and by the limited knowledge, relative to other organs, of its physiology. Moreover, adult neurogenesis in rodents, the main animal model for experimental research, greatly differs from neurogenesis in humans, and very few neural pathologies are faithfully reproduced in the laboratory, which makes it even harder to test novel strategies for therapy. Nevertheless, the

price at stake for developing effective therapies for neural disorders, a main cause of disease in rich countries, is great and first attempts in this direction have already been undertaken. In most cases, however, their efficacy is difficult to evaluate for it is unfeasible to "use" patients as negative control.

Admittedly, even if based on circumstantial evidence and a relatively scarce knowledge of human CNS physiology and pathology, the first attempts in this direction are promising as they, in some cases, have lead to major improvement of clinical conditions. For example, transplantation of human embryonic brain tissue into patients with Parkinson Disease allowed one of six patients to suspend medical treatment for more than a year (Wenning et al. 1997). Others works, though also in principle successful, have instead raised more controversy. For example, neural cells have been cultured for several passages in vitro and then transplanted into brains of patients with stroke (Kondziolka et al. 2000). More recently, a similar approach has been used to treat patients with open brain injury (Zhu et al. 2006). The fact that cells passaged for several weeks, if not months, in culture may undergo transformation and lead to cancer after transplantation makes these attempts hazardous, if not ethically questionable.

Thanks to the revolutionary advent of induced pluripotency (Takahashi and Yamanaka 2006) we can now envision safer and more efficient systems to generate *patient-customized* stem cells for therapy. Clearly, more work needs to be done to be able to control their differentiation into the desired cell type and to explore their use in therapy. This is the reason why experiments on animal models of neural disorders, including Alzheimer (Brinton and Wang 2006), Parkinson (Arias-Carrion and Yuan 2009), stroke (Zhang et al. 2008b) and spinal cord lesions (Okano and Sawamoto 2008) are so important.

Finally, it should be mentioned that certain brain tumors have now been shown to originate from an altered proliferation of neural precursors (Alcantara Llaguno et al. 2009; Wang et al. 2009) and, thus, studying neural stem cells may open up possibilities for understanding cancer and, perhaps, design new therapies for it (Colleoni and Torrente 2008).

7.4.6 Conclusions and Future Development in Research

It should be evident from this chapter that understanding the mechanisms underlying adult neurogenesis and its physiological function and manipulating this process for therapeutic purposes are all in a very preliminary phase. However, the momentum created by recent breakthroughs in the field and the great hopes that somatic stem cells carry for future therapies largely justify the huge investments devoted to it. While it is too early to predict the real usefulness of neural stem cell in therapy, there is little doubt that future basic research in this field will be crucial to better understand brain development and evolution.

References

Abrous DN, Koehl M, Le Moal M (2005) Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85:523–569

Ackman JB, Siddiqi F, Walikonis RS, LoTurco JJ (2006) Fusion of microglia with pyramidal neurons after retroviral infection. J Neurosci 26:11413–11422

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437:894–897

Aigner S, Denli AM, Gage FH (2007) A novel model for an older remodeler: the BAF swap in neurogenesis. Neuron 55:171–173

Alcantara Llaguno S, Chen J, Kwon CH, Jackson EL, Li Y, Burns DK, Alvarez-Buylla A, Parada LF (2009) Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. Cancer Cell 15:45–56

Alonso M, Viollet C, Gabellec MM, Meas-Yedid V, Olivo-Marin JC, Lledo PM (2006) Olfactory discrimination learning increases the survival of adult-born neurons in the olfactory bulb. J Neurosci 26:10508–10513

Altman J (1962) Are new neurons formed in the brains of adult mammals? Science 135:1127–1128

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41:683–686

Ambros V (2004) The functions of animal microRNAs. Nature 431:350–355

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442:823–826

Arai Y, Funatsu N, Numayama-Tsuruta K, Nomura T, Nakamura S, Osumi N (2005) Role of Fabp7, a downstream gene of Pax6, in the maintenance of neuroepithelial cells during early embryonic development of the rat cortex. J Neurosci 25:9752–9761

Arias-Carrion O, Yuan TF (2009) Autologous neural stem cell transplantation: a new treatment option for Parkinson's disease? Med Hypotheses 73:757–759

Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284:770–776

Attardo A, Calegari F, Haubensak W, Wilsch-Bräuninger M, Huttner WB (2008) Live imaging at the onset of cortical neurogenesis reveals differential appearance of the neuronal phenotype in apical versus basal progenitor progeny. PLoS One 3:e2388

Barry D, McDermott K (2005) Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. Glia 50:187–197

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215–233

Bautch VL, James JM (2009) Neurovascular development: the beginning of a beautiful friendship. Cell Adh Migr 3:199–204

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ (2003) Dicer is essential for mouse development. Nat Genet 35:215–217

Biron VL, McManus KJ, Hu N, Hendzel MJ, Underhill DA (2004) Distinct dynamics and distribution of histone methyl-lysine derivatives in mouse development. Dev Biol 276:337–351

Borello U, Cobos I, Long JE, McWhirter JR, Murre C, Rubenstein JL (2008) FGF15 promotes neurogenesis and opposes FGF8 function during neocortical development. Neural Dev 3:17

BoulderComm (1970) Embryonic vertebrate central nervous system: revised terminology. The Boulder Committee. Anat Rec 166:257–261

Breunig JJ, Arellano JI, Macklis JD, Rakic P (2007a) Everything that glitters isn't gold: a critical review of postnatal neural precursor analyses. Cell Stem Cell 1:612–627

Breunig JJ, Silbereis J, Vaccarino FM, Sestan N, Rakic P (2007b) Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. Proc Natl Acad Sci U S A 104:20558–20563

Breunig JJ, Sarkisian MR, Arellano JI, Morozov YM, Ayoub AE, Sojitra S, Wang B, Flavell RA, Rakic P, Town T (2008) Primary cilia regulate hippocampal neurogenesis by mediating sonic hedgehog signaling. Proc Natl Acad Sci U S A 105:13127–13132

Brinton RD, Wang JM (2006) Therapeutic potential of neurogenesis for prevention and recovery from Alzheimer's disease: allopregnanolone as a proof of concept neurogenic agent. Curr Alzheimer Res 3:185–190

Briscoe J, Pierani A, Jessell TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101:435–445

Calegari F, Haubensak W, Haffner C, Huttner WB (2005) Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. J Neurosci 25:6533–6538

Cameron HA, McKay RD (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 435:406–417

Cao L, Jiao X, Zuzga DS, Liu Y, Fong DM, Young D, During MJ (2004) VEGF links hippocampal activity with neurogenesis, learning and memory. Nat Genet 36:827–835

Cao X, Yeo G, Muotri AR, Kuwabara T, Gage FH (2006) Noncoding RNAs in the mammalian central nervous system. Annu Rev Neurosci 29:77–103

Cao X, Pfaff SL, Gage FH (2007) A functional study of miR-124 in the developing neural tube. Genes Dev 21:531–536

Capela A, Temple S (2002) LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron 35:865–875

Carlen M, Meletis K, Goritz C, Darsalia V, Evergren E, Tanigaki K, Amendola M, Barnabe-Heider F, Yeung MS, Naldini L, Honjo T, Kokaia Z, Shupliakov O, Cassidy RM, Lindvall O, Frisen J (2009) Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke. Nat Neurosci 12:259–267

Castro DS, Skowronska-Krawczyk D, Armant O, Donaldson IJ, Parras C, Hunt C, Critchley JA, Nguyen L, Gossler A, Göttgens B, Matter JM, Guillemot F (2006) Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev Cell 11:831–844

Caviness VS, Takahashi T, Nowakowski RS (1995) Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. Trends Neurosci 18:379–383

Cayuso J, Martí E (2005) Morphogens in motion: growth control of the neural tube. J Neurobiol 64:376–387

Cayuso J, Ulloa F, Cox B, Briscoe J, Martí E (2006) The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity. Development 133:517–528

Cheng LC, Tavazoie M, Doetsch F (2005) Stem cells: from epigenetics to microRNAs. Neuron 46:363–367

Cheng LC, Pastrana E, Tavazoie M, Doetsch F (2009) miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat Neurosci 12:399–408

Chenn A, McConnell SK (1995) Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell 82:631–641

Chenn A, Walsh CA (2003) Increased neuronal production, enlarged forebrains and cytoarchitectural distortions in beta-catenin overexpressing transgenic mice. Cereb Cortex 13:599–606

Chesnutt C, Burrus LW, Brown AM, Niswander L (2004) Coordinate regulation of neural tube patterning and proliferation by TGFbeta and WNT activity. Dev Biol 274:334–347

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383:407–413

Chiasson BJ, Tropepe V, Morshead CM, van der Kooy D (1999) Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. J Neurosci 19:4462–4471

Chojnacki AK, Mak GK, Weiss S (2009) Identity crisis for adult periventricular neural stem cells: subventricular zone astrocytes, ependymal cells or both? Nat Rev Neurosci 10:153–163

Colleoni F, Torrente Y (2008) The new challenge of stem cell: brain tumour therapy. Cancer Lett 272:1–11

Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtas S, van Roon-Mom WM, Bjork-Eriksson T, Nordborg C, Frisen J, Dragunow M, Faull RL, Eriksson PS (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315:1243–1249

Dahmane N, Sánchez 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

Dayer AG, Cleaver KM, Abouantoun T, Cameron HA (2005) New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol 168:415–427

De Pietri Tonelli D, Pulvers JN, Haffner C, Murchison EP, Hannon GJ, Huttner WB (2008) miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. Development 135:3911–3921

Dehay C, Kennedy H (2007) Cell-cycle control and cortical development. Nat Rev Neurosci 8:438–450

Dickinson ME, Krumlauf R, McMahon AP (1994) Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. Development 120:1453–1471

Diez del Corral R, Breitkreuz DN, Storey KG (2002) Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. Development 129:1681–1691

Diez del Corral R, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K (2003) Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40:65–79

Doe CQ, Skeath JB (1996) Neurogenesis in the insect central nervous system. Curr Opin Neurobiol 6:18–24

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046–5061

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999a) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1999b) Regeneration of a germinal layer in the adult mammalian brain. Proc Natl Acad Sci U S A 96:11619–11624

Du T, Zamore PD (2005) microPrimer: the biogenesis and function of microRNA. Development 132:4645–4652

Ekholm SV, Reed SI (2000) Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 12:676–684

Erecińska M, Silver IA (2001) Tissue oxygen tension and brain sensitivity to hypoxia. Respir Physiol 128:263–276

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317

Estivill-Torrus G, Pearson H, van Heyningen V, Price DJ, Rashbass P (2002) Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. Development 129:455–466

Ever L, Gaiano N (2005) Radial 'glial' progenitors: neurogenesis and signaling. Curr Opin Neurobiol 15:29–33

Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. Eur J Neurosci 18:2803–2812

Fan G, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L, Hattori D, Ge W, Shen Y, Wu H, ten Hoeve J, Shuai K, Sun YE (2005) DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 132:3345–3356

Farkas LM, Huttner WB (2008) The cell biology of neural stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. Curr Opin Cell Biol 20:707–715

Filippov V, Kronenberg G, Pivneva T, Reuter K, Steiner B, Wang LP, Yamaguchi M, Kettenmann H, Kempermann G (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Mol Cell Neurosci 23:373–382

Firket H, Verly WG (1958) Autoradiographic visualization of synthesis of deoxyribonucleic acid in tissue culture with tritium-labelled thymidine. Nature 181:274–275

Fish JL, Kosodo Y, Enard W, Pääbo S, Huttner WB (2006) Aspm specifically maintains symmetric proliferative divisions of neuroepithelial cells. Proc Natl Acad Sci U S A 103:10438–10443

Fish JL, Dehay C, Kennedy H, Huttner WB (2008) Making bigger brains-the evolution of neural-progenitor-cell division. J Cell Sci 121:2783–2793

Fode C, Ma Q, Casarosa S, Ang SL, Anderson DJ, Guillemot F (2000) A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev 14:67–80

Frielingsdorf H, Schwarz K, Brundin P, Mohapel P (2004) No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A 101:10177–10182

Fukuda S, Kato F, Tozuka Y, Yamaguchi M, Miyamoto Y, Hisatsune T (2003) Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. J Neurosci 23:9357–9366

Gavin BJ, McMahon JA, McMahon AP (1990) Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. Genes Dev 4:2319–2332

Gerdes JM, Davis EE, Katsanis N (2009) The vertebrate primary cilium in development, homeostasis, and disease. Cell 137:32–45

Givogri MI, de Planell M, Galbiati F, Superchi D, Gritti A, Vescovi A, de Vellis J, Bongarzone ER (2006) Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. Dev Neurosci 28:81–91

Götz M, Huttner WB (2005) The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788

Götz M, Stoykova A, Gruss P (1998) Pax6 controls radial glia differentiation in the cerebral cortex. Neuron 21:1031–1044

Gould E, Reeves AJ, Fallah M, Tanapat P, Gross CG, Fuchs E (1999a) Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A 96:5263–5267

Gould E, Reeves AJ, Graziano MS, Gross CG (1999b) Neurogenesis in the neocortex of adult primates. Science 286:548–552

Gould E, Vail N, Wagers M, Gross CG (2001) Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A 98:10910–10917

Gross CG (2000) Neurogenesis in the adult brain: death of a dogma. Nat Rev Neurosci 1:67–73

Gueneau G, Privat A, Drouet J, Court L (1982) Subgranular zone of the dentate gyrus of young rabbits as a secondary matrix. A high-resolution autoradiographic study. Dev Neurosci 5:345–358

Guillemot F (2007) Cell fate specification in the mammalian telencephalon. Prog Neurobiol 83:37–52

Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, Ruas JL, Poellinger L, Lendahl U, Bondesson M (2005) Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell 9:617–628

Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A (2008) Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. Nat Neurosci 11:277–284

Hatakeyama J, Bessho Y, Katoh K, Ookawara S, Fujioka M, Guillemot F, Kageyama R (2004) Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development 131:5539–5550

Haubensak W, Attardo A, Denk W, Huttner WB (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc Natl Acad Sci U S A 101:3196–3201

Hayes NL, Nowakowski RS (2000) Exploiting the dynamics of S-phase tracers in developing brain: interkinetic nuclear migration for cells entering versus leaving the S-phase. Dev Neurosci 22:44–55
Hayes NL, Nowakowski RS (2002) Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. Brain Res Dev Brain Res 134:77–85
He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW (1998) Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512
Hevner RF (2006) From radial glia to pyramidal-projection neuron: transcription factor cascades in cerebral cortex development. Mol Neurobiol 33:33–50
Honjo T (1996) The shortest path from the surface to the nucleus: RBP-J kappa/Su(H) transcription factor. Genes Cells 1:1–9
Houbaviy HB, Murray MF, Sharp PA (2003) Embryonic stem cell-specific MicroRNAs. Dev Cell 5:351–358
Hu G, Lee H, Price SM, Shen MM, Abate-Shen C (2001) Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. Development 128:2373–2384
Huttner WB, Brand M (1997) Asymmetric division and polarity of neuroepithelial cells. Curr Opin Neurobiol 7:29–39
Ichikawa M, Shiga T, Hirata Y (1983) Spatial and temporal pattern of postnatal proliferation of glial cells in the parietal cortex of the rat. Brain Res 285:181–187
Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11:1153–1161
Imayoshi I, Sakamoto M, Ohtsuka T, Kageyama R (2009) Continuous neurogenesis in the adult brain. Dev Growth Differ 51:379–386
Ingham PW, McMahon AP (2001) Hedgehog signaling in animal development: paradigms and principles. Genes Dev 15:3059–3087
Ishibashi M, McMahon AP (2002) A sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordia in the early mouse embryo. Development 129:4807–4819
Jacob J, Briscoe J (2003) Gli proteins and the control of spinal-cord patterning. EMBO Rep 4:761–765
Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1:20–29
Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci U S A 99:11946–11950
Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J (1999) Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96:25–34
Kageyama R, Nakanishi S (1997) Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr Opin Genet Dev 7:659–665
Kageyama R, Ohtsuka T, Kobayashi T (2008) Roles of Hes genes in neural development. Dev Growth Differ 50(Suppl 1):S97–S103
Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092–1094
Kempermann G (2008) The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? Trends Neurosci 31:163–169
Kempermann G, Gage FH (2002) Genetic influence on phenotypic differentiation in adult hippocampal neurogenesis. Brain Res Dev Brain Res 134:1–12
Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495
Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447–452

Kenney AM, Rowitch DH (2000) Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol Cell Biol 20:9055–9067
Kenney AM, Cole MD, Rowitch DH (2003) Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. Development 130:15–28
Kim VN (2005) MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6:376–385
Klein ME, Impey S, Goodman RH (2005) Role reversal: the regulation of neuronal gene expression by microRNAs. Curr Opin Neurobiol 15:507–513
Koketsu D, Mikami A, Miyamoto Y, Hisatsune T (2003) Nonrenewal of neurons in the cerebral neocortex of adult macaque monkeys. J Neurosci 23:937–942
Komada M, Saitsu H, Kinboshi M, Miura T, Shiota K, Ishibashi M (2008) Hedgehog signaling is involved in development of the neocortex. Development 135:2717–2727
Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, Jannetta P, DeCesare S, Elder EM, McGrogan M, Reitman MA, Bynum L (2000) Transplantation of cultured human neuronal cells for patients with stroke. Neurology 55:565–569
Konno D, Shioi G, Shitamukai A, Mori A, Kiyonari H, Miyata T, Matsuzaki F (2008) Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. Nat Cell Biol 10:93–101
Korada S, Zheng W, Basilico C, Schwartz ML, Vaccarino FM (2002) Fibroblast growth factor 2 is necessary for the growth of glutamate projection neurons in the anterior neocortex. J Neurosci 22:863–875
Kornack DR, Rakic P (1999) Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A 96:5768–5773
Kornack DR, Rakic P (2001) Cell proliferation without neurogenesis in adult primate neocortex. Science 294:2127–2130
Kosodo Y, Röper K, Haubensak W, Marzesco AM, Corbeil D, Huttner WB (2004) Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. EMBO J 23:2314–2324
Kriegstein A, Alvarez-Buylla A (2009) The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149–184
Kriegstein AR, Götz M (2003) Radial glia diversity: a matter of cell fate. Glia 43:37–43
Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027–2033
Kuwabara T, Hsieh J, Muotri A, Yeo G, Warashina M, Lie DC, Moore L, Nakashima K, Asashima M, Gage FH (2009) Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. Nat Neurosci 12:1097–1105
Lai K, Kaspar BK, Gage FH, Schaffer DV (2003) Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6:21–27
Lange C, Mix E, Rateitschak K, Rolfs A (2006) Wnt signal pathways and neural stem cell differentiation. Neurodegener Dis 3:76–86
Lange C, Huttner WB, Calegari F (2009) Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. Cell Stem Cell 5:320–331
Lathia JD, Mattson MP, Cheng A (2008) Notch: from neural development to neurological disorders. J Neurochem 107:1471–1481
Lessard J, Wu JI, Ranish JA, Wan M, Winslow MM, Staahl BT, Wu H, Aebersold R, Graef IA, Crabtree GR (2007) An essential switch in subunit composition of a chromatin remodeling complex during neural development. Neuron 55:201–215
Leuner B, Mendolia-Loffredo S, Kozorovitskiy Y, Samburg D, Gould E, Shors TJ (2004) Learning enhances the survival of new neurons beyond the time when the hippocampus is required for memory. J Neurosci 24:7477–7481

Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926
Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437:1370–1375
Liem KF, Tremml G, Jessell TM (1997) A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell 91:127–138
Liu Y, Helms AW, Johnson JE (2004) Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development 131:1017–1028
Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179–193
Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810
Lois C, Alvarez-Buylla A (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A 90:2074–2077
Lok J, Gupta P, Guo S, Kim WJ, Whalen MJ, van Leyen K, Lo EH (2007) Cell-cell signaling in the neurovascular unit. Neurochem Res 32:2032–2045
Lopez-Munoz F, Boya J, Alamo C (2006) Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramon y Cajal. Brain Res Bull 70:391–405
Louissaint A Jr, Rao S, Leventhal C, Goldman SA (2002) Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 34:945–960
Lukaszewicz A, Savatier P, Cortay V, Kennedy H, Dehay C (2002) Contrasting effects of basic fibroblast growth factor and neurotrophin 3 on cell cycle kinetics of mouse cortical stem cells. J Neurosci 22:6610–6622
Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G (2003) Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 39:937–950
Machon O, van den Bout CJ, Backman M, Kemler R, Krauss S (2003) Role of beta-catenin in the developing cortical and hippocampal neuroepithelium. Neuroscience 122:129–143
Martí E, Takada R, Bumcrot DA, Sasaki H, McMahon AP (1995) Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. Development 121:2537–2547
Martinez-Marcos A, Ubeda-Banon I, Deng L, Halpern M (2000) Neurogenesis in the vomeronasal epithelium of adult rats: evidence for different mechanisms for growth and neuronal turnover. J Neurobiol 44:423–435
Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, Fan G, Sun YE (2003) DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science 302:890–893
Mason I (2007) Initiation to end point: the multiple roles of fibroblast growth factors in neural development. Nat Rev Neurosci 8:583–596
McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP (1998) Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev 12:1438–1452
Megason SG, McMahon AP (2002) A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development 129:2087–2098
Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9:729–731
Mirzadeh Z, Merkle FT, Soriano-Navarro M, Garcia-Verdugo JM, Alvarez-Buylla A (2008) Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. Cell Stem Cell 3:265–278
Mission JP, Takahashi T, Caviness VS Jr (1991) Ontogeny of radial and other astroglial cells in murine cerebral cortex. Glia 4:138–148

Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T, Ogawa M (2004) Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development 131:3133–3145

Morin X, Jaouen F, Durbec P (2007) Control of planar divisions by the G-protein regulator LGN maintains progenitors in the chick neuroepithelium. Nat Neurosci 10:1440–1448

Morshead CM, van der Kooy D (1992) Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. J Neurosci 12:249–256

Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D (1994) Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13:1071–1082

Nakayama KI, Nakayama K (2006) Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer 6:369–381

Ninkovic J, Gotz M (2007) Signaling in adult neurogenesis: from stem cell niche to neuronal networks. Curr Opin Neurobiol 17:338–344

Noctor SC, Martínez-Cerdeño V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136–144

Noctor SC, Martínez-Cerdeño V, Kriegstein AR (2008) Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 508:28–44

Nottebohm F (1985) Neuronal replacement in adulthood. Ann N Y Acad Sci 457:143–161

Nusse R, Fuerer C, Ching W, Harnish K, Logan C, Zeng A, ten Berge D, Kalani Y (2008) Wnt signaling and stem cell control. Cold Spring Harb Symp Quant Biol 73:59–66

Nyfeler Y, Kirch RD, Mantei N, Leone DP, Radtke F, Suter U, Taylor V (2005) Jagged1 signals in the postnatal subventricular zone are required for neural stem cell self-renewal. EMBO J 24:3504–3515

Ochiai W, Nakatani S, Takahara T, Kainuma M, Masaoka M, Minobe S, Namihira M, Nakashima K, Sakakibara A, Ogawa M, Miyata T (2009) Periventricular notch activation and asymmetric Ngn2 and Tbr2 expression in pair-generated neocortical daughter cells. Mol Cell Neurosci 40:225–233

Ohnuma S, Harris WA (2003) Neurogenesis and the cell cycle. Neuron 40:199–208

Ohnuma S, Philpott A, Harris WA (2001) Cell cycle and cell fate in the nervous system. Curr Opin Neurobiol 11:66–73

Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J 18:2196–2207

Okano H (2002) Stem cell biology of the central nervous system. J Neurosci Res 69:698–707

Okano H, Sawamoto K (2008) Neural stem cells: involvement in adult neurogenesis and CNS repair. Philos Trans R Soc Lond B Biol Sci 363:2111–2122

Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99:247–257

Okano H, Sakaguchi M, Ohki K, Suzuki N, Sawamoto K (2007) Regeneration of the central nervous system using endogenous repair mechanisms. J Neurochem 102:1459–1465

Olson AK, Eadie BD, Ernst C, Christie BR (2006) Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. Hippocampus 16:250–260

Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M (2008) Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells 26:1663–1672

Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479–494

Panchision DM (2009) The role of oxygen in regulating neural stem cells in development and disease. J Cell Physiol 220:562–568

Panchision DM, Pickel JM, Studer L, Lee SH, Turner PA, Hazel TG, McKay RD (2001) Sequential actions of BMP receptors control neural precursor cell production and fate. Genes Dev 15:2094–2110

Parr BA, Shea MJ, Vassileva G, McMahon AP (1993) Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development 119:247–261

Parras CM, Schuurmans C, Scardigli R, Kim J, Anderson DJ, Guillemot F (2002) Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. Genes Dev 16:324–338

Perez SE, Rebelo S, Anderson DJ (1999) Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. Development 126:1715–1728

Pierani A, Brenner-Morton S, Chiang C, Jessell TM (1999) A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97:903–915

Pierani A, Moran-Rivard L, Sunshine MJ, Littman DR, Goulding M, Jessell TM (2001) Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron 29:367–384

Pilaz LJ, Patti D, Marcy G, Ollier E, Pfister S, Douglas RJ, Betizeau M, Gautier E, Cortay V, Doerflinger N, Kennedy H, Dehay C (2009) Forced G1-phase reduction alters mode of division, neuron number, and laminar phenotype in the cerebral cortex. Proc Natl Acad Sci U S A 21:21924

Pistollato F, Chen HL, Schwartz PH, Basso G, Panchision DM (2007) Oxygen tension controls the expansion of human CNS precursors and the generation of astrocytes and oligodendrocytes. Mol Cell Neurosci 35:424–435

Pollard SM, Wallbank R, Tomlinson S, Grotewold L, Smith A (2008) Fibroblast growth factor induces a neural stem cell phenotype in foetal forebrain progenitors and during embryonic stem cell differentiation. Mol Cell Neurosci 38:393–403

Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 28:69–80

Raballo R, Rhee J, Lyn-Cook R, Leckman JF, Schwartz ML, Vaccarino FM (2000) Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. J Neurosci 20:5012–5023

Ramon y Cajal S (1913) Degeneracion y regeneracion de los niervos. Imprenta de hijos de Nicolas Moya, Madrid

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710

Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1–13

Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565–4574

Ringe J, Kaps C, Burmester GR, Sittinger M (2002) Stem cells for regenerative medicine: advances in the engineering of tissues and organs. Naturwissenschaften 89:338–351

Robertson KD, Wolffe AP (2000) DNA methylation in health and disease. Nat Rev Genet 1:11–19

Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. Neuron 39:13–25

Rottach A, Leonhardt H, Spada F (2009) DNA methylation-mediated epigenetic control. J Cell Biochem 108:43–51

Sanai N, Berger MS, Garcia-Verdugo JM, Alvarez-Buylla A (2007) Comment on "Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension". Science 318:393; author reply 393

Sansom SN, Griffiths DS, Faedo A, Kleinjan DJ, Ruan Y, Smith J, van Heyningen V, Rubenstein JL, Livesey FJ (2009) The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet 5:e1000511

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proc Natl Acad Sci U S A 103:17501–17506

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153–7160
Seroogy KB, Gall CM, Lee DC, Kornblum HI (1995) Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. Brain Res 670:157–164
Shastri VP (2006) Future of regenerative medicine: challenges and hurdles. Artif Organs 30:828–834
Shen Q, Wang Y, Kokovay E, Lin G, Chuang SM, Goderie SK, Roysam B, Temple S (2008) Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. Cell Stem Cell 3:289–300
Shi Y, Massagué J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685–700
Shi Y, Sun G, Zhao C, Stewart R (2008) Neural stem cell self-renewal. Crit Rev Oncol Hematol 65:43–53
Shimogori T, Banuchi V, Ng HY, Strauss JB, Grove EA (2004) Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. Development 131:5639–5647
Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 96:5522–5527
Simpson TI, Price DJ (2002) Pax6; a pleiotropic player in development. Bioessays 24:1041–1051
Smirnova L, Gräfe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG (2005) Regulation of miRNA expression during neural cell specification. Eur J Neurosci 21:1469–1477
Stanfield BB, Trice JE (1988) Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections. Exp Brain Res 72:399–406
Steiner B, Wolf S, Kempermann G (2006) Adult neurogenesis and neurodegenerative disease. Regen Med 1:15–28
Storm EE, Garel S, Borello U, Hebert JM, Martinez S, McConnell SK, Martin GR, Rubenstein JL (2006) Dose-dependent functions of Fgf8 in regulating telencephalic patterning centers. Development 133:1831–1844
Stoykova A, Fritsch R, Walther C, Gruss P (1996) Forebrain patterning defects in Small eye mutant mice. Development 122:3453–3465
Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403:41–45
Suh MR, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, Cha KY, Chung HM, Yoon HS, Moon SY, Kim VN, Kim KS (2004) Human embryonic stem cells express a unique set of microRNAs. Dev Biol 270:488–498
Suh H, Deng W, Gage FH (2009) Signaling in adult neurogenesis. Annu Rev Cell Dev Biol 25:253–275
Sun G, Yu RT, Evans RM, Shi Y (2007) Orphan nuclear receptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation. Proc Natl Acad Sci U S A 104:15282–15287
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Takahashi T, Nowakowski RS, Caviness VS (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J Neurosci 15:6046–6057
Takizawa T, Nakashima K, Namihira M, Ochiai W, Uemura A, Yanagisawa M, Fujita N, Nakao M, Taga T (2001) DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 1:749–758
Tavazoie M, Van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, Garcia-Verdugo JM, Doetsch F (2008) A specialized vascular niche for adult neural stem cells. Cell Stem Cell 3:279–288
Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422–426
Toresson H, Potter SS, Campbell K (2000) Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development 127:4361–4371

Turner BM (2002) Cellular memory and the histone code. Cell 111:285–291
Ulloa F, Briscoe J (2007) Morphogens and the control of cell proliferation and patterning in the spinal cord. Cell Cycle 6:2640–2649
Vaccarino FM, Schwartz ML, Raballo R, Nilsen J, Rhee J, Zhou M, Doetschman T, Coffin JD, Wyland JJ, Hung YT (1999a) Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. Nat Neurosci 2:246–253
Vaccarino FM, Schwartz ML, Raballo R, Rhee J, Lyn-Cook R (1999b) Fibroblast growth factor signaling regulates growth and morphogenesis at multiple steps during brain development. Curr Top Dev Biol 46:179–200
van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030–1034
Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78:99–107
Visvanathan J, Lee S, Lee B, Lee JW, Lee SK (2007) The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. Genes Dev 21:744–749
Viti J, Gulacsi A, Lillien L (2003) Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. J Neurosci 23:5919–5927
Voigt T (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol 289:74–88
Wang Y, Yang J, Zheng H, Tomasek GJ, Zhang P, McKeever PE, Lee EY, Zhu Y (2009) Expression of mutant p53 proteins implicates a lineage relationship between neural stem cells and malignant astrocytic glioma in a murine model. Cancer Cell 15:514–526
Warren N, Caric D, Pratt T, Clausen JA, Asavaritikrai P, Mason JO, Hill RE, Price DJ (1999) The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. Cereb Cortex 9:627–635
Watanabe D, Suetake I, Tada T, Tajima S (2002) Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech Dev 118:187–190
Watanabe D, Uchiyama K, Hanaoka K (2006) Transition of mouse de novo methyltransferases expression from Dnmt3b to Dnmt3a during neural progenitor cell development. Neuroscience 142:727–737
Wenning GK, Odin P, Morrish P, Rehncrona S, Widner H, Brundin P, Rothwell JC, Brown R, Gustavii B, Hagell P, Jahanshahi M, Sawle G, Bjorklund A, Brooks DJ, Marsden CD, Quinn NP, Lindvall O (1997) Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. Ann Neurol 42:95–107
Wine-Lee L, Ahn KJ, Richardson RD, Mishina Y, Lyons KM, Crenshaw EB (2004) Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. Development 131:5393–5403
Wolpert L (1969) Positional information and the spatial pattern of cellular differentiation. J Theor Biol 25:1–47
Yoon K, Nery S, Rutlin ML, Radtke F, Fishell G, Gaiano N (2004) Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 24:9497–9506
Zechner D, Fujita Y, Hülsken J, Müller T, Walther I, Taketo MM, Crenshaw EB, Birchmeier W, Birchmeier C (2003) beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev Biol 258:406–418
Zhang ZG, Chopp M (2009) Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. Lancet Neurol 8:491–500
Zhang RL, Zhang ZG, Lu M, Wang Y, Yang JJ, Chopp M (2006) Reduction of the cell cycle length by decreasing G1 phase and cell cycle reentry expand neuronal progenitor cells in the subventricular zone of adult rat after stroke. J Cereb Blood Flow Metab 26:857–863
Zhang CL, Zou Y, He W, Gage FH, Evans RM (2008a) A role for adult TLX-positive neural stem cells in learning and behaviour. Nature 451:1004–1007

Zhang RL, Zhang ZG, Chopp M (2008b) Ischemic stroke and neurogenesis in the subventricular zone. Neuropharmacology 55:345–352
Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645–660
Zhu J, Zhou L, XingWu F (2006) Tracking neural stem cells in patients with brain trauma. N Engl J Med 355:2376–2378
Zuccato C, Cattaneo E (2009) Brain-derived neurotrophic factor in neurodegenerative diseases. Nat Rev Neurol 5:311–322

Chapter 8
Liver Stem Cells

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Abstract The liver is an essential organ for life, serving as the center for metabolism and playing various critical functions in controlling systemic homeostasis. Among multiple types of cells comprising the liver, hepatocytes and cholangiocytes are the two epithelial cell lineages in the organ, and commonly originate from hepatoblasts during organogenesis in developing embryos. Thus, hepatoblasts possess bi-lineage differentiation potential into hepatocytes and cholangiocytes, a phenotypic feature that can best distinguish and define liver stem cells. While the liver is considered not to rely on any resident stem cell population for their homeostatic maintenance, facultative stem/progenitor cells with bi-lineage differentiation potential, referred to as oval cells in rodents, may emerge under severe damage conditions and play critical roles in promoting the regenerative processes. However, the extent to which these putative stem/progenitor cells contribute to regeneration has been debated, as recent studies have shown that most regenerative responses are hepatocyte-derived, particularly in mice. Evidence for both scenarios will be discussed in later sections. Nevertheless, identification of specific markers has enabled researchers to isolate and characterize these fetal and adult stem/progenitor cell populations. *In vitro* culture systems as well as *in vivo* studies using animal models have allowed elucidation of detailed molecular mechanisms, including intercellular signaling webs and intracellular transcriptional regulatory networks, which coordinately regulate development, differentiation and behavior of these cells. Understanding the cellular and molecular basis of liver development and regeneration from the perspective of the embryonic and adult stem/progenitor cells should make invaluable contributions to future development of technologies to produce fully functional hepatocytes *in vitro* that are applicable for cell therapy and pharmaceutical screening.

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_8

Keywords Hepatocyte • Cholangiocyte • Hepatoblast • Oval cell • Liver regeneration

8.1 Introduction

The liver is the largest organ in the adult human, and accounts for approximately one fiftieth of the body weight. The mammalian liver plays multiple critical roles in maintaining vital activity of the organisms, including metabolism of amino acids, lipids, and carbohydrates, serum protein synthesis, detoxification of xenobiotic compounds, production and secretion of bile, immune regulation, and so forth. To achieve these complex biological functions, the liver possesses a sophisticated and well organized structure composed of several different types of cells (Fig. 8.1). Hepatocytes, also known as the liver parenchymal cells, account for 80 % of the total volume of the organ and serve as the primary cell type to execute the majority of the organ's functions. The other cell types, collectively identified as non-parenchymal cells (NPCs), include cholangiocytes (bile duct epithelial cells, or BECs), Kupffer cells, hepatic stellate cells, endothelial cells, coelomic epithelial cells (mesothelial cells), and several kinds of immune cells. While each of these cell types has its own embryonic origin (Asahina et al. 2011), hepatocytes and cholangiocytes, the two epithelial lineages in the organ, derive from a common precursor cell population, so-called hepatoblasts, in the developing liver (Lemaigre 2009; Tanaka et al. 2011; Tanimizu and Miyajima 2007; Zhao and Duncan 2005) (Fig. 8.2a). Thus, the term "liver stem cell" (or "hepatic stem cell") is most generally applied to represent this type of bi-potential progenitor cells that can differentiate to both hepatocytes and cholangiocytes. Hepatoblasts, however, are usually considered a cell population found only during the fetal period, and it is not clear whether and how these cells are related to the putative stem/progenitor cell populations in the adult liver. In other words, the capacity for self renewal of hepatoblasts *in vivo* remains undetermined. Thus, it would be safer to distinguish these cells as the fetal liver "stem/progenitor" cells, and we would like to adopt this description in this chapter.

In contrast to the situation in the developing liver, where hepatoblasts are fairly well established as the bipotential liver stem/progenitor cell, the one regarding the adult liver still has considerable controversy. In many other organs, such as the hematopoietic and epidermal systems and the small intestine, the tissue stem cells can be defined, and have indeed been isolated and/or anatomically located, as the cells that are responsible for normal tissue turnover. Thus, those stem cells, under the physiological condition, continue self-renewal while producing progeny that give rise to the mature cell types and eventually replace the aging cells in the organ to maintain homeostasis. In the liver, hepatocyte turnover occurs very slowly, and it is still unclear and under debate whether such a "stem cell" also exists and is actively involved in homeostatic maintenance of the organ.

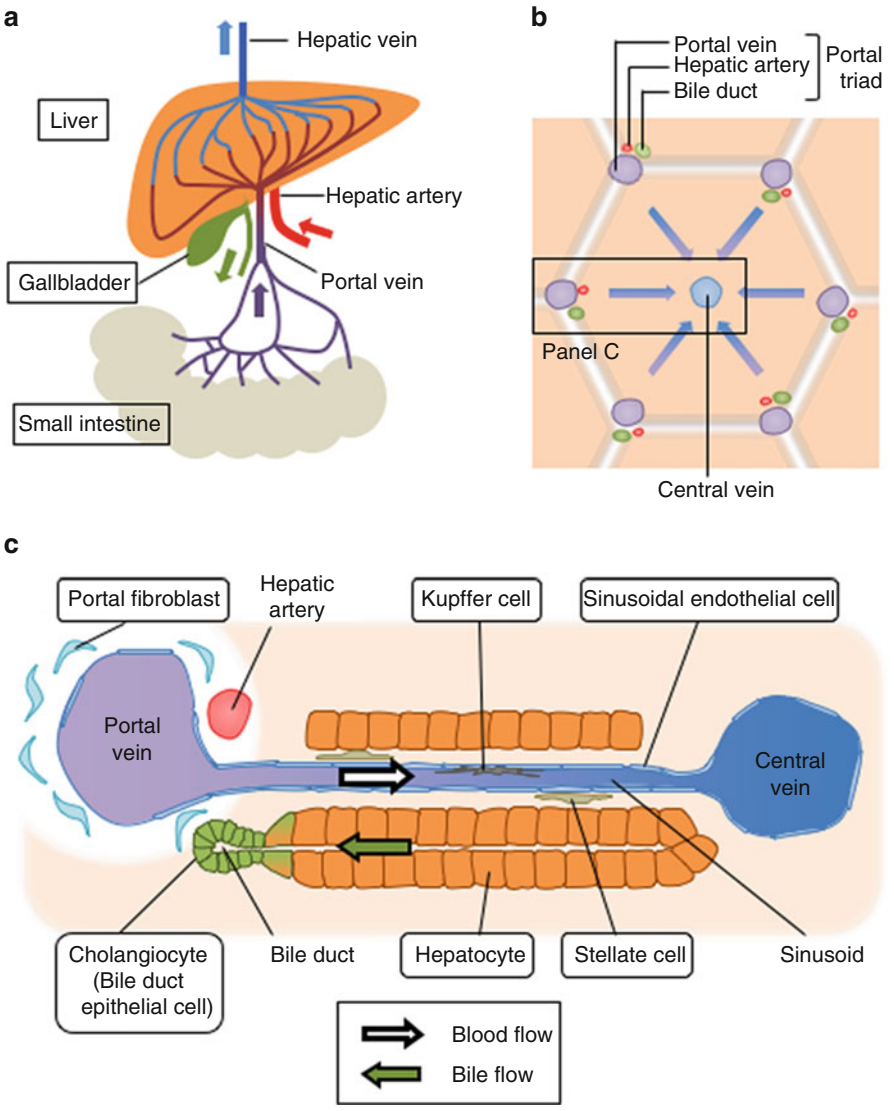

Fig. 8.1 The liver structure and its constituent cells. (**a**) The liver has a dual blood supply via the portal vein and the hepatic artery. The portal vein delivers the venous blood from the intestines as well as from the pancreas and spleen, while the hepatic artery supplies oxygen to the organ. As an exocrine gland, the liver excretes bile via the bile duct, where the gallbladder is associated as the reservoir, leading to the duodenum. (**b**) The liver is composed of the functional unit, also known as the liver lobule. Within the lobule, the arterial and portal venous bloods enter via the portal triad located at the vertices of this polygonal structure, mix, and flow through the parenchyma towards the central efferent vein. (**c**) Magnified view of the liver parenchyma, where cords of hepatocytes are lined by sinusoidal capillaries. Hepatocytes produce bile and excrete it into canaliculi that are linked to bile ducts in the portal triad

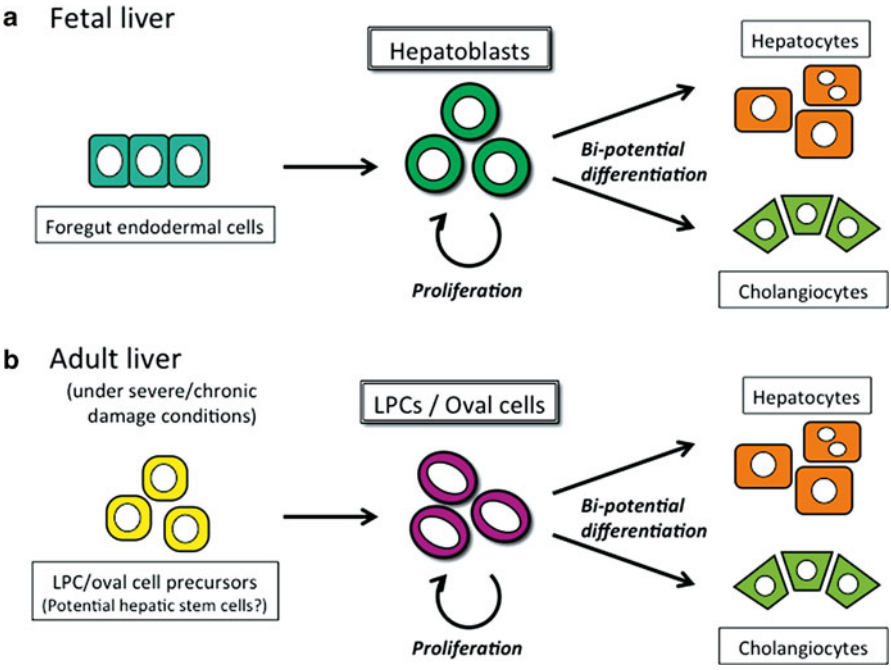

Fig. 8.2 Stem/progenitor cell populations in the fetal and adult livers. In the course of fetal liver development (**a**), hepatoblasts are derived from foregut endodermal cells, proliferate, and then undergo differentiation into two epithelial cell lineages, hepatocytes and cholangiocytes (bile duct epithelial cells). This bi-lineage differentiation potential is regarded as the hallmark of liver stem/progenitor cells. In the adult liver, regeneration can usually be achieved by replication of differentiated, mature cell populations (not shown here). Under severe/chronic liver damage conditions (**b**), however, facultative stem/progenitor cells, called LPCs or oval cells, emerge from hitherto unidentified precursor cells and expand. These cells also possess bi-lineage differentiation potential and are considered to contribute to the regeneration process

Nevertheless, apart from this relatively complicated situation regarding the *bona fide* stem cell in the adult liver, researchers in this field have been quite successful in identifying and characterizing several different classes of putative adult liver stem cells, which should be of significant importance particularly in view of therapeutic applications. Classification of these different adult liver stem cells will be briefly described in Sect. 8.2.2.

8.2 Derivation/Classification

8.2.1 Hepatoblasts

Among the three germ layers generated during gastrulation (i.e., ectoderm, mesoderm, and endoderm), the liver derives principally from the endoderm. The endoderm differentiates into the primitive gut, which in turn gives rise to the gastrointestinal

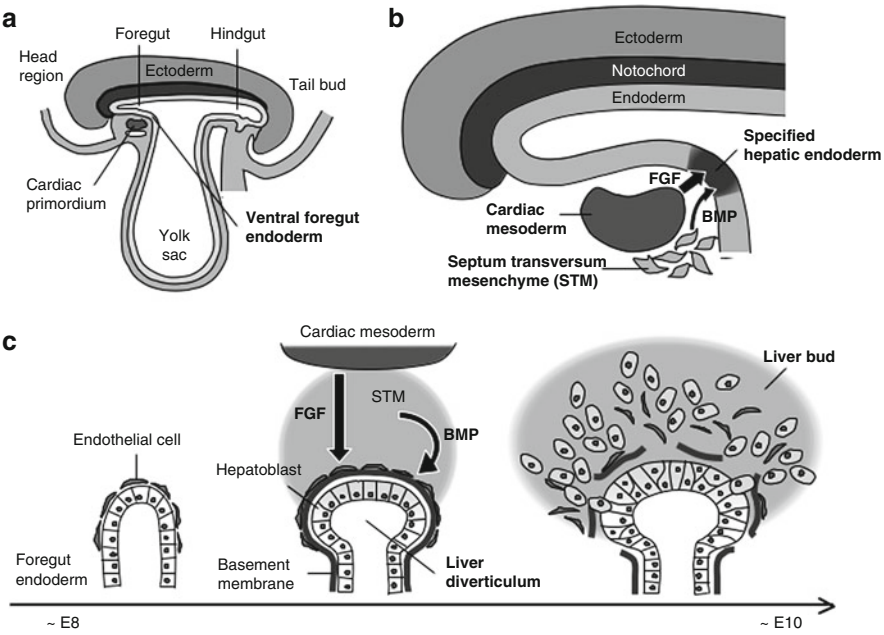

Fig. 8.3 Early stages in the liver development. At the beginning of the liver development, the ventral portion of the foregut endoderm is positioned close to the heart primordium (**a**), which provides the inductive FGF signal. This, together with the BMP signal from the adjacent septum transversum mesenchyme (STM), drives the liver developmental program, leading to specification of the hepatic endoderm (**b**). In the mouse embryo, this initial inductive step occurs at around E8, leading to formation of the hepatic endoderm consisting of hepatoblasts, the embryonic liver stem/progenitor cells. They subsequently undergo dynamic morphological changes and form the liver bud (**c**)

tract as well as various associated organs, including the thyroid, lung, pancreas, as well as liver. During the early stages of liver development, interaction between the endoderm and the adjacent mesoderm plays a key role in induction of the organ (Fig. 8.3).

The processes and mechanisms of mammalian liver development have been most extensively studied in the mouse embryos. Liver organogenesis begins at embryonic day (E) 8.0–8.5 in mice, which corresponds to approximately 3 weeks of gestation in humans. At this stage, the ventral foregut endoderm, a part of the endoderm from which the liver forms, faces the developing heart and receives inductive signals for the hepatic fate from the cardiac mesoderm. The fibroblast growth factor (FGF) family of secreted proteins has been shown to mediate the signaling for organogenesis (Jung et al. 1999). In addition, septum transversum mesenchyme (STM), a mesodermal tissue located adjacently to both of these organ primordia, also contributes to hepatic fate induction by providing another soluble factor, bone morphogenetic protein (BMP) (Rossi et al. 2001). Coordinated action of both FGF and BMP drives the liver developmental program with concomitant induction of several hepatic lineage-specific genes, such as *Albumin* and *Transthyretin*. This process is

called “hepatic specification” and leads to generation of hepatoblasts, the fetal liver stem/progenitor cells, initially lining up to form the hepatic endoderm.

Soon after the hepatic endoderm formation, hepatoblasts undergo dynamic changes in their morphology and localization, in a process that can be divided into three stages (Bort et al. 2006). In stage I (E8.5), along with the expression of liver-specific genes, the initially cuboidal hepatoblasts become columnar in shape, leading to the formation of a thickened epithelium. In stage II (E9.0–E9.5), the hepatoblasts further change their morphology to become a pseudo-stratified epithelium. A study using mice deficient for the hematopoietically expressed homeobox (Hhex, or Hex) gene has shown that this transcription factor is critical for this stage (Bort et al. 2006). In stage III (E9.5+), the basal lamina that has covered the epithelium breaks down and the hepatoblasts start to delaminate and then migrate into the surrounding stroma, the STM, to form the liver bud. This step is also controlled by the functions of homeobox transcription factors, prospero-related homeobox 1 (Prox1), hepatocyte nuclear factor (HNF) 6 (HNF6; also known as Onecut-1 or Oc1) and Onecut2 (Oc2) (Margagliotti et al. 2007; Sosa-Pineda et al. 2000).

After liver bud formation, hepatoblasts continuously proliferate throughout embryonic development. Proliferation and survival of hepatoblasts is known to be regulated by various extracellular signals, such as hepatocyte growth factor (HGF), transforming growth factor beta (TGFbeta), Wnt/beta-catenin, and Sonic hedgehog (Hirose et al. 2009; Micsenyi et al. 2004; Schmidt et al. 1995; Tanimizu and Miyajima 2007; Weinstein et al. 2001). These signals may act on hepatoblasts either in an autocrine fashion, or by being supplied from the surrounding mesenchymal cells. Endothelial cells also play a critical role in hepatoblast regulation, as mice lacking endothelial cells show a defect in liver bud outgrowth (Matsumoto et al. 2001). The molecular nature of the signal provided by endothelial cells still remains elusive.

8.2.2 Adult Liver Stem/Progenitor Cells

In the field of liver biology, the term “liver stem cells” (or “hepatic stem cells”) has been defined and used by researchers in several different ways. As exemplified by Grompe (2003), the definitions can include, but may not be limited to, the following:

(a) Cells responsible for normal tissue turnover
(b) Cells which give rise to regeneration after partial hepatectomy (PHx)
(c) Cells responsible for progenitor-dependent regeneration
(d) Transplantable liver repopulating cells
(e) Cells which result in hepatocyte and bile duct phenotype *in vitro*

As is the case with stem cells in several other tissues/organs, clonogenic potential (colony forming activity) *in vitro* and long-term label-retaining activity *in vivo* have also been utilized as hallmarks to identify putative liver stem cells. Notably,

these definitions are not mutually exclusive, and a given "liver stem cell" population may fulfill several of these criteria simultaneously. For each definition, stem cells are classified according to different and specific types of assays, either *in vitro* or *in vivo*.

Hepatocyte turnover under the physiological condition is relatively slow, which makes it quite difficult to investigate the cellular behavior in the course of homeostatic maintenance of the liver. Nevertheless, hepatocyte replacement does occur, and there must be a mechanism that ensures this tissue turnover. One of the long-standing models is the so-called "streaming liver hypothesis", where a "flow" of hepatocytes is assumed, just analogous to the well-appreciated crypt-to-villi movement of the intestinal epithelial cells. In this model, hepatocytes are newly formed in the periportal region and then gradually move, while undergoing lineage maturation, toward the central vein (Zajicek et al. 1985). Although appealing, much of the evidence accumulated so far argues against this hypothesis, and it is more favorably considered that the liver maintenance is achieved by simple division of the preexisting hepatocytes. Interestingly, a study in the human liver using mitochondrial mutations as a genetic marker identified that clonal patches of hepatocytes did emerge from the periportal regions and extended toward the central veins, supporting the presence of the hepatocyte flow as assumed by the streaming liver hypothesis (Fellous et al. 2009). Moreover, a genetic lineage-tracing study in mice provided striking evidence supporting the hypothesis. In those experiments, SRY-box containing gene 9 (Sox9)-CreERT2 knock-in mice were crossed with a reporter strain where Cre-mediated recombination enables permanent cell labeling and subsequent fate tracking, and cholangiocytes were subsequently specifically pulse-labeled in adulthood by tamoxifen-dependent transient Cre activation. While lineage-labeled cells were initially confined to bile ducts, they gradually spread out to hepatocytes from the periportal toward pericentral regions as time progressed and, after around 1 year, occupied the whole parenchyma nearly completely. The labeled cells also remained present in bile ducts, thereby indicating that the Sox9-expressing biliary cells can continuously supply mature hepatocytes for normal tissue turnover while possessing self-renewing activity as well (Furuyama et al. 2011). However, genetic lineage-tracing studies in mice by other groups, using a different Sox9-CreERT2 strain to label biliary cells or a Cre-expressing adeno-associated viral (AAV) vector to specifically label hepatocytes, have both provided rather conflicting results with the above study and thus strongly argue against the streaming liver hypothesis (Carpentier et al. 2011; Malato et al. 2011). Further studies are needed to solve this seeming discrepancy and elucidate the exact nature and the underlying mechanisms for physiological maintenance of the liver.

The characteristic feature of the liver is its unique and remarkably high capacity to regenerate upon various injuries, such as those caused by partial hepatectomy or toxic insults. In rodent models, for example, after 70 % PHx, the liver can completely recover its initial volume and function within a week or so. During this recovery process, hepatocytes, as well as cholangiocytes, in the remaining liver undergo a few cycles of cell division to sufficiently restore the lost tissue (Michalopoulos and DeFrances 1997). Thus, liver regeneration can usually be achieved by differentiated

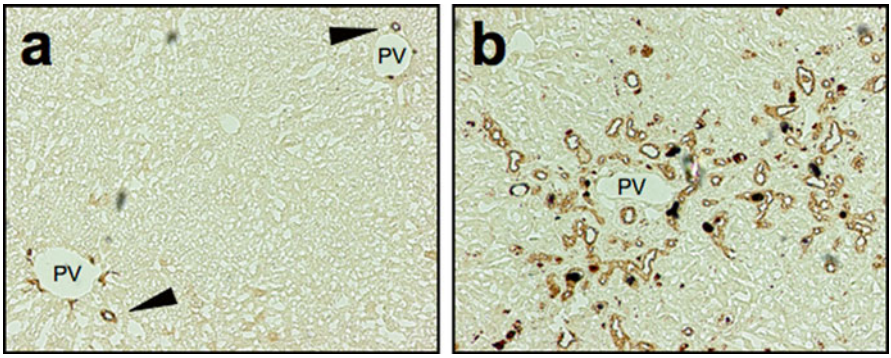

Fig. 8.4 Emergence of LPCs in a mouse model of chronic liver injury. Sections of the liver prepared from a normal mouse (**a**) and a mouse fed DDC diet for 8 weeks (**b**) were immunostained with anti-CK19 antibody. In the liver of the mouse fed DDC diet, $CK19^{+}$ LPCs emerge from the periportal area, forming duct-like structures (**b**; *brown signals*). Note that CK19 marks cholangiocytes comprising bile ducts in the normal liver (**a**; *arrowheads*). *PV* portal vein

postmitotic hepatocytes that remain uninjured, without necessitating an involvement of stem/progenitor cell populations. Interestingly, the immediate regenerative response leading to restoration of liver mass has been shown to be the result of hepatocyte hypertrophy rather than proliferation (Miyaoka et al. 2012). In mice, hypertrophy of hepatocytes appears to be sufficient to regenerate the liver after 30 % PHx. Within 7 days post-PHx, hepatocytes had increased their size by 1.4-fold to restore liver mass, with negligible contribution from proliferation. However, regeneration from more drastic reduction of liver mass after 70 % hepatectomy involved an initial hypertrophic response followed by compensatory proliferation from existing hepatocytes. This suggests that hepatocytes themselves are playing a major role in liver regeneration following injury induced by PHx.

When the liver is subjected to severe and/or chronic damage, however, hepatocyte proliferation is suppressed. It is under this condition when the facultative stem/progenitor cells are known to emerge and contribute to the liver regeneration process. Those stem/progenitor cells, often referred to as oval cells in rodent models, are characterized by their potential to proliferate as well as to differentiate into both hepatocytes and cholangiocytes, the two epithelial lineages in the liver (Duncan et al. 2009; Grompe 2003; Matthews and Yeoh 2005; Newsome et al. 2004; Tanaka et al. 2011; Tanimizu and Miyajima 2007) (Fig. 8.2b). The most popular model to induce oval cells is the 2-acetylaminofluorene (2-AAF)/PHx system in rats, where hepatocyte proliferation is blocked by 2-AAF prior to PHx. This model, however, is not applicable to induce oval cells in mice. Other procedures, such as the administration of a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-containing diet (Preisegger et al. 1999) and a choline-deficient ethionine-supplemented diet (CDE) (Akhurst et al. 2001) have been established and used in mice, as well as in rats (Fig. 8.4). Notably, most of the experimental procedures used to induce oval cell emergence and proliferation in the rodent liver eventually lead to tumorigenesis.

Although the term "oval cells" are used specifically in rodents, cells with similar characteristics have also been reported in various human liver diseases, such as chronic viral hepatitis, alcoholic liver disease (ALD), and nonalcoholic fatty liver disease (NAFLD), and are also implicated in tumorigenesis (Fausto 2004; Lee et al. 2006; Roskams et al. 2003). In humans, these cells are usually referred to as "hepatic progenitor cells" or "intermediate hepatobiliary cells". The use of these different terms seems to have caused substantial confusion in the field. In addition, it has become more and more recognized that the disease etiologies and as well as the phenotypes of the induced cells are not necessarily the same among various injury conditions and experimental settings. In this chapter, the term "LPC (liver progenitor cell)" is used hereafter to broadly describe all the various disease-activated, putative stem/progenitor cell populations that have been observed in adult livers regardless of species or injury model. It should be emphasized that the definition of LPCs here is based solely on their immunological (e.g., expression of BEC-related markers such as cytokeratin [CK] 19) and histological (e.g., ectopic emergence in the parenchymal region) phenotypes, but not on their functional characteristics in terms of differentiation potentials and/or contribution to liver regeneration.

While LPCs are well known to emerge from the periportal area, the cellular origin of these cells is still not clarified (Fig. 8.5). Ever since their initial characterization, phenotypic resemblance between oval cells/LPCs and BECs has suggested that putative progenitors for these cells originate from the biliary tree. The fact that most of the molecular markers for LPCs are also expressed in cholangiocytes supports this notion. In a recently published study, a novel method for three-dimensional (3D) imaging of the biliary tree has been used to demonstrate that the emergence and expansion of LPCs actually represent expansion of the biliary tree upon chronic liver injury (Kaneko et al. 2015). Thus, injection of ink into the common bile duct allowed visualization of the biliary tree architecture in the mouse liver. Diverse patterns of biliary tree remodeling reflect the liver's robust response to different types of damage induced by toxins such as DDC, CDE, carbon tetrachloride (CCl_4) and thioacetamide (TAA). In general, extension of the biliary tree appears to be directed to the area of injury in a continuous structure, and suggests an intimate correlation between the LPC population and the bile ducts. Further evidence exists supporting the ability of the biliary network to contribute to liver regeneration. In zebrafish models, hepatocytes were selectively destroyed after administration of metronidazole using hepatocyte-specific bacterial nitroreductase transgenic (Tg) lines (Choi et al. 2014; He et al. 2014). This induced proliferation of biliary epithelial cells and subsequent conversion into hepatocytes, which were then able to repopulate the liver. Although this is not a common scenario in the mammalian liver, it does provide evidence of the potential for BEC mediated liver regeneration in severe liver damage.

It is not clear, however, whether most if not all cholangiocytes can equally or similarly behave as progenitors for LPCs, or if there is a specialized "cell-of-origin for LPCs" located somewhere among the cholangiocytes. Potentially as an extension of the latter scenario is the model that the canal of Hering, a structure where interlobular bile ducts and hepatocytes are connected, is the origin of LPCs, as has been

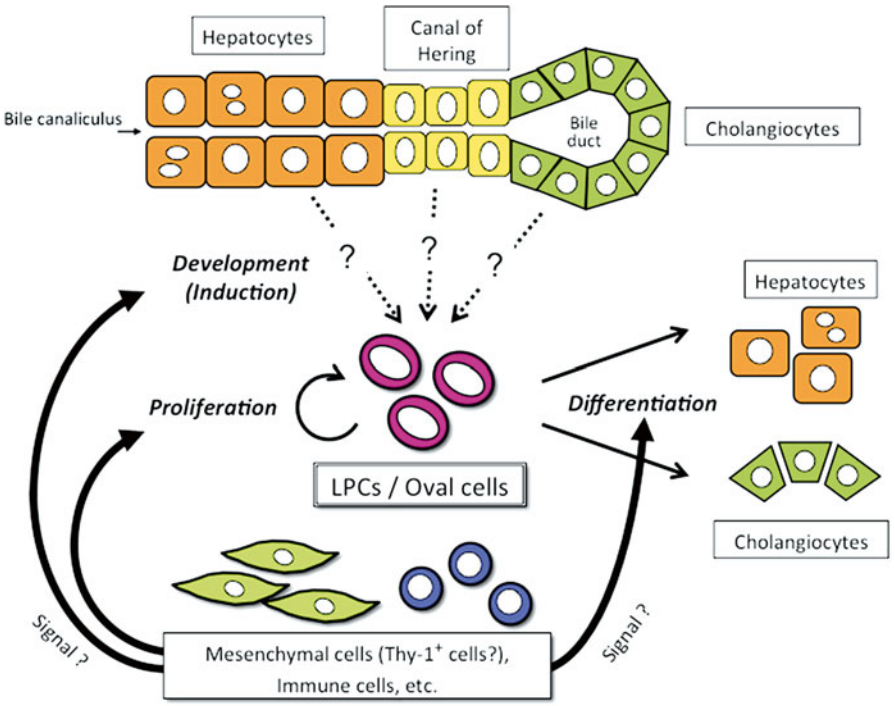

Fig. 8.5 Relationship among cells involved in the LPC response. Upon severe/chronic liver damages, LPCs/oval cells emerge from the periportal region. The exact origin of these cells has not yet been identified, but is supposed to be the canals of Hering, cholangiocytes, or hepatocytes. Together with LPCs/oval cells, several types of mesenchymal cells as well as immune cells accumulate in the injured liver and are often observed surrounding them. These cells are likely to modulate induction, proliferation, migration, and/or differentiation of LPCs/oval cells by means of various signaling mechanisms including direct cell-cell interaction, secretion of soluble factors (cytokines), and deposition of extracellular matrices, thereby playing key roles in regulation of the LPC response

suggested for rat oval cells (Paku et al. 2001). Given its anatomical location in between cholangiocytes and hepatocytes, it appears reasonable to assume that this structure may serve as a niche for putative stem cells for these two cell lineages. Unfortunately, direct proof for this model is hampered by a lack of specific marker for cells constituting the canal of Hering. Identification of such a molecule and a subsequent genetic lineage-tracing study should help clarifying this issue.

Another likely source of progenitor cells is the mature hepatocytes (Nagahama et al. 2014; Sekiya and Suzuki 2014; Tarlow et al. 2014b; Yanger et al. 2013). As an example, recent work published by Tarlow et al. (2014b) has clearly shown that hepatocytes are able to convert to a duct-like phenotype upon DDC diet induced injury in mice. The livers of $Fah^{-/-}$ mice, a model for tyrosinemia type I, were repopulated with genetically labeled hepatocytes. After 6 weeks of DDC-induced injury, the authors detected donor hepatocyte-derived labeled cells (hepPDs) which were

also positive for classical biliary ductal markers. However, these cells are distinct from host-derived biliary epithelial cells (bilPDs) and retained expression of hepatocyte-specific genes, albeit at low levels. The hepPDs were functionally different to bilPDs, as they were incapable of organoid formation *in vitro*. Transplantation of human hepatocytes into $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ (FRG) mice, followed by subsequent DDC feeding induced formation of duct-like cells of donor origin, which were shown to express both hepatic and biliary markers. This suggests that hepatocytes themselves can be the origin for bipotential LPCs in chronic liver injury and that there exists considerable heterogeneity among LPCs even in a particular injury setting. In another study, the same group showed that Sox9 positive ductal cells did not contribute to hepatocyte formation *in vivo* (Tarlow et al. 2014a). In this instance, labeling of Sox9 positive progenitors by tamoxifen mediated Cre recombinase expression followed by various types of liver injuries revealed that the resulting progeny cells were invariably biliary cells. $Sox9^+$ cells may contribute to the LPC activation, but this in turn did not lead to hepatocyte differentiation.

Not surprisingly, emergence and expansion of LPCs upon liver injury is not an autonomous process within these cells but involves various other types of cells, which interact either directly or indirectly with LPCs, and also possibly with their putative precursor cells. Thus, together they shape the entire phenomenon often termed as "LPC response (oval cell response)". Mesenchymal cells, such as stellate cells, have long been suggested to physically interact with oval cells/LPCs and exert some signals on them. It has been shown that a population of mesenchymal cells expressing thymus cell antigen 1 (Thy1), which is distinct from stellate cells or myofibroblasts, reside in close proximity to oval cells in rat liver (Yovchev et al. 2009). Further characterization of this unique population may provide a clue to understand the nature of signals controlling oval cell/LPC behaviors. Indeed, a recent study in mice has elucidated a molecular basis for interaction between these cells, showing that fibroblast growth factor 7 (FGF7) is a key signal for inducing LPC expansion in DDC injured mouse livers (Takase et al. 2013). FGF7 signaling originated from the $Thy1^+$ cell population localized near LPCs in the DDC injured liver. The significant expression of fibroblast growth factor 7 (FGF7) in $Thy1^+$ cells and correspondingly specific expression of the FGF7 receptor FGFR2b in LPCs of DDC injured livers suggests that there is a strong correlation between $Thy1^+$ cell-mediated FGF7 signaling and LPC expansion.

Chronic injury conditions in the liver are usually associated with induction of inflammation, and the role of lymphocytes and inflammatory responses have also been suggested (Knight et al. 2007; Strick-Marchand et al. 2008). In accordance with this notion, several inflammatory cytokines, such as tumor necrosis factor (TNF)-alpha and interferon-gamma, have been shown to modulate LPC response, although their modes of action are not fully clarified. A cytokine well known to be involved in LPC regulation is TNF-like weak inducer of apoptosis (TWEAK); transgenic mice overexpressing TWEAK in the liver exhibited periportal LPC hyperplasia, while DDC diet-induced oval cell expansion was significantly reduced in mice lacking the TWEAK receptor Fn14, as well as in wild-type mice administrated with a blocking anti-TWEAK monoclonal antibody (Jakubowski et al. 2005).

As a signal related to LPC response, several studies have identified activation of the canonical Wnt/beta-catenin pathway in LPCs (Apte et al. 2008; Hu et al. 2007; Itoh et al. 2009; Yang et al. 2008). The Wnt/beta-catenin pathway is well known to be involved in stem cell regulation in various organs and tissues, and further characterization of the role of this pathway in oval cells, including its relevant target genes and interaction with other signaling pathways, has been investigated. In one instance, Wnt signaling was shown to be crucial for LPC differentiation to the hepatocyte lineage (Boulter et al. 2012). To demonstrate the importance of this pathway, stabilization of beta-catenin was achieved in mice by conditional deletion of exon3 to render it resistant to degradation. This was sufficient to activate the canonical Wnt pathway, leading to conversion of the progenitor cell population to a hepatocyte phenotype. It was hypothesized that Wnt signaling was derived from liver macrophages. Selective deletion of F4/80 positive cells within the liver by liposomal clodronate administration caused formation of progenitor-derived ductules in the liver. The biliary route seems to be the default pathway for progenitor cells, which supports the notion of a close relationship between LPCs and the biliary network. In this study, Notch signaling was shown to be vital during biliary regeneration, with increased expression of the Notch pathway ligand Jagged1 during the ductular reaction. The Wnt and Notch signaling pathways are crucial for proper liver development and will be discussed in later sections. Similarly, a study which used an ethanol induced fibrotic zebrafish model has shown that antagonistic interaction between Wnt and Notch signaling during fibrosis may induce hepatocyte neogenesis from the hepatic stem cell compartment (Huang et al. 2014a). Wnt agonists were shown to induce Numb, a membrane associated protein that prevents Notch signaling. As previously discussed, Notch signaling has been shown to be vital for biliary development and maintenance.

Another study has shown that despite inactivating Notch signaling and activating Wnt/beta-catenin signaling in a lineage tracing model, LPCs could not be directed towards the hepatocyte fate (Jors et al. 2015). Notch inactivation did not affect the biliary compartment under physiological conditions, and though it did impair the ductular reaction after DDC administration, there was no evidence of LPCs converting to hepatocytes. Rather than acting as a source of progenitor cells for producing new hepatocytes, the authors suggest that the role of the ductular reaction is to provide an environment which encourages existing hepatocytes to proliferate. This again raises the argument that ductular cells are limited in their plasticity and are different to truly bipotential stem/progenitor cells.

8.3 Characteristics/Properties

8.3.1 Hepatoblasts

In order to characterize a particular cell population of interest, the cell sorting method using antibodies against specific surface markers is a powerful tool, as cells can be viably isolated and thus can be subjected to *in vitro* culture and/or *in vivo*

transplantation experiments. In the last decade, much effort has been made to identify such specific cell surface antigens expressed on fetal hepatoblasts, leading to successful identification of several markers as well as establishment of protocols to isolate and culture these cells.

Delta-like 1 homolog (Dlk1; also known as Pref-1 or fetal antigen 1) was initially identified as a marker for mouse hepatoblasts, and was later shown to be useful for enriching and purifying rat fetal liver progenitor cells with liver repopulating activity (Oertel et al. 2008; Tanimizu et al. 2004). DLK1 is also known to be expressed in human fetal liver (Floridon et al. 2000). In mouse embryos, Dlk1 expression in the liver is initially observed around E9.0 in the developing liver bud and is maintained at a high level until E16, which then declines significantly and disappears at the neonatal and adult stages. $Dlk1^+$ cells isolated from E14.5 livers expressed albumin and formed colonies composed of hetpatocyte ($Albumin^+$) and cholangiocyte ($CK19^+$) lineages *in vitro*. Moreover, 7 % of the colony-forming $Dlk1^+$ cells formed large colonies containing more than 100 cells during 5 days of culture, thus indicating that Dlk1 serves as a useful marker to enrich for highly proliferative, bipotential hepatoblasts from fetal liver.

E-cadherin is also widely used as a fetal hepatoblast marker (Nitou et al. 2002), although its expression is not necessarily limited to these cells but persists even in the differentiated epithelial lineages. Using a specific monoclonal antibody against E-cadherin, hepatoblasts can be isolated form E12.5 mouse liver with high purity and efficiency. Other hepatoblast markers include Liv-2 in the mouse, whose antigen has not yet been molecularly identified (Watanabe et al. 2002).

In addition to these relatively simple methods for isolation of hepatoblasts based on the expression of single positive selection markers, several other groups have established protocols employing combinations of multiple markers, sometimes including those for negative selection. In the rat system, the $RT1A1^-$ $OX18^{low}$ $ICAM1^+$ fraction of E13 fetal liver has been shown to contain hepatoblasts (Kubota and Reid 2000). Suzuki et al. designated as "hepatic colony-forming unit in culture (H-CFU-C)" a putative self-renewing stem cell population in the developing mouse liver. Thus, based on an *in vitro* single cell-based assay of sorted cells, clonogenic cells capable of both self-renewal and multilineage differentiation were identified. They separated fetal liver cells based on expression of several markers including alpha6- and beta1-integrin subunits (CD49f and CD29, respectively) and demonstrated that the $CD45^-$ $Ter119^-$ $c\text{-}Kit^-$ $CD29^+$ $CD49f^+$ and $CD45^-$ $Ter119^-$ $c\text{-}Kit^-$ $c\text{-}Met^+$ $CD49f^{+/low}$ fractions of E13.5 mouse liver contained the H-CFU-C activity (Suzuki et al. 2000, 2002). Recently, CD13 (aminopeptidase N) has been identified as a surface marker expressed on a subset of the $Dlk1^+$ hepatoblasts. Colony formation assays have revealed that the $CD13^+$ fraction contains an enriched population of cells with clonogenic liver stem/progenitor activity, compared with the $Dlk1^+$ fraction (Kakinuma et al. 2009).

Using a combination of surface markers, the phenotypic transition of hepatoblasts during the course of mouse fetal liver development has been elucidated (Tanaka et al. 2009). Thus, upon liver bud formation at approximately E9, hepatoblasts expressing both Dlk1 and epithelial cell adhesion molecule (EpCAM; also known

as CD326, Tacstd1, or Trop1), a known marker for cholangiocytes and LPCs (see below), emerge from EpCAM$^+$ Dlk1$^-$ foregut endodermal cells. The EpCAM$^+$ Dlk1$^+$ cells contain highly proliferative hepatoblasts at E11.5, and thereafter undergo dramatic reduction in expression of EpCAM concomitantly with loss of proliferative potential. At around E16.5, EpCAM expression is upregulated in ductal plates around the portal vein, while absent in immature hepatocytes.

8.3.2 Adult Liver Stem/Progenitor Cells

Oval cells were initially described by Farber, using a rat model of liver carcinogenesis, as "small oval cells about the ducts and vessels in the portal areas" having "scanty, lightly basophilic cytoplasm and pale blue-staining nuclei (by hematoxylin and eosin stain)" (Farber 1956). Since then, many studies have further characterized these cells and have established them as facultative liver stem/progenitor cells in adult livers that are likely to play a relevant role in liver regeneration from various types of injuries (Duncan et al. 2009; Grompe 2003; Matthews and Yeoh 2005; Newsome et al. 2004; Tanaka et al. 2011; Tanimizu and Miyajima 2007). Thus, oval cells/LPCs are considered to be capable of differentiating into two hepatic epithelial lineages, i.e., hepatocyte and cholangiocyte. In relation to this notion, oval cells/LPCs express both hepatocyte (Albumin) and cholangiocyte (CK19) markers. The immature hepatocyte marker alpha-fetoprotein (Afp) is known to be expressed in oval cells in rats, but not in LPCs in mice (Jelnes et al. 2007). Similarly, expression of the hepatoblast marker Dlk1 has been shown in a subpopulation of rat oval cells, but is not found in mouse LPCs (Jelnes et al. 2007; Jensen et al. 2004; Tanimizu et al. 2004). There are several monoclonal antibodies that have long been used as "golden standards" to recognize oval cell/LPC markers, such as OV-1 and OV-6 in rats (Dunsford and Sell 1989) and A6 in mice (Engelhardt et al. 1990). OV-1 antibody reacts with an unknown antigen expressed on the surface of oval cells and thus can be used to isolate these cells, while OV-6 antibody recognizes a common epitope in the cytoskeleton components CK14 and CK19 (Bisgaard et al. 1993). Unfortunately, the A6 antibody used for mouse studies recognizes some intracellular antigen and thus is not suitable to be used for sorting of viable LPCs.

Similar to the situation with fetal liver hepatoblasts, much effort has been made in recent years to explore cell surface molecules that can be used to identify and isolate oval cells/LPCs. This has led to the identification of EpCAM and CD133 (also known as prominin 1) as novel oval cell/LPC markers in both mice and rats (Okabe et al. 2009; Rountree et al. 2007; Suzuki et al. 2008b; Yovchev et al. 2007). The oncofetal protein glypican-3 has also been documented as a rat oval cell marker (Grozdanov et al. 2006). Notably, however, these molecules, as well as the OC-1/OC-6 and A6 antigens, are all expressed also in cholangiocytes in the normal liver. This fact strongly implies a close relationship between cholangiocytes and oval cells/LPCs as mentioned earlier, with the former possibly being an origin of the latter.

Interestingly, Trop2 (Tacstd2), a transmembrane molecule that is structurally similar to EpCAM, has been found to be expressed exclusively in LPCs in the injured liver, but not in cholangiocytes in the normal liver (Okabe et al. 2009). Thus, Trop2 may serve as a genuine "LPC marker" and would be advantageous for further characterization of LPCs. Similarly, the transcription factor Foxl1 has been identified as another potential LPC-specific marker (Sackett et al. 2009). Although this molecule is not a cell-surface antigen, a Tg mouse line expressing Cre recombinase under the control of the Foxl1 promoter has been generated. Thus, a lineage tracing study using the Foxl1-Cre Tg mice suggested that both hepatocytes and cholangiocytes were found as descendants of Foxl1^{+} LPCs. This does not necessarily indicate that single oval cells can clonally differentiate into these two lineages, but strongly supports the notion that oval cells are bipotential progenitors for hepatocytes and cholangiocytes. In a more recent study, the Foxl1-Cre; Rosa$^{YFP/iDTR}$ mice were generated for use in a study where Foxl1 expressing cells could be traced by Cre-mediated recombination and YFP expression and subsequently deleted by administration of diphtheria toxin (Shin et al. 2015). The authors showed that Foxl1-expressing progenitors were the origin of newly formed cholangiocytes and hepatocyte after injury induced by the CDE diet, and that the extent of conversion was dependent on the severity of liver injury. These cells contributed in ameliorating hepatic steatosis, and selective ablation of the Foxl1 progenitor derived cells with diphtheria toxin was shown to prolong the disease state. In contrast, DDC diet-injured livers showed Foxl1 progenitor contribution to the formation of cholangiocytes only. These cholangiocytes do not participate in the regenerative response. It should be noted that there have not been extensive studies to compare between the progenitor cell populations in recent reports by many different groups, so there are conflicting results regarding the extent to which the stem/progenitor cell compartment contributes to liver regeneration.

A panel of surface reactive monoclonal antibodies has been established, including MIC1-1C3, that can each detect different populations of ductal and periductal cells in the mouse LPC response (Dorrell et al. 2008). Intriguingly, some of them seem to label cell populations that are apparently enriched or reside specifically in the LPC-induced livers, with little or no reactivity shown in the normal liver. Identification of the corresponding antigen molecules, as well as further characterization of these cell populations, should increase our understanding of the mechanisms of the LPC response at the cellular and molecular levels.

Using flow cytometry-based cell separation methods in combination with the aforementioned cell surface markers, LPCs can be viably isolated and subjected to *in vitro* culture to evaluate their proliferation and differentiation potentials. Studies based on the expression of EpCAM or CD133, and also of MIC1-1C3 or Foxl1-Cre-mediated fluorescent reporter, have consistently demonstrated that LPCs isolated from injured livers proliferate to form colonies *in vitro* in the presence of certain combinations of growth factors, and the clonally expanded cells are capable of differentiating into both hepatocyte and cholangiocyte lineages under appropriate culture conditions (Dorrell et al. 2011; Okabe et al. 2009; Shin et al. 2011; Suzuki et al. 2008b). These results strongly suggest that LPCs indeed possess clonal bi-lineage

differentiation potential, at least *in vitro*, a notion which needs to be evaluated using *in vivo* experimental systems as well. As most of the LPC antigens including EpCAM and CD133 are also expressed in cholangiocytes under uninjured conditions, the cells positive for these markers were also isolated from normal adult livers and similarly subjected to *in vitro* culture experiments (Kamiya et al. 2009; Okabe et al. 2009; Suzuki et al. 2008b). Interestingly, both EpCAM$^+$ cells and CD133$^+$ cells isolated from the normal liver also formed colonies and underwent differentiation into hepatocytes and cholangiocytes. Essentially the same results were also obtained with MIC1-1C3 (Dorrell et al. 2011). Thus, the normal adult liver harbors "potential hepatic stem cells", which can be defined as those with clonogenicity and bi-lineage differentiation potential *in vitro*, similar to H-CFU-C in the embryonic liver. Notably, EpCAM$^+$ cells isolated from human postnatal livers, as well as fetal livers, have also been found to contain closely related hepatic stem cells (hHpSCs) that can be defined *in vitro* (Schmelzer et al. 2007). The exact location and characteristics of these potential hepatic stem cell populations *in vivo*, as well as their possible contribution to homeostasis and/or regenerative process of the liver, remain to be elucidated. In particular, it is tempting to speculate that these cells may serve as the precursors for LPCs, which needs to be addressed in future studies.

More recently, Lgr5, a well-established marker for tissue stem cells in the intestine and many other organs, has also been demonstrated to be expressed specifically in damage-activated LPCs in the mouse liver (Huch et al. 2013). Viably isolated Lgr5$^+$ cells can be clonally expanded *in vitro* as transplantable organoids in a 3D culture system, and those organoids possess bi-lineage differentiation potential and retain many characteristics of the original epithelial architecture. The relationship between Lgr5$^+$ cells and those expressing the common LPC markers such as CK19 and EpCAM, however, has not been clearly determined. Nevertheless, through optimization of this culture condition, phenotypically and functionally equivalent epithelial organoids can also be formed from BECs in the human liver (Huch et al. 2015). This allows clonal long-term expansion of primary human adult liver cells with the stem cell property and, intriguingly, the expanded cells have been shown to be highly stable at the chromosome and structural level, thereby paving a way for establishing a novel platform for therapeutic applications and disease modeling.

8.4 Differentiation Capacity and Their Precursors

As mentioned in the preceding sections, the characteristic feature of the liver stem/progenitor cells is their potential to differentiate into two lineages, i.e., hepatocytes and cholangiocytes. In addition to these two hepatic cell lineages, much evidence has accumulated supporting that the liver stem/progenitor cells are also capable of differentiating into pancreatic and other cell lineages both *in vitro* and *in vivo* under appropriate experimental settings. So far, circumstantial understanding of the mechanisms of liver stem/progenitor cell differentiation has been accomplished with

regard to hepatoblasts in the developing liver, and herein we will focus mostly on this issue. Although oval cells/LPCs have also been shown to possess differentiation capacity to hepatocytes, cholangiocytes and other cell types, the underlying mechanisms have not been fully characterized.

8.4.1 Differentiation into Hepatocytes

By definition, hepatoblasts undergo during their development a fate decision between the hepatocyte and cholangiocyte lineages. The molecular basis of this hepato-biliary lineage decision still remains largely unknown. Several molecules have been implicated in differentiation of hepatoblasts into the cholangiocyte lineage, which will be discussed in the next section.

While the adult liver performs various metabolic activities, the fetal liver lacks such functions and instead serves as a hematopoietic organ. Around E10 in the mouse embryo, hematopoietic stem cells migrate into the fetal liver from the aorta-gonad-mesonephros region and the placenta, and expand their population tremendously in the microenvironment provided by the fetal liver till birth. During this period of time, hematopoietic cells enhance differentiation of hepatoblasts into hepatocytes by producing cytokines (Kinoshita et al. 1999). As hematopoiesis switches from the fetal liver to the bone marrow, liver development progresses so that the organ becomes a center for metabolism.

Several *in vitro* primary culture systems for fetal liver cells, and more specifically for sorted hepatoblasts, have been established and extensively used to characterize the cellular and molecular mechanisms of hepatocyte differentiation. In many cases, oncostatin M (OSM), one of the interleukin 6-family cytokines, shows potent activity for inducing differentiation of hepatoblasts and immature hepatocytes to functional hepatocytes, as evidenced by expression of various hepatocyte-specific marker genes and acquisition of metabolic functions such as cytosolic glycogen accumulation and ammonia clearance from the culture medium (Kamiya et al. 1999). OSM transduces signals through a specific receptor complex containing the gp130 subunit, and the livers of mice lacking gp130 show defects in functional differentiation of hepatocytes (Kamiya et al. 1999). As OSM receptor-deficient mice exhibit no obvious anomaly in the liver development, other cytokines may play a similar or redundant role (Tanaka et al. 2003). In addition to the OSM signals, HGF, extracellular matrices (ECMs), and cell-to-cell contacts have also been implicated in stimulating hepatocyte differentiation (Kamiya et al. 2002; Kojima et al. 2000; Suzuki et al. 2003). On the other hand, TNF-alpha has been shown to antagonize the differentiation-promoting activities of OSM and control the timing of hepatocyte maturation (Kamiya and Gonzalez 2004). Thus, TNF-alpha expression is detected in the liver until perinatal stages, and then decreases after birth. Concomitantly with this transition, hepatocytes are relieved from the inhibitory effect of TNF-alpha and then strongly induced to acquire mature metabolic functions.

In addition to these extracellular signals, hepatocyte differentiation and maturation are regulated by cell-intrinsic machineries involving various transcription factors. A set of transcription factors, such as HNF1alpha, HNF4, and CCAAT/enhancer binding protein (C/EBP) alpha, are known to be abundantly and characteristically expressed in hepatocytes, and thus are collectively termed as "liver-enriched transcription factors". While studies using gene knockout mice have demonstrated that each of these molecules has its own unique functions as suggested by observed specific phenotypes (Costa et al. 2003; Schrem et al. 2002, 2004), it has become evident that they function cooperatively to form a dynamic transcriptional network of autoregulatory and cross-regulatory loops (Kyrmizi et al. 2006; Lemaigre 2009). In addition, these liver-enriched transcription factors also interact with various other transcription factors and/or regulatory molecules in a context-dependent manner to achieve specific target gene expression. For example, C/EBPalpha is an essential factor for glucose metabolism during the perinatal stage, and mice lacking this transcription factor die soon after birth due to hypoglycemia caused by defective gluconeogenic gene expression (Wang et al. 1995). Despite of this specific functional requirement at the perinatal stage, C/EBPalpha is already expressed in E14.5 fetal liver, suggesting that an additional factor may function cooperatively to ensure its temporally-regulated activity. Indeed, the forkhead family transcription factor Foxo1 has been found to be inducibly expressed in the perinatal liver, physically interact with C/EBPalpha, and augment C/EBPalpha-dependent transcription of a gluconeogenic gene, phosphoenolpyruvate carboxykinase (PEPCK) (Sekine et al. 2007). On the other hand, C/EBPalpha is also critical for ammonia detoxification activity of hepatocytes, as the knockout mice lack expression of carbamoyl phosphate synthetase-I (CPS1), a key enzyme in the urea cycle, leading to hyperammonemia (Kimura et al. 1998). Again, CPS1 is expressed only after the neonatal stage, and hence an involvement of some regulatory factor for C/EBPalpha-dependent CPS1 expression was suspected. In this case, Y-box binding protein-1 (YB-1) has been identified to be a molecule that suppresses C/EBPalpha function and negatively regulates CPS1 expression in the fetal liver (Chen et al. 2009). YB-1 is highly expressed in E14.5 fetal liver, and the expression significantly declines before birth. This results in the release of C/EBPalpha from YB-1-mediated suppression on the CPS1 promoter, leading to expression of CPS1 and ammonia clearance activity. Taken together, transcriptional activities of C/EBPalpha are differently controlled by expression and cooperative function of specialized "gatekeeper" molecules, Foxo1 and YB-1, for gluconeogenic and urea cycle enzymes, respectively.

As hepatocytes acquire mature metabolic functions, they also undergo structural maturation and establish specialized tissue architectures that are associated with their functions (Tanimizu and Miyajima 2007). They construct so-called "hepatocyte-type" epithelial polarity, where the apical surface, termed the bile canaliculus, is formed between neighboring hepatocytes, and the polarized hepatocytes are organized to form a cord-like structure. A study using gene knockout mice has suggested that the small GTPase ARF6 plays a critical role in the latter process (Suzuki et al. 2006).

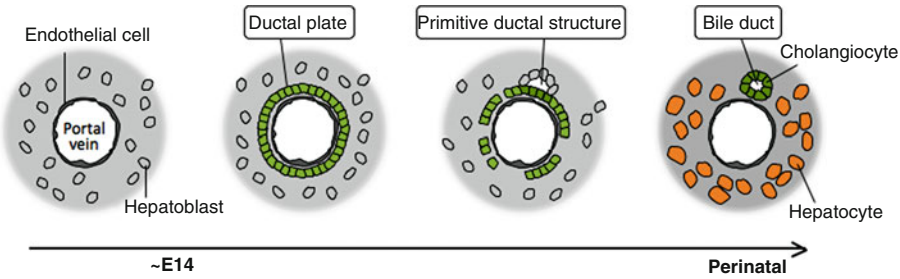

Fig. 8.6 Bile duct formation. Some hepatoblasts around the portal vein are specified to the cholangiocyte fate and align to form a single cell layer called the ductal plate. Then, the ductal plate becomes focally bilayered to form the asymmetric, primitive ductal structure (PDS) with a luminal space. By the perinatal period, the hepatoblasts lining the parenchymal side of the PDS differentiate to cholangiocytes, leading to formation of radially symmetrical duct structures

8.4.2 Differentiation into Cholangiocytes

Cholangiocytes are epithelial cells that line the biliary tract. The biliary tract can be separated ontogenetically into two parts; the extrahepatic bile duct and the intrahepatic bile duct (IHBD). The extrahepatic bile duct is comprised of the hepatic ducts, the cystic duct, the common bile duct, and the gallbladder, and develops from the endoderm independently of the hepatoblast formation. On the other hand, cholangiocytes forming the intrahepatic bile ducts derive from hepatoblasts, as mentioned earlier. It is not clear how the extrahepatic and intrahepatic biliary tracts, developing separately, eventually anastomose.

The process of IHBD formation from hepatoblasts involves cholangiocyte differentiation (lineage specification) and morphogenesis of ductal structures. In mouse embryos, the initial sign of cholangiocyte specification can be recognized at E11.5, when the cholangiocyte marker Sox9 is expressed in liver cells that are located a short distance from the branches of the portal vein (Antoniou et al. 2009). These cells align around the portal vein to form a single-layered structure, called the ductal plate (Fig. 8.6). At E15.5, the ductal plate becomes focally bilayered to form the primitive ductal structures (PDS), and lumens can be detected between the two layers. A recent study has suggested that the PDS are transiently asymmetrical, in that the cells on the portal-side layer express Sox9 but not the hepatoblast marker HNF4, while those on the parenchymal-side layer express HNF4 but not Sox9 (Antoniou et al. 2009). By E18.5, the hepatoblasts lining the parenchymal side of the PDS differentiate to cholangiocytes, leading to formation of radially symmetrical duct structures entirely delineated by cholangiocytes. During this process, the ductal plate cells that are not involved in tubulogenesis regress and eventually disappear, and the remaining ducts become surrounded by periportal mesenchymal cells. Although it has long been considered that this regression of ductal plate cells that do not contribute to the mature bile duct structure is mediated by apoptosis, a recent

study has shown that they undergo not apoptosis but rather differentiation to a subset of hepatocytes in the periportal region (Carpentier et al. 2011).

With regard to the molecular mechanisms involved in cholangiocyte differentiation, the roles of several transcription factors have been implicated, such as Sal-like 4 (Sall4), T-box transcription factor 3 (Tbx3), the Onecut transcription factors HNF6 and OC2, and HNF1beta. Based on gene expression profile as well as overexpression and knockdown experiments in purified fetal mouse hepatoblasts, Sall4 has been shown to play a role in regulating the lineage commitment of hepatoblasts by inhibiting their differentiation into hepatocytes while driving them towards the cholangiocyte lineage (Oikawa et al. 2009). In Tbx3 knockout mouse embryos, hepatoblast proliferation is severely impaired, with biliary differentiation promoted at the expense of hepatocyte differentiation, suggesting that Tbx3 plays a role in hepato-biliary lineage decision (Ludtke et al. 2009; Suzuki et al. 2008a). Gene expression analyses have shown that Tbx3 functions to maintain expression of hepatocyte transcription factors, HNF4alpha and C/EBPalpha, while suppressing that of cholangiocyte transcription factors, HNF6 and HNF1beta (Ludtke et al. 2009). Mice deficient of HNF6 shows bile duct malformation, and this phenotype is further enhanced by combined knockout of HNF6 and OC2 (Clotman et al. 2002, 2005). A direct and critical target of HNF6 is HNF1beta, and mice with liver-specific inactivation of HNF1beta shows defect in bile duct development (Coffinier et al. 2002). The Onecut transcription factors also regulate hepato-biliary lineage decision of hepatoblasts by modulating transforming growth factor (TGF) beta signaling. In the normal liver, TGFbeta signaling is found to be strongly activated near the portal veins but weakly in the rest of the parenchyma. In the livers of HNF6 and OC2 double knockout mice, increased TGFbeta signaling is observed in the parenchymal region, where "hybrid" hepatic cells that display characteristics of both hepatocytes and cholangiocytes are generated (Clotman et al. 2005). Thus, the Oncecut transcription factors play a role in shaping correct zonation of TGFbeta signaling activity to ensure induction of cholangiocytes only in the periportal region.

Another molecular mechanism well known to be involved in bile duct formation is the Notch signaling pathway. In humans, mutations in JAGGED 1 (JAG1), a ligand for the Notch receptors, are associated with Alagille syndrome (ALGS or ALGS1; Online Mendelian Inheritance in Man #118450), an autosomal dominant disorder characterized by multiple developmental defects including neonatal cholestasis caused by paucity of the IHBD (Li et al. 1997; Oda et al. 1997). In addition, another form of Alagille syndrome has been found to be caused by mutations in the NOTCH2 gene (ALGS2; Online Mendelian Inheritance in Man #610205) (McDaniell et al. 2006). In accord with these notions, mice doubly heterozygous for a Jag1 null allele and a Notch2 hypomorphic allele recapitulate many characteristics of the human syndrome, including the bile duct paucity (McCright et al. 2002). A study using an *in vitro* culture of mouse hepatoblasts has shown that activation of the Notch signaling pathway promotes differentiation of hepatoblasts into the cholangiocyte lineage by coordinating a network of liver-enriched transcription factors including HNF1alpha and beta, HNF4, and C/EBPalpha (Tanimizu and Miyajima 2004). Indeed, conditional knockout of RBP-Jkappa, an essential downstream

signal component of the Notch receptor, results in a reduced number of cholangiocytes at E16.5, confirming a role of this signaling pathway in cholangiocyte cell fate specification (Zong et al. 2009). In contrast, studies using the aforementioned compound (doubly heterozygous) mouse mutant for Jag1 and Notch2, or the liver-specific Notch2 knockout mice, have shown that Notch2 signaling is required for bile duct morphogenesis, but is likely dispensable for biliary specification (Geisler et al. 2008; Lozier et al. 2008). Similarly, in fetal livers of mice lacking hairy and enhancer of split 1 (Hes1), a target of the Notch signaling, the ductal plate formation occurs normally but the subsequent remodeling and tubular structure formation is completely blocked (Kodama et al. 2004). This discrepancy may result from the presence of multiple Notch signaling components, including the ligands, receptors and targets, which can play redundant and compensatory roles in biliary differentiation. It is also possible that conditional deletion of Notch2 in the developing liver was not complete or early enough. In the periportal region of the developing liver, the ligand Jag1 is expressed in portal fibroblasts and the endothelium of the portal vein, as well as in cholangiocytes at later stages (Geisler et al. 2008; Kodama et al. 2004; Loomes et al. 2007; Lozier et al. 2008; Suzuki et al. 2008c; Zong et al. 2009). An elaborate study employing cell type-specific knockout mouse models has clearly demonstrated that deletion of Jag1 specifically in SM22alpha-expressing portal vein mesenchyme, but not in the endothelium, leads to hepatic defects reminiscent of Alagille syndrome. In mice lacking Jag1 in the SM22alpha-positive cells, the initial formation of the ductal plate occurs normally, yet those biliary-specified cells are unable to undergo subsequent morphological changes, leading to paucity of bile duct formation (Hofmann et al. 2010). Although the nature of the SM22alpha-expressing cells is not fully characterized, it is considered most plausible that portal fibroblasts stimulate cholangiocytes lining the ductal plate via the Jag1/Notch2 interaction and the downstream Hes1 expression, which leads to induction of ductal morphogenesis.

8.4.3 Differentiation into Non-hepatic Lineages

The liver and the pancreas share a common developmental origin, and a bipotential precursor cell population for these organs has been identified within the embryonic endoderm (Deutsch et al. 2001). In addition, hepatocytes and pancreatic beta-cells are known to have similarities in gene expression profiles and possess similar inherent glucose sensing systems, thereby being capable of responding to changes in blood glucose concentrations. Consistent with these facts, many studies have demonstrated that liver stem/progenitor cells from both embryonic and adult origins as well as hepatocytes can be converted to insulin-producing cells, functional pancreatic beta-cell-like cells, and/or to islet-like cell clusters containing other pancreatic lineages under certain conditions.

Clonally expanded H-CFU-C derived from fetal mouse liver shows expression of pancreatic endocrine and exocrine lineage markers in culture, and can be integrated

into and form pancreatic ducts and acinar cells when transplanted into pancreas of recipient mice (Suzuki et al. 2002). Notably, H-CFU-C is also shown to be capable of differentiating into gastric and intestinal cells *in vivo*. Purified adult rat hepatic oval cells can be differentiated into pancreatic endocrine hormone-producing cells when cultured in a high-glucose environment (Yang et al. 2002). Rat liver epithelial WB cells, which represent the cultured counterpart of stem-like cells derived from normal adult liver, can be reprogrammed into functional insulin-producing cells by stable expression of pancreatic duodenal homeobox 1 (Pdx1) or its super-active form (Pdx1-VP16) (Tang et al. 2006). Epithelial progenitor cells derived from human fetal liver (FH cells) can also be induced to differentiate into insulin-producing cells after expression of the PDX1 gene (Zalzman et al. 2003).

In addition to these *in vitro* experiments, several studies employing *in vivo* gene delivery systems have shown that adenoviral vector-mediated transduction of pancreatic transcription factors, such as Pdx1, Neurogenin3 (Ngn3), NeuroD, and MafA, can induce formation of ectopic islet-like cells and production of insulin in the adult liver (Ferber et al. 2000; Kojima et al. 2003; Song et al. 2007; Wang et al. 2007). Although these phenomena have been considered to represent trans-differentiation of mature hepatocytes into pancreatic cells, a recent study employing Ngn3 gene transfer in combination with a genetic lineage tracing have suggested an alternative possibility. Thus, introduction of this transcription factor can sufficiently induce emergence of ectopic, periportal islet-like cell clusters in streptozotocin (STZ)-induced diabetic model mice, and these clusters do not originate from differentiated hepatocytes but are rather likely produced by "trans-determination" of LPC-like progenitor cells, which are lineage-determined but not terminally differentiated (Yechoor et al. 2009). In view of this, it is noteworthy that DDC-induced activation of LPCs *in vivo* has been reported to ameliorate STZ-induced diabetes in mice (Kim et al. 2007).

8.5 Potential Application for Therapies

At present, orthotopic liver transplantation is the most commonly used procedure to treat various liver diseases. This, however, has always been hampered by persistent shortage of donor organs. Although isolated mature hepatocytes when transplanted have been shown to successfully repopulate the recipient liver with considerably high efficiency at least in rodent models, the rates of engraftment and survival of transplanted hepatocytes in human liver is often very limited. Furthermore, despite mature hepatocytes showing tremendous proliferative activity in response to regenerative stimuli *in vivo*, they usually lose this capacity immediately once isolated and cultured *in vitro*. Thus, the ability to obtain an unlimited supply of human hepatocytes from an expandable source should significantly improve the development and clinical application of hepatocyte transplantation. In addition, it will also facilitate the studies on the basic mechanisms of human liver diseases, as well as evaluation of drugs for their actions and toxicities due to the metabolism of

xenobiotics in hepatocytes. Considering the strong proliferative potential and amenability for *in vitro* manipulation, the liver stem/progenitor cells may be attractive candidates for these applications. These cells may also be useful for cell therapy to treat diabetic patients, given their potential to be effectively reprogrammed toward pancreatic lineages. However, isolation of fetal hepatoblasts and adult hepatic stem/progenitor cells from human liver for therapeutic use will be technically and practically challenging, even though the aforementioned liver organoid culture system could be a promising alternative.

In the last decade, much effort and concomitant progress have been made in establishing the protocols to generate various types of functionally differentiated cells, including mature hepatocytes, *in vitro* from pluripotent or multipotent stem cells (Snykers et al. 2009), particularly from embryonic stem (ES) cells (Agarwal et al. 2008; Basma et al. 2009; Cai et al. 2007) and more recently from induced pluripotent stem (iPS) cells (Gai et al. 2010; Si-Tayeb et al. 2010; Song et al. 2009; Sullivan et al. 2010). This has led to the notion that application of the precise conditions that recapitulate the normal developmental process within the embryo is generally the best way to achieve highly functional derivatives. Thus, to produce hepatocytes for example, these pluripotent stem cells can be sequentially induced to differentiate to the definitive endoderm, then the hepatic lineage cells with the character of hepatoblasts, and finally to functional hepatocytes, directed by the timed use of appropriate amounts and combinations of cytokines (Gouon-Evans et al. 2006). In view of this, studies elucidating the mechanisms of the normal liver organogenesis, and particularly of hepatoblast development and differentiation, should provide an important clue to future development of an optimized protocol to induce functional hepatocytes *in vitro*. Conversely, *in vitro* differentiation systems from human ES or iPS cells to hepatocytes can offer a means of elucidating the mechanisms of human liver development *ex vivo* (DeLaForest et al. 2011). Notably, use of specific surface markers for hepatoblasts or other hepatic cells to enrich for particular cell lineages in the course of induced differentiation would be advantageous for obtaining hepatocytes with better quality and quantity. Moreover, this will also be beneficial to eliminate contamination from undifferentiated stem cells that remain, as these cells may potentially cause tumors such as teratomas upon transplantation into recipients. One example is the combined use of the cell surface markers CD13 and CD133 to enrich for human hepatoblasts generated from iPS cells (Yanagida et al. 2013). Basically, common approaches for enrichment of hepatoblast or hepatocyte-like cells are based on the combined use of lineage specific markers with stem cell markers. Caution must be exercised, however, as markers optimized for mouse-derived progenitors may not always be appropriate for use in isolating human iPS-derived hepatoblasts.

Upon the emergence of the iPS cell technology, the fact that terminally differentiated somatic cells can be converted to a totally different, pluripotent state by a relatively small number of defined factors urged many researchers to test the possibility that they could also be reprogrammed directly to different cell lineages without going through a pluripotent intermediate. This approach, so-called direct reprogramming, has indeed been shown to be quite promising with various target

cell lineages, including hepatocytes. By forcedly introducing a small number of endodermal and hepatic transcription factors, two groups have independently demonstrated that mouse fibroblasts can be converted to hepatocyte-like cells (Huang et al. 2011; Sekiya and Suzuki 2011). Functionally, they are still not fully mature hepatocytes, yet are able to repopulate *in vivo* when transplanted into a mouse model. These "induced hepatocytes (iHep)" may provide an alternative, relatively simple platform to realize gene and cell therapy to treat liver diseases.

More recently, there has been considerable success in generating human hepatocyte-like cells using similar approaches. In one such study, expression of the factors FOXA3, HNF1A and HNF4A (collectively called 3T) was induced in primary human fetal limb fibroblasts (Huang et al. 2014b). The resulting human induced hepatocytes (hiHeps) increasingly expressed genes specific for hepatocytes, with a corresponding decrease in expression of fibroblast-specific genes. Absence of markers for biliary epithelium or hepatoblasts indicated that the hiHeps were stably differentiated into mature hepatocytes. They were also shown to have normal hepatocyte detoxification and biliary excretion functions. However, due to the extent of differentiation, the cells could not be expanded to generate large numbers for *in vivo* validation. It was necessary to utilize a lentivirus vector to induce expression of SV40 large T antigen, though this caused a subsequent reduction in metabolic activity compared to nontransformed hiHep cells. Another similar study utilized a broader array of transcription factors for direct reprogramming of fibroblasts (HNF1A, HNF4A, HNF6, ATF5, PROX1, CEBPA, p53 ShRNA, c-myc) by mimicking conditions for liver bud induction and hepatocyte maturation (Du et al. 2014). Like the previously described reprogramming protocol, the hiHeps show a high degree of functional activity comparable to primary hepatocytes. The advantage of direct reprogramming compared to the inclusion of an intermediate pluripotent state is that there is theoretically a reduced risk of tumorigenesis. As each hiHep is the result of a unique reprogramming event, however, there may be a certain level of heterogeneity among the cell population in terms of the functional activity of each cell. Nevertheless, in general, both studies show amazing progress in the generation of functional human hepatocytes.

8.6 Conclusion and Future Development in Research

In recent years, considerable progress has been made in our understanding of the mechanisms of liver development at the cellular and molecular levels. Establishment of methods for *in vitro* culture of fetal liver cells, in particular the isolated hepatoblasts, as well as various mouse models with genetic modifications have invaluably contributed to identify and elucidate the role of genes involved in fetal liver development. Although characterization of adult liver stem/progenitor cells represented by oval cells has been less successful compared to that of hepatoblasts, it has been accelerated by the finding and availability of useful marker molecules. Further analyses on the extrinsic signals and the intrinsic genetic and epigenetic programs

regulating these cell populations should lead to clarification of the molecular basis of liver regeneration, as well as its similarities and differences with that of liver development. In-depth understanding of the mechanisms governing these complicated and elaborate processes is crucial for establishing an efficient protocol to generate functional hepatic cells amenable to therapeutic cell transplantation and pharmaceutical drug development.

References

Agarwal S, Holton KL, Lanza R (2008) Efficient differentiation of functional hepatocytes from human embryonic stem cells. Stem Cells 26(5):1117–1127

Akhurst B, Croager EJ, Farley-Roche CA, Ong JK, Dumble ML, Knight B, Yeoh GC (2001) A modified choline-deficient, ethionine-supplemented diet protocol effectively induces oval cells in mouse liver. Hepatology 34(3):519–522

Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, Jacquemin P, Pierreux CE, Clotman F, Lemaigre FP (2009) Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136(7):2325–2333

Apte U, Thompson MD, Cui S, Liu B, Cieply B, Monga SP (2008) Wnt/beta-catenin signaling mediates oval cell response in rodents. Hepatology 47(1):288–295

Asahina K, Zhou B, Pu WT, Tsukamoto H (2011) Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. Hepatology 53(3):983–995

Basma H, Soto-Gutierrez A, Yannam GR, Liu L, Ito R, Yamamoto T, Ellis E, Carson SD, Sato S, Chen Y, Muirhead D, Navarro-Alvarez N, Wong RJ, Roy-Chowdhury J, Platt JL, Mercer DF, Miller JD, Strom SC, Kobayashi N, Fox IJ (2009) Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. Gastroenterology 136(3):990–999

Bisgaard HC, Parmelee DC, Dunsford HA, Sechi S, Thorgeirsson SS (1993) Keratin 14 protein in cultured nonparenchymal rat hepatic epithelial cells: characterization of keratin 14 and keratin 19 as antigens for the commonly used mouse monoclonal antibody OV-6. Mol Carcinog 7(1):60–66

Bort R, Signore M, Tremblay K, Martinez Barbera JP, Zaret KS (2006) Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. Dev Biol 290(1):44–56

Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, Ridgway RA, Seo SS, Spee B, Van Rooijen N, Sansom OJ, Iredale JP, Lowell S, Roskams T, Forbes SJ (2012) Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 18(4):572–579

Cai J, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology 45(5):1229–1239

Carpentier R, Suner RE, van Hul N, Kopp JL, Beaudry JB, Cordi S, Antoniou A, Raynaud P, Lepreux S, Jacquemin P, Leclercq IA, Sander M, Lemaigre FP (2011) Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 141(4):1432–1438

Chen YR, Sekine K, Nakamura K, Yanai H, Tanaka M, Miyajima A (2009) Y-box binding protein-1 down-regulates expression of carbamoyl phosphate synthetase-I by suppressing CCAAT enhancer-binding protein-alpha function in mice. Gastroenterology 137(1):330–340

Choi TY, Ninov N, Stainier DY, Shin D (2014) Extensive conversion of hepatic biliary epithelial cells to hepatocytes after near total loss of hepatocytes in zebrafish. Gastroenterology 146(3):776–788. doi:10.1053/j.gastro.2013.10.019

Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P, Roskams T, Rousseau GG, Lemaigre FP (2002) The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129(8):1819–1828

Clotman F, Jacquemin P, Plumb-Rudewiez N, Pierreux CE, Van der Smissen P, Dietz HC, Courtoy PJ, Rousseau GG, Lemaigre FP (2005) Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev 19(16):1849–1854

Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, Pontoglio M, Yaniv M, Barra J (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 129(8):1829–1838

Costa RH, Kalinichenko VV, Holterman AX, Wang X (2003) Transcription factors in liver development, differentiation, and regeneration. Hepatology 38(6):1331–1347

DeLaForest A, Nagaoka M, Si-Tayeb K, Noto FK, Konopka G, Battle MA, Duncan SA (2011) HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. Development 138(19):4143–4153

Deutsch G, Jung J, Zheng M, Lora J, Zaret KS (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128(6):871–881

Dorrell C, Erker L, Lanxon-Cookson KM, Abraham SL, Victoroff T, Ro S, Canaday PS, Streeter PR, Grompe M (2008) Surface markers for the murine oval cell response. Hepatology 48(4):1282–1291

Dorrell C, Erker L, Schug J, Kopp JL, Canaday PS, Fox AJ, Smirnova O, Duncan AW, Finegold MJ, Sander M, Kaestner KH, Grompe M (2011) Prospective isolation of a bipotential clonogenic liver progenitor cell in adult mice. Genes Dev 25(11):1193–1203

Du Y, Wang J, Jia J, Song N, Xiang C, Xu J, Hou Z, Su X, Liu B, Jiang T, Zhao D, Sun Y, Shu J, Guo Q, Yin M, Sun D, Lu S, Shi Y, Deng H (2014) Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem Cell 14:394–403

Duncan AW, Dorrell C, Grompe M (2009) Stem cells and liver regeneration. Gastroenterology 137(2):466–481

Dunsford HA, Sell S (1989) Production of monoclonal antibodies to preneoplastic liver cell populations induced by chemical carcinogens in rats and to transplantable Morris hepatomas. Cancer Res 49(17):4887–4893

Engelhardt NV, Factor VM, Yasova AK, Poltoranina VS, Baranov VN, Lasareva MN (1990) Common antigens of mouse oval and biliary epithelial cells. Expression on newly formed hepatocytes. Differentiation 45(1):29–37

Farber E (1956) Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylamino-fluorene, and 3′-methyl-4-dimethylaminoazobenzene. Cancer Res 16(2):142–148

Fausto N (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 39(6):1477–1487

Fellous TG, Islam S, Tadrous PJ, Elia G, Kocher HM, Bhattacharya S, Mears L, Turnbull DM, Taylor RW, Greaves LC, Chinnery PF, Taylor G, McDonald SA, Wright NA, Alison MR (2009) Locating the stem cell niche and tracing hepatocyte lineages in human liver. Hepatology 49(5):1655–1663

Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 6(5):568–572

Floridon C, Jensen CH, Thorsen P, Nielsen O, Sunde L, Westergaard JG, Thomsen SG, Teisner B (2000) Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. Differentiation 66(1):49–59

Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T, Uemoto S (2011) Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet 43(1):34–41

Gai H, Nguyen DM, Moon YJ, Aguila JR, Fink LM, Ward DC, Ma Y (2010) Generation of murine hepatic lineage cells from induced pluripotent stem cells. Differentiation 79(3):171–181

Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, Radtke F, Schmid RM, Siveke JT (2008) Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48(2):607–616

Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G (2006) BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. Nat Biotechnol 24(11):1402–1411

Grompe M (2003) Pancreatic-hepatic switches in vivo. Mech Dev 120(1):99–106

Grozdanov PN, Yovchev MI, Dabeva MD (2006) The oncofetal protein glypican-3 is a novel marker of hepatic progenitor/oval cells. Lab Invest 86(12):1272–1284

He J, Lu H, Zou Q, Luo L (2014) Regeneration of liver after extreme hepatocyte loss occurs mainly via biliary transdifferentiation in zebrafish. Gastroenterology 146(3):789–800 e788. doi:10.1053/j.gastro.2013.11.045

Hirose Y, Itoh T, Miyajima A (2009) Hedgehog signal activation coordinates proliferation and differentiation of fetal liver progenitor cells. Exp Cell Res 315(15):2648–2657

Hofmann JJ, Zovein AC, Koh H, Radtke F, Weinmaster G, Iruela-Arispe ML (2010) Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development 137(23):4061–4072

Hu M, Kurobe M, Jeong YJ, Fuerer C, Ghole S, Nusse R, Sylvester KG (2007) Wnt/beta-catenin signaling in murine hepatic transit amplifying progenitor cells. Gastroenterology 133(5):1579–1591

Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475(7356):386–389

Huang M, Chang A, Choi M, Zhou D, Anania FA, Shin CH (2014a) Antagonistic interaction between Wnt and Notch activity modulates the regenerative capacity of a zebrafish fibrotic liver model. Hepatology 60(5):1753–1766. doi:10.1002/hep.27285

Huang P, Zhang L, Gao Y, He Z, Yao D, Wu Z, Cen J, Chen X, Liu C, Hu Y, Lai D, Hu Z, Chen L, Zhang Y, Cheng X, Ma X, Pan G, Wang X, Hui L (2014b) Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. Cell Stem Cell 14:370–384

Huch M, Dorrell C, Boj SF, van Es JH, Li VS, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ, Haft A, Vries RG, Grompe M, Clevers H (2013) In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. Nature 494(7436):247–250

Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Wenum M, Fuchs SA, de Ligt J, van de Wetering M, Sasaki N, Boers SJ, Kemperman H, de Jonge J, Ijzermans JN, Nieuwenhuis EE, Hoekstra R, Strom S, Vries RR, van der Laan LJ, Cuppen E, Clevers H (2015) Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell 160(1–2):299–312. doi:10.1016/j.cell.2014.11.050

Itoh T, Kamiya Y, Okabe M, Tanaka M, Miyajima A (2009) Inducible expression of Wnt genes during adult hepatic stem/progenitor cell response. FEBS Lett 583(4):777–781

Jakubowski A, Ambrose C, Parr M, Lincecum JM, Wang MZ, Zheng TS, Browning B, Michaelson JS, Baetscher M, Wang B, Bissell DM, Burkly LC (2005) TWEAK induces liver progenitor cell proliferation. J Clin Invest 115(9):2330–2340

Jelnes P, Santoni-Rugiu E, Rasmussen M, Friis SL, Nielsen JH, Tygstrup N, Bisgaard HC (2007) Remarkable heterogeneity displayed by oval cells in rat and mouse models of stem cell-mediated liver regeneration. Hepatology 45(6):1462–1470

Jensen CH, Jauho EI, Santoni-Rugiu E, Holmskov U, Teisner B, Tygstrup N, Bisgaard HC (2004) Transit-amplifying ductular (oval) cells and their hepatocytic progeny are characterized by a novel and distinctive expression of delta-like protein/preadipocyte factor 1/fetal antigen 1. Am J Pathol 164(4):1347–1359

Jors S, Jeliazkova P, Ringelhan M, Thalhammer J, Durl S, Ferrer J, Sander M, Heikenwalder M, Schmid RM, Siveke JT, Geisler F (2015) Lineage fate of ductular reactions in liver injury and carcinogenesis. J Clin Invest 125(6):2445–2457. doi:10.1172/JCI78585

Jung J, Zheng M, Goldfarb M, Zaret KS (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284(5422):1998–2003

Kakinuma S, Ohta H, Kamiya A, Yamazaki Y, Oikawa T, Okada K, Nakauchi H (2009) Analyses of cell surface molecules on hepatic stem/progenitor cells in mouse fetal liver. J Hepatol 51(1):127–138

Kamiya A, Gonzalez FJ (2004) TNF-alpha regulates mouse fetal hepatic maturation induced by oncostatin M and extracellular matrices. Hepatology 40(3):527–536

Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A (1999) Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J 18(8):2127–2136

Kamiya A, Kojima N, Kinoshita T, Sakai Y, Miyajima A (2002) Maturation of fetal hepatocytes in vitro by extracellular matrices and oncostatin M: induction of tryptophan oxygenase. Hepatology 35(6):1351–1359

Kamiya A, Kakinuma S, Yamazaki Y, Nakauchi H (2009) Enrichment and clonal culture of progenitor cells during mouse postnatal liver development in mice. Gastroenterology 137(3):1114–1126

Kaneko K, Kamimoto K, Miyajima A, Itoh T (2015) Adaptive remodeling of the biliary architecture underlies liver homeostasis. Hepatology 61(6):2056–2066. doi:10.1002/hep.27685

Kim S, Shin JS, Kim HJ, Fisher RC, Lee MJ, Kim CW (2007) Streptozotocin-induced diabetes can be reversed by hepatic oval cell activation through hepatic transdifferentiation and pancreatic islet regeneration. Lab Invest 87(7):702–712

Kimura T, Christoffels VM, Chowdhury S, Iwase K, Matsuzaki H, Mori M, Lamers WH, Darlington GJ, Takiguchi M (1998) Hypoglycemia-associated hyperammonemia caused by impaired expression of ornithine cycle enzyme genes in C/EBPalpha knockout mice. J Biol Chem 273(42):27505–27510

Kinoshita T, Sekiguchi T, Xu MJ, Ito Y, Kamiya A, Tsuji K, Nakahata T, Miyajima A (1999) Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. Proc Natl Acad Sci U S A 96(13):7265–7270

Knight B, Akhurst B, Matthews VB, Ruddell RG, Ramm GA, Abraham LJ, Olynyk JK, Yeoh GC (2007) Attenuated liver progenitor (oval) cell and fibrogenic responses to the choline deficient, ethionine supplemented diet in the BALB/c inbred strain of mice. J Hepatol 46(1):134–141

Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T (2004) The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 127(6):1775–1786

Kojima N, Kinoshita T, Kamiya A, Nakamura K, Nakashima K, Taga T, Miyajima A (2000) Cell density-dependent regulation of hepatic development by a gp130-independent pathway. Biochem Biophys Res Commun 277(1):152–158

Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 9(5):596–603

Kubota H, Reid LM (2000) Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proc Natl Acad Sci U S A 97(22):12132–12137

Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I (2006) Plasticity and expanding complexity of the hepatic transcription factor network during liver development. Genes Dev 20(16):2293–2305

Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS (2006) A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 12(4):410–416

Lemaigre FP (2009) Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 137(1):62–79

Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont ME, Rand EB, Piccoli DA, Hood L, Spinner NB (1997) Alagille syndrome

is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 16(3):243–251
Loomes KM, Russo P, Ryan M, Nelson A, Underkoffler L, Glover C, Fu H, Gridley T, Kaestner KH, Oakey RJ (2007) Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology 45(2):323–330
Lozier J, McCright B, Gridley T (2008) Notch signaling regulates bile duct morphogenesis in mice. PLoS One 3(3):e1851
Ludtke TH, Christoffels VM, Petry M, Kispert A (2009) Tbx3 promotes liver bud expansion during mouse development by suppression of cholangiocyte differentiation. Hepatology 49(3):969–978
Malato Y, Naqvi S, Schurmann N, Ng R, Wang B, Zape J, Kay MA, Grimm D, Willenbring H (2011) Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. J Clin Invest 121(12):4850–4860
Margagliotti S, Clotman F, Pierreux CE, Beaudry JB, Jacquemin P, Rousseau GG, Lemaigre FP (2007) The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. Dev Biol 311(2):579–589
Matsumoto K, Yoshitomi H, Rossant J, Zaret KS (2001) Liver organogenesis promoted by endothelial cells prior to vascular function. Science 294(5542):559–563
Matthews VB, Yeoh GC (2005) Liver stem cells. IUBMB Life 57(8):549–553
McCright B, Lozier J, Gridley T (2002) A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development 129(4):1075–1082
McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA, Spinner NB (2006) NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet 79(1):169–173
Michalopoulos GK, DeFrances MC (1997) Liver regeneration. Science 276(5309):60–66
Micsenyi A, Tan X, Sneddon T, Luo JH, Michalopoulos GK, Monga SP (2004) Beta-catenin is temporally regulated during normal liver development. Gastroenterology 126(4):1134–1146
Miyaoka Y, Ebato K, Kato H, Arakawa S, Shimizu S, Miyajima A (2012) Hypertrophy and unconventional cell division of hepatocytes underlie liver regeneration. Curr Biol 22(13):1166–1175
Nagahama Y, Sone M, Chen X, Okada Y, Yamamoto M, Xin B, Matsuo Y, Komatsu M, Suzuki A, Enomoto K, Nishikawa Y (2014) Contributions of hepatocytes and bile ductular cells in ductular reactions and remodeling of the biliary system after chronic liver injury. Am J Pathol 184(11):3001–3012. doi:10.1016/j.ajpath.2014.07.005
Newsome PN, Hussain MA, Theise ND (2004) Hepatic oval cells: helping redefine a paradigm in stem cell biology. Curr Top Dev Biol 61:1–28
Nitou M, Sugiyama Y, Ishikawa K, Shiojiri N (2002) Purification of fetal mouse hepatoblasts by magnetic beads coated with monoclonal anti-e-cadherin antibodies and their in vitro culture. Exp Cell Res 279(2):330–343
Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS, Chandrasekharappa SC (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 16(3):235–242
Oertel M, Menthena A, Chen YQ, Teisner B, Jensen CH, Shafritz DA (2008) Purification of fetal liver stem/progenitor cells containing all the repopulation potential for normal adult rat liver. Gastroenterology 134(3):823–832
Oikawa T, Kamiya A, Kakinuma S, Zeniya M, Nishinakamura R, Tajiri H, Nakauchi H (2009) Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. Gastroenterology 136(3):1000–1011
Okabe M, Tsukahara Y, Tanaka M, Suzuki K, Saito S, Kamiya Y, Tsujimura T, Nakamura K, Miyajima A (2009) Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver. Development 136(11):1951–1960
Paku S, Schnur J, Nagy P, Thorgeirsson SS (2001) Origin and structural evolution of the early proliferating oval cells in rat liver. Am J Pathol 158(4):1313–1323
Preisegger KH, Factor VM, Fuchsbichler A, Stumptner C, Denk H, Thorgeirsson SS (1999) Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the

3,5-diethoxycarbonyl-1,4-dihydrocollidine mouse model for chronic alcoholic liver disease. Lab Invest 79(2):103–109
Roskams TA, Libbrecht L, Desmet VJ (2003) Progenitor cells in diseased human liver. Semin Liver Dis 23(4):385–396
Rossi JM, Dunn NR, Hogan BL, Zaret KS (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 15(15):1998–2009
Rountree CB, Barsky L, Ge S, Zhu J, Senadheera S, Crooks GM (2007) A CD133-expressing murine liver oval cell population with bilineage potential. Stem Cells 25(10):2419–2429
Sackett SD, Li Z, Hurtt R, Gao Y, Wells RG, Brondell K, Kaestner KH, Greenbaum LE (2009) Foxl1 is a marker of bipotential hepatic progenitor cells in mice. Hepatology 49(3):920–929
Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, Moss N, Melhem A, McClelland R, Turner W, Kulik M, Sherwood S, Tallheden T, Cheng N, Furth ME, Reid LM (2007) Human hepatic stem cells from fetal and postnatal donors. J Exp Med 204(8):1973–1987
Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C (1995) Scatter factor/hepatocyte growth factor is essential for liver development. Nature 373(6516):699–702
Schrem H, Klempnauer J, Borlak J (2002) Liver-enriched transcription factors in liver function and development. Part I: the hepatocyte nuclear factor network and liver-specific gene expression. Pharmacol Rev 54(1):129–158
Schrem H, Klempnauer J, Borlak J (2004) Liver-enriched transcription factors in liver function and development. Part II: the C/EBPs and D site-binding protein in cell cycle control, carcinogenesis, circadian gene regulation, liver regeneration, apoptosis, and liver-specific gene regulation. Pharmacol Rev 56(2):291–330
Sekine K, Chen YR, Kojima N, Ogata K, Fukamizu A, Miyajima A (2007) Foxo1 links insulin signaling to C/EBPalpha and regulates gluconeogenesis during liver development. EMBO J 26(15):3607–3615
Sekiya S, Suzuki A (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475(7356):390–393
Sekiya S, Suzuki A (2014) Hepatocytes, rather than cholangiocytes, can be the major source of primitive ductules in the chronically injured mouse liver. Am J Pathol 184(5):1468–1478. doi:10.1016/j.ajpath.2014.01.005
Shin S, Walton G, Aoki R, Brondell K, Schug J, Fox A, Smirnova O, Dorrell C, Erker L, Chu AS, Wells RG, Grompe M, Greenbaum LE, Kaestner KH (2011) Foxl1-Cre-marked adult hepatic progenitors have clonogenic and bilineage differentiation potential. Genes Dev 25(11):1185–1192
Shin S, Upadhyay N, Greenbaum LE, Kaestner KH (2015) Ablation of Foxl1-Cre-labeled hepatic progenitor cells and their descendants impairs recovery of mice from liver injury. Gastroenterology 148(1):192–202 e193. doi:10.1053/j.gastro.2014.09.039
Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51(1):297–305
Snykers S, De Kock J, Rogiers V, Vanhaecke T (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. Stem Cells 27(3):577–605
Song YD, Lee EJ, Yashar P, Pfaff LE, Kim SY, Jameson JL (2007) Islet cell differentiation in liver by combinatorial expression of transcription factors neurogenin-3, BETA2, and RIPE3b1. Biochem Biophys Res Commun 354(2):334–339
Song Z, Cai J, Liu Y, Zhao D, Yong J, Duo S, Song X, Guo Y, Zhao Y, Qin H, Yin X, Wu C, Che J, Lu S, Ding M, Deng H (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell Res 19(11):1233–1242
Sosa-Pineda B, Wigle JT, Oliver G (2000) Hepatocyte migration during liver development requires Prox1. Nat Genet 25(3):254–255
Strick-Marchand H, Masse GX, Weiss MC, Di Santo JP (2008) Lymphocytes support oval cell-dependent liver regeneration. J Immunol 181(4):2764–2771

Sullivan GJ, Hay DC, Park IH, Fletcher J, Hannoun Z, Payne CM, Dalgetty D, Black JR, Ross JA, Samuel K, Wang G, Daley GQ, Lee JH, Church GM, Forbes SJ, Iredale JP, Wilmut I (2010) Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology 51(1):329–335

Suzuki A, Zheng Y, Kondo R, Kusakabe M, Takada Y, Fukao K, Nakauchi H, Taniguchi H (2000) Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. Hepatology 32(6):1230–1239

Suzuki A, Zheng YW, Kaneko S, Onodera M, Fukao K, Nakauchi H, Taniguchi H (2002) Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. J Cell Biol 156(1):173–184

Suzuki A, Iwama A, Miyashita H, Nakauchi H, Taniguchi H (2003) Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. Development 130(11):2513–2524

Suzuki T, Kanai Y, Hara T, Sasaki J, Sasaki T, Kohara M, Maehama T, Taya C, Shitara H, Yonekawa H, Frohman MA, Yokozeki T, Kanaho Y (2006) Crucial role of the small GTPase ARF6 in hepatic cord formation during liver development. Mol Cell Biol 26(16):6149–6156

Suzuki A, Sekiya S, Buscher D, Izpisua Belmonte JC, Taniguchi H (2008a) Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. Development 135(9):1589–1595

Suzuki A, Sekiya S, Onishi M, Oshima N, Kiyonari H, Nakauchi H, Taniguchi H (2008b) Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. Hepatology 48(6):1964–1978

Suzuki K, Tanaka M, Watanabe N, Saito S, Nonaka H, Miyajima A (2008c) p75 Neurotrophin receptor is a marker for precursors of stellate cells and portal fibroblasts in mouse fetal liver. Gastroenterology 135(1):270–281, e273

Takase HM, Itoh T, Ino S, Wang T, Koji T, Akira S, Takikawa Y, Miyajima A (2013) FGF7 is a functional niche signal required for stimulation of adult liver progenitor cells that support liver regeneration. Genes Dev 27(2):169–181

Tanaka M, Hirabayashi Y, Sekiguchi T, Inoue T, Katsuki M, Miyajima A (2003) Targeted disruption of oncostatin M receptor results in altered hematopoiesis. Blood 102(9):3154–3162

Tanaka M, Okabe M, Suzuki K, Kamiya Y, Tsukahara Y, Saito S, Miyajima A (2009) Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. Mech Dev 126(8–9):665–676

Tanaka M, Itoh T, Tanimizu N, Miyajima A (2011) Liver stem/progenitor cells: their characteristics and regulatory mechanisms. J Biochem 149(3):231–239

Tang DQ, Lu S, Sun YP, Rodrigues E, Chou W, Yang C, Cao LZ, Chang LJ, Yang LJ (2006) Reprogramming liver-stem WB cells into functional insulin-producing cells by persistent expression of Pdx1- and Pdx1-VP16 mediated by lentiviral vectors. Lab Invest 86(1):83–93

Tanimizu N, Miyajima A (2004) Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. J Cell Sci 117(Pt 15):3165–3174

Tanimizu N, Miyajima A (2007) Molecular mechanism of liver development and regeneration. Int Rev Cytol 259:1–48

Tanimizu N, Tsujimura T, Takahide K, Kodama T, Nakamura K, Miyajima A (2004) Expression of Dlk/Pref-1 defines a subpopulation in the oval cell compartment of rat liver. Gene Expr Patterns 5(2):209–218

Tarlow BD, Finegold MJ, Grompe M (2014a) Clonal tracing of Sox9+ liver progenitors in mouse oval cell injury. Hepatology 60(1):278–289. doi:10.1002/hep.27084

Tarlow BD, Pelz C, Naugler WE, Wakefield L, Wilson EM, Finegold MJ, Grompe M (2014b) Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. Cell Stem Cell 15(5):605–618. doi:10.1016/j.stem.2014.09.008

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR, Darlington GJ (1995) Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269(5227):1108–1112

Wang AY, Ehrhardt A, Xu H, Kay MA (2007) Adenovirus transduction is required for the correction of diabetes using Pdx-1 or Neurogenin-3 in the liver. Mol Ther 15(2):255–263

Watanabe T, Nakagawa K, Ohata S, Kitagawa D, Nishitai G, Seo J, Tanemura S, Shimizu N, Kishimoto H, Wada T, Aoki J, Arai H, Iwatsubo T, Mochita M, Watanabe T, Satake M, Ito Y, Matsuyama T, Mak TW, Penninger JM, Nishina H, Katada T (2002) SEK1/MKK4-mediated SAPK/JNK signaling participates in embryonic hepatoblast proliferation via a pathway different from NF-kappaB-induced anti-apoptosis. Dev Biol 250(2):332–347

Weinstein M, Monga SP, Liu Y, Brodie SG, Tang Y, Li C, Mishra L, Deng CX (2001) Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. Mol Cell Biol 21(15):5122–5131

Yanagida A, Ito K, Chikada H, Nakauchi H, Kamiya A (2013) An in vitro expansion system for generation of human iPS cell-derived hepatic progenitor-like cells exhibiting a bipotent differentiation potential. PLoS One 8(7):e67541

Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB (2002) In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 99(12):8078–8083

Yang W, Yan HX, Chen L, Liu Q, He YQ, Yu LX, Zhang SH, Huang DD, Tang L, Kong XN, Chen C, Liu SQ, Wu MC, Wang HY (2008) Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. Cancer Res 68(11):4287–4295

Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, Thung SN, Wells RG, Greenbaum LE, Stanger BZ (2013) Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes Dev 27(7):719–724

Yechoor V, Liu V, Espiritu C, Paul A, Oka K, Kojima H, Chan L (2009) Neurogenin3 is sufficient for transdetermination of hepatic progenitor cells into neo-islets in vivo but not transdifferentiation of hepatocytes. Dev Cell 16(3):358–373

Yovchev MI, Grozdanov PN, Joseph B, Gupta S, Dabeva MD (2007) Novel hepatic progenitor cell surface markers in the adult rat liver. Hepatology 45(1):139–149

Yovchev MI, Zhang J, Neufeld DS, Grozdanov PN, Dabeva MD (2009) Thymus cell antigen-1-expressing cells in the oval cell compartment. Hepatology 50(2):601–611

Zajicek G, Oren R, Weinreb M Jr (1985) The streaming liver. Liver 5(6):293–300

Zalzman M, Gupta S, Giri RK, Berkovich I, Sappal BS, Karnieli O, Zern MA, Fleischer N, Efrat S (2003) Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci U S A 100(12):7253–7258

Zhao R, Duncan SA (2005) Embryonic development of the liver. Hepatology 41(5):956–967

Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, Stanger BZ (2009) Notch signaling controls liver development by regulating biliary differentiation. Development 136(10):1727–1739

Chapter 9
Intestinal Stem Cells in Homeostasis and Cancer

Sandhya Singh, Mitnala Sasikala, G.V. Rao, and D. Nageshwar Reddy

Abstract Intestinal stem cell (ISC) population at crypts base displays extraordinary capability for organized renewal and regeneration of intestinal epithelium. Complex interplay among stem cells, its progeny and niche balances between self-renewal and differentiation to maintain intestinal homeostasis. Involvement of various interactions to regulate intestinal epithelium rapid renewal, presents with high risk of developing cancer. Intestinal stem cells (ISCs) biology and cancer development is closely related in various aspects. Studies have shown, ISCs as the cells of origin for majority of intestinal cancer where signaling pathways regulating ISC are often deregulated, giving rise to cancer stem cell (CSC). Moreover, intestinal cancers are shown to maintain cellular hierarchy similar to intestinal epithelium with presence of CSC at apex. CSCs are cell subpopulation with ISC like features involved in tumor genesis. Here we present common and different features of ISC and CSC with special emphasis on differential regulation of Wnt, Notch and BMP signaling pathways in both stem cell populations. Recent identification of both ISC and CSC markers along with technological development to track stem cell lineage and endogenous activity in vivo with possibility to generate ex vivo intestinal organoids, has broaden our understanding regarding ISC driven intestinal epithelium homeostasis, repair and cancer. Basic understanding of intestinal stem cell biology and its role in carcinogenesis opens up exciting opportunity to develop stem cell based therapeutics for cancer treatment.

Keywords Intestinal stem cell • Paneth cell • Cancer stem cell • Transit-amplifying cell • Enterocyte • Subepithelial myofibroblasts • Niche • Wnt pathway • Notch pathway • Carcinogenesis • miRNA

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_9

9.1 Introduction

Rapid renewal of intestinal epithelium, which requires accurate tissue organization, presents it with higher risk of developing cancer (Creamer et al. 1961). Intestinal epithelial homeostasis is maintained by intestinal stem cell (ISC) present at the bottom of the crypts where it undergoes asymmetric cell division to renew itself, and give rise to transit-amplifying cells (TA cells). Stem cell cease to divide while TA cells divide further and migrate toward crypt villus junction, and differentiating into different lineages simultaneously. Regulated intestinal stem cell activity maintains homeostasis of intestinal epithelium. by linking proliferative capacity of stem cell and TA cells as per tissue requirements (Barker 2014). Understanding of proliferation and migration in the maintenance of intestinal epithelial homeostasis involving various intrinsic and extrinsic factors is emerging. Identification of signaling pathways involved in intestinal epithelial homeostasis, will not only provide basic aspects in ISC regulation, but also help us understand various disease pathologies, including cancer. Mutational studies in ISC of mouse model have shown similarities between ISC and CSC (cancer stem cell), which provides strong evidence that regulated ISC proliferation prevent tumor genesis (Ashley 2013; Tajbakhsh 2014).

Stem cell (SC) population from different tissue shows distinct diversity in their proliferation capacity, indicating different regenerative requirement. Intestinal stem cell, identified by Lgr5+ marker, falls into rapidly dividing SC, whose proliferation also increases further on tissue injury or infection similar to slow dividing stem cell from other localization. This similarity indicates common mechanism for proliferative plasticity regulation, for SC from different localization. Homeostasis maintenance in intestinal epithelium requires regulation of ISC activity at two levels: (1) SC proliferation by asymmetric SC division for self-renewal, and differentiation, to maintain pluripotent SC population; (2) Increase in SC proliferation in response to tissue injury and again entry into quiescent/non proliferative state after tissue repair and regeneration (Cheng and Leblond 1974; Barker et al. 2008; Metcalfe et al. 2014).

Involvement ISCs and its tight regulation based on its microenvironment, and its differentiated lineage, indicates its dysregulation will lead to cancer initiation and regeneration failure. The complex ISC lineages in mammals presents with major bottleneck in identification of regulatory processes at stem cell level in vivo. Identification of ISCs in posterior midgut from Drosophila, shows proliferative plasticity similar to mammalian ISCs. Presence of well defined genetic manipulation tools, identification of factors involved in crypt homeostasis is very rapid in this model organism. Characterization of factors involved in ISC regulation in these simpler model organisms, provides strong mechanistic framework to study intestinal tissue homeostasis maintenance in mammals. Further studies are required in mammals to understand the intricate interplay between intestinal epithelium and its microenvironment for in homeostasis, regeneration and carcinogenesis (Barker et al. 2009; Biteau et al. 2011; Andriatsilavo et al. 2013; Barker 2014; Baker et al. 2015).

Recent breakthrough discovery with generation of intestinal organoid culture system, an in vitro model system can be utilized to study in vivo Intestinal organization. This method utilizes ISC from Intestinal crypts that grows into organoids in presence of matrigel (extracellular matrix proteins) that supports three-dimensional growth. ISC and CSC culture to generate intestinal organoids will come handy in understanding normal crypt intestinal homeostasis, and it's role in cancer development (Chandler and Lagasse 2010; Barker 2014).

This chapter provides current concept on ISC identity, signaling pathways and microenvironment signals that regulate intestinal homeostasis. We also discuss recent development on similarities and dissimilarities in normal and cancer intestinal stem cell, with special emphasis on disturbed intestinal homeostasis in cancer initiation and cancer stem cell maintenance.

9.2 Intestinal Epithelium Architecture

Intestinal tract epithelium architecture and its different segment's developmental features are well studied. Intestinal tract primary functions include digestion, nutrients absorption, secretions, and immune response. The elegant intestinal mucosal architecture with multiple infoldings, facilitates increase of surface area which supports its function. Intestinal wall is made up of four layers: epithelium, lamina propria, muscular mucosae, muscularis propria and the serosa. Mucosa forms the innermost layer further subdivided into three layers, with an important functional and structural role. The first layer faces the intestinal lumen and is made up of single layer epithelial cells. Lamina propria forms the second layer and consists of connective tissue and lymph nodes. Muscularis mucosae made of smooth muscle cells forms the third layer, and rest on submucosa, under which muscularis propria layer is present. The outermost layer is known as serosa and adventitia, if it lacks mesothelial cells in outer layer. Different types of cell populate sub mucosal layer such as inflammatory cells, nerve fibers, and lymphatics. Small arteries and venous also branches and distribute blood from this area (Montgomery et al. 1999).

The intestinal epithelium consists of various cell types under constant renewal process while maintaining a pattern. Under normal condition, large number of cells lost in intestinal lumen tract, are efficiently replaced by ISC compartment. ISCs along with progenitor cells present in crypt give rise to differentiated epithelial cells while migration toward lumen. The basic intestinal architecture depends on the dynamic homeostasis between cell generation at intestinal crypt and cell loss at lumen. Different parameters including dietary, growth factors, and transcriptional factors plays key role in regulation of intestinal homeostasis. A complex interplay within various signaling pathways maintains homeostasis by appropriate cell loss of apoptotic cells at intestinal epithelium. Disruption of homeostatic balance either leads to mucosal atrophy or mucosal hyperplasia, caused by less or overproduction of cells. Mucosal atrophy causes mal-absorption of nutrients while hyperplasia

leads to hypersecretion with increased risk of cancer development (Barker et al. 2008; Chandler and Lagasse 2010; Barker 2014).

9.3 Intestinal Stem Cells

The rapid intestinal tracts turn over and regeneration after injury depends on resident intestinal stem cell in crypt of Lieberkuhn. Intestinal stem cells are cells undergoing asymmetric cell division to maintain there own number with capability of self renewal over longer period and supply all undifferentiated cell types specific to intestinal tissue (Figs. 9.1 and 9.2). It also serve as internal repair system through aiding regeneration, by replacing lost or damaged cells in case of injury as long as organism is alive to maintain tissue homeostasis and maintain its architecture. Recent studies, provides strong evidences about existence of dedicated stem cell in different adult tissues with an important role in respective tissue maintenance. However, inspite of rapid identification of adult stem cells in different tissue; it is still not established if various tissue-specific stem cells behaviors converge on common molecular and cell biological concepts. Stem cells originate either from embryos formed during embryogenesis termed as embryonic stem cells or tissue specific somatic "Adult stem cells". Both stem cells type are known for there

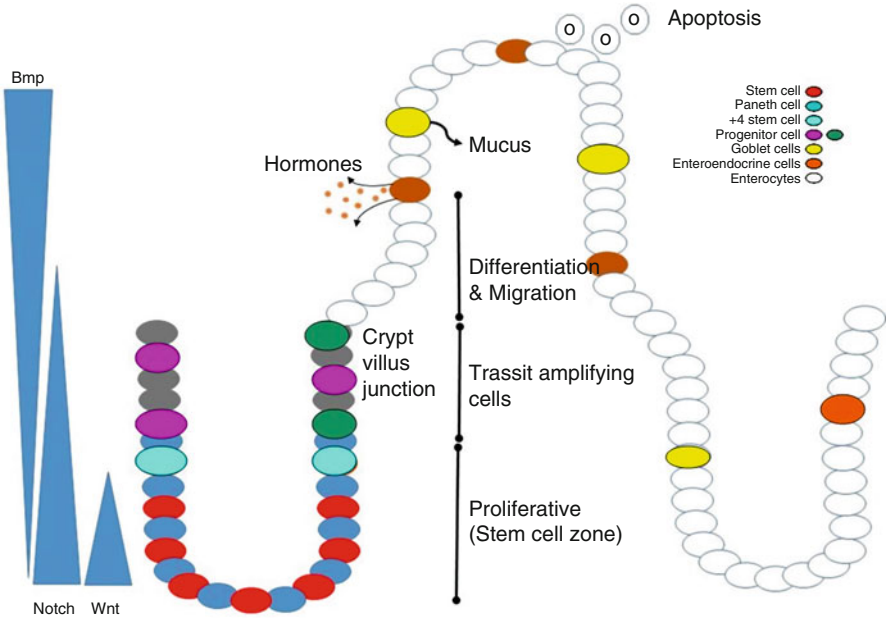

Fig. 9.1 Intestinal epithelium architecture showing intestinal stem cell (marked in *red*) at crypt base alternating with paneth cells (marked in *blue*) contribute to maintenance of intestinal epithelium homeostasis

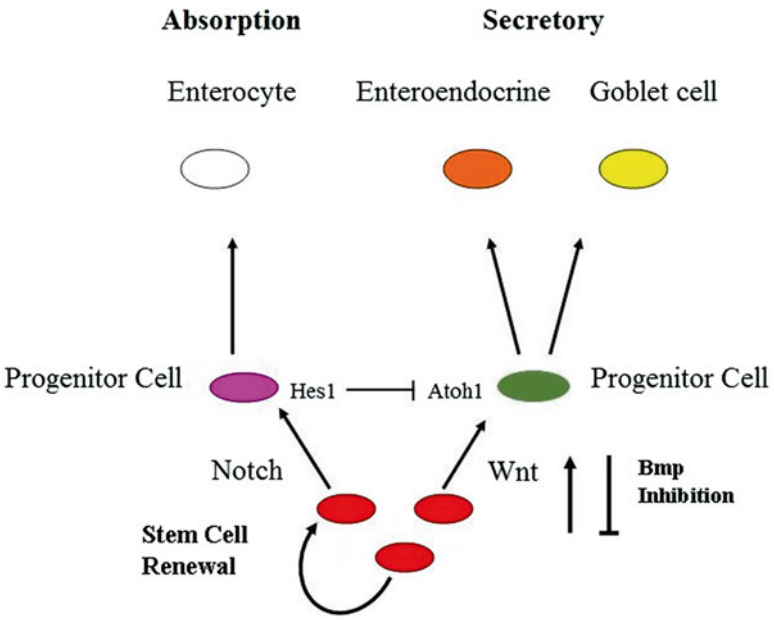

Fig. 9.2 Intestinal stem cell (marked in *red*) produce all differentiated intestinal epithelium cell (marked in *white, orange & yellow*), while maintaining its own self renewal. It also shows regulatory role of Wnt, Bmp & Notch pathway in cell fate decision of progenitor cell (marked in *pink & green*)

potential to differentiate into another tissue specific cell type, like epithelial cells of different origin, red blood cells, muscle cells or nerve cells. Two features specific to stem cell differentiate it from other cell types; firstly, stem cells are non specialized cells capable of self renewal even after long quiescent state. Secondly, stem cells can be differentiated into tissue specific differentiated cells with specialized function under different physiological or experimental conditions, induced with different stimulus. In rapidly renewing organs like intestinal mucosa, stem cells regularly divide to maintain homeostasis and replace lost cells under normal physiological situation or damaged cells during regeneration after tissue injury. Whereas, certain organs, like brain or heart, their tissue specific resident adult stem cells have a major role to play, in regeneration to replace cells lost during injury or disease. Unique regenerative stem cells property holds tremendous therapeutic potential in various diseases treatment like diabetes or heart disease (Weissman 2000; Fuchs 2009; Todaro et al. 2010).

The intestinal stem cell in the base of crypt surrounded by fibroblasts, and supporting mesenchymal tissue form a basic anatomical unit, that leads to its differentiation into four cell type of secretory lineages of absorptive enterocytes, enteroendocrine, Paneth and goblet cells. Intestinal stem cells (ISCs) in crypt base are continuously activated by various intrinsic and extrinsic factor to generate progenitor or transit amplifying (TA) cells which further differentiates into mature

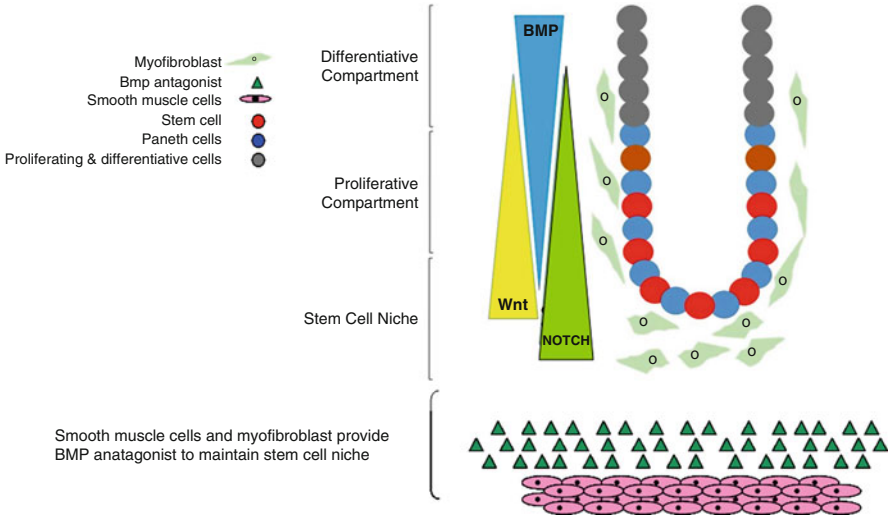

Fig. 9.3 Intestinal stem cell and its differentiated progeny maintains intestinal epithelium homeostasis in its appropriate niche

tissue specific cell lineages capable of performing specialized functions (Barker et al. 2008; van der Flier and Clevers 2009; Schwitalla et al. 2013) (Fig. 9.3). Under normal conditions, TA cells reside for 2–3 days in crypts and undergo cell division for approximately six times to reach crypt villus junction, where it differentiate into all four terminally differentiated cell types. Intestinal crypt is usually populated by undifferentiated cells; but differentiated Paneth cells known to secrete antibacterial agents, is exceptionally is located at crypt base and also escape it migration towards lumen. Under physiological situation, ISCs undergoes asymmetric division to generate one stem cell and other TA cell, which eventually differentiate into mature epithelial cells (Figs. 9.1 and 9.2). However, after injury, damaged ISCs are replaced by one daughter stem cell from ISCs, undergoing symmetrical division to generate two stem cells. Tightly regulated ISC symmetrical and asymmetrical division, to maintain self renewal and differentiation of TA cell while migrating upwards to lumen from crypt, suggest various intrinsic and extrinsic factors involved like its ISC niche, signaling pathways, microenvironment induced etc. (Scoville et al. 2008) (Figs. 9.1, 9.2 and 9.3).

9.4 Intestinal Stem Cell Niche

Intestinal stem cell niche is defined as an anatomical unit composed of intestinal stem cells, its progeny, and its microenvironment, which together maintain normal homeostasis by appropriate differentiation into mature cells (Barker and Clevers 2007). It protects ISC from various stimuli that might lead to excessive ISC

differentiation and thus prevent from risk of developing cancer. The intestinal homeostasis depends on intricate balance between self-renewal and differentiation. Fibroblasts surrounding intestines, known as subepithelial myofibroblasts play an important role in ISC niche formation through various cytokines and growth factors secretions, which promote TA cells differentiation to differentiated mature cell lineages of enterocytes, enteroendocrine, goblet, and Paneth cells (Giannakis et al. 2006; Moore and Lemischka 2006) (Fig. 9.3).

Two different models exists to explain crypts structure maintenance by ISC, which are "+4 position" and "stem cell zone" model located either at crypts bottom or below the +4 position respectively (Potten et al. 1974; Barker et al. 2008). The "+4 position" model, supported by Chris Potten et al., suggest terminally differentiated Paneth cells populate crypt base. Therefore, the ISCs locates itself at +4 position just above Paneth cells, which differentiates into enteroendocrine, goblet and enterocytes cells while migrating out of the crypts onto the vill, and as Paneth cells on moving down toward the crypt base. The model proposed by Leblond et al. (Cheng and Leblond 1974) suggest that crypt base columnar cells, an undifferentiated cycling cells lying alternatively with Paneth cells, are most likely the true intestinal stem cells. However, exact identity for intestinal stem cell still remains controversial due to lack of specific ISC markers for these cells (Fig. 9.4). Every crypt is supposed to harbor at least 4–6 independent stem cells. Recent, BrdU-labeling studies observed label-retaining cells localized at +4 positions with respect to crypt bottom, while first three positions was taken by terminally differentiated Paneth cells. Moreover, +4 cells were also found to be very sensitive to radiation, a stem cell property, known to prevent genetic modification in stem cell (Potten et al. 2002). Lineage tracing studies in Bmi-Cre-ER knock-in allele mice model, further supports "+4 position" ISC model (Sangiorgi and Capecchi 2008; Barker and Clevers 2010). Studies from Barker et al. support "stem cell zone" model where Prominin and Lgr5/GPR49 (Wnt target gene) staining label CBC stem cells alternating with paneth cells in the mouse intestinal tract (Barker et al. 2007, 2009). CBC cells were also shown to maintain intestinal epithelial cell self-renewal and generation of differentiated mature intestinal epithelial cells for long time. Later, lineage-tracing experiments identified both CBC and +4 cells with feature of adult multipotent stem cells showing self renewal. Therefore, these two stem cell population presents with strong possibility of combined role to play in intestinal epithelial tissue homeostasis maintenance and regeneration under physiological and various pathological conditions (Sancho et al. 2003; Barker et al. 2008).

9.5 Intestinal Stem Cell Marker

The majority of intestinal stem cell marker identified, show non specific expression throughout the proliferative crypt compartment. Recent breakthrough studies by Clevers et al. identified Lgr5/Gpr49 gene as specific intestinal stem cell marker confined to intestinal stem cell in crypts bottom (Barker and Clevers 2010).

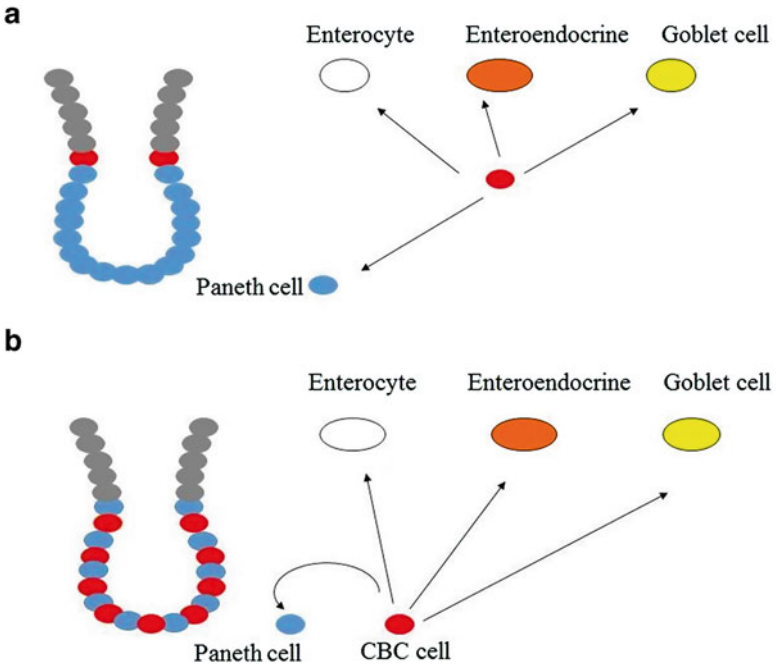

Fig. 9.4 Intestinal stem cell models; (**a**) "+4 position" model proposed by Chris Potten and colleagues suggest that crypt base is mainly occupied by Paneth Cells (marked in *blue*) with stem cells (marked in *red*) at the +4 position, placed above Paneth cells. (**b**) "stem cell zone" model proposed by Leblond et al. suggest undifferentiated crypt base columnar cells stem cell (marked in *red*) alternating with Paneth cells (marked in *blue*) are real intestinal stem cells. Due to lack of specific intestinal stem cell marker, accurate evidence in support of either model is still lacking

The Lgr5 gene expresses an orphan G protein-coupled receptor (GPCR) and exhibits similarity to GPCRs like FSH, TSH with glycoprotein ligands. It consists of large leucine-rich extracellular domain; known to express in other tissue stem cells also driven by wnt signaling pathway. Restriction of Lgr5 expression in APC min mouse in crypt bottom cells and adenomas supports involvement of Wnt pathway (Chandler and Lagasse 2010) (Fig. 9.5). Studies have shown regeneration of functional, self renewing intestinal crypt like structures in vitro on exposure of Lgr5 ISC to appropriate signaling pathways and extracellular matrix (Sato et al. 2009) (Fig. 9.8). ISC identified based on other stem cell markers like Bmi, Prominin-1 also showed similar results (Munoz et al. 2012; Metcalfe et al. 2014). Additionally, lineage-tracing experiments also confirm Lgr5+ cells as self renewing ISCs that maintain the intestinal epithelium architecture. Lgr5 represent a common adult stem cell marker since it identifies stem cells from other tissues like mammary gland, stomach epithelium and hair follicles too. However, functional stem cell features of Lgr5 cell is well studied in intestine compared to other tissues. Identification of ISC for stem cell

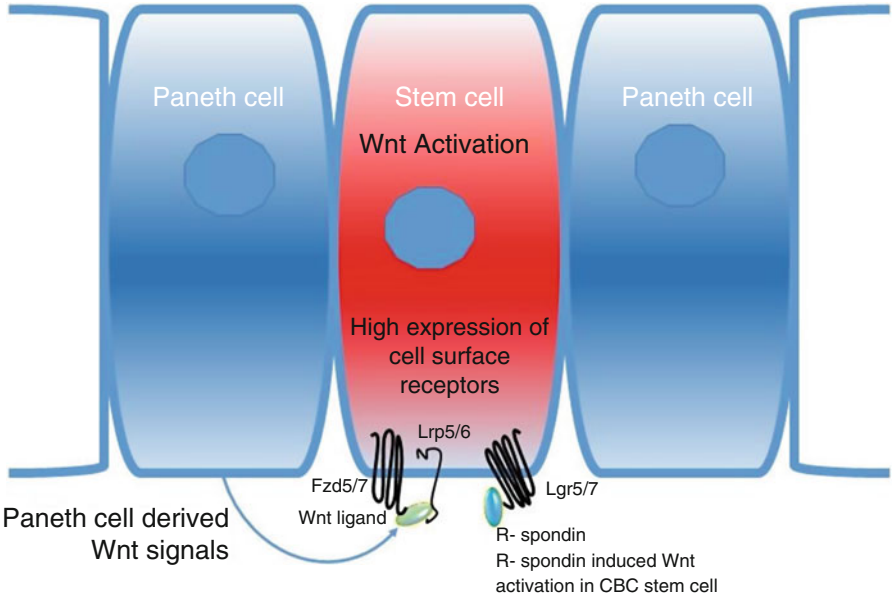

Fig. 9.5 Paneth cells (marked in *blue*) produce various wnt ligands which act on adjacent Lgr5+ intestinal stem cell (marked in *red*) through various cell surface receptors, to activate Wnt pathway and maintain stem cell phenotype and functions. R-spondins also maintains synergistic high wnt activity in stem cell zone via highly expressed cell surface receptors

lineage tracing or its identity is based on many markers, like Lgr5, Prominin 1, and Bmi (Sangiorgi and Capecchi 2008; van der Flier and Clevers 2009; Zhu et al. 2009). Apart from ISC, CSC can also be identified based on markers, like Lgr5, Prominin, CD29, and CD24, which can be used efficiently to identify therapeutic targets to design therapies to eradicate them, a major cause for recurrence and metastasis (Merlos-Suarez et al. 2011; Yu et al. 2012a) (Fig. 9.6).

9.6 Signaling Pathways Regulating Intestinal Stem Cell

Inspite of debate regarding ISC identity, common consensus exist about its localization in niche that presents them with constant stimulus from various morphogenetic pathways like Wnt, Notch, BMP, JAK/STAT1, PTEN, AKT, PI3K and Ephrin. Different studies using animal model, confirms important role of these signaling pathways in ISC regulation required for epithelial cell renewal and differentiation to maintain intestinal homeostasis (Figs. 9.1 and 9.7). Deregulation of any signaling pathway disturbs intricate delicate balance required to maintain intestinal homeostasis, which in turn can lead to carcinogenesis (Scoville et al. 2008; van der Flier and Clevers 2009; Barker 2014).

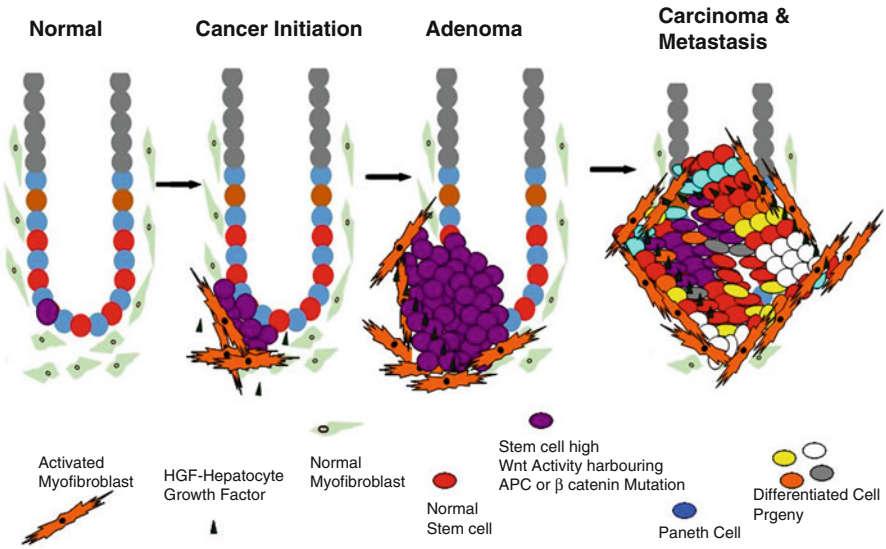

Fig. 9.6 Intestinal carcinogenesis is initiated by genetic mutations of APC or β-catenin or epigenetic modification to dysregulate balance among various signaling pathway causing high wnt activity of stem cell (marked in *purple*) leading to polyp formation. These intestinal stem cell modifications disturbs homeostasis which activates surrounding niche giving rise to activated myofibroblast (marked in *orange*) secreting various growth factor like HGF (marked in *black*). This disturbed homeostasis further activates Wnt pathway supporting clonal expansion of initial maintains synergistic high wnt activity in stem cell zone via highly expressed cell surface receptors

9.6.1 Wnt Signaling Pathway

The canonical Wnt pathway regulates ISC and epithelial cell renewal to maintain intestinal tissue homeostasis (Pinto et al. 2003). Utilizing mouse model, role of Wnt pathway activation in intestinal tissue homeostasis maintenance and its inhibition in epithelial cell renewal blockage is well studied. Wnt pathway inhibition blocks epithelial renewal while its activation promotes epithelial proliferation. High wnt activity at base along with its appropriate gradient along the crypts axis maintains stem/progenitor phenotype, proliferation, differentiation and migration. Wnt target genes like MYC, EPHB2 and CDCA7 show high expression at crypt base, while expression of genes driven by wnt suppression like P21 and SMAD2 is high at crypt top. Wnt receptors and co-receptors, involved in wnt signaling pathways are well expressed by intestinal epithelium. Activation of Wnt pathway utilizing Wnt agonist like r spondin over expression, further confirms its active role in epithelial proliferation in intestinal homeostasis maintenance (Kosinski et al. 2007) (Figs. 9.1 and 9.5).

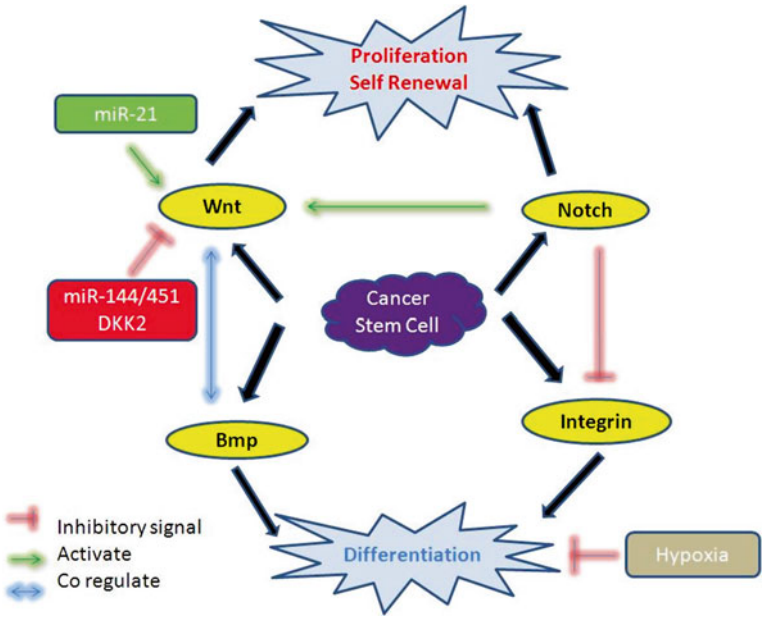

Fig. 9.7 Dysregulated signaling pathway regulates cancer stem cell (CSCs)

Recently, its involvement in CSC identity and carcinogenesis has also gained momentum. Stabilization of Wnt pathway in mouse intestinal stem cells is sufficient to generate adenomas, in contrast to differentiated cells (Pinto and Clevers 2005). Majority of colorectal cancers presents with constitutive wnt activation, harboring activating mutations of oncogene, β catenin or inactivating mutation of tumor suppressor, APC (Schwitalla et al. 2013). A study by Vermeulen et al. also reported, Wnt signaling gradient in tumors using Wnt reporter constructs, which correlated with differentiation markers like MUC2 inversely (Vermeulen et al. 2010). Presence of cells exhibiting high Wnt activity near stromal myofibroblasts in crypts base, depict cancer stem cell population. This indicates role of external factors, secreted from myofibroblast like hepatocyte growth factors in β catenin stabilization to activate Wnt pathway, which in turn regulates CSC phenotype (Fig. 9.6). Moreover, Wnt pathway activation in differentiated cells by secreted myofibroblast factors also pushes them to acquire CSC phenotype both in vitro and in vivo, which confirms dynamic involvement of microenvironment (Powell et al. 2005; Sato et al. 2011).

In absence of Wnt ligands, β catenin is degraded by proteosomal degradation complex after phosphorylation by casein kinase 1 (CK1) and glycogen synthase 3 (GSK3) mediated by multiprotein degradation complex including other proteins like APC and Axin-1. In presence of wnt ligands, it binds to the frizzled cell receptor which associate Axin-1 to the plasma surface and prevents β catenin degradation. Stabilized β catenin translocates to nucleus to interact with T-cell factor/Lymphoid Enhancer Factors (TCF/LEFs), involved in expression of Wnt target genes (Schwitalla et al. 2013).

Wnt pathway is of utmost importance in intestinal homeostasis maintenance since it is involved in regulating ISC pools (Figs. 9.1, 9.3 and 9.6). However, wnt pathway works in cooperation with both inhibitory and stimulating ephrin gradients its receptor tyrosine kinase receptors known to maintain ISC niches. Wnt activation by APC inactivation (Wnt pathway tumor suppersor gene) or β-catenin activation (stabilized mutated form ovexpression) leads to intestinal hyperplasia. On the other hand, wnt activator silencing like TCF4 or wnt inhibitor overexpression like Dickkopf homologue-1 (Dkk-1) (Fig. 9.7), impairs ISC proliferation, which disturbs intestinal epithelium homeostasis involved in carcinogenesis. In addition to ISC maintenance, Wnt pathway is also involved in differentiation of paneth and progenitor cell, their maturation and migration along villus (Fig. 9.7). Ephrin B receptors and its ligands both identified as wnt target genes guide differentiation and migration. Both wnt receptors and its ligands depicts disparate expression pattern across intestinal epithelium, but β-catenin shows constant nuclear localization at crypts bottom. Constant ß-catenin nuclear localization of ISC at crypts base occurs as a result of constant wnt activation by presence of adjacent WNT 3a secreting paneth cell (Sato et al. 2011) (Fig. 9.5). Depletion of stem cell pool on loss of paneth cell, confirms its role in maintenance of intestinal stem cell pool. Further research is needed on differential regulation of wnt pathways across intestinal epithelium but consensus exists on its important role in regulating intestinal stem cell involved in intestinal homeostasis maintenance.

9.6.2 BMP Pathway

BMP pathway plays an important role in intestinal epithelium development by acting as a negative regulator in regulation of intestinal epithelial cell proliferation at crypts bottom (Haramis et al. 2004). BMP belongs to transforming growth factor-β family which binds to BMP receptors. Presence of BMP isoforms in mesenchyme and its receptor in intestinal epithelium further indicates its active involvement. Deletion of BMP receptor in intestinal epithelium leads to hyperproliferation of epithelial cell. BMP antagonist, "Noggin" is expressed around crypts and +4 cells, and its overexpression leads to ectopic crypt formation. Mice lacking BMP-2 and BMP-4 null were found to be embryonically lethal (Batts et al. 2006) (Figs. 9.1, 9.3 and 9.7). Additionally conditional depletion of BMP receptor confirmed BMP act to inhibit crypt formation and intestinal stem cell renewal. β-catenin nuclear translocation on inactivation of BMP receptor or overexpression of its antagonist like Noggin indicates interaction among BMP and Wnt signaling pathways (Figs. 9.1, 9.2, 9.3 and 9.7). Studies also provide evidence on microenvironment related Wnt and Bmp pathways role in regulation of intestinal stem cell in maintenance of intestinal epithelium (He et al. 2004). Recent study by Guezguez A et al., elegantly shows BMP pathway modulates stemness of normal human intestinal epithelial crypt (HIEC) cell line by activation of Wnt pathway (Guezguez et al. 2014). Activation of wnt pathway in HIEC cells by Wnt activator like R-spondin 1 induces β-catenin nuclear

translocation to switch on WNT target genes. BMP pathway inactivation by its antagonist "Noggin" activates wnt pathway to induce intestinal stem cell features, characterized by expression of stem cell marker like LGR5. This model can serve as a suitable model to further investigate intestinal stem cell regulation in self-renewal and differentiation (Guezguez et al. 2014).

9.6.3 Notch Pathway

Notch pathway also plays an important role in maintenance of crypt compartment by regulating intestinal stem cell self renewal in cooperation with both Wnt and BMP pathway (Fre et al. 2005). Notch is a single transmembrane receptors involved in cell fate differentiation. Various components involved in Notch pathway are known to express in crypts base. Studies utilizing knock out animal further proves its indispensible role in ISC regulation. Notch pathway activation in intestinal epithelium induces cell proliferation, while its inactivation causes reduction of differentiated secretory cells. Along with its role in ISC proliferation, recent studies shows its functional role in TA compartment in regulating different cell fate in the intestinal epithelium (Fre et al. 2009) (Figs. 9.1, 9.2, 9.3 and 9.7).

Role of other signaling pathways like JAK/STAT, PTEN, PI3K in regulation of ISC phenotype is limiting, however various evidence exist in literature about their involvement (Biteau et al. 2008).

9.7 Intestinal Stem Cell in Carcinogenesis

Intestinal stem cell role in the maintenance of intestinal epithelium renewal and repair is well established (Leblond and Walker 1956; Barker et al. 2008). Recent identification of stem cell marker along with robust lineage tracing technologies to keep track of endogenous stem cell function in vivo, has further confirmed its indispensible role in intestinal homeostasis and regeneration (Barker et al. 2012; Blanpain and Simons 2013). Stem cell driven intestinal homeostasis is tightly regulated at various level, which when deregulated is also known to lead intestinal carcinogenesis. These recent developments in intestinal stem cell biology presents with an immense opportunity to exploit its potential to develop stem cell based therapeutics for effective cancer therapy (Buske et al. 2011; Munoz et al. 2012).

Identification of stem cell marker, techniques to evaluate their behavior in vivo and development of ex vivo intestinal organoid culture from patients normal and disease tissue, paved the path to understand their behavior in homeostasis and disease (Sato and Clevers 2013). Studies in intestinal cancer identify derailed intestinal stem cell interaction with its niche, lead to cancer initiation and progression (Fig. 9.6). Intestinal tumors harbour a small population of cells with stem cell features known as 'cancer stem cells', responsible for carcinogenesis. Both normal and cancer stem

cell depicts many similarities and differences guided by similar signaling pathway, however, differentially regulated (Schwitalla et al. 2013).

Cancer is considered as a stem cell disease since stem cells undergoes self-renewal and cancer is disease of dysregulated self-renewal (Radtke and Clevers 2005). Moreover, stem cells have a long life span sufficient enough to acquire different sequential mutations to become malignant, whereas for a normal differentiating cell to become malignant and escape apoptosis need to acquire multiple mutations effecting cell proliferation, cell adhesion, and migration collectively, instead serially. Strong evidences exist in literature which supports existence of cellular heterogeneity within tumor with CSC at the apex (Al-Hajj et al. 2003; Pinto and Clevers 2005). Among heterogeneous cell population of tumor, only CSCs harbor self renewal ability required to sustain the tumor growth (Barker et al. 2009).

9.7.1 Cancer Stem Cell

Long life span and self renewal nature of intestinal stem cells present them as an ideal candidate for cell of origin for intestinal cancer (Barker et al. 2009). Wnt stabilization in intestinal stem cell is sufficient for adenoma generation in contrast to differentiated cells. Literature presents with strong evidence regarding existence of subpopulation of cells in intestinal cancer with stem cell properties termed as 'cancer stem cells' (CSCs) (Clevers 2011) They show feature similar to normal stem cell like self-renewal, generation of different cell lineage, capacity of tumor initiation and progression. Single cells obtained from same primary tumor shows different cancer initiation properties. CSC like their normal stem cell counterparts shows different levels of differentiation (Fig. 9.6). Therefore, intestinal cancer retains differentiation potential and 'crypt-like' morphology, along with expression markers for differentiated cells like enterocyte, endocrine and goblet cell markers. Different in vitro and in vivo experiment utilizing cell lines and primary tumors confirm presence of CSC capable of cancer initiation and multipotent differentiation potential (Ricci-Vitiani et al. 2007; Vermeulen et al. 2008; Yeung et al. 2010). Based on normal and cancer stem cell similarity, CSCs can be isolated by normal intestinal stem cells markers like CD133 and CD44, with potential to regenerate original tumor in mouse. However, there exist some tumors which fall in poorly differentiated category, incapable of recapitulate crypt like structures are very aggressive with poor prognosis (Yokoyama et al. 2010; Dalerba et al. 2011; Kim et al. 2015). Gene expression studies reports that tumors and cell lines with more immature stem cell like cells were found to be more aggressive with poor prognosis. CSC like normal stem cell maintains self-renewing properties but lack some or most of its capacity to differentiate. Therefore, unlike normal stem cell, CSC is more in number, often forming majority of tumor. Stem cell population dynamics mathematical modeling supports this idea (Johnston et al. 2007, 2010).

Over expression of ABC transporters in CSC marks them more resistant to chemotherapy while Il4 over expression provides enhanced DNA repair and mark them

apoptosis resistant. However, as an alternative mechanism, CSCs chemotherapeutic resistance is also reported to be mediated by differentiated cells in tumor with high expression of ABCB1 multidrug efflux pumps (Dean et al. 2005; Todaro et al. 2007; Houthuijzen et al. 2012). In colorectal cancer studies, primary tumor harboring CSC with marker CD26 depicts high metastatic potential and early stage patients with CD26 + CSCs presnt with metastasis in follow up (Pang et al. 2010).

A major limitation to CSC studies is presence of a unique definitive CSC marker, inspite of fact existing markers like CD44 and CD133 are capable of CSC enrichment under specific conditions (Dieter et al. 2011). Existence of many, possibly interchanging stem cell populations along with presence of subpopulation, suggests toward non existence of universal CSC marker (Takeda et al. 2011). This scenario is further complicated by heterogeneous nature of tumor like epigenetic or miRNA mediated silencing of stem cell marker reported in intestinal cancer (de Sousa et al. 2011b).

9.7.2 Deregulated Signaling Pathway Regulates CSCs

Various signaling pathways mentioned above coordinately regulate ISC for efficient epithelial cell renewal and differentiation to maintain intestinal homeostasis. Abrupt deregulation of these signaling pathways disturbs intricate delicate balance required to maintain intestinal homeostasis, which in turn can lead to carcinogenesis. Among all pathways, Wnt, Notch and BMP actively participate in regulating ISC self renewal and differentiation is of utmost importance.

The canonical Wnt pathway deregulation is recently established as a regulator of colon CSCs (Figs. 9.1, 9.2, 9.3 and 9.7). Wnt maintains intestinal homeostasis by regulating intestinal stem cell pools. Stabilized wnt signaling in ISC in contrast to other differentiated cell, is sufficient to generate adenomas. Wnt stabilization can occur in response to mutation in any protein involved in multi protein degradation complex formation (Korinek et al. 1998; Pinto et al. 2003; Kemper et al. 2010; de Sousa et al. 2011a). Approximately, about 90 % of colorectal cancers were found to harbor APC or β catenin mutation which leads to wnt activation. Intestinal tumor shows heterogeneous level of wnt signaling which correlate inversely with differentiation markers like MUC2. Cell with high wnt signaling express stem cell marker CD133 with increased in vitro colonogenicity and highly enriched in tumor initiating cells. High expression of wnt inhibitors at crypt top while activator at crypt bottom, indicates tight regulation of wnt pathway in maintenance of normal crypt stem cells (Gregorieff et al. 2005; Davies et al. 2008). Recent discovery of intestinal stem cell marker, a wnt LGR5 positive, was identified in screening Wnt target genes in crypt base. Isolated single LGR5 cells are shown to form in vitro differentiated crypt-like organoids in 3D in vitro culture (Kosinski et al. 2007; Sato et al. 2009).

Loss of LGR5 in vitro culture of mouse crypt inhibits its growth while wnt administration in vitro rescued inhibition. LGR5 mediate wnt signaling in intestinal epithelium by direct interaction with frizzled receptor complex, mediated by

r-spondin signaling (Fig. 9.5). Interestingly, loss of Lgr5 in cancer cell line leads to increase in Wnt target gene expression [51], which might be explained by possible role of Lgr5 in r-spondin signaling, lacking in vitro culture unlike in vivo. Therefore, wnt activation on Lgr5 knock down in cancer cell points toward its possible negative regulatory role in absence of r-spondin signaling. Role of Lgr5 as wnt pathway negative regulator is further supported by intestines of LGR5 null mouse, most probably a stage before r-spondin signaling sets in (Carmon et al. 2011; de Lau et al. 2011; Walker et al. 2011).

Inspite of literature available for LGR5 in normal intestinal stem cell, its definitive role in CSCs is not clear. A heterogeneous Lgr5 expression is reported in primary colorectal tumors as well as metastatic tumors. Similar expression pattern is also observed for Olfm4, a surrogate marker for Lgr5 stem cells in various in human adenocarcinomas. However, many tumor tissues and cancer cell lines were also found not expressing Lgr5. Moreover, CRC patient's studies correlating survival with LGR5 expression showed no significant correlation (van der Flier et al. 2009a; Kleist et al. 2011; Takahashi et al. 2011).

LGR5 mRNA expression studies in colorectal cancer tissues showed 95 % to less then 1 % LGR5+ cells and absent LGR5 expression in one third of tumors analyzed. Functional studies in cancer cell lines knocking out LGR5 increased tumour initiating property, while its over-expression exerts reverse effect (Merlos-Suarez et al. 2011). LGR5 was also shown to have role in regulating cell adhesion and effect cell migration negatively, suggests important function beyond mediating only Wnt signaling (Walker et al. 2011).

In normal intestinal epithelium of mouse, LGR5 stem cells were shown to be regulated by the Wnt target transcription factor, Achaete scute-like2 (ASCL2) [57]. Knock down of ASCL2 in mouse intestine leads to rapid loss of LGR5 cells, while over expression depicts crypt hyperplasia with ectopic crypts on villi (van der Flier et al. 2009b).

Similar to Lgr5, exact role of ASCL2 colorectal CSCs regulation is also not clear, however it is reported to be upregulated in early stage in colorectal cancer, following wnt activation, showing similar frequency in both adenomas and cancer. Due to lack of appropriate antibodies and reagents lacking sensitivity, current studies on role of Ascl2, Olfm4 and Lgr5 in CSCs are not up to the mark (Reed et al. 2012). The Ephrin Receptor B2 (Eph Receptor B2/EPHB2), another Wnt target gene expressed in crypts base act as better stem cell marker for normal human adult intestinal stem cells. Similar to LGR5 cells, EPHB2 expressing cells from normal human intestinal epithelium are multipotent, and capable of formation of crypt-like structures in vitro under appropriate in vitro 3D growth conditions. CRC tumors were also shown to coexpress EPHB2, LGR5, CD44 and ASCL2. CSC expressing EPHB2 shows high clonogenicity and tumour initiating capacity, which correlates negatively with differentiation markers analyzed in tumours. Both LGR5 and EPHB2 over expressing CSCs cells were found localized in crypt-like structures surrounding lumens, suggests existence of spatial relationship pattern in tumor similar to normal stem cell in crypt architecture (Jung et al. 2011; Merlos-Suarez et al. 2011).

Notch signaling pathway is known to regulate Intestinal stem cell through expression of four Notch genes which act as receptors for ligands like Jagged 1, Jagged2, and Delta Like (Dll) 1,3 and 4. Binding of ligand to Notch receptor leads to its cleavage by csecretase to release intracellular Notch domain (NICD) (Fre et al. 2005). Released NICD localize in nucleus to interact with CBF1/RBPJ to switch on Notch target genes like HES-1, -5,-7, Hey-1, Hey-2, HEYL (Miyamoto and Rosenberg 2011) (Fig. 9.2) Notch is also shown to enhance NFκB expression. High expression of Notch1 and Jagged-1 is observed in transit amplifying colon cells whereas expression of DLL-1 and 4 mediated Notch target genes are required mouse intestinal stem cells homeostasis (Pellegrinet et al. 2011). Inhibition of Notch pathway in mouse intestine leads to reduction in crypt cell proliferation, which promotes conversion of secretory endocrine and goblet cell at the cost absorptive cells. In contrast, activation of Notch-1 signaling causes differentiation reduction with increased proliferation of progenitor. Notch pathway is also known to regulate Wnt signalling via Akt (Anderson and Wong 2010), even though mechanistic details are controversial; Notch/Wnt interactions are likely to play important role in stem cell regulation (Figs. 9.2 and 9.7). Jag1 expression in progenitor cell is down-stream target of Wnt signalling while Wnt antagonist Dkk2 is also known to be regulated by Notch (Katoh 2007) Constitutive Notch activation involved in mouse intestinal stem cells proliferation is disturbed by lack of Tcf4 activity, indicates cooperative involvement of active Wnt signaling, for effective regulation of stem cells by Notch signaling (Fre et al. 2009).

Involvement of Notch signaling pathway in differentiation suppression and its constructive interaction with Wnt pathway mark it as important parameter involved in CSCs regulation (Fig. 9.2).

Constitutive Notch pathway activation in APC mutant mice presents with large number of adenomas even in colon, an area normally resistant to adenoma formation in mice cancer models (Fig. 9.6) (Laurent et al. 2008). Compared to normal intestinal tissue, Notch-1 and Hes-1 are over expressed in colorectal cancers (Zagouras et al. 1995). Stem cell like cells derived from in vitro primary cell culture from colorectal tumours showed high Notch signalling in comparison to normal tissue (Sikandar et al. 2010). Further notch pathway inhibition in these stem cell like cells pushed them towards differentiation with high apoptosis and reduced clonogenicity (Yeung et al. 2010, 2011). In a different study, knockdown of Notch in established colorectal cancer cell line causes reduced proliferation and clonogenicity with increased apoptosis, while Notch overexpression promotes reverse effect (Zhang et al. 2010). Interaction among Notch and Wnt pathways is mediated by voltage-gated proton channel NADPH Oxidase 1 (NOX1), expressed in normal colon and frequently over expressed in colorectal cancer, which interestingly correlates with K-Ras mutations (Laurent et al. 2008) Mice deficient of NOX1, down regulates both wnt and notch pathway which promotes bulk differentiation of intestinal progenitors cells into goblet cells, which indicates possible role of NOX1 in CSC by preventing its differentiation.

The possible interactions of various signaling pathways in cancer stem cells maintenance is shown in Fig. 9.7.

Notch and Wnt signalling pathway are on forefront of research involved in CSC regulation, however different other pathways also have an important role to play. BMP pathway consists of various multifunctional growth factors from TGFβ superfamily known to regulate intestinal stem cell differentiation. Conditional knockdown of BMPR1 in mice intestine leads to development of multiple intestinal polyps, as a result of Wnt signaling activation and increased proliferation of stem cells (He et al. 2004). Comparative gene expression analysis of human colon crypt tops and crypt bottoms, showed high expression of different BMP components in differentiated cells present at top (Kosinski et al. 2007). Whereas, different BMP inhibitors, like Gremlin-1, were found to be highly expressed at crypts bottom, indicates indispensible role of BMP pathway in intestinal differentiation regulation. BMP4 is highly expressed in colorectal cancer but not expressed in vitro expanded culture of CD133+ colorectal CSCs (Lombardo et al. 2011). Simulation of in vitro expanded CSCs with BMP4 induced differentiation by downregulating Wnt and LGR5 through PI3K/AKT signalling inhibition, along with decreased colonogenicity and increased sensitivity to 5-fluorouracil. In a similar way, exposure of differentiating $CaCO_2$ to BMP inhibitors Gremlin-1, pushed them to stem cell like features with reduced expression of differentiation markers (Kosinski et al. 2007). Reduced BMP signaling in CSC to avoid differentiation must be maintained by disturbed complex interplay between different pathways, in a fashion similar to ISC (He et al. 2004).

9.8 Epigenetic Regulation of Cancer Stem Cell Related Genes in Cancer

Different studies have shown synergistic role of both genetic and epigenetic alternation in cancer initiation and progression, including intestinal cancer. Abrupt DNA methylation of CpG island in promoter region of tumor suppressor genes inactivates it to initiate and promote cancer development. Inactivation of tumor suppressor APC known to induce aberrant DNA methylation disrupts normal intestinal epithelial cell differentiation as a major contributor of intestinal carcinogenesis. Epigenetic modification including DNA methylation and histone modifications, leads to abrupt response to various stimuli involved in maintenance of normal intestinal epithelium, which causes gene expression dysregulation which promotes cancer initiation and progression.

Gene signatures identified independently from normal intestinal stem cell with high wnt activity and EPHB2 expression indicates poor prognosis in patients (de Sousa et al. 2011b; Merlos-Suarez et al. 2011) However, in one of study by Medema et al. correlates increased expression of stem cell markers LGR5 and ASCL2 (Wnt targets gene) with good prognosis. Patient's tumors exhibiting Wnt target genes poor expression falls with immature stem cell (Merlos-Suarez et al. 2011). Detailed analysis identified silencing of wnt target stem cell marker genes in later stages

epigenetically through CpG island methylation. Ectopic overexpression of silenced gene or CpG demethylation by 5-azacytidine, inactivates Wnt signalling in cell lines, which leads to reduction in tumor growth in xenografts developed. These observations provide strong evidence for wnt target gene promoter methylation as good prognostic factor for tumor recurrence, and indicate that CSC gene signature reflects tumor's differentiation status and not CSC numbers. Therefore, expression of general stem cell related genes in tumour mark it more aggressive compared to tumors with high numbers of intestinal stem cell markers expressing cells. Absence of cells expressing wnt mediated intestinal stem cell markers can not confirm absence of CSCs. However, in contrast such tumours may consist of more CSCs with high expression other genes known to impart stem cell features like OCT4 (Merlos-Suarez et al. 2011), even though such CSCs will be not under Wnt signaling regulation. Poorly differentiated tumours with silenced Wnt stem cell marker genes, represent only limited patient tumours. Therefore for moderately differentiated tumours, presence of EPHB2/Wnt high CSCs may represent poor prognosis, but require further studies comparing actual number of CSC with patients prognosis. These observations lead to various open end question regarding reduction of wnt signaling by silencing of individual wnt target genes in later cancer stages and its correlation with poor prognosis. One possible explanation can be LGR5 methylation lead to reduction in Wnt signalling through lack of response to R-spondin. Nevertheless, Wnt target genes role beyond stem cell signaling, favoring tumor growth can not be ignored.

Epigenetic regulation by methylating DNA to promote CSC phenotype is reported for non wnt regulated genes also (Fig. 9.7). The intestinal stem cell marker BMI1, member of Polycomb group (PcG) proteins, are known for their role regulating transcription mediated by chromatin methylation. Gene expression analysis of highly methylated gene in colorectal cancer, identified more number of polycomb target gene silenced then and non-polycomb targets (Widschwendter et al. 2007). Therefore, epigenetic silencing take over for reversible chromatin methylation to switch off genes involved in differentiation to CSC phenotype to maintain a stem-like state in colorectal tumours. This observation is further confirmed by a study where embryonic stem cell genes overlap with adult intestinal stem cell signature and found to correlate with poor prognosis (de Sousa et al. 2011b; Merlos-Suarez et al. 2011). Role of BMI1 is also confirmed in intestinal CSCs. Moreover, BMI1 is also reported to be influenced by Wnt signaling indirectly, and directly by Krüppel-like factor 4 (KLF4), involved in goblet cell differentiation (Yu et al. 2012a). BMI1 mediated H2A ubiquitination is repressed by KLF4, and in vitro knock down in tumor xenografts promoted goblet cell differentiation and reduction in proliferation. These studies indicate important link between BMI1 and Wnt in regulating CSC, where inhibition of stem cell related gene like BMI1 promote CSC differentiation, an important therapeutic implication that can be explored in cancer treatment.

Mechanistic studies aimed at identification of epigenetic modifications in cancerous intestinal tissue in comparison to normal intestinal epithelium provides an opportunity to explore intestinal stem cell biology, diagnostic, prognostic and therapeutic intervention.

9.9 miRNA Regulation of CSCs

Research on exploring role of microRNAs (miRNA) in CSCs regulation is emerging. miRNAs are small non coding RNA molecules capable of regulating gene expression. Comparative miRNA expression analysis between normal intestinal and cancerous mucosa, identified overlapping miRNA expression pattern of cancer mucosa with embryonic tissues (Monzo et al. 2008). Members belonging to miR-17-92 cluster and miR17-5p were found to falls in the overlapping pattern.

In vitro inhibition studies of miR17-5p in DLD1 colon cancer cell line showed reduction in clongenicity and cell proliferation. Moreover, intestinal in situ miR-17-5p labeling, shows high expression in crypts base with reducing gradient at crypt top, favors its role in ISCs regulation.

A different in vitro studies on intestinal cancer cell line cultures in stem cell promoting media shows reduced expression of miR-144/451, and its over expression decelerate its tumor initiating capacity (Bitarte et al. 2011) (Fig. 9.7). Further studies identified miR-144/451 cluster role in Wnt pathway regulation, a major pathway involved in ISC regulation. Another miRNA identified in cancer cells lines miR-21 was also found to activate Wnt and inhibit TGFbR2 expression, pathways involved in ISC regulation and differentiation (Yu et al. 2012b) (Fig. 9.7). These initial studies about role of miRNAs in CSC regulation performed in cancer cell lines, provides strong evidence. However, further work is required to analyze their role in isolated CSC population, to explore CSC regulation by miRNAs (Fig. 9.7).

9.10 Tumor Microenvironment Maintains CSCs

Along with internal factors, external microenvironment plays an important role in regulation of both normal intestinal stem cell and CSCs. Normal intestinal stem cell are localized in crypts bottom surrounded by myofibroblasts and smooth muscle cells lying near basement membrane (Richman et al. 1987; Richman and Bodmer 1988). All these components form a niche involved in maintenance of intestinal stem cell compartment (Powell et al. 2005). Paneth cells adjacent to Lgr5 positive stem cell provides appropriate niche environment in the mouse small intestine (Sato et al. 2011), while in absence of paneth cell in large intestinal crypt, myofibroblasts surrounding crypt constitute the stem cell niche. Myofibroblasts and smooth muscle cells surrounding crypts bottom is known to secrete various signaling molecules and BMP inhibitors involved in differentiation suppression and wnt activation to maintain ISC at crypt base (Kosinski et al. 2007) (Figs. 9.3 and 9.6).

Intestinal tumor initiation, progression and metastasis occurrence in the area closer to myofibroblasts, (Richman et al. 1987; Richman and Bodmer 1988) owes to an inflammatory microenvironment maintained by cytokine secretion during various inflammatory diseases (Andoh et al. 2005, 2007; Pinchuk et al. 2011). CSCs are also found localized near myofibroblasts, moreover, myofibroblast conditioned

medium was shown to activate wnt signaling and suppress differentiation to maintain stem cell generated spheroids culture in vitro (Vermeulen et al. 2010). Among various factor secreted by myofibroblast, hepatocyte growth factor is known for maintaining stemness while TGF promotes differentiation (Chakrabarty et al. 1990). Interestingly, cancer cell and myofibroblast coculture studies shows mutual promotion of differentiation by each other, confirming their active complex interaction to coexist (Lewis et al. 2004; Webber et al. 2010). Adjoining extracellular matrix is also known to regulate ISC by influencing differentiation, which plays an important role in intestinal homeostasis maintenance. During carcinogenesis, extracellular matrix integrity is disrupted which in turn regulates CSCs, by dysregulated differentiation. Inhibition of integrin signaling utilizing blocking peptides disrupts formation of differentiated crypt like structure by cancer cells, further confirms indispensible role of extracellular matrix in intestinal tissue homeostasis and maintenance (Pignatelli and Bodmer 1989), while, integrin signaling activation promotes differentiation into mucous and endocrine cell lineages (Kirkland and Ying 2008) (Figs. 9.3, 9.6 and 9.7).

Tissue microenvironment oxygen level serves as another important external parameter, known to regulate CSC. A study shows regulation of ISC by oxygen level through wnt pathway modulation using hypoxic in vitro cell culture model system (Keith and Simon 2007; Mazumdar et al. 2010). As a tumor grows, it becomes hypoxic leading to HIF transcription factors activation. Expression of HIF transcription factors in intestinal cancer as well as other cancer, is known to induce expression of embryonic stem cell markers gene like OCT4 (Mathieu et al. 2011) (Fig. 9.7). In vitro studies on colorectal cancer cell lines demonstrate that cells become more clonogenic with decreased expression of differentiation inducing transcription factor like CDX1, under hypoxic condition (Yeung et al. 2011). Furthermore, cancer cells with high HIF1 expression suppresses differentiation while shows BMI1 up regulation. Ectopic expression of differentiation inducing transcription factor like CDX1 was sufficient to reduce colonogenicity and induce differentiation even in hypoxic condition. Therefore, these studies proves beyond doubt that hypoxic tumor microenvironment suppresses differentiation and maintains CSCs.

9.11 Intestinal Stem Cell in Tissue Injury and Regeneration

The intestinal epithelium undertakes various distinct functional roles which include absorption, digestion, secretion and immunological. Moreover, the entire human intestinal epithelium undergoes complete regeneration every week. This magnificent regenerative ability is possible by existence of active intestinal stem cell (ISC) populations in crypts base (Barker et al. 2008).

Bmi1 and Lgr5 positive ISCs identified independently by mice lineage tracing, represents two functionally different ISC populations in Vivo. Lgr5 positive ISCs are crypt–based actively dividing, Wnt sensitive and radiosensitive involved in

homeostasis maintenance and intestinal epithelium regeneration on daily basis. On the other hand, Bmi1 represents quiescent ISCs, insensitive to Wnt activation and radioresistant, weakly involved in homeostatic regeneration. However, these quiescent Bmi1$^+$ ISCs are actively involved in intestinal regeneration after tissue injury like radiotherapy, where they proliferate quickly to repopulate crypts and villi. In vitro, clonogenic culture using isolated Bmi1$^+$ ISCs generates self-renewing intestinal epithelial spheroids that consist of Lgr5-positive ISCs, provides strong evidence regarding lineage relationship between theses two ISCs. Therefore, these studies confirm existence of quiescent Bmi1 ISCs as tissue injury induced reserve ISCs with different functional role compared to Lgr5$^+$ ISCs to support a system where different ISC populations promote both homeostatic and tissue injury induced regeneration (Li and Clevers 2010).

Lgr5$^+$ ISCs respond to Wnt activation by R Spondin and Wnt inhibition by Dkk1, whereas Bmi1$^+$ ISCs are resistant to Wnt stimulus. On radiation insult, actively dividing Lgr5$^+$ ISCs are lost, while quiescent Bmi1$^+$ ISCs get induced to rapidly proliferate to regenerate lost intestinal epithelium. Presence of two functionally distinct ISC populations supports model for their differential involvement during homeostasis versus tissue injury induced regeneration (Scoville et al. 2008; Greco and Guo 2010).

An elegant study by Tian et al. reported, an important role for Lgr5$^+$ ISC intestinal homeostasis maintenance by ablating them utilizing diphtheria toxin receptor (dTR) knock-in genetic strategy (Tian et al. 2011). They also showed simultaneous expansion of Bmi1$^+$ ISCs on Lgr5$^+$ ISC ablation, which in turn produce Lgr5 positive cells in vivo (Tian et al. 2011). These findings further support injury induced proliferation of quiescent Bmi1$^+$ ISCs to replace lost Lgr5$^+$ ISCs, and their lineage relationship. Experimental model of Lgr5$^+$ ISCs ablation by dTR knock-in genetic strategy differs from radiation injury model with respect to crypt loss observed only in former one. However, intestinal epithelial regeneration by Bmi1$^+$ ISCs occurs in similar fashion in both experimental models, which indicates either functional redundancy or alternative possession of distinct function of these two intestinal stem cell populations. Baseline functional differences between Bmi1$^+$ and Lgr5$^+$ ISCs with respect to quiescence, response after injury, effect of wnt pathway manipulation and radio sensitivity, strongly support functional distinct nature of two ISC stem cell populations. Bmi1$^+$ ISC recruitment following injury further provides strong evidence regarding heterogeneity of ISC population involved in intestinal epithelial regeneration.

However, use of Bmi1 only as ISC marker to identify quiescent ISC activated on tissue injury, might present with the possibility with exclusion of ISC that can be identified by inclusion of other putative ISCs markers (May et al. 2009; Levin et al. 2010; Montgomery et al. 2011; von Furstenberg et al. 2011). Additionally, presence of proliferating cells not expressing Bmi1, 2 days post irradiation suggest involvement of ISC populations in regeneration post injury. Moreover, relative low number of Bmi1$^+$ ISCs are insufficient to regenerate complete intestinal epithelium post irradiation (Sangiorgi and Capecchi 2008; Tian et al. 2011). Other ISCs markers identified to mark +4 quiescent cell includes Hopx, Dcamkl1 and mTert (May et al. 2009; Montgomery et al. 2011; Takeda et al. 2011). mTert localized at +4 position mobilize after tissue injury, and coexpress both Lgr5 and Bmi1 indicates it as ideal

quiescent ISC marker (Montgomery et al. 2011; Schepers et al. 2011). However, further extensive research is required to identify other ISC markers and their relationship with existing +4 ISC markers like Bmi1^{+}, mTert^{+}, Dcamkl1 and Hopx.

Intestinal organoid cultures obtained from single isolated Bmi1-YFP+ cells was shown to consist of all differentiated cells of intestinal lineages along with Lgr5+ cells, further supports lineage relationship between two functionally distinct intestinal stem cell population (Tian et al. 2011). Interestingly, both FACS sorted Bmi1+ ISCs and Lgr5+ ISCs after depletion of local tissue microenvironment, generates intestinal organoids of similar nature (Sato et al. 2009). Intestinal organoids self renewal and proliferation, derived from Bmi1+ ISCs and Lgr5+ ISCs, responded equally to Rspo1 stimulus, similar to reported earlier while tracing Bmi1+ lineage tracing in vitro, however, Bmi1+ ISCs are comparatively insensitive to R spondin stimulus in vivo (Ootani et al. 2009). These observations support the role of ISC niche in guiding in vivo quiescence for Bmi1+ ISCs, missing in vitro set up (Sato et al. 2009). Additionally, possibility of Lgr5 expression among Bmi1+ ISCs can not be excluded completely, inspite of their functionally distinct role in vivo. Further investigation is needed to explain differential in vivo and in vitro behavior of Bmi1+ ISCs and Lgr5+ ISCs are dependent on tissue microenvironment or it's an inherent cell nature.

9.12 Future Perspectives

Despite recent technological and therapeutic development, in diagnosis and treatment options, intestinal cancer still present itself as a major challenge. Inoperable tumor or metastatic disease develops therapy resistance, finally leading to death. Presence of CSC, responsible for tumor initiation, its maintenance and development of therapy resistance, leads to tumor recurrence and metastasis, a major reason for mortality. Even though direct evidence for existence of CSC is still missing, research is being conducted for identification, isolation and characterization of CSC. ISC were identified as the cell of origin in intestinal cancer, owing to its long life span and occurrence of mutagenesis as a rare event. A recent study showed loss of APC in Lgr5 positive ISC leads to formation of macroscopic adenomas, in contrast similar mutation in differentiated progeny develops microscopic adenomas CSC originates from normal ISC, undergoes self renewal and differentiation in similar fashion, and also shares complex regulation by signaling pathways like Wnt, BMP and Notch pathways, however dysregulated. Indispensible role of tissue microenvironment and stroma in regulating ISC and CSC in vivo, provides an important area for future research. Identification of factors, specifically and differentially regulated in CSC maintenance, will serve as therapeutic target to prevent tumor growth by eradicating them. Last decade, tremendous progress was achieved in identification of normal intestinal stem cells and its role in maintenance of intestinal tissue homeostasis. Research in next decade will be more focused on identification of marker to isolate and characterize intestinal CSCs, a major requirement for development of better diagnostic, prognostic and therapeutic targets (Fig. 9.8).

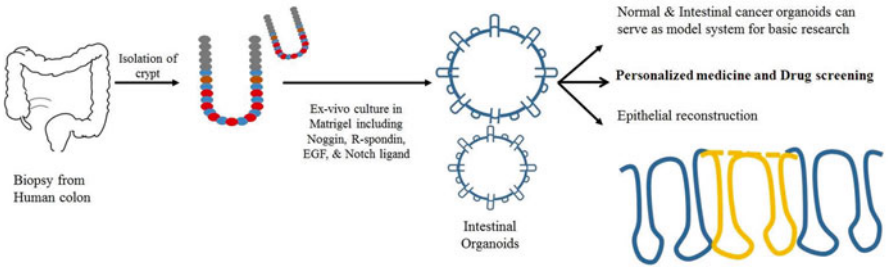

Fig. 9.8 Intestinal organoid culture from human intestinal epithelium including matched normal mucosa from patients to reconstruct damaged epithelia in different diseases like ulcers, additionally matched normal and intestinal cancer organoids can serve as model system for basic research to understand intestinal stem cell biology along with various translational research like personalized medicine and drug screening

Recent work by Barker et al. showed generation of all intestinal cell lineages from single isolated Lgr5 positive cells, present at the bottom of the crypts. Moreover, Lgr5 positive ISCs genetic transformation gives rise to tumor initiating cells, a feature of CSC (Barker et al. 2009). An ex vivo technique to isolate, maintain and expand ISCs to generate functional intestinal organoids with all cell lineages (Sato et al. 2009) will provide an excellent opportunity to study ISC biology to understand the link between ISCs and CSCs (Fig. 9.8).

The functional role of genes mutated in intestinal cancer development, have been extensively studied using genetic mouse models and cancer cell lines. With possibility of ISC isolation, its genetic manipulation followed by its culture to generate functional intestinal organoids in 3D, provides an appropriate model system for basic research to investigate mutational events involved in development of intestinal carcinogenesis.

Cancer stem cells are identified as tumour initiating cells capable of promoting tumorigenesis by their self-renewal and generation of non tumorigenic differentiated cells. At present, stemness property for CSC is determined by xenograft assay in immunodeficient mice. This assay is usually performed on fluorescence activated cell sorted cancer cells expressing putative CSC marker, for their capability to generate tumor similar to original tumor. However, it exhibit various limitation such as non consensus over specific CSC marker, tumor microenvironment differences between the original and transplanted site and species barrier among rodents and humans. Therefore, current thrust in cancer research is on identification of markers that can distinguish between CSC from non CSC population. Recent studies are performed to isolate and characterize CSC using various CSC markers like CD133, CD44, CD166 ALDH1 and their combination (Dalerba et al. 2007; Shmelkov et al. 2008; Huang et al. 2009; Levin et al. 2010). Normal ISC markers, like LGR5or EPHB2are also reported to identify CSC population in tumor (Merlos-Suarez et al.

2011; Kemper et al. 2012). Recently, Lgr5 genetic lineage tracing study identified a sub population (5–10 %) in adenoma, capable of its self renewal and generation of differentiated adenoma cells (Schepers et al. 2012). Therefore, instead of FACS based isolation by cell detachment and dissociation from its microenvironment, a forward genetic approach to identify candidate genes function in CSC development from ISC due to disturbed homeostasis utilizing various genetically engineered mice model (Barker et al. 2009). Nevertheless, development of mice model requires huge efforts in terms of time and cost, especially if analysis includes multiple genes. Even studies in mice suffer with limitation considering interspecies variation, additionally, similar studies in humans are impossible.

In this scenario, intestinal Organoids cultures from normal ISC and CSC from patient's normal and diseases tissue can serve as an ideal model system to explore intestinal stem cell biology. Possibility of genetic manipulation of intestinal organoids by viral transductions (Koo et al. 2012), provides an important tool to study effect of common mutations in human ISCs on intestinal epithelium maintenance and cancer development. Successful application of CRISPR/Cas9 technology, to mend faulty gene is reported for cystic fibrosis transmembrane conductor receptor (CFTR) gene for intestinal organoids, obtained from cystic fibrosis patients (Schwank et al. 2013). Similar technology can be also be utilized to mend faulty oncogenes or tumor suppressor genes. Use of organoids culture with possibility of genetic manipulation associated with intestinal carcinogenesis, provides a strong model system to explore mechanism involved in CSC generation, which can be used as therapeutic target to eradicate them, a major culprit involved in recurrence and metastasis.

More recently, Watson et al. reported, generation of human intestinal organoids (HIOs) in vitro from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (McCracken et al. 2011; Spence et al. 2011; Forster et al. 2014). These HIOs develop into mature human intestinal epithelium, where intestinal stem cell maintains crypt villus architecture and human mesenchyme, supported well by mouse vasculature on its grafting. In vivo grafting in mice showed appropriate intestinal epithelium maturation and expansion. Intestinal epithelium was functionally active and consists of all differentiated intestinal cell lineages. Human intestinal epithelium derived from transplanted HIO in mice, responded well to host systemic signals following intestinal resection, suggests important role of paracrine factors adaptive intestinal response (Juno et al. 2002; Juno et al. 2003). Therefore, it provides an ideal in vivo model to study human intestinal epithelium, which was restricted to primary cell cultures of digested surgical biopsies including mesenchymal cells transplantation on biodegradable scaffolds (Lahar et al. 2011; Levin et al. 2013; Watson et al. 2014; Wells and Spence 2014). This model of human intestine further opens promising avenue to explore intestinal epithelium embryonic development, physiology, disease including cancer, translational studies pharmacological and therapeutic transplantation.

References

Al-Hajj M, Wicha MS et al (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100(7):3983–3988

Anderson EC, Wong MH (2010) Caught in the Akt: regulation of Wnt signaling in the intestine. Gastroenterology 139(3):718–722

Andoh A, Bamba S et al (2005) Colonic subepithelial myofibroblasts in mucosal inflammation and repair: contribution of bone marrow-derived stem cells to the gut regenerative response. J Gastroenterol 40(12):1089–1099

Andoh A, Bamba S et al (2007) Role of intestinal subepithelial myofibroblasts in inflammation and regenerative response in the gut. Pharmacol Ther 114(1):94–106

Andriatsilavo M, Gervais L et al (2013) The Drosophila midgut as a model to study adult stem cells. Med Sci (Paris) 29(1):75–81

Ashley N (2013) Regulation of intestinal cancer stem cells. Cancer Lett 338(1):120–126

Baker A-M, Graham TA et al (2015) Characterization of LGR5 stem cells in colorectal adenomas and carcinomas. Sci Rep 5:8654

Barker N (2014) Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. Nat Rev Mol Cell Biol 15(1):19–33

Barker N, Clevers H (2007) Tracking down the stem cells of the intestine: strategies to identify adult stem cells. Gastroenterology 133(6):1755–1760

Barker N, Clevers H (2010) Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology 138(5):1681–1696

Barker N, van Es JH et al (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449(7165):1003–1007

Barker N, van de Wetering M et al (2008) The intestinal stem cell. Genes Dev 22(14):1856–1864

Barker N, Ridgway RA et al (2009) Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 457(7229):608–611

Barker N, van Oudenaarden A et al (2012) Identifying the stem cell of the intestinal crypt: strategies and pitfalls. Cell Stem Cell 11(4):452–460

Batts LE, Polk DB et al (2006) Bmp signaling is required for intestinal growth and morphogenesis. Dev Dyn 235(6):1563–1570

Bitarte N, Bandres E et al (2011) MicroRNA-451 is involved in the self-renewal, tumorigenicity, and chemoresistance of colorectal cancer stem cells. Stem Cells 29(11):1661–1671

Biteau B, Hochmuth CE et al (2008) JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut. Cell Stem Cell 3(4):442–455

Biteau B, Hochmuth CE et al (2011) Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. Cell Stem Cell 9(5):402–411

Blanpain C, Simons BD (2013) Unravelling stem cell dynamics by lineage tracing. Nat Rev Mol Cell Biol 14(8):489–502

Buske P, Galle J et al (2011) A comprehensive model of the spatio-temporal stem cell and tissue organisation in the intestinal crypt. PLoS Comput Biol 7(1):e1001045

Carmon KS, Gong X et al (2011) R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc Natl Acad Sci U S A 108(28):11452–11457

Chakrabarty S, Fan D et al (1990) Modulation of differentiation and proliferation in human colon carcinoma cells by transforming growth factor beta 1 and beta 2. Int J Cancer 46(3):493–499

Chandler JM, Lagasse E (2010) Cancerous stem cells: deviant stem cells with cancer-causing misbehavior. Stem Cell Res Ther 1(2):13

Cheng H, Leblond CP (1974) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. III. Entero-endocrine cells. Am J Anat 141(4):503–519

Clevers H (2011) The cancer stem cell: premises, promises and challenges. Nat Med 17(3):313–319

Creamer B, Shorter RG et al (1961) The turnover and shedding of epithelial cells. I. The turnover in the gastro-intestinal tract. Gut 2:110–118
Dalerba P, Dylla SJ et al (2007) Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 104(24):10158–10163
Dalerba P, Kalisky T et al (2011) Single-cell dissection of transcriptional heterogeneity in human colon tumors. Nat Biotechnol 29(12):1120–1127
Davies PS, Dismuke AD et al (2008) Wnt-reporter expression pattern in the mouse intestine during homeostasis. BMC Gastroenterol 8:57
de Lau W, Barker N et al (2011) Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 476(7360):293–297
de Sousa EM, Vermeulen L et al (2011a) Targeting Wnt signaling in colon cancer stem cells. Clin Cancer Res 17(4):647–653
de Sousa EMF, Colak S et al (2011b) Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. Cell Stem Cell 9(5):476–485
Dean M, Fojo T et al (2005) Tumour stem cells and drug resistance. Nat Rev Cancer 5(4):275–284
Dieter SM, Ball CR et al (2011) Distinct types of tumor-initiating cells form human colon cancer tumors and metastases. Cell Stem Cell 9(4):357–365
Forster R, Chiba K et al (2014) Human intestinal tissue with adult stem cell properties derived from pluripotent stem cells. Stem Cell Rep 2(6):838–852
Fre S, Huyghe M et al (2005) Notch signals control the fate of immature progenitor cells in the intestine. Nature 435(7044):964–968
Fre S, Pallavi SK et al (2009) Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. Proc Natl Acad Sci U S A 106(15):6309–6314
Fuchs E (2009) The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 137(5):811–819
Giannakis M, Stappenbeck TS et al (2006) Molecular properties of adult mouse gastric and intestinal epithelial progenitors in their niches. J Biol Chem 281(16):11292–11300
Greco V, Guo S (2010) Compartmentalized organization: a common and required feature of stem cell niches? Development 137(10):1586–1594
Gregorieff A, Pinto D et al (2005) Expression pattern of Wnt signaling components in the adult intestine. Gastroenterology 129(2):626–638
Guezguez A, Pare F et al (2014) Modulation of stemness in a human normal intestinal epithelial crypt cell line by activation of the WNT signaling pathway. Exp Cell Res 322(2):355–364
Haramis AP, Begthel H et al (2004) De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. Science 303(5664):1684–1686
He XC, Zhang J et al (2004) BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nat Genet 36(10):1117–1121
Houthuijzen JM, Daenen LG et al (2012) The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression. Br J Cancer 106(12):1901–1906
Huang EH, Hynes MJ et al (2009) Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res 69(8):3382–3389
Johnston MD, Edwards CM et al (2007) Mathematical modeling of cell population dynamics in the colonic crypt and in colorectal cancer. Proc Natl Acad Sci U S A 104(10):4008–4013
Johnston MD, Maini PK et al (2010) On the proportion of cancer stem cells in a tumour. J Theor Biol 266(4):708–711
Jung P, Sato T et al (2011) Isolation and in vitro expansion of human colonic stem cells. Nat Med 17(10):1225–1227
Juno RJ, Williams JL et al (2002) A serum factor after intestinal resection stimulates epidermal growth factor receptor signaling and proliferation in intestinal epithelial cells. Surgery 132(2):377–383

Juno RJ, Knott AW et al (2003) A serum factor(s) after small bowel resection induces intestinal epithelial cell proliferation: effects of timing, site, and extent of resection. J Pediatr Surg 38(6):868–874

Katoh M (2007) WNT antagonist, DKK2, is a Notch signaling target in intestinal stem cells: augmentation of a negative regulation system for canonical WNT signaling pathway by the Notch-DKK2 signaling loop in primates. Int J Mol Med 19(1):197–201

Keith B, Simon MC (2007) Hypoxia-inducible factors, stem cells, and cancer. Cell 129(3):465–472

Kemper K, Grandela C et al (2010) Molecular identification and targeting of colorectal cancer stem cells. Oncotarget 1(6):387–395

Kemper K, Prasetyanti PR et al (2012) Monoclonal antibodies against Lgr5 identify human colorectal cancer stem cells. Stem Cells 30(11):2378–2386

Kim JE, Lee JM et al (2015) Differentiation of poorly differentiated colorectal adenocarcinomas from well- or moderately differentiated colorectal adenocarcinomas at contrast-enhanced multidetector CT. Abdom Imaging 40(1):1–10

Kirkland SC, Ying H (2008) Alpha2beta1 integrin regulates lineage commitment in multipotent human colorectal cancer cells. J Biol Chem 283(41):27612–27619

Kleist B, Xu L et al (2011) Expression of the adult intestinal stem cell marker Lgr5 in the metastatic cascade of colorectal cancer. Int J Clin Exp Pathol 4(4):327–335

Koo BK, Stange DE et al (2012) Controlled gene expression in primary Lgr5 organoid cultures. Nat Methods 9(1):81–83

Korinek V, Barker N et al (1998) Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 19(4):379–383

Kosinski C, Li VS et al (2007) Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. Proc Natl Acad Sci U S A 104(39):15418–15423

Lahar N, Lei NY et al (2011) Intestinal subepithelial myofibroblasts support in vitro and in vivo growth of human small intestinal epithelium. PLoS One 6(11):e26898

Laurent E, McCoy JW 3rd et al (2008) Nox1 is over-expressed in human colon cancers and correlates with activating mutations in K-Ras. Int J Cancer 123(1):100–107

Leblond CP, Walker BE (1956) Renewal of cell populations. Physiol Rev 36(2):255–276

Levin TG, Powell AE et al (2010) Characterization of the intestinal cancer stem cell marker CD166 in the human and mouse gastrointestinal tract. Gastroenterology 139(6):2072–2082. e2075

Levin DE, Barthel ER et al (2013) Human tissue-engineered small intestine forms from postnatal progenitor cells. J Pediatr Surg 48(1):129–137

Lewis MP, Lygoe KA et al (2004) Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. Br J Cancer 90(4):822–832

Li L, Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. Science 327(5965):542–545

Lombardo Y, Scopelliti A et al (2011) Bone morphogenetic protein 4 induces differentiation of colorectal cancer stem cells and increases their response to chemotherapy in mice. Gastroenterology 140(1):297–309

Mathieu J, Zhang Z et al (2011) HIF induces human embryonic stem cell markers in cancer cells. Cancer Res 71(13):4640–4652

May R, Sureban SM et al (2009) Doublecortin and CaM kinase-like-1 and leucine-rich-repeat-containing G-protein-coupled receptor mark quiescent and cycling intestinal stem cells, respectively. Stem Cells 27(10):2571–2579

Mazumdar J, O'Brien WT et al (2010) O2 regulates stem cells through Wnt/beta-catenin signalling. Nat Cell Biol 12(10):1007–1013

McCracken KW, Howell JC et al (2011) Generating human intestinal tissue from pluripotent stem cells in vitro. Nat Protoc 6(12):1920–1928

Merlos-Suarez A, Barriga FM et al (2011) The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell 8(5):511–524
Metcalfe C, Kljavin NM et al (2014) Lgr5+ stem cells are indispensable for radiation-induced intestinal regeneration. Cell Stem Cell 14(2):149–159
Miyamoto S, Rosenberg DW (2011) Role of Notch signaling in colon homeostasis and carcinogenesis. Cancer Sci 102(11):1938–1942
Montgomery RK, Mulberg AE et al (1999) Development of the human gastrointestinal tract: twenty years of progress. Gastroenterology 116(3):702–731
Montgomery RK, Carlone DL et al (2011) Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. Proc Natl Acad Sci U S A 108(1):179–184
Monzo M, Navarro A et al (2008) Overlapping expression of microRNAs in human embryonic colon and colorectal cancer. Cell Res 18(8):823–833
Moore KA, Lemischka IR (2006) Stem cells and their niches. Science 311(5769):1880–1885
Munoz J, Stange DE et al (2012) The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. EMBO J 31(14):3079–3091
Ootani A, Li X et al (2009) Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. Nat Med 15(6):701–706
Pang R, Law WL et al (2010) A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell 6(6):603–615
Pellegrinet L, Rodilla V et al (2011) Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. Gastroenterology 140(4):1230–1240. e1231–e1237
Pignatelli M, Bodmer WF (1989) Integrin-receptor-mediated differentiation and growth inhibition are enhanced by transforming growth factor-beta in colorectal tumour cells grown in collagen gel. Int J Cancer 44(3):518–523
Pinchuk IV, Beswick EJ et al (2011) Human colonic myofibroblasts promote expansion of CD4+ CD25high Foxp3+ regulatory T cells. Gastroenterology 140(7):2019–2030
Pinto D, Clevers H (2005) Wnt, stem cells and cancer in the intestine. Biol Cell 97(3):185–196
Pinto D, Gregorieff A et al (2003) Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev 17(14):1709–1713
Potten CS, Kovacs L et al (1974) Continuous labelling studies on mouse skin and intestine. Cell Tissue Kinet 7(3):271–283
Potten CS, Owen G et al (2002) Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci 115(Pt 11):2381–2388
Powell DW, Adegboyega PA et al (2005) Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. Am J Physiol Gastrointest Liver Physiol 289(1):G2–G7
Radtke F, Clevers H (2005) Self-renewal and cancer of the gut: two sides of a coin. Science 307(5717):1904–1909
Reed KR, Tunster SJ et al (2012) Entopic overexpression of Ascl2 does not accelerate tumourigenesis in ApcMin mice. Gut 61(10):1435–1438
Ricci-Vitiani L, Lombardi DG et al (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445(7123):111–115
Richman PI, Bodmer WF (1988) Control of differentiation in human colorectal carcinoma cell lines: epithelial-mesenchymal interactions. J Pathol 156(3):197–211
Richman PI, Tilly R et al (1987) Colonic pericrypt sheath cells: characterisation of cell type with new monoclonal antibody. J Clin Pathol 40(6):593–600
Sancho E, Batlle E et al (2003) Live and let die in the intestinal epithelium. Curr Opin Cell Biol 15(6):763–770
Sangiorgi E, Capecchi MR (2008) Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet 40(7):915–920
Sato T, Clevers H (2013) Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. Science 340(6137):1190–1194

Sato T, Vries RG et al (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459(7244):262–265
Sato T, van Es JH et al (2011) Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469(7330):415–418
Schepers AG, Vries R et al (2011) Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes. EMBO J 30(6):1104–1109
Schepers AG, Snippert HJ et al (2012) Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science 337(6095):730–735
Schwank G, Koo BK et al (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell 13(6):653–658
Schwitalla S, Fingerle AA et al (2013) Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. Cell 152(1–2):25–38
Scoville DH, Sato T et al (2008) Current view: intestinal stem cells and signaling. Gastroenterology 134(3):849–864
Shmelkov SV, Butler JM et al (2008) CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. J Clin Invest 118(6):2111–2120
Sikandar SS, Pate KT et al (2010) NOTCH signaling is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancer. Cancer Res 70(4):1469–1478
Spence JR, Mayhew CN et al (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470(7332):105–109
Tajbakhsh S (2014) Ballroom dancing with stem cells: placement and displacement in the intestinal crypt. Cell Stem Cell 14(3):271–273
Takahashi H, Ishii H et al (2011) Significance of Lgr5(+ve) cancer stem cells in the colon and rectum. Ann Surg Oncol 18(4):1166–1174
Takeda N, Jain R et al (2011) Interconversion between intestinal stem cell populations in distinct niches. Science 334(6061):1420–1424
Tian H, Biehs B et al (2011) A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature 478(7368):255–259
Todaro M, Alea MP et al (2007) Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 1(4):389–402
Todaro M, Francipane MG et al (2010) Colon cancer stem cells: promise of targeted therapy. Gastroenterology 138(6):2151–2162
van der Flier LG, Clevers H (2009) Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol 71:241–260
van der Flier LG, Haegebarth A et al (2009a) OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. Gastroenterology 137(1):15–17
van der Flier LG, van Gijn ME et al (2009b) Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell 136(5):903–912
Vermeulen L, Todaro M et al (2008) Single-cell cloning of colon cancer stem cells reveals a multilineage differentiation capacity. Proc Natl Acad Sci U S A 105(36):13427–13432
Vermeulen L, De Sousa EMF et al (2010) Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 12(5):468–476
von Furstenberg RJ, Gulati AS et al (2011) Sorting mouse jejunal epithelial cells with CD24 yields a population with characteristics of intestinal stem cells. Am J Physiol Gastrointest Liver Physiol 300(3):G409–G417
Walker F, Zhang HH et al (2011) LGR5 is a negative regulator of tumourigenicity, antagonizes Wnt signalling and regulates cell adhesion in colorectal cancer cell lines. PLoS One 6(7):e22733
Watson CL, Mahe MM et al (2014) An in vivo model of human small intestine using pluripotent stem cells. Nat Med 20(11):1310–1314
Webber J, Steadman R et al (2010) Cancer exosomes trigger fibroblast to myofibroblast differentiation. Cancer Res 70(23):9621–9630

Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. Cell 100(1):157–168

Wells JM, Spence JR (2014) How to make an intestine. Development 141(4):752–760

Widschwendter M, Fiegl H et al (2007) Epigenetic stem cell signature in cancer. Nat Genet 39(2):157–158

Yeung TM, Gandhi SC et al (2010) Cancer stem cells from colorectal cancer-derived cell lines. Proc Natl Acad Sci U S A 107(8):3722–3727

Yeung TM, Gandhi SC et al (2011) Hypoxia and lineage specification of cell line-derived colorectal cancer stem cells. Proc Natl Acad Sci U S A 108(11):4382–4387

Yokoyama S, Takifuji K et al (2010) Moderately differentiated colorectal adenocarcinoma as a lymph node metastatic phenotype: comparison with well differentiated counterparts. BMC Surg 10:8

Yu T, Chen X et al (2012a) Regulation of the potential marker for intestinal cells, Bmi1, by beta-catenin and the zinc finger protein KLF4: implications for colon cancer. J Biol Chem 287(6):3760–3768

Yu Y, Kanwar SS et al (2012b) MicroRNA-21 induces stemness by downregulating transforming growth factor beta receptor 2 (TGFbetaR2) in colon cancer cells. Carcinogenesis 33(1):68–76

Zagouras P, Stifani S et al (1995) Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc Natl Acad Sci U S A 92(14):6414–6418

Zhang Y, Li B et al (2010) Notch1 regulates the growth of human colon cancers. Cancer 116(22):5207–5218

Zhu L, Gibson P et al (2009) Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 457(7229):603–607

Chapter 10
Cancer Stem Cells: Perspectives Beyond Immunophenotypes and Markers

Sharmila A. Bapat

Abstract Decades of cancer research have failed to resolve therapeutic refractoriness and tumor dormancy that leads to disease recurrence. This presents formidable obstacles in achieving total remission for patients in several cancers. It is however realized that residual tumor regenerative potential resides in a rare population of cells with properties of self-renewal that permit them to remain quiescent yet contribute to recurrent disease. These cells are referred to as either Cancer stem cells (CSCs) or Tumor Initiating Cells (TICs), and their isolation, identification and extensive characterization followed through the establishment of several phenotypic and functional *in vitro* and *in vivo* assays. Notably, similarities with normal tissue stem cells have emphasized the need of developing new approaches for their specific targeting as opposed to current chemo- or radio-therapy. Thereby considerable interest and research has culminated in elucidating the behavior of CSCs vis-à-vis their deviations from normal stem cell performance, which might provide therapeutic novel cues. However, their identification, characterization and understanding of the cellular contexts in which they can be formidable yet have not been truly achieved beyond the development of convenient tools. This chapter outlines the present challenges in the field of CSC biology.

Keywords Cancer stem cells • Asymmetric cell division • Quiescence • Dedifferentiation • Transdifferentiation

10.1 Introduction

According to Greek mythology, when Prometheus defied the gods and stole fire for mankind, he was chained to Mount Caucasus where each day an eagle (emblem of Zeus, king of gods) would prey on his liver (believed to be the centre of emotions,

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_10

not the heart!). Luckily for him, the high cell regeneration in this organ allowed him to tide over circumstances until he was freed by Hercules (Rosenthal 2003). Tumors sometimes appear to harbor such Promethean regenerative homeostasis that makes them virtually indestructible. This germ of an idea could well be the genesis of Cancer Stem Cells (CSCs), a concept supported by clinical observations of regeneration, unfortunately running awry. CSCs were termed so since they were believed to arise through transformation of normal tissue-derived stem cells. Hence similarities between these and normal stem cells were noted *vis-à-vis* expression of surface markers, derivation of a regenerative cell hierarchy comprising of CSCs purported to be a rare fraction within tumors that retains the capability of reversible quiescence; progenitors that constitute the proliferative tumor fraction and differentiated cells that represent the phenotypic and functional heterogeneity present in the organ in which the tumor establishes itself (Reya et al. 2001). Such extrapolation of cancer being a derivative of normal repair functions with CSCs driving tumor heterogeneity was first convincingly demonstrated in leukemia, wherein aberrant regeneration bestowed imbalanced growth advantages within the HSC hierarchy along with compromised tissue functioning from deficits in progenitor maturation (Bonnet and Dick 1997). This understanding also correlated with the clinical pattern of the disease and was hence accepted by researchers and clinicians alike, thus establishing a framework for further studies. CSCs in solid tumors were prospectively identified almost a decade thereafter (Al-Hajj et al. 2003; Singh et al. 2004; Bapat et al. 2005), with the leukemia model providing the prototype for initial studies drawing similarities of marker expression between normal tissue and tumor-derived stem cells and their regenerative capabilities.

10.1.1 Prospective Isolation and Identification of Cells in Tumors with Stem-Like Properties

Another decade thereafter has witnessed a deluge of research literature in the field with convictions though becoming stronger, also giving rise to severe criticisms. The most emphatic of these initially concerned isolation of CSCs through application of normal stem surface markers (Woodward and Sulman 2008; Duan et al. 2013). This strategy is widely applied and appreciated as a relatively easy and convenient tool; however excessive reliance on these techniques alone led to dilution of the basic concept. Since the regenerative CSC hierarchy is known to be associated with differentiation arrest and compromised tissue functions resulting from various combinations of genomic alterations, it also raises concerns of ambiguous correlations between normal and aberrant cellular subsets as identified by the same markers (Jaggupilli and Elkord 2012). Most importantly, in solid tumors the use of surface markers in discriminating between mature and immature cells is not established (Schulenburg et al. 2015). Thus the precision of prospective CSC isolation was thought to be compromised in certain tumors wherein marker correlation with

specific cell subsets is uncertain on the background of lack of similar functional correlations.

Yet another difficulty was that since normal stem cells and resolution of their functionalities is not achieved in several organs, isolation of CSCs in tumors arising in those was not possible. This problem was circumvented through development of 'universal' immunophenotyping approaches wherein screening was achieved using generic marker cocktails comprising of c-kit, CD44, CD24, CD133, Epcam, nestin, nanog, Oct-4, Aldh activity and their combinations (Medema et al. 2013). An understanding of such correlation however is unclear since none of these markers are exclusively associated with CSCs, and some of them are not even expressed in the normal tissue. While current understanding suggests acquisition of self-renewal and blocked differentiation to be most important in reverting to a stem cell state, these functions are not known to be associated with any known surface markers, further making such correlations rather incomprehensible. Thus 'marker-independent' approaches including sorting of side population (SP) stem cells within tumors that have a specific capability to efflux Hoechst dyes, growth of tumor derived cells as non-adherent spheroid cultures that could enrich CSCs within the population, label chase to identify differences in growth kinetics (quiescence vs. proliferation), etc. came to be explored for prospective CSC studies (Tirino et al. 2013).

Lack of information relating to normal homeostasis in certain organs also limits understanding the presence of CSCs in their tumor derivatives. In some tumors, normal stem cells may serve as the targets of oncogenic transformation to yield CSCs, while in others, transit-amplifying progenitors and/or differentiated cells may present the initial oncogenic genomic rearrangements. An essential feature accompanying the implied dedifferentiation in the latter is acquisition of self-renewal capabilities along with maturation defects (Quintana et al. 2010). Although such reprogramming is demonstrated at a functional level, convincing correlations with altered immunophenotypes are not established. During tumor formation and maintenance, CSCs like their normal counterparts continue to self-renew and generate differentiated derivatives that constitute the major bulk of a tumor. Disease progression further is believed to follow Darwinian principles of evolution in which continual genetic instability could establish several CSC clones of differential capabilities within the same tumor, to provide an increasing complexity of possibilities for survival. These complexities cannot be resolved through studies with surface markers.

10.1.2 Distinction Between CSCs, TICs and Cell-of-Origin of Tumors

A basic requisite for CSC isolation is that variability arising from execution of techniques such as differences in tumor digestion, source of tumors (cell lines vs. tumors, human vs. xenografts, extensively passed vs. early xenografts), stringency

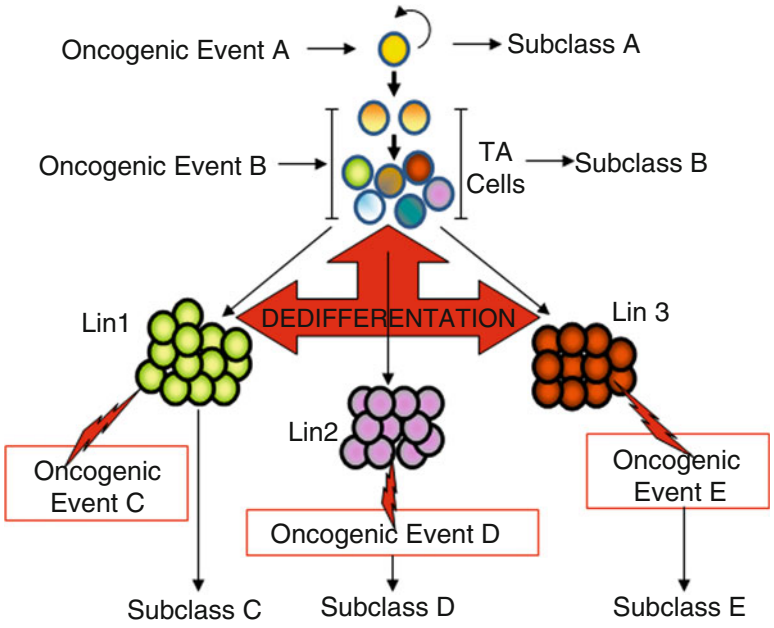

Fig. 10.1 Diverse cell(s)-of-origin and pathways to emergence of CSCs result in different sub-classes of tumors and deem implausible assigning of a single CSC immunophenotype (Lin1, Lin2, Lin3 – Lineage1, Lineage2, Lineage3 respectively)

of assays used in evaluation, etc. should be maintained at minimal. The diversity of CSC phenotypes (based on surface markers, SP sorting, label quenching, etc.) however leads to confounding interpretations when viewed across tumors from patients with the same histological and molecular subtype. This perspective of inter-tumor heterogeneity encompasses differences arising from ethnicity and genetic variability between individuals, environmental factors, cell-of-origin and type of oncogenic events (Marusyk et al. 2012). Such principles of tumor stratification rely on the type of oncogenic mutations or genomic rearrangements involved and/or their targeted lineage (cell-of-origin) within the organ (Visavader and Lindemann 2012; Fig. 10.1). Correlating across this variability to identify discrete sub-classes of tumors with similar molecular features forms the basis of tumor/patient stratification and is achieved in several cancers. In such a situation, it is difficult to comprehend how a single CSC phenotype could be associated with all known tumor subtypes arising from diverse cell(s)-of-origin and oncogenic events.

Tumor regeneration in immune compromised animal models from human tumor derived sorted cells thus increasingly gained recognition as a universal 'proof-of-function', besides providing a robust assay for functional validation of CSC self-renewal. Soon thereafter, Tumor Initiating Cells (TICs) became the new buzz word round the block that indicated definite emphasis on cell functions over phenotype.

While in most cases CSCs qualified as TICs, the latter were also reportedly isolated in instances wherein tumors do not conform to classical hierarchical regeneration (Rehe et al. 2013). This revealed that the frequency of TICs could vary from being relatively rare to comprising a significant fraction of tumors, thereby making their distinction from the rare CSCs (Bapat et al. 2009), implying that establishment of mitotic quiescence than expression of 'stemness' features was a more important characteristic of TICs (Wang et al. 2015).

However while functional readouts are indeed important and suggest non-exclusivity, CSCs nevertheless still retain an edge over TICs by providing a reasonable account of intra-tumor heterogeneity that assigns varying regenerative potential within a hierarchy, and cellular plasticity that is a key feature of stem cells besides self-renewal and regeneration. Under different conditions of stress or microenvironmental cross-talk, the coexistence of discrete CSC pools may be evident that reflects on emerging phenotypic heterogeneity within the same tumor. To address these basic questions in the field today, it becomes necessary to map out all possible correlates between the differentiation hierarchy, clonal selection capabilities and various molecular and cellular phenotypes within an organ/tumor that will better elucidate the subtle differences and identities between CSCs, TICs and cell-or-origin of tumors.

10.2 Understanding the Cellular State

10.2.1 Asymmetric Cell Division

Asymmetric cell division is central to stem cell capabilities of long-term regeneration and maintenance of homeostasis, while remaining a fundamental means of generating cell diversity. Such functionality is manifested through generation of two daughter cells with discrete alternative fates of self-renewal (implying return to the stem cell state) vs. lineage commitment and differentiation (defining the state). While asymmetric division is the convention in normal homeostasis, adult stem cells may also undergo symmetric divisions under conditions wherein stem-cell pools are depleted by injury or disease (Kahn 2011). Two modes of regulation of asymmetric cell division are recognized, *intrinsic mechanisms* are driven by altered cell polarity and asymmetric segregation of cellular components during division, while *extrinsic mechanisms* are manifested by the reliance of a cell on its niche to receive cues that will drive it to self-renew or differentiate (Kelsom and Wange 2012). The exclusivity of these two modes remains to be determined. More important is an emerging concept that failure of asymmetric cell division could have widespread consequences in neoplastic growth. Perturbed stem cell activation and functioning is demonstrated to lead to imbalanced regeneration followed by tumorigenesis in several model systems including *Drosophila* neuroblasts (Siegrist and Doe 2006; Izumi and Kaneko 2012).

10.2.1.1 Intrinsic Regulation – Polarity, Asymmetric Segregation of Cellular Components, EMT

Establishment of cell fate suggests the subsistence of intrinsic features that drive asymmetric division, and extends beyond the simple principles of mitosis wherein equal segregation of sister chromatids ensures that each daughter cell receives a single, complete copy of the parent genome. Stem cells in their specific niche often exhibit an apical-basal axis of polarity. During mitosis, polarity establishes an asymmetrical localization of self-renewal regulators; concurrently asymmetric mitotic spindle positioning regulates the polarization of other determinants (Yamashita et al. 2007). Within the daughter cells thus formed, one inherits most of the polar and self-renewal determinants to re-establish polarity and revert back to the 'stem-cell' state, while the other loses these regulators and generates a critical mass of cells necessary for differentiation (Sugioka and Sawa 2012; Inaba and Yamashita 2012). Such differential distribution of determinants is reported at the RNA as well as protein expression levels (Gómez-López et al. 2014; Ganguly et al. 2012). Thereby, coordination of asymmetric protein localization and polarity with cell cycle progression through mitosis are important contributions of asymmetric cell division in maintenance of homeostasis. A large number of proteins including numb, par, pon, brat, Miranda, prospero, stuaufen, pins, gai, loco, inscuteable, aPKC, lgl, polo, aurora A, polo, pp2a, dpn, zif, etc. associated with asymmetric localization, involvement with spindle orientation and/or cell polarity are currently recognized as being determinants of asymmetric division (Kelsom and Wange 2012; Poulson and Lechler 2012). Significantly, specific mutations in the tumor suppressor Apc can influence spindle alignments and planar cell polarities that regulate daughter cell anisotropic movements away from niche-supporting cells (Quyn et al. 2010 ; Chang et al. 2012). Such processes are relatively easy to understand in simple epithelial layers like the ovarian surface epithelium; however, stratified epithelia further require cross-talk between layers to establish a delicate balance of proliferation, mitotic spindle orientation, differentiation and cell loss (Graham et al. 2010; Muthuswamy and Xue 2012). Failure to do so can trigger dysplasia through cooperation with other predisposing factors and progress to transformation.

In cancer, epithelial to mesenchymal transition (EMT) that was initially correlated with invasion and metastases, is now also being associated with disrupted cell polarity (Zheng and Kang 2014; Moreno-Bueno et al. 2008). This process wherein epithelial cells lose their characteristic features and undergo dissolution of cell–cell contacts to acquire a migratory, mesenchymal phenotype, is crucial to normal embryonic development wherein a tightly controlled program at the epigenetic and transcriptional levels achieves specific developmental milestones within specified parameters of time and space (Kerosuo and Bronner-Fraser 2012). This ensures formation of the three germ layers, differentiation and generation of organ structures within the developing embryo, thereby protecting against transformation. In the context of cancer, the process contributes to dissemination of tumor cells and metastases in a manner speculated to be an aberrant derivative of the normal program. The establishment of EMT has also been associated with re-acquisition of

'stemness' features in tumor cells and an indication of moving towards a recalcitrant, invasive stage of disease; aggressive tumors appear to be markedly associated with EMT (Beuran et al. 2015). Considerable diversity in the molecular profiles of this program in different tumors lends further support for this perception. For example, the conservation of definite EMT networks driven by specific transcriptional programs in a particular tumor type may define distinctive molecular classes (Scheela and Weinberg 2012). Expression of EMT- and CSC-associated genes in cells at the invasive edge of the tumor as well as within blood vessels further suggests EMT to be a first step in generation of migrating CSCs that ensure tumor regeneration at distant sites (Klymkowsky and Savagner 2009).

10.2.1.2 Extrinsic Regulation – Niche Effects

Paget's famous report outlining the "seed and soil" concept of metastases was amongst the first to recognize the contribution of tumor microenvironment in disease progression (Paget 1889). Even in the normal state, stem cells are localized at specialized positions within organs termed as niche, contact with which is crucial for its self-renewal and maintenance (Morrison and Spradling 2008). Initial equivalent daughter cells are impressed with different future fates following interactions with each other and with their environment (Jan and Jan 1998). Tumor niches comprise of cellular components including stromal, endothelial, inflammatory, mesenchymal stem cells and the extracellular matrix (ECM) that is a rich source of epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGFβ), stem cell factor (SCF), chemokines etc. besides factors involved in Notch, Wnt and SHH signaling that promote stem/progenitor cell traits (Zhu et al. 2011). Niche cells may either be derived from the transformed stem cell lineage, another stem cell clone or normal host cells including the stroma, immune cells, etc. Significantly, heterogeneity within the tumor stroma generates a diversity of signals that influence the niche and CSCs (Costea et al. 2013). Some of the niche-associated ECM components are expressed by CSCs themselves (CD44, hyaluron, etc.), thereby ensuring self-sufficiency or independence of growth signals (Sebens and Schafer 2012). CSC niches are most frequently identified at hypoxic and/or perivascular locations or invasive fronts of tumors (Mimeault and Batra 2013; Filatova et al. 2012; Beck et al. 2011; Calabrese et al. 2007). In some subpopulations within gastrointestinal tumors, asymmetric cell division with non-random chromosomal cosegregation independent of cell-to-cell contact is regulated by Wnt pathway signals from the tumor niche in a heat-sensitive paracrine fashion (Xin et al. 2013). Another mechanism for ECM mediated influences on asymmetric division involves parallel alignment of the mitotic spindle axis mediated by hyaluronan-CD44 signaling from the apical surface cell membrane, while the same signaling from the basal surface-membrane aligns the mitotic spindle along an oblique-perpendicular axis. Fibronectin – integrinαβ6 signaling from the basal surface-membrane could also mediate similar alignments (Fujiwara et al. 2008). Such variations perturb the distribution of intrinsic factors. Stem cell (and CSC) niches

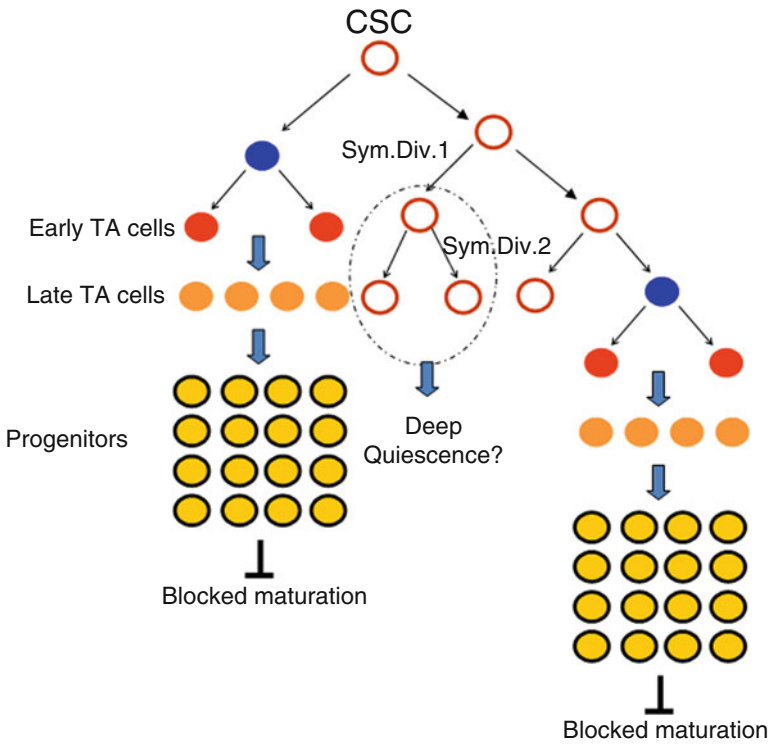

Fig. 10.2 States of asymmetric and symmetric divisions influencing quiescence and proliferation (Sym.Div. – symmetric division)

thus are gaining recognition as specialised ecological settings that generate the instructive cues for sustenance of specific metabolic states, signaling, feedback control and coordination between heterogeneous populations within the organ/tumor (Lander et al. 2012; Schepers et al. 2015).

10.2.1.3 Consequences of Asymmetric and Symmetric Divisions in Cancer

Studies in mouse models demonstrate perturbed asymmetric divisions of normal stem cells to lead to abnormal self-renewal and neoplastic transformation (Powell et al. 2010). The frequency of asymmetric division in tumors also negatively correlates with their proliferative capacity; more proliferative tumors are likely to harbor less asymmetric than symmetric divisions (Fig. 10.2). Thus the relevance of asymmetric cell divisions in maximizing tumor cell proliferation and generation of genetic diversity in a regenerative hierarchy is questionable since the basic caveat of such an arrangement is to balance cell generation with death that suggests effective apoptotic and homeostatic mechanisms. On the other hand, increased symmetric

divisions correlate with higher proliferative capacity and undifferentiated tumors, thereby appearing to be a more obvious path of regeneration. However, too rapid tumor growth itself can be self-limiting (due to stem cell exhaustion). Hence arriving at a balance between asymmetric and symmetric cell divisions may be a reasonable modality of retaining long-term regeneration in tumors. It has been shown that asymmetric division is more frequent in early- than late-stage tumors suggesting a major mechanistic shift in long-term regenerative capabilities during disease progression. Various factors including Akt, p53, EGFR and microenvironmental signaling can affect the balance of cell fate choice between symmetry and asymmetry (Dey-Guha et al. 2011). Recently, a tightly controlled miR-34a circuit has been shown to control the decision of a CSC to perform either symmetric or asymmetric division (Bu et al. 2013).

10.2.2 Quiescence

Stem cells exist in a transient state of cell cycle arrest from which they can be 'awakened' to re-enter into the cell cycle and perform regenerative functions. Such reversible quiescence distinguishes stem cells from differentiated cells that have permanently exited from the cell cycle. Asymmetric cell division is contextually linked to quiescence; conversely, symmetric cell division may be followed by a loss of quiescence. Surprisingly, despite the recognition that disrupted asymmetric cell divisions lead to tumorigenesis, evidences of quiescent cells in tumors are seriously lacking. This is largely due to a paucity of well-defined phenotypic quiescence-determining markers that could provide a convenient read-out, as well as experimental model systems to study the phenomenon. A large majority of hematopoietic stem cells (HSCs) are reported to be in the G0 stage of the cell cycle, whereas very few committed multipotent progenitor cells are actually quiescent although they initially appear to be slow-cycling (Wilson et al. 2008). Whether such distribution of quiescent fractions in stem vs. progenitor cells does actually exist within tumors and is modulated as in regenerative tissues, remains to be addressed. CSC quiescence *in situ* is often defined by their niche components and localization within the tumor. Perivascular and osteoblastic niches as well as several ECM components such as CD44, POSTN, tenascin C, etc. have been described as being essential for establishing and maintaining quiescence in CSCs.

Profiling of differential cell cycle kinetics within HSCs led to recognition of two discrete pools of slow-cycling/homeostatic stem cells vs. dormant/deeply quiescent stem cells (Wilson et al. 2009) both of which are likely to have distinct functions and underlying regulatory mechanisms. Slow-cycling HSCs exhibit relatively higher metabolic activities and active replication machinery than dormant HSCs in which metabolism is at basal maintenance levels and the replication machinery is almost shut off (van der Wath et al. 2009). The former contribute to homeostatic organ regeneration in a definite cyclic pattern, while the latter may be evoked only on drastic depletion of the stem cell pool under extreme circumstances of injury or

stress. Similar differential pools of CSCs with specific functionalities are yet to be completely resolved in tumors, although recent, label-chase studies for examining cycling over time through either thymidine analogues (bromodeoxyuridine – BrdU) incorporation, or chromatin-associated green fluorescent protein (GFP) expression under the control of a doxycyclin regulated transgenic allele, or lipophilic membrane labeling dyes such as PKH or CFSE have provided differential cycling-based, marker-free approaches for the identification of quiescent CSCs in tumors (Kusumbe and Bapat 2009; Foudi et al. 2009; Park et al. 2013; Terskikh et al. 2012; Mani et al. 2008). Such tumors kinetics suggest switching between slow-cycling and deep quiescence, especially on exposure to chemotherapy in mouse models. A hypothetical hierarchy of such CSC pools and their progeny is represented in Fig. 10.2. Such plasticity could be crucial in generating population asymmetry between different derivatives of a founder CSC, and becomes an effective mechanism to offset replicative aging with differential rates of regeneration. Quiescent cells are non-responsive to therapy; hence activation from a quiescent state could be a crucial first step of sensitization to chemotherapy. Deep quiescence however, presents an indeterminable, recalcitrant situation for a cancer patient in whom a state of remission is achieved but with an uncertain caveat. Tracing the subtle differences between the different modes of cell divisions in homeostasis *vs.* deep quiescence within tumors at the molecular level would unravel the differential mechanisms in quiescence and dormancy likely to be highly relevant at the clinical level, yet remain a challenge.

10.2.3 Dedifferentiation and Transdifferentiation

Dedifferentiation and transdifferentiation represent two extreme opposing options that a tumor cell can acquire during tumor progression. Dedifferentiation is suggested to arise from the plasticity acquired by tumor cells to mediate transition between invasive and proliferative states (phenotype switching), in which EMT plays an important role. In reconciling such events with the CSC hierarchy, it appears that the cross-talk of EMT with other pathways in response to microenvironmental stress leads to acquisition of stemness features and dedifferentiation of non-stem cells (Morel et al. 2008 ; Kurrey et al. 2009; Brabletz et al. 2005). Thus the EMT program not only enables physical dissemination of cells from primary tumors but through dedifferentiation, confers on them regenerative capabilities crucial for subsequent establishment of secondary metastases at sites of dissemination (Choi et al. 2012). Dedifferentiation is also derived from expression of embryonal markers like cancer testis antigens in tumors, which was one of the earliest suggestions of activation of developmental programs by tumor cells and considered as an indication of the acquisition of pluripotency in an aberrant setting.

Similarly, transdifferentiation is a pathologically established process in differentiated teratocarcinomas. From a wider perspective, CSCs may transdifferentiate into cell lineages other than that those from which the tumor arose including adipocyte, neuronal, vasculogenic, neuroendocrine and even along immune cell lineages

(Ramakrishnan et al. 2013). Another possibility is that transition of tumor cells into a mature, differentiated mesenchymal phenotype through EMT contributes to the generation of reactive stroma that is crucial for CSC maintenance. Transdifferentiation could be triggered by fusion events as recently demonstrated between tumor infiltrating hematopoietic cells and epithelial cancer cells (Shekhani et al. 2013). Further, the fact that tumor progenitors which are in a state of maturation arrest can be induced to progress into a post-mitotic terminally differentiated state on exposure to appropriate chemicals, presents an attractive therapeutic strategy which is not as yet fully exploited (Nör et al. 2013). All trans-retinoic acid (ATRA) has shown promise in such an approach in differentiation of acute promyelocytic leukemia blasts into mature granulocytes; along with demethylating agents or histone deacetylase inhibitors, it can also differentiate solid tumor cells along neuronal lineages (Politis et al. 2008; Zelivianski et al. 2001). Similarly, unsaturated fatty acids may drive CSC differentiation into adipocyte-like cells; while neuroendocrine differentiation is reported from prostate CSCs and in small cell lung carcinoma (Hanahan and Weinberg 2000). Transdifferentiation is evinced in the generation of reactive tumor stroma and vasculogenic mimicry, while dedifferentiation supports tumor survival and progression through multidrug resistance. Hence both necessitate the development of individualistic strategies. Understanding of such cellular plasticity through dedifferentiation and transdifferentiation may mediate reconciliation between the hierarchical and stochastic models of CSC emergence and tumor establishment (Hanahan and Weinberg 2011; Plaks et al. 2015).

10.3 CSCs and the Hallmarks of Cancer

By virtue of their nomenclature, besides an association with stem cell characteristics, it is expected that CSCs would also exhibit capabilities of tumor cells. Six primary hallmark features are proposed to be associated with transformation and progression of cancer (Gunes and Rudolph 2013); more recently, four additional enabling capabilities are identified (Gatti et al. 2011). It thus becomes pertinent to explore their association(s) with CSCs.

10.3.1 Primary Hallmark Features

10.3.1.1 Enabling Replicative Immortality

Cellular immortality is an intrinsic quality of stem cells achieved through maintenance of telomere length by telomerase. In tumor cells, telomere shortening often leads to genetic instability that reactivates telomerase to avoid genetic chaos thereby generating CSCs (Mo and Zhang 2013). While this suggests a reversal of direction towards reacquisition of immortalization and cell proliferation, it is yet distinct

from that of intrinsic telomerase expression in a stem cell which is targeted by oncogenic mutation(s) to undergone transformation. Thereby although the end result(s) are similar in the two states, a subtle and distinct difference exists in the pathways leading to self-renewal.

10.3.1.2 Resistance to Cell Death

Self-renewal in turn, defines capabilities of quiescence and regeneration of a cell. Most of the mechanisms mediating tumor cell death such as metabolic-, oxidative-, differentiation- or therapy-induced stress are ineffective in a quiescent stem cell which can ensure survival under adverse conditions. Stem cells and CSCs express several types of membrane ATP-binding cassette transporters including ABCG2, that provide an active mechanism for elimination of toxic intracellular components and drugs (Januchowski et al. 2013; Hanahan and Coussens 2012). The involvement of aldehyde dehydrogenase (Aldh) expressed by several CSCs is also demonstrated in tumor cell drug resistance (Faust et al. 2012). Thereby, acquisition of self-renewal possibly involves a multitude of mechanisms that complement and ensure establishment of stable intrinsic quiescence and impart to CSCs an option to select the most effective tool for combating specific adverse microenvironments during cancer progression.

10.3.1.3 Sustained Proliferation

The regenerative capability of a CSC provides the primary impetus for building up a primary mass of tumor cells. Continuing oncogenic mutations in several signaling pathways and transcriptional networks complements this aberrant growth, while disruption of feedback mechanisms further triggers expansion by blocking maturation of progenitors into functionally relevant, differentiated cells. This shifts the self-renewal kinetics of CSCs towards a state of frequent activation that culminates in accumulation of committed, yet proliferating progenitors with compromised functionality. Paracrine cross-talk between the transformed regenerative hierarchy and tumor stroma is also an important accessory in maintaining this state of sustained proliferation (Liu and Dean 2010).

10.3.1.4 Evasion of Growth Suppression

Maturation arrest consequently implies longer retention of progenitors and a slower turnover into terminally differentiated cells that disrupts homeostatic mechanisms that attempt to restore tissue functions. Such efforts at homeostasis may involve acquisition of resistance to apoptotic pathways that tissue cells normally commit to, or loss of contact inhibition that consequently leads to cell proliferation and has since long been associated with cancer cells grown in

culture. *In situ*, such homeostatic mechanisms regulate organ size and in cancer may be driven by Ras and FoxM1 mutations or activated EMT and Hippo-Yap pathways (Fuxe and Karlsson 2012; Kusumbe et al. 2009). One of the most significantly altered cellular responses involved in evasion of growth suppression is towards the growth factor TGFβ, which establishes extensive cross-talk with diverse downstream pathways involved in EMT, inflammation and immune evasion (Bao et al. 2006).

10.3.1.5 Inducing Angiogenesis

A rapidly proliferating tumor is slated to encounter the stresses imposed by limited availability of nutrients and gaseous exchange/diffusion. CSCs and progenitors are activated by such microenvironmental factors including hypoxia, hypoglycemia and iron deficiency into triggering an angiogenic switch in growing tumors. This effectively mediates the recruitment of primitive endothelial stem and progenitor cells by CSCs to primary and metastatic tumors in a paracrine manner through secretion of angiogenic growth factors such as VEGF (Doan and Chute 2012). CSCs also contribute to establishment of vasculature through their potential to transdifferentiate along endothelial and vascular smooth muscle-like lineages and give rise to non-endothelial channels through the process described as vasculogenic mimicry (Ping and Bian 2011). This active role of CSCs in establishing angiogenesis and/or vasculogenesis significantly contributes to niche development, and may be essential for their sustenance within tumors since both processes support survival of cells during tumor progression. Notably, the perivascular niche is known to play an important role in regulation and maintenance of CSC quiescence in leukemia, breast, skin and brain tumors (Ghajar et al. 2013). This further assigns an indispensable role to tumor vasculature in acquiring long-term dormancy.

10.3.1.6 Activating Invasion and Metastasis

EMT has been considered as the primary mediator of the program of invasion and metastasis in several tumor types. Epigenetic and microenvironmental influences on hypoxia, autocrine – paracrine signals from Wnt, tyrosine kinases, NF-κB and growth factor (TGFβ, EGF) pathways etc. trigger a transcriptional program that initiates intercellular junction dissolution and modulate phenotypic changes. Effectively this leads to enhanced plasticity within the regenerative hierarchy that strikingly generates variations in the development-associated invasive program. Acquisition of a mesenchymal phenotype is stated to facilitate invasion through the basement membrane and/or tumor stroma into blood vessels and lymphatics. Such dissemination poses a challenge to a migrating cell that relies on several intrinsic and acquired survival mechanisms of CSCs. On homing to secondary sites, these stem-like cells mediate a regenerative program that is inclusive of mesenchymal to epithelial transition (MET) to establish metastatic colonization.

Surprisingly, in some cancers EMT is *not* indicated to be a modality of invasion and metastases of tumor cells and CSCs. Cohesive or cooperative cell migration (CCM) of cell groups that exhibit intact cell junctions (a characteristic epithelial morphology) and dissemination through passive implanting in lymphatic spaces and blood vessels have been described. Such features may provide a survival advantage to the migrating cell clusters with respect to protection from immune attack and sheer forces of circulation, to increase the probabilities of generating micrometastases. The relevance of CCM in a clinicopathological setting is presently considered as being more robust than that of EMT (Chui 2013). These differing metastases modalities *viz.* EMT vs CCM have been recently applied as a classifier in high-grade serous ovarian carcinoma to achieve patient stratification into discrete subtypes (Gardi et al. 2014). Notably, in each subtype, different pathways of stem cell activation and regulation are noted.

As in the case of CSCs/TICs, the terminology of EMT is often used rather ambiguously to imply any process associated with loss of cell polarity, invasion, metastasis, acquisition of stemness, resistance to apoptosis and generation of cancer associated fibroblasts. A specific cellular shape/morphology may not sufficient to achieve all these effects without a defined molecular context in terms of the associated genes and activation of specific networks. This is important since the apparently diverse outcomes reported in association with EMT are reached under specific cellular contexts, and hence it becomes pertinent to define specific tissue and systems network associations wherein some functions either complement each other or may be exclusive. In doing so, one could achieve improved understanding of context-specific EMT pathways and their cross-talk in driving de-differentiation and/or trans-differentiation.

10.3.2 Enabling Characteristics and Emerging Hallmarks

10.3.2.1 Genome Instability and Mutation

Maintenance of genome integrity is a critical mission of the stem cell hierarchy; compromise of the same results in transformation and posits the generation of CSCs. Random oncogenic insults in progenitors/differentiated cells also trigger cellular reprogramming towards dedifferentiation that is accompanied by acquisition of self-renewal properties and a ‘stem-like’ phenotype. Several tumors are established as being monoclonal, which ensures faithful inheritance and propagation of the oncogenic mutation(s) within the CSC progeny, besides imparting a specific molecular identity to the tumor. In the last few years, considerable evidence has been additionally presented that genetic instability is crucial in the generation of intratumor heterogeneity and clonal evolution (Naik et al. 2015). Differential clone dominance is established through the principles of Darwinian selection in which clones exhibiting genomic rearrangements in addition to the founder mutations are selected for by the microenvironment and may interact in either a competitive and mutualistic manner (Ng et al. 2012). Consequently, genome protection from further

oxidative damage by the niche may be achieved through synthesis of hyaluron that scavenges ROS, maintenance of the tumor cell at low hypoxic levels, etc. that play a major role in protecting the transformed clone against continual DNA damage and (mis)repair cycles that could lead to genetic chaos (Darzynkiewicz and Balazs 2012).

10.3.2.2 Tumor-Promoting Inflammation

Several chronic inflammatory states are considered high risk factors for the onset of cell transformations likely to progress towards malignancy. Persistent tissue injury and repair leads to increased localized levels of several inflammatory cytokines and growth factors like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, TGFβ, nuclear factor kB (NFkB), etc (Shigdar et al. 2014). Such cyclic/recurrent activation of developmental signaling and regenerative pathways also perturbs stem cell self-renewal and tissue homeostasis resulting in aberrant cell expansion and hyperplasia. Production of ROS and hypoxia in the reactive stroma further sets the stage for transformation of those CSCs that are continuously activated (Tanno and Matsui 2011). Tumor-promoting inflammatory niches also recruit components of the innate immune system that precipitate and support progression of incipient wounds associated with inflammation into full-blown tumors.

10.3.2.3 Reprogramming Energy Metabolism

The metabolic switch of cancer cells to glycolysis even in the presence of oxygen first reported by Warburg is paradoxical given its poor efficiency of energy generation (Warburg 1956). More recently, it has been reported that CSCs overexpress metabolism-related genes like glycine decarboxylase and pyruvate kinase M2 along with metabolites such as 2-hydroxyglutarate, lactate and kynurenine that are suggested to act as continual oncogenic metabolic triggers that contribute to disease progression by mediating epigenetic and genetic reprogramming (Zhang et al. 2013).

10.3.2.4 Evading Immune Destruction

Overcoming the host immune surveillance mechanisms is now recognized to be essential for tumor establishment and progression. CSCs have been demonstrated to lose MHC class I molecules and selectively silence expression of tumor-associated antigens towards such an evasion (Guerry et al. 1984). Yet other mechanisms of immune evasion of CSCs include altered immunogenicity, production of regulatory molecules by tumor cells, interactions with tumor-infiltrating immune cells (Qi et al. 2012), functional inactivation of antigen-reactive T lymphocytes, activation of Treg cells by secretion of immunosuppressive cytokines, including IL-4, IL-10 and TGF-β (Dancescu et al. 1992). Tolerance may also be achieved through clonal

anergy of macrophages and DCs, or expression of tolerogenic molecules including B7-H1, B7.1, CD47, SIRPa, CD200 by CSCs that facilitate immune evasion (Quesnel 2008).

10.4 Future Perspectives

The diversity of specific mechanisms involved in the generation of tumors through reprogramming of the intracellular circuitry to perturb regenerative homeostasis as well as acquire hallmark capabilities suggest that these may be achieved in a tumor – and tissue- specific manner. Feed-forward mechanisms of CSC emergence potentiate normal stem cell transformation and generation of maturation arrested derivatives; while feedback mechanisms suggest dedifferentiation or reprogramming towards a loss of differentiated identity. Both mechanisms emphasize that fidelity of either the quiescent state or a stable differentiated identity cannot be taken for granted and is susceptible to loss of intuitive, instructive cellular networks that could present either as transient ablation of homeostasis, or more seriously as stable transformation (Holmberg and Perlmann 2012). While there is increasing knowledge in the field regarding cell fate determination, lineage commitment and differentiation, mechanisms ensuring long-term stable stem cell state maintenance or phenotypic and functional differentiation remains poorly understood, yet can be highly relevant to cancer biology.

The existence of CSCs is convincing enough in certain cancers, yet mere expression of a certain phenotype and aberrant regeneration provides a narrow comprehension for inter-tumor heterogeneity between different tumor subtypes, and intra-tumor heterogeneity between specific cellular subsets within the same tumor. These phenomena in turn represent a wide range of variation of cellular and molecular cross-talks. While the present review emphasizes cellular aspects of CSCs, an equal if not more extensive research efforts currently focus on understanding how such cellular states and phenomena are regulated in tumors that are notoriously heterogeneous at the cellular and molecular levels. Hard wiring of gene expression is known to control normal tissue homeostasis; in contrast in tumors these are suggested to be flexible and completely erratic. Further integrative analyses across the various levels of gene regulation including epigenetic, genetic, proteomic, protein translation and signaling may thus be necessary to define specific tissue contexts.

While understanding a cellular state *in situ* is the objective of most research programs, development and use of appropriate *in vitro* model systems is necessitated on logistic and ethical grounds. In doing so, it should be ensured that such models are as close to the *in situ* state as is possible through addressing various issues. Can immortalized cell lines be termed as CSC lines? Do these or prospectively isolated CSCs retain a memory of the complex developmental process by which the tissue was first constructed, and can it be reproduced in experimental models? Are patient-derived xenografts more relevant than cell line derived xenografts? In the latter are humanized animal models harbouring human stroma and/or human

immune cells more relevant? Given the complexity of inter-tumor and intra-tumor heterogeneity how could one develop the most relevant models of specific cancers? Although related processes such as asymmetric cell division are currently addressed using models, their systemic homology in human disease opens a whole new door of complexities (Matsumura and Toyoshima 2012). This list of newly arising queries obviously is relentless, but it is quite likely that integration of inter-disciplinary studies including simulation, computation and mathematical modelling to channelize information from reductionism approaches towards construction of a systems view will address them in the long run.

Most if not all the above discussions regarding the future of CSCs seems to be centered on elucidation of their very existence and underpinning various characters crucial for their identity and functions. However, these have serious clinical implications that can contribute to development of cancer therapy in eliminating the regenerative tumor hierarchy to improve patient prognosis. Translation of these basic concepts thus is imperative and involve the development of specific drugs, inhibitors and antibodies to stabilize for example, disrupted polarity mechanisms, restore homeostasis mechanisms, identify 'druggable molecular targets' within the cellular systems networks, restrict migrating CSCs, stabilize innate immune responses to CSCs, neutralize the reactive tumor niche (Lathia et al. 2011; Bliss et al. 2014; Fan et al. 2015; Pan et al. 2015; Ajani et al. 2015), etc. The wider expectation of these efforts is to achieve optimal combinatorial therapies that would improve drug tolerance and patient prognosis in a personalized manner. Thus the process of pursuing the elusive quiescent CSC promises to be an exciting endeavor, and leads to an appreciation of the intrinsic drive of these populations in surviving against all odds in a manner akin to the Darwinian principles of survival-of-the-fittest. Being fraught with frustrations it might at times appear to be an academic exercise; however such information will be of immense important in view of developing appropriate personalized, specific and more effective therapies in cancer.

References

Ajani JA, Song S, Hochster HS et al (2015) Cancer stem cells: the promise and the potential. Semin Oncol 42(Suppl 1):S3–S17

Al-Hajj M, Wicha MS, Benito-Hernandez A et al (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100:3983–3988

Bao S, Wu Q, Sathornsumetee S et al (2006) Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. Cancer Res 66:7843–7848

Bapat SA, Mali AM, Koppikar CB et al (2005) Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 65:3025–3029

Bapat S, Collins A, Dean M et al (2009) Cancer Stem Cells (CSCs): similarities and variation of the theme of normal stem cells. In: Bapat S (ed) Cancer stem cells: identification and targets. Wiley, Hoboken, pp 1–26

Beck B, Driessens G, Goossens S et al (2011) A vascular niche and a VEGF-Nrp1 loop regulate the initiation and stemness of skin tumours. Nature 478:399–403

Beuran M, Negoi I, Paun S et al (2015) The epithelial to mesenchymal transition in pancreatic cancer: a systematic review. Pancreatology 15:217–225, S1424-3903(15)00038-1

Bliss SA, Greco SJ, Rameshwar P (2014) Hierarchy of breast cancer cells: key to reverse dormancy for therapeutic intervention. Stem Cells Transl Med 3:782–786
Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730–737
Brabletz T, Jung A, Spaderna S et al (2005) Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. Nat Rev Cancer 5:744–749
Bu P, Chen KY, Chen JH et al (2013) A microRNA miR-34a-regulated bimodal switch targets notch in colon cancer stem cells. Cell Stem Cell 12:602–615
Calabrese C, Poppleton H, Kocak M et al (2007) A perivascular niche for brain tumor stem cells. Cancer Cell 11:69–82
Chang KC, Wang C, Wang H (2012) Balancing self-renewal and differentiation by asymmetric division: insights from brain tumor suppressors in Drosophila neural stem cells. Bioessays 34:301–310
Choi SY, Gout PW, Collins CC et al (2012) Epithelial immune cell-like transition (EIT): a proposed transdifferentiation process underlying immune-suppressive activity of epithelial cancers. Differentiation 83:293–298
Chui MH (2013) Insights into cancer metastasis from a clinicopathologic perspective: epithelial-Mesenchymal Transition is not a necessary step. Int J Cancer 132:1487–1495
Costea DE, Hills A, Osman AH et al (2013) Identification of two distinct carcinoma-associated fibroblast subtypes with differential tumor-promoting abilities in oral squamous cell carcinoma. Cancer Res 73:3888–3901
Dancescu M, Rubio-Trujillo M, Biron G et al (1992) Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. J Exp Med 176:1319–1326
Darzynkiewicz Z, Balazs EA (2012) Genome integrity, stem cells and hyaluronan. Aging (Albany NY) 4:78–88
Dey-Guha I, Wolfer A, Yeh AC et al (2011) Asymmetric cancer cell division regulated by AKT. Proc Natl Acad Sci U S A 108:12845–12850
Doan PL, Chute JP (2012) The vascular niche: home for normal and malignant hematopoietic stem cells. Leukemia 26:54–62
Duan JJ, Qiu W, Xu SL et al (2013) Strategies for isolating and enriching cancer stem cells-well begun is half done. Stem Cells Dev 22:2221–2239
Fan D, Zhou X, Li Z et al (2015) Stem cell programs are retained in human leukemic lymphoblasts. Oncogene 34:2083–2093
Faust D, Al-Butmeh F, Linz B et al (2012) Involvement of the transcription factor FoxM1 in contact inhibition. Biochem Biophys Res Commun 426:659–663
Filatova A, Acker T, Garvalov BK (2012) The cancer stem cell niche(s): the crosstalk between glioma stem cells and their microenvironment. Biochim Biophys Acta 1830:2496–2508
Foudi A, Hochedlinger K, Van Buren D et al (2009) Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nat Biotechnol 27:84–90
Fujiwara T, Kawakatsu T, Tayama S et al (2008) Hyaluronan-CD44 pathway regulates orientation of mitotic spindle in normal epithelial cells. Genes Cells 13:759–770
Fuxe J, Karlsson MC (2012) TGF-β-induced epithelial-mesenchymal transition: a link between cancer and inflammation. Semin Cancer Biol 22:455–461
Ganguly A, Sasayama D, Cho HT (2012) Regulation of the polarity of protein trafficking by phosphorylation. Mol Cells 33:423–430
Gardi NL, Deshpande TU, Kamble SC et al (2014) Discrete molecular classes of ovarian cancer suggestive of unique mechanisms of transformation and metastases. Clin Cancer Res 20:87–99
Gatti L, Cossa G, Beretta GL et al (2011) Novel insights into targeting ATP-binding cassette transporters for antitumor therapy. Curr Med Chem 18:4237–4249
Ghajar CM, Peinado H, Mori H et al (2013) The perivascular niche regulates breast tumour dormancy. Nat Cell Biol 15:807–817

Gómez-López S, Lerner RG, Petritsch C (2014) Asymmetric cell division of stem and progenitor cells during homeostasis and cancer. Cell Mol Life Sci 71:575–597
Graham TA, Jawad N, Wright NA (2010) Spindles losing their bearings: does disruption of orientation in stem cells predict the onset of cancer? Bioessays 32:468–472
Guerry D, Alexander MA, Herlyn MF et al (1984) HLA-DR histocompatibility leukocyte antigens permit cultured human melanoma cells from early but not advanced disease to stimulate autologous lymphocytes. J Clin Invest 73:267–271
Gunes C, Rudolph KL (2013) The role of telomeres in stem cells and cancer. Cell 152:390–393
Hanahan D, Coussens LM (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21:309–322
Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 144:646–674
Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674
Holmberg J, Perlmann T (2012) Maintaining differentiated cellular identity. Nat Rev Genet 13:429–439
Inaba M, Yamashita YM (2012) Asymmetric stem cell division: precision for robustness. Cell Stem Cell 11:461–469
Izumi H, Kaneko Y (2012) Evidence of asymmetric cell division and centrosome inheritance in human neuroblastoma cells. Proc Natl Acad Sci U S A 109:18048–18053
Jaggupilli A, Elkord E (2012) Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity. Clin Dev Immunol 2012:708036
Jan YN, Jan LY (1998) Asymmetric cell division. Nature 392:775–778
Januchowski R, Wojtowicz K, Zabel M (2013) The role of aldehyde dehydrogenase (ALDH) in cancer drug resistance. Biomed Pharmacother 67:669–680
Kahn M (2011) Symmetric division versus asymmetric division: a tale of two coactivators. Future Med Chem 3:1745–1763
Kelsom C, Wange L (2012) Uncovering the link between malfunctions in Drosophila neuroblast asymmetric cell division and tumorigenesis. Bioscience 2:38
Kerosuo L, Bronner-Fraser M (2012) What is bad in cancer is good in the embryo: importance of EMT in neural crest development. Semin Cell Dev Biol 23:320–332
Klymkowsky MW, Savagner P (2009) Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. Am J Pathol 174:1588–1593
Kurrey NK, Jalgaonkar SP, Joglekar AV et al (2009) Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer. Stem Cells 27:2059–2068
Kusumbe AP, Bapat SA (2009) Cancer stem cells and aneuploid populations within developing tumors are the major determinants of tumor dormancy. Cancer Res 69:9245–9253
Kusumbe AP, Mali AM, Bapat SA (2009) CD133-expressing stem cells associated with ovarian metastases establish an endothelial hierarchy and contribute to tumor vasculature. Stem Cells 27:498–508
Lander AD, Kimble J, Clevers H et al (2012) What does the concept of the stem cell niche really mean today? BMC Biol 10:19
Lathia JD, Venere M, Rao MS et al (2011) Seeing is believing: are cancer stem cells the Loch Ness monster of tumor biology? Stem Cell Rev Rep 7:227–237
Liu Y, Dean DC (2010) Tumor initiation via loss of cell contact inhibition versus Ras mutation: do all roads lead to EMT? Cell Cycle 9:897–900
Mani SA, Guo W, Liao MJ et al (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133:704–715
Marusyk A, Almendro V, Polyak K (2012) Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 12:323–334
Matsumura S, Toyoshima F (2012) ABL1 joins the cadre of spindle orientation machinery. Cell Struct Funct 37:81–87
Medema JP et al (2013) Cancer stem cells: the challenges ahead. Nat Cell Biol 15:338–344

Mimeault M, Batra SK (2013) Hypoxia-inducing factors as master regulators of stemness properties and altered metabolism of cancer- and metastasis-initiating cells. J Cell Mol Med 17:30–54
Mo W, Zhang JT (2013) Human ABCG2: structure, function, and its role in multidrug resistance. Int J Biochem Mol Biol 3:1–27
Morel AP, Lièvre M, Thomas C et al (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One 3:e2888
Moreno-Bueno G, Portillo F, Cano A (2008) Transcriptional regulation of cell polarity in EMT and cancer. Oncogene 27:6958–6969
Morrison SJ, Spradling AC (2008) Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132:598–611
Muthuswamy SK, Xue B (2012) Cell polarity as a regulator of cancer cell behavior plasticity. Annu Rev Cell Dev Biol 28:599–625
Naik RR, Singh AK, Mali AM et al (2015) A tumor deconstruction platform identifies definitive end-points in the evaluation of drug responses. Oncogene. doi:10.1038/onc.2015.130. [Epub ahead of print]
Ng CK, Pemberton HN, Reis-Filho JS (2012) Breast cancer intratumor genetic heterogeneity: causes and implications. Expert Rev Anticancer Ther 12:1021–1032
Nör C, Sassi FA, de Farias CB, Schwartsmann G et al (2013) The histone deacetylase inhibitor sodium butyrate promotes cell death and differentiation and reduces neurosphere formation in human medulloblastoma cells. Mol Neurobiol 48:533–543
Paget S (1889) The distribution of secondary growths in cancer of the breast. Lancet 1:571–573
Pan Q, Li Q, Liu S et al (2015) Targeting cancer stem cells using immunologic approaches. Stem Cells. doi:10.1002/stem.2039. [Epub ahead of print]
Park JP, Raafat A, Feltracco JA et al (2013) Differential gene expression in nuclear label-retaining cells in the developing mouse mammary gland. Stem Cells Dev 22:1297–1306
Ping YF, Bian XW (2011) Cancer stem cells switch on tumor neovascularization. Curr Mol Med 11:69–75
Plaks V, Kong N, Werb Z (2015) The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? Cell Stem Cell 16:225–238
Politis PK, Akrivou S, Hurel C et al (2008) BM88/Cend1 is involved in histone deacetylase inhibition-mediated growth arrest and differentiation of neuroblastoma cells. FEBS Lett 582:741–748
Poulson ND, Lechler T (2012) Asymmetric cell divisions in the epidermis. Int Rev Cell Mol Biol 295:199–232
Powell AE, Shung CY, Saylor KW et al (2010) Lessons from development: a role for asymmetric stem cell division in cancer. Stem Cell Res 4:3
Qi Y, Li RM, Kong FM et al (2012) How do tumor stem cells actively escape from host immunosurveillance? Biochem Biophys Res Commun 420:699–703
Quesnel B (2008) Tumor dormancy and immunoescape. APMIS 116:685–694
Quintana E, Shackleton M, Foster HR et al (2010) Phenotypic heterogeneity among tumorigenic melanoma cells from patients that is reversible and not hierarchically organized. Cancer Cell 18:510–523
Quyn AJ, Appleton PL, Carey FA et al (2010) Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. Cell Stem Cell 6:175–181
Ramakrishnan M, Mathur SR, Mukhopadhyay A (2013) Fusion derived epithelial cancer cells express hematopoietic markers and contribute to stem cell and migratory phenotype in ovarian carcinoma. Cancer Res 73:5360–5370
Rehe K, Wilson K, Bomken S et al (2013) Acute B lymphoblastic leukaemia-propagating cells are present at high frequency in diverse lymphoblast populations. EMBO Mol Med 5:38–51
Reya T, Morrison SJ, Clarke MF et al (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
Rosenthal N (2003) Prometheus's vulture and the stem-cell promise. N Engl J Med 349:267–274

Scheela C, Weinberg RA (2012) Cancer stem cells and epithelial–mesenchymal transition: concepts and molecular links. Semin Cancer Biol 22:396–403
Schepers K, Campbell TB, Passegue´ E (2015) Normal and leukemic stem cell niches: insights and therapeutic opportunities. Cell Stem Cell 16:254–267
Schulenburg A, Blatt K, Cerny-Reiterer S et al (2015) Cancer stem cells in basic science and in translational oncology: can we translate into clinical application? J Hematol Oncol 8:16
Sebens S, Schafer H (2012) The tumor stroma as mediator of drug resistance – a potential target to improve cancer therapy? Curr Pharm Biotechnol 13:2259–2272
Shekhani MT, Jayanthy AS, Maddodi N et al (2013) Cancer stem cells and tumor transdifferentiation: implications for novel therapeutic strategies. Am J Stem Cells 2:52–61
Shigdar S, Li Y, Bhattacharya S et al (2014) Inflammation and cancer stem cells. Cancer Lett 345:271–278
Siegrist SE, Doe CQ (2006) Extrinsic cues orient the cell division axis in Drosophila embryonic neuroblasts. Development 133:529–536
Singh SK, Clarke ID, Hide T et al (2004) Cancer stems cells in nervous system tumors. Oncogene 23:7267–7273
Sugioka K, Sawa H (2012) Formation and functions of asymmetric microtubule organization in polarized cells. Curr Opin Cell Biol 24:517–525
Tanno T, Matsui W (2011) Development and maintenance of cancer stem cells under chronic inflammation. J Nihon Med Sch 78:138–145
Terskikh VV, Vasiliev AV, Vorotelyak EA (2012) Label retaining cells and cutaneous stem cells. Stem Cell Rev 8:414–425
Tirino V, Desiderio V, Paino F et al (2013) Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. FASEB J 27:13–24
van der Wath RC, Wilson A, Laurenti E et al (2009) Estimating dormant and active hematopoietic stem cell kinetics through extensive modeling of bromodeoxyuridine label-retaining cell dynamics. PLoS One 4:e6972
Visavader JE, Lindemann GJ (2012) Cancer stem cells: current status and evolving complexities. Cell Stem Cell 10:717–728
Wang N, Docherty F, Brown HK et al (2015) Mitotic quiescence, but not unique "stemness," marks the phenotype of bone metastasis-initiating cells in prostate cancer. FASEB J. pii: fj.14-266379. [Epub ahead of print]
Warburg O (1956) On the origin of cancer cells. Science 123:309–314
Wilson A, Laurenti E, Oser G et al (2008) Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135:1118–1129
Wilson A, Laurenti E, Trumpp A (2009) Balancing dormant and self-renewing hematopoietic stem cells. Curr Opin Genet Dev 19:461–468
Woodward WA, Sulman EP (2008) Cancer stem cells: markers or biomarkers? Cancer Metastasis Rev 27:459–470
Xin HW, Ambe CM, Ray S et al (2013) Wnt and the cancer niche: paracrine interactions with gastrointestinal cancer cells undergoing asymmetric cell division. Cancer 4:447–457
Yamashita YM, Mahowald AP, Perlin JR et al (2007) Asymmetric inheritance of mother versus daughter centrosome in stem cell division. Science 315:518–521
Zelivianski S, Verni M, Moore C et al (2001) Multipathways for transdifferentiation of human prostate cancer cells into neuroendocrine-like phenotype. Biochim Biophys Acta 1539:28–43
Zhang G, Yang P, Guo P et al (2013) Biochim Biophys Acta 1836:49–59
Zheng H, Kang Y (2014) Multilayer control of the EMT master regulators. Oncogene 33:1755–1763
Zhu X, Zhou X, Lewis MT et al (2011) Cancer stem cell, niche and EGFR decide tumor development and treatment response: a bio-computational simulation study. J Theor Biol 269:138–149

Chapter 11
Mesenchymal Stromal Cells (MSC)

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Abstract Mesenchymal stem or stromal cells (MSC) are plastic-adherent fibroblast-like cells that can readily be isolated from various tissues and expanded in vitro. *Per definitionem*, they are able to differentiate into bone, cartilage and adipose tissue. In the last 15 years, a huge number of different preparative protocols have been developed to yield MSC-like cell lines from starting materials as diverse as bone marrow, fat tissue, umbilical cord blood and peripheral blood. However, these protocols as well as the resulting cell populations are heterogeneous. Furthermore, the composition of the cell products and their differentiation potential may change in the course of long-term culture expansion. There is still a need for standardized protocols and universal criteria for quality control of the starting cell populations as well as for the cell products after expansion. Nevertheless, MSCs have already found their way into a huge number of clinical studies addressing a broad variety of diseases. Even though there is no convincing evidence that MSCs are involved in the process of tissue repair by true transdifferentiation, they probably contribute to the repair process by immunomodulatory effects and interaction with other cell types.

Keywords MSC • Cell culture • GMP • Cell based therapy • Regenerative medicine

11.1 Introduction

Almost 40 years ago, in the early 1970s, Friedenstein et al. described discrete fibroblast-like colonies in monolayer cultures of bone marrow, spleen and thymus that could be easily maintained under culture conditions and that demonstrated

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DOI 10.1007/978-3-319-27610-6_11

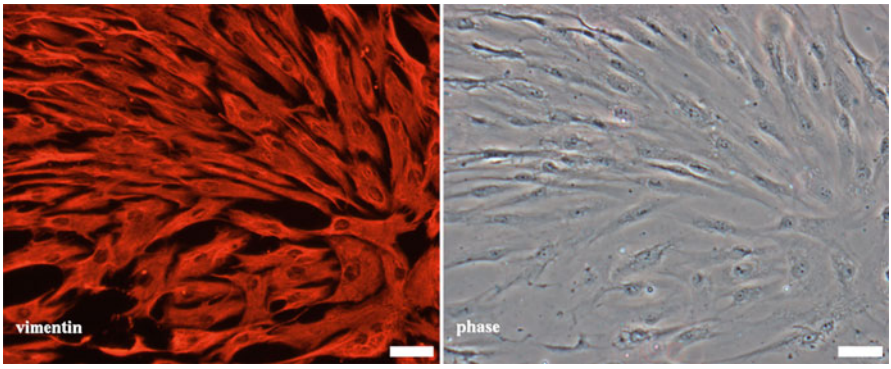

Fig. 11.1 MSC express vimentin, an intermediate filament typical for cells of mesenchymal origin. The cells are showing a fibroblastoid morphology. Immunofluorescent staining (*left*) and phase contrast (*right*) (scale bar 150 µm)

differentiation characteristics in vitro as well as in vivo upon their re-transplantation (Friedenstein et al. 1968, 1974). The term "mesenchymal stem cells" (MSCs) however, has been made popular in the early 1990s by Caplan (1991). Caplan and others used periosteal cells from young chicken, which were transplanted into athymic mice and demonstrated their osteo-chondrogenic differentiation potential (Horwitz and Keating 2000; Nakahara et al. 1990). Today, the term "MSC" is commonly applied to plastic-adherent cell preparations isolated from bone marrow or other tissues that are able to differentiate into bone, cartilage and adipose tissue under specific conditions (see Fig. 11.1). Although there exist no specific markers for these cells, they are usually positive for several antigens such as CD73, CD90, CD105 and lack expression of hematopoietic antigens (Dominici et al. 2006; Wagner et al. 2005a).

The multilineage differentiation potential of MSCs is still under debate. It is commonly accepted that a rare mesenchymal progenitor cell population is present in bone marrow, that possesses differentiation potential towards different cell lineages. However, mounting evidence indicates that the described adherent cell populations are highly heterogeneous and actually consist of several subpopulations, which gradually overgrow under certain culture conditions and might mimick the phenomenon of differentiation. Therefore, these cells might not fulfill all the criteria to be named "stem cells" and should therefore be named "mesenchymal stromal cells" (Horwitz and Keating 2000). Consequently, the acronym "MSC" stays the same, whereas the term "mesenchymal stem cells" should only be reserved for cells that meet specified criteria for stem cells, i.e. unlimited self-renewal capacity. Alternatively they have been named "multipotent mesenchymal stromal cells" or "multipotent stromal cells" to indicate the multipotent differentiation capacity of these cell preparations (Horwitz et al. 2005). Numerous authors have described protocols for the isolation and cultivation of MSCs from tissues other than the bone marrow, mainly from umbilical cord blood and adipose tissue.

11.2 Derivation of MSCs

The source and the property of MSC preparations from different laboratories vary significantly and the resulting cell products are highly heterogeneous. The lack of standardization considerably hampers the comparability of results among different research groups (Wagner and Ho 2007; Bieback et al. 2012).

We and others have demonstrated that slight differences of the culture conditions could favor the expansion of certain subsets and might contribute to genetic instability. Based on the morphology of MSC preparations, three distinct cell types could be distinguished: spindle-shaped cells, large flat cells and small round-cell subpopulations (Colter et al. 2001; Horn et al. 2008; Schallmoser et al. 2010). MSC cultures are continuously unstable and can give rise to individual cells – and subsequently cell colonies – producing, for example, smooth muscle-typical α-actin filaments and myofilaments containing cardiac α-actin (Ho et al. 2008). Even other cells in the culture start to form sub-lines positive for cytokeratin filaments. An entirely different program may be characteristic for other MSCs, which begin to synthesize special types of fat storage such as adipophilin-positive fat droplets in adipocytes (Heid et al. 1998).

Altogether, these results again emphasize the importance of standardizing the isolation of the initial cell material, culture media and methods. At the same time it is of utmost importance to develop quality control systems for MSC preparations for clinical applications and guidelines for "Good Manufacturing Practice" (GMP) have to be fulfilled (Wuchter et al. 2015). To this end, the following variables have to be taken into consideration.

11.2.1 Species

MSCs have been isolated from many different species such as mouse, guinea pig, chick, rabbit, dog, pigs and human. Knowledge gained from animal models cannot always be extrapolated for human cells. It seems as if there existed many similarities with human MSCs, but a systematic comparison of MSCs from different species is yet elusive. Experimental data of MSCs from animal models have to be validated in the human system prior to clinical application. In this chapter we will focus on human MSCs.

11.2.2 Isolation of MSCs from Different Sources

MSCs were originally isolated from bone marrow (Friedenstein et al. 1966; Pittenger et al. 1999). In the last decade however, MSC-like cell lines could be derived from various other tissues such as umbilical cord blood (Bieback et al. 2004; Erices et al.

2000), umbilical cord matrix (Secco et al. 2008a, b; Zeddou et al. 2010), adipose tissue (Baptista et al. 2009; de Girolamo et al. 2007; Lee et al. 2004; Zuk et al. 2001), peripheral blood (Kuznetsov et al. 2001; Zvaifler et al. 2000) and skeletal muscle (Jiang et al. 2002b). Furthermore, cell preparations that fulfill the minimal criteria for MSCs have also been isolated from other tissue of adult mice such as brain, liver, kidney, lung, thymus and pancreas (da Silva et al. 2006). There is little doubt that multipotent cell populations of mesenchymal derivation reside in many tissues. Our gene expression analysis has provided clear evidence that a significant number of genes is differentially expressed in MSCs isolated from specific tissue (Wagner et al. 2005a). Correspondingly, the differentiation potentials and functional implications varied widely among MSC preparations derived from different origins (Kern et al. 2006; Wagner et al. 2007a). This fact has to be taken into account when comparing results from different research groups.

11.2.3 Isolation/Depletion Using Surface Markers

Various surface markers such as STRO-1, CD73, CD105 and CD271 have been used for positive selection of MSCs. Alternatively, negative selection was performed using hematopoietic surface markers such as CD34, CD45, Ter119 and glycophorin. These markers have been used alone or in combination for enrichment of fibroblast colony forming units (CFU-F). However, they do not allow direct isolation of multipotent MSCs. A sophisticated comparison of the molecular features of MSCs that were isolated with different enrichment methods is elusive, but it is likely that the composition of heterogenic cell preparations is significantly affected by these separation steps (Horn et al. 2008; Wagner and Ho 2007).

11.2.4 Coating of Surface and Biomaterials

Adherence to the surface of culture dishes is the most prominent feature of MSCs. Properties of the surface (e.g. roughness, hydrophobicity and elasticity) significantly affect selection or differentiation potential of cell preparations (Anderson et al. 2004; Engler et al. 2006). Many protocols have applied additional protein coating of the surface (e.g. fibronectin, gelatine or collagen) to enhance cell adhesion and to mimic certain aspects of the natural extracellular microenvironment. Culture on either fibronectin or gelatine can have an affect upon the morphology of the cell products after culture.

11.2.5 Culture Media and Serum Supplements

Culture media have a tremendous impact on gene expression and proteome of MSCs (Wagner et al. 2005a, 2006). A huge arsenal of basal culture media is available and many different media have successfully been used for isolation of MSCs in different laboratories. Furthermore, there is evidence that oxygen tension plays an important role and that hypoxia accelerates MSC differentiation (Ren et al. 2006).

So far most culture protocols for MSC preparation contain serum additives. Serum concentrations usually vary between 2 and 20 %. Most studies have used fetal calf serum (FCS). Concerns regarding BSE, other infectious complications and host immune reactions have fueled investigation of alternative culture supplements. Several groups have developed alternative culture protocols for the expansion of MSCs based on reagents of human origin (i.e. serum, plasma, platelet lysate etc.) that replaced fetal bovine serum (Bieback et al. 2009; Kocaoemer et al. 2007; Lange et al. 2007; Müller et al. 2006; Schallmoser et al. 2007; Stute et al. 2004). The impact of these supplements on the composition of cell preparations is yet unknown but different growth kinetics and cell morphology indicate their relevance. The development of a chemically defined and serum free growth medium would therefore substantially contribute to standardized MSC preparations.

11.2.6 In Vitro Cultivation (Passage, Density and Cryopreservation)

MSCs can be passaged in vitro for a limited number of times before they become senescent and stop proliferation. As a matter of fact, molecular profiles and functional features of MSCs are significantly affected by this process of cellular aging (DiGirolamo et al. 1999; Fehrer et al. 2006; Javazon et al. 2004; Wagner et al. 2008, 2009). Cell density of cultures seems to be crucial, too. Once grown to confluence, MSCs have been shown to lose some of their differentiation potential (Colter et al. 2001; Sotiropoulou et al. 2006). Furthermore, MSCs are often cryopreserved with DMSO in liquid nitrogen. There is evidence that cryopreserved and non-cryopreserved MSCs possess the same differentiation potential, but an effect on their biological properties cannot be excluded (Kotobuki et al. 2005; Wang and Scott 1993).

11.3 Characteristics and Properties of MSCs

11.3.1 Cellular Markers

MSCs are often isolated from the marrow as plastic-adherent cell fraction without specific enrichment. Some groups however described markers for the isolation of MSCs from primary human and murine tissues, such as STRO-1 (Simmons and Torok-Storb 1991), CD271 (low-affinity nerve growth factor receptor) (Quirici et al. 2002), CD73 (SH3, SH4) and CD105 (endoglin, SH2) (Sabatini et al. 2005), whereas CD45, Ter119 and glycophorin A (CD 235) are used for negative selections of MSCs (Jiang et al. 2002a). Buhring et al. described another panel of surface markers, including platelet-derived growth factor receptor-D (CD140b), HER-2/erbB2 (CD340) and frizzled-9 (CD349), within the CD271-bright population (Buhring et al. 2007). All these markers might lead to an enrichment of MSCs, but the resulting cell populations are still heterogeneous and the majority of isolated cells will not give rise to fibroblast colony-forming units (CFU-F). So far, there is no commonly accepted set of markers that distinctively describes MSCs.

To address this problem the International Society for Cellular Therapy (ISCT) has proposed three minimal criteria to define MSCs (Dominici et al. 2006):

1. MSCs must be plastic-adherent if maintained in standard culture conditions;
2. MSCs must express CD73, CD90 and CD105, and lack expression of hematopoietic markers such as CD14 or CD11b, CD19 or CD79a, CD34, CD45, HLA-DR;
3. MSCs must be capable of differentiation into osteoblasts, adipocytes and chondroblasts under in vitro differentiating conditions.

Neither morphologic characteristics nor specific surface markers can reliably discern the multipotent subset in MSC preparations. Using a panel of 22 surface markers including the above mentioned, there was no significant phenotypic difference between MSC and human fibroblast cell lines (HS68 and NHDF) detectable (Wagner et al. 2005a). Osteogenic, adipogenic and chondrogenic differentiation was exclusively observed in MSC preparations, but not in differentiated fibroblasts (Wagner et al. 2006). Thus, MSC-populations cannot be identified by these surface markers. Taken together, there remains an urgent need for the standardization of isolation- and culture-protocols in order to gain comparable results among different laboratories. Hence, the above mentioned minimal criteria of the ISCT are necessary and helpful, but not sufficient.

11.3.2 Gene Expression Profiling and Proteomics

Gene expression analysis has provided another dimension for the molecular characterization of cell preparations. We have compared gene expression profiles of MSCs derived from bone marrow, adipose tissue and cord blood (Wagner et al. 2006, 2007a). Initial analysis demonstrated a consistent up-regulation of at least 25 well-characterized genes in all MSC preparations, irrespective of origin and culture conditions. These genes included fibronectin 1 (FN1) and other extracellular matrix proteins (GPC4, LTBP1, ECM2, CSPG2) as well as transcription factors (nuclear factor I/B [NFIB]), homeo box genes (HOXA5 and HOXB6) and inhibitor of differentiation/DNA binding (ID1). However, none of these genes alone was specific for MSCs and we have not been able to define a unique marker or marker constellation for MSCs. Furthermore, we analyzed the proteome of MSCs. One hundred thirty-six protein-spots were unambiguously identified by MALDI-TOF-MS. Most of the identified proteins up-regulated in MSCs play a role in cytoskeleton, protein folding and metabolism. Candidate genes should be highly expressed and localized on the cell surface. In contrast, transcription factors and regulators of signal transduction are often scarcely expressed and the use of extracellular proteins is unfavourable for quality control purposes.

These results indicate that a single genomic or proteomic marker is not sufficient to define multipotent cell populations. It seems more likely, that it takes a combination of markers to reliably define MSCs.

11.3.3 A Special Type of Cell-Junctions Between MSCs

It has been demonstrated that bone marrow derived MSCs under in vitro conditions are interconnected by special villiform-to-vermiform cytoplasmatic protrusions and invaginations, termed *processus adhaerentes*, which tight-fittingly insert into deep plasma membrane invaginations, often forming batteries of interdigitating cell-cell connections with long cuff-like junctions (Wuchter et al. 2007). Cell junctions connect MSCs in the intercellular space with small puncta adhaerentia. Tentacle-like cell processes could be observed that made junctional contacts with up to 8 other MSCs, and over distances exceeding 400 μm. Alternatively, they can also form deep plasma membrane invaginations in neighbouring cells (*recessus adhaerentes*) (Fig. 11.2). This type of cell junctions is characterized by a molecular complement comprising N-cadherin and cadherin-11, in combination with the cytoplasmic plaque proteins α- and β-catenin, together with p120ctn and plakoglobin. The long *processus adhaerentes* interconnect several distant MSCs to formations of a closer packed cell assembly. The frequency and morphology of these junctional complexes are greatly affected by culture conditions (unpublished observation). A similar type of homotypic cell-cell interaction has previously been described by Werner W. Franke and co-workers in studies of primary mesenchymal cells of the mouse

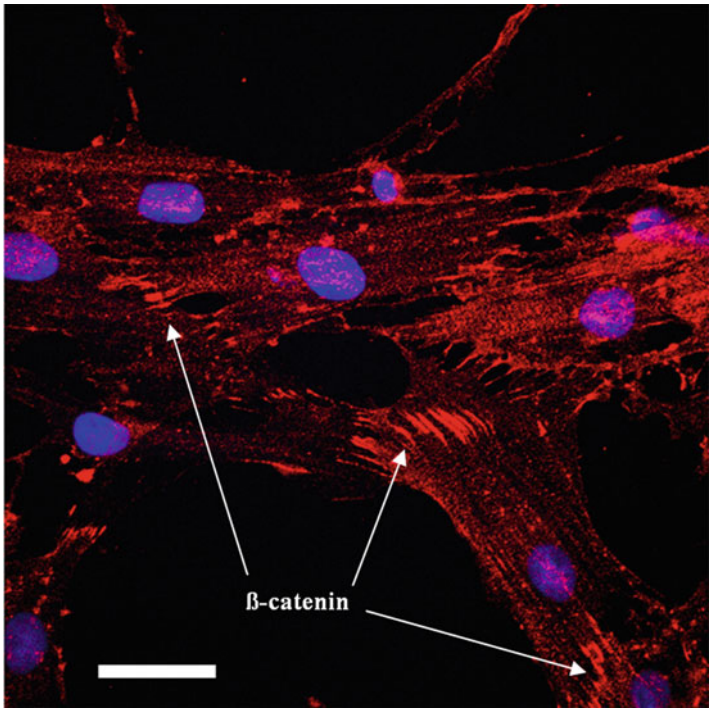

Fig. 11.2 MSC are interconnected by junctional complexes. Immunofluorescent staining of ß-catenin (*red*), nuclei are stained in *blue* (scale bar 100 µm)

embryo (Franke et al. 1983). These findings indicate that this special type of cell junctions is more wide spread in embryonal and other tissues and might be relevant for the primitive function of MSCs and heterotypic interaction with other cell types.

11.3.4 Co-culture of MSCs and Hematopoietic Stem Cells

The interaction between human hematopoietic stem cells (HSC) and their niche plays a key role in regulating maintenance of "stemness" and differentiation. MSC feeder-layer can serve as surrogate model for the hematopoietic stem cell (HSC) niche in vitro (Wagner et al. 2005b, 2007a, c; Walenda et al. 2010). Intercellular connections between HSC and MSC are mainly realized by podia formation of the HSC linking to the adjacent MSC. These podia vary greatly in length and shape (uropodia, filopodia). Along these podia and especially at the contact zone to the MSC, the cytoplasmic plaque proteins alpha- and beta-catenin and protein $p120^{ctn}$ could be identified, as well as the transmembrane glycoprotein N-cadherin (Wuchter et al. 2008). Cell division kinetic of HSC was increased when cocultured with MSC and the rate of CD34+ cells remained higher compared to monoculturing of HSC in

the same culture-medium (Wagner et al. 2007b; Ludwig et al. 2014). These results underline that direct cellular contact is essential for homing and adhesion of HSC to the cellular niche and subsequently for the regulation of self-renewal versus differentiation in HSC.

11.4 Differentiation Capacity

"Pluripotent" stem cells give rise to diverse cell types of all three germ layers. In contrast, "multipotent" stem cells can only produce related cell types of the same germinal layer.

A subtype of bone marrow derived cells, called "multipotent adult progenitor cells" (MAPC) has been suggested by the group of Verfaillie to be able to generate cells with characteristics of visceral mesoderm, neuro-ectoderm and endoderm (Jiang et al. 2002b, 2003; Zeng et al. 2006). However, the validity and reproducability of these data has been discussed controversely (Check 2007).

Kogler et al. described another subset of MSCs derived from human cord blood that they called "unrestricted somatic stem cells" (USSC) (Kogler et al. 2004). These cells were able to differentiate into many cell types, even hepatocytes and cardiomyocytes. These experiments suggest that culture conditions and specific modifications of the isolation protocols have a tremendous impact on the developmental potential of the populations generated, albeit the starting cell populations could be phenotypically identical. Ratajczak and coworkers recently identified a population of CXCR4(+) "very small embryonic like stem cells" (VSEL) in murine bone marrow and human cord blood (Halasa et al. 2008; Kucia et al. 2006a). They hypothesized that these cells are deposited during development in BM as a mobile pool of circulating pluripotent stem cells that play a pivotal role in postnatal tissue turnover, both of non-hematopoietic and hematopoietic tissues (Kucia et al. 2008a). These cells could be mobilized from BM and circulate in peripheral blood during tissue/organ injury in an attempt to regenerate damaged organs (Kucia et al. 2008b). However, if these cells are mobilized at the wrong time and migrate to the wrong place they might contribute to the development of several pathologies, including tumor formation (Kucia et al. 2006b).

On the other hand, the validity of all the initial experiments on transdifferentiation potentials of other adult stem cells, for example hematopoietic stem cells (HSC), has been severely challenged in the last few years. Some of the experiments could not be reproduced by other groups (Check 2007; Morshead et al. 2002; Raedt et al. 2007; Ying et al. 2002). In other cases, the assumed process of transdifferentiation under closer examination finally turned out to be a product of spontaneous cell fusion (Terada et al. 2002).

Taken together, it is commonly accepted, that MSCs are multipotent with differentiation potential towards bone, cartilage and adipose lineage. Nevertheless, this does not rule out the possibility that scarce mesenchymal progenitor cell populations in bone marrow may exist, that truly possess further differentiation potential.

11.5 Replicative Senescence and Aging of MSCs

Culture expansion of MSCs is limited as well as for any other normal, somatic cell. After a certain number of cell divisions they enter a senescent state and ultimately stop proliferation. These cells are not dead and can be maintained in this non-proliferative state for months. This phenomenon was first described in the 1960s by Leonard Hayflick (1965). Cellular senescence is accompanied by morphologic changes: cell enlargement and a flat "fried egg morphology". Notably, the in vitro differentiation potential also decays after long-term culture expansion. Furthermore, replicative senescence of MSCs is accompanied by various gene expression changes that are even consistent under different culture conditions (Schallmoser et al. 2010; Wagner et al. 2008)

Various molecular pathways have been implicated in senescence such as DNA damage, accumulation of the cyclin-dependent kinase inhibitor p16INK4a and oxidative stress (Ho et al. 2005; Janzen et al. 2006; Kiyono et al. 1998). Progressive shortening of the telomeres or modified telomeric structures have been proposed to be the main trigger for replicative senescence – with every cell division the number of telomere repeats decreases and this has been suggested as a kind of internal clock (Bonab et al. 2006; Fehrer and Lepperdinger 2005; Lansdorp 2008). It is however still controversially discussed if telomere shortening is the only initiating mechanism for replicative senescence or if it rather resembles an effect of senescence (Di Donna et al. 2003; Kiyono et al. 1998; Masutomi et al. 2003; Zimmermann et al. 2004). Alternatively, it has been proposed that molecular switches such as epigenetic modifications might play a central role for regulation of cellular aging (Bork et al. 2010; Chambers et al. 2007; Nilsson et al. 2005; Shibata et al. 2007; Suzuki et al. 2008; Wilson and Jones 1983; Young and Smith 2001).

Since the first discovery of the Hayflick limit it has been speculated if replicative senescence is involved in aging of the whole organism. Indeed, several authors have shown an inverse relationship between donor age and the replicative life span *in vitro* for fibroblasts as well as for MSCs (Mareschi et al. 2006; Schneider and Mitsui 1976; Stenderup et al. 2003). These studies are hampered by large inter-individual differences and MSC content and therefore necessitate high numbers of donor samples (Bonab et al. 2006; Cristofalo et al. 1998; Wagner et al. 2009). Overall, MSCs from elderly people seem to have a slower proliferation rate already at the initial cell passage and that they contain larger and flatter cells in comparison to cells from younger donors (Roobrouck et al. 2008). Zhou and co-workers demonstrated that the number of cells that staining positive for senescence-associated beta-galactosidase is significantly higher in samples from elderly donors in comparison to younger donors (Zhou et al. 2008). Another observation, which supports age-related effects on replicative senescence is that the frequency of cells with colony forming potential declines at higher ages (Baxter et al. 2004; Stolzing et al. 2008). The two processes are also related on a molecular basis: genes which are up-regulated in long-term culture are also up-regulated in elderly people (Wagner et al. 2008, 2009).

We have recently analyzed DNA methylation profiles of MSC using the HumanMethylation27 BeadChip (Bork et al. 2010). This platform represents 27,578 CpG sites that are associated with promoter regions of more than 13,500 annotated genes. Our results revealed that overall the methylation remained rather constant throughout long-term culture for 2–3 month. However, specific CpG islands were either hyper-methylated or hypo-methylated and the same changes were also verified in independent donor samples. Differentially methylated regions correlated with various developmental genes and there was an association with differential methylation between samples from young and elderly donors. These results support the notion, that replicative senescence and aging represent developmental processes that are regulated by similar epigenetic modifications.

Despite such molecular insights it is still only scarcely understood how long-term culture affects the composition of MSC preparations and five processes seem to be involved (Wagner et al. 2010a, b): (1) MSCs are composed of sub-populations with different proliferation rates and therefore the heterogeneity notoriously changes in the course of *in vitro* culture; (2) cells in culture acquire mutations and other stochastic cellular defects; (3) self-renewal of MSCs may be impaired in the artificial environment of a culture dish leading to gradual differentiation; (4) the number of cell divisions might be restricted – for example by telomere loss under culture conditions or (5) replicative senescence might be associated with the aging process of the organism as mentioned above.

Due to the functional implications of long-term culture there is a growing perception that this process has to be taken into account – especially for clinical applications. On the other hand the state of replicative senescence is poorly defined by the number of population doublings or even by the number of passages. Reliable markers for cellular aging are urgently needed.

11.6 Potential Applications for Therapies

Theoretically, MSCs can be isolated from a small aspirate of BM or tissue samples and expanded in vitro. Preliminary studies suggested that MSCs preferentially home to damaged tissue and therefore might have therapeutic potential (Le Blanc 2006). The website www.ClinicalTrials.gov of the National Institutes of Health (Bethesda, MD, USA) is currently listing more than 1000 active clinical trials in which MSCs are involved. Clinical applications include treatment of such different entities as steroid refractory graft versus host disease (GvHD), osteonecrosis, articular cartilage defects, severe chronic myocardial ischemia, decompensated liver cirrhosis, multiple sclerosis, Type I and II diabetes mellitus, Lupus nephritis, Crohn's disease and more. So far the studies revealed no serious side effects upon transplantation of MSCs. However, it seems very unlikely that the beneficial effects that have been observed in some studies are really due to transdifferentiation of MSCs into the damaged tissue cells. For example, clinical trials using MSCs for myocardial infarction have proceeded rapidly, but there is little or no evidence for the

differentiation of MSCs to coupled cardiomyocytes (Caplan and Dennis 2006; Grinnemo et al. 2006; Stamm et al. 2006). The beneficial effects shown in some of these studies might be attributable to paracrine anti-inflammatory signalling or stimulation of endogenous repair processes by the injected cells (Mazhari and Hare 2007). The precise underlying mechanisms are yet unknown. This lack of knowledge might not prevent application of MSCs in clinical settings if there are benefits for the patient and if there are no or minimal side effects.

At present, the most promising clinical studies make use of the immunomodulatory effects of MSCs. In vitro data suggest that MSCs have low inherent immunogenicity as they induce little, if any, proliferation of allogeneic lymphocytes. Instead, MSCs appear to be immunosuppressive in vitro (Le Blanc and Ringdén 2007; Malcherek et al. 2014). A multicenter, phase II experimental study enrolled 55 patients with steroid-resistant, severe, acute GvHD. Patients were treated with mesenchymal stem cells, derived with the European Group for Blood and Marrow Transplantation ex-vivo expansion procedure. 30 patients had a complete response and nine showed improvement. Complete responders had lower transplantation-related mortality 1 year after infusion than did patients with partial or no response (37 % vs 72 %; $p=0.002$) and higher overall survival 2 years after haematopoietic stem-cell transplantation (53 % vs 16 %; $p=0.018$) (Le Blanc et al. 2008). In a pilot study LeBlanc et al. could furthermore demonstrate, that transplantation of mesenchymal stem cells enhanced the engraftment of hematopoietic stem cells: seven patients underwent treatment with mesenchymal stem cells together with allogeneic hematopoietic stem cell transplantation, resulting in fast engraftment of absolute neutrophil count and platelets and 100 % donor chimerism, even in three patients regrafted for graft failure/rejection (Le Blanc et al. 2007).

11.7 Conclusions and Future Development in Research

The lack of common standards and universal guidelines for the preparation of MSCs has greatly hampered further progress. The quality of preparations from different laboratories varies significantly and the cell products are notoriously heterogeneous regarding the source and freshness of starting material, isolation protocols, culture-conditions, number of passages upon culture, etc. There is an urgent need for the development of molecular markers and universal criteria for quality control of the starting cell populations as well as for the cell products after expansion. For clinical use, a clear definition of the transplanted cell populations in conjunction with serum-free culture media and close quality controls according to GMP-standards throughout the whole production-process is essential (Wuchter et al. 2015).

Even though there is so far no convincing evidence that MSCs themselves are involved in the process of tissue repair by transdifferentiation, they probably contribute to this process by providing a supportive microenvironment for other cell types that are directly involved, including other types of adult stem cells. At present, three major fields can be identified, in which MSCs can and will be used for

therapeutic purposes: (I) skin and tissue repair (Le Blanc 2006; Mazhari and Hare 2007; Müller et al. 2008b); (II) therapy of chronic graft vs. host disease (GvHD) (Aksu et al. 2008; Le Blanc et al. 2008; Tian et al. 2008), and (III) enhancement of hematopoietic stem cell engraftment in an allogeneic transplant setting (Le Blanc et al. 2007; Müller et al. 2008a). The underlying key mechanism in all the three fields is not completely understood, but probably a result of MSC-induced immunosuppression. We therefore need aggressive attempts to better understand the immunomodulatory mechanisms of MSC.

Acknowledgements This work was supported by the German Research Foundation DFG (SFB 873), the Network for Aging Research (NAR, Heidelberg), the Heidelberg Academy of Sciences (WIN-Kolleg) and the Stem Cell Network North Rhine Westphalia. We would like to thank Dr. Rainer Saffrich for excellent image acquisition.

Disclosures There are no potential conflicts of interest to disclose, except the following:

Patrick Wuchter: Consultancy, honoraria and membership on Advisory Boards of Sanofi-Aventis.

Wolfgang Wagner is involved in the company Cygenia that provides service for epigenetic aging and senescence signatures (www.cygenia.com).

Anthony D. Ho: Consultancy, honoraria and membership on Advisory Boards of Genzyme/Sanofi-Aventis.

References

Aksu AE, Horibe E, Sacks J, Ikeguchi R, Breitinger J, Scozio M, Unadkat J, Feili-Hariri M (2008) Co-infusion of donor bone marrow with host mesenchymal stem cells treats GvHD and promotes vascularized skin allograft survival in rats. Clin Immunol 127(3):348–358

Anderson DG, Levenberg S, Langer R (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 22:863–866

Baptista LS, do Amaral RJ, Carias RB, Aniceto M, Claudio-da-Silva C, Borojevic R (2009) An alternative method for the isolation of mesenchymal stromal cells derived from lipoaspirate samples. Cytotherapy 11(6):706–715

Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ, Bellantuono I (2004) Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 22:675–682

Bieback K, Kern S, Kluter H et al (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625–634

Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, Klüter H (2009) Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells 27(9):2331–2341

Bieback K, Wuchter P, Besser D, Franke W, Becker M, Ott M, Pacher M, Ma N, Stamm C, Klüter H, Müller A, Ho AD, START-MSC consortium (2012) Mesenchymal stromal cells (MSCs): science and f(r)iction. J Mol Med (Berl) 90(7):773–782

Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B (2006) Aging of mesenchymal stem cell in vitro. BMC Cell Biol 7:14

Bork S, Pfister S, Witt H, Horn P, Korn B, Ho AD, Wagner W (2010) DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. Aging Cell 9(1):54–63

Buhring HJ, Battula VL, Treml S et al (2007) Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262–271

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

Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem 98:1076–1084

Chambers SM, Shaw CA, Gatza C, Fisk CJ, Donehower LA, Goodell MA (2007) Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS Biol 5:201

Check E (2007) The hard copy. News feature. Nature 446:485–486

Colter DC, Sekiya I, Prockop DJ (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci U S A 98:7841–7845

Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC (1998) Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation. Proc Natl Acad Sci 95:10614–10619

da Silva ML, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213

de Girolamo L, Sartori MF, Albisetti W, Brini AT (2007) Osteogenic differentiation of human adipose-derived stem cells: comparison of two different inductive media. J Tissue Eng Regen Med 1(2):154–157

Di Donna S, Mamchaoui K, Cooper RN, Seigneurin-Venin S, Tremblay J, Butler-Browne GS, Mouly V (2003) Telomerase can extend the proliferative capacity of human myoblasts, but does not lead to their immortalization. Mol Cancer Res 1:643–653

DiGirolamo CM, Stokes D, Colter D et al (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107:275–281

Dominici M, Le Blanc K, Mueller I et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317

Engler AJ, Sen S, Sweeney HL et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689

Erices A, Conget P, Minguell JJ (2000) Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 109:235–242

Fehrer C, Lepperdinger G (2005) Mesenchymal stem cell aging. Exp Gerontol 40:926–930

Fehrer C, Laschober G, Lepperdinger G (2006) Aging of murine mesenchymal stem cells. Ann N Y Acad Sci 1067:235–242

Franke WW, Grund C, Jackson BW et al (1983) Formation of cytoskeletal elements during mouse embryogenesis. IV. Ultrastructure of primary mesenchymal cells and their cell-cell interactions. Differentiation 25:121–141

Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16:381–390

Friedenstein AJ, Petrakova KV, Kurolesova AI et al (1968) Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 6:230–247

Friedenstein AJ, Chailakhyan RK, Latsinik NV et al (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17:331–340

Grinnemo KH, Månsson-Broberg A, Leblanc K, Corbascio M, Wärdell E, Siddiqui AJ, Hao X, Sylvén C, Dellgren G (2006) Human mesenchymal stem cells do not differentiate into cardiomyocytes in a cardiac ischemic xenomodel. Ann Med 38:144–153

Halasa M, Baskiewicz-Masiuk M, Dabkowska E et al (2008) An efficient two-step method to purify very small embryonic-like (VSEL) stem cells from umbilical cord blood (UCB). Folia Histochem Cytobiol 46(2):239–243

Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 37:614–636

Heid HW, Moll R, Schwetlick I, Rackwitz HR, Keenan TW (1998) Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. Cell Tissue Res 294(2):309–321

Ho AD, Wagner W, Mahlknecht U (2005) Stem cells and ageing. The potential of stem cells to overcome age-related deteriorations of the body in regenerative medicine. EMBO Rep 6:35–38

Ho AD, Wagner W, Franke WW (2008) Heterogeneity of mesenchymal stromal cell preparations. Cytotherapy 10:320–330

Horn P, Bork S, Diehlmann A, Walenda T, Eckstein V, Ho AD (2008) Wagner W (2008) Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy 10(7):676–685

Horwitz EM, Keating A (2000) Nonhematopoietic mesenchymal stem cells: what are they? Cytotherapy 2:387–388

Horwitz EM, Le BK, Dominici M et al (2005) Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 7:393–395

Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT (2006) Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443:421–426

Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32:414–425

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002a) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49. Corrigendum (2007) Nature 447:880–881

Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM (2002b) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 30:896–904

Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM (2003) Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci U S A 100(Suppl 1):11854–11860

Kern S, Eichler H, Stoeve J et al (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294–1301

Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396:84–88

Kocaoemer A, Kern S, Kluter H et al (2007) Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells 25:1270–1278

Kogler G, Sensken S, Airey JA et al (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123–135

Kotobuki N, Hirose M, Machida H et al (2005) Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. Tissue Eng 11:663–673

Kucia M, Reca R, Campbell FR et al (2006a) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20(5):857–869

Kucia M, Zuba-Surma E, Wysoczynski M et al (2006b) Physiological and pathological consequences of identification of very small embryonic like (VSEL) stem cells in adult bone marrow. J Physiol Pharmacol 57(Suppl 5):5–18

Kucia M, Wysoczynski M, Ratajczak J et al (2008a) Identification of very small embryonic like (VSEL) stem cells in bone marrow. Cell Tissue Res 331(1):125–134. Epub 2007 Sep 9. Review

Kucia M, Wysoczynski M, Wu W et al (2008b) Evidence that very small embryonic-like stem cells are mobilized into peripheral blood. Stem Cells 26(8):2083–2092. Epub 2008 May 29

Kuznetsov SA, Mankani MH, Gronthos S et al (2001) Circulating skeletal stem cells. J Cell Biol 153:1133–1140

Lange C, Cakiroglu F, Spiess AN et al (2007) Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. J Cell Physiol 213(1):18–26

Lansdorp PM (2008) Telomeres, stem cells, and hematology. Blood 111:1759–1766

Le Blanc K (2006) Mesenchymal stromal cells: tissue repair and immune modulation. Cytotherapy 8(6):559–561

Le Blanc K, Ringdén O (2007) Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med 262(5):509–525

Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J, Ljungman P, Lönnies H, Nava S, Ringdén O (2007) Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia 21(8):1733–1738

Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371(9624):1579–1586

Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675

Ludwig A, Saffrich R, Eckstein V, Bruckner T, Wagner W, Ho AD, Wuchter P (2014) Functional potentials of human hematopoietic progenitor cells are maintained by mesenchymal stromal cells and not impaired by Plerixafor. Cytotherapy 16(1):111–121

Malcherek G, Jin N, Hückelhoven AG, Mani J, Wang L, Gern U, Diehlmann A, Wuchter P, Schmitt A, Chen B, Ho AD, Schmitt M (2014) Mesenchymal stromal cells inhibit proliferation of virus-specific CD8+ T cells. Leukemia 28(12):2388–2394

Mareschi K, Ferrero I, Rustichelli D, Aschero S, Gammaitoni L, Aglietta M, Madon E, Fagioli F (2006) Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. J Cell Biochem 97:744–754

Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC (2003) Telomerase maintains telomere structure in normal human cells. Cell 114:241–253

Mazhari R, Hare JM (2007) Mechanisms of action of mesenchymal stem cells in cardiac repair: potential influences on the cardiac stem cell niche. Nat Clin Pract Cardiovasc Med 4(Suppl 1):S21–S26

Morshead CM, Benveniste P, Iscove NN et al (2002) Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. Nat Med 8:268–273

Müller I, Kordowich S, Holzwarth C, Spano C, Isensee G, Staiber A, Viebahn S, Gieseke F, Langer H, Gawaz MP, Horwitz EM, Conte P, Handgretinger R, Dominici M (2006) Animal serum-free culture conditions for isolation and expansion of multipotent mesenchymal stromal cells from human BM. Cytotherapy 8:437–444

Müller I, Kordowich S, Holzwarth C, Isensee G, Lang P, Neunhoeffer F, Dominici M, Greil J, Handgretinger R (2008a) Application of multipotent mesenchymal stromal cells in pediatric patients following allogeneic stem cell transplantation. Blood Cells Mol Dis 40(1):25–32

Müller I, Vaegler M, Holzwarth C, Tzaribatchev N, Pfister SM, Schütt B, Reize P, Greil J, Handgretinger R, Rudert M (2008b) Secretion of angiogenic proteins by human multipotent mesenchymal stromal cells and their clinical potential in the treatment of avascular osteonecrosis. Leukemia 22(11):2054–2061

Nakahara H, Bruder SP, Haynesworth SE et al (1990) Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. Bone 11:181–188

Nilsson O, Mitchum RD Jr, Schrier L, Ferns SP, Barnes KM, Troendle JF, Baron J (2005) Growth plate senescence is associated with loss of DNA methylation. J Endocrinol 186:241–249

Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Quirici N, Soligo D, Bossolasco P et al (2002) Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. Exp Hematol 30:783–791

Raedt R, Pinxteren J, Van Dycke A et al (2007) Differentiation assays of bone marrow-derived multipotent adult progenitor cell (MAPC)-like cells towards neural cells cannot depend on morphology and a limited set of neural markers. Exp Neurol 203:542–554

Ren H, Cao Y, Zhao Q et al (2006) Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. Biochem Biophys Res Commun 347:12–21

Roobrouck VD, Ulloa-Montoya F, Verfaillie CM (2008) Self-renewal and differentiation capacity of young and aged stem cells. Exp Cell Res 314:1937–1944

Sabatini F, Petecchia L, Tavian M, Jodon de Villeroché V, Rossi GA, Brouty-Boyé D (2005) Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. Lab Invest 85:962–971

Schallmoser K, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, Drexler C, Lanzer G, Linkesch W, Strunk D (2007) Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion 47(8):1436–1446

Schallmoser K, Bartmann C, Rodhe E, Bork S, Guelly C, Obenauf AC, Reinisch A, Horn P, Ho AD, Strunk D, Wagner W (2010) Replicative senescence-associated gene expression changes in mesenchymal stromal cells are similar under different culture conditions. Haematologica 95(6):867–874

Schneider EL, Mitsui Y (1976) The relationship between in vitro cellular aging and in vivo human age. Proc Natl Acad Sci 73:3584–3588

Secco M, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M (2008a) Mesenchymal stem cells from umbilical cord: do not discard the cord! Neuromuscul Disord 18(1):17–18

Secco M, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M (2008b) Multipotent stem cells from umbilical cord: cord is richer than blood! Stem Cells 26(1):146–150

Shibata KR, Aoyama T, Shima Y, Fukiage K, Otsuka S, Furu M, Kohno Y, Ito K, Fujibayashi S, Neo M, Nakayama T, Nakamura T, Toguchida J (2007) Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. Stem Cells 25:2371–2382

Simmons PJ, Torok-Storb B (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 78:55–62

Sotiropoulou PA, Perez SA, Salagianni M et al (2006) Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 24(2):462–471

Stamm C, Liebold A, Steinhoff G et al (2006) Stem cell therapy for ischemic heart disease: beginning or end of the road? Cell Transplant 15(Suppl 1):S47–S56

Stenderup K, Justesen J, Clausen C, Kassem M (2003) Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone 33:919–926

Stolzing A, Jones E, McGonagle D, Scutt A (2008) Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. Mech Ageing Dev 129:163–173

Stute N, Holtz K, Bubenheim M et al (2004) Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol 32:1212–1225

Suzuki T, Farrar JE, Yegnasubramanian S, Zahed M, Suzuki N, Arceci RJ (2008) Stable knockdown of PASG enhances DNA demethylation but does not accelerate cellular senescence in TIG-7 human fibroblasts. Epigenetics 3:281–291

Terada N, Hamazaki T, Oka M et al (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545

Tian Y, Deng YB, Huang YJ, Wang Y (2008) Bone marrow-derived mesenchymal stem cells decrease acute graft-versus-host disease after allogeneic hematopoietic stem cells transplantation. Immunol Invest 37(1):29–42

Wagner W, Ho AD (2007) Mesenchymal stem cell preparations – comparing apples and oranges. Stem Cell Rev 3:239–248

Wagner W, Wein F, Seckinger A et al (2005a) Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 33:1402–1416

Wagner W, Saffrich R, Wirkner U et al (2005b) Hematopoietic progenitor cells and cellular microenvironment: behavioral and molecular changes upon interaction. Stem Cells 23:1180–1191

Wagner W, Feldmann RE Jr, Seckinger A, Maurer MH, Wein F, Blake J, Krause U, Kalenka A, Burgers HF, Saffrich R, Wuchter P, Kuschinsky W, Ho AD (2006) The heterogeneity of human mesenchymal stem cell preparations – evidence from simultaneous analysis of proteomes and transcriptomes. Exp Hematol 34:536–548

Wagner W, Roderburg C, Wein F et al (2007a) Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. Stem Cells 10:2638–2647

Wagner W, Wein F, Roderburg C et al (2007b) Adhesion of hematopoietic progenitor cells to human mesenchymal stem cells as a model for cell-cell interaction. Exp Hematol 35:314–325

Wagner W, Wein F, Roderburg C et al (2007c) Adhesion of human hematopoietic progenitor cells to mesenchymal stromal cells involves CD44. Cells Tissues Organs 188(1-2):160–169

Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD (2008) Replicative senescence of mesenchymal stem cells – a continuous and organized process. PLoS One 5:e2213

Wagner W, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, Benes V, Blake J, Huber FX, Eckstein V, Boukamp P, Ho AD (2009) Aging and replicative senescence have related effects on human stem and progenitor cells. PLoS One 4:e5846

Wagner W, Bork S, Lepperdinger G, Joussen S, Ma N, Strunk D, Koch C (2010a) How to track cellular aging of mesenchymal stromal cells. Aging (Albany NY) 2:224–230

Wagner W, Ho AD, Zenke M (2010b) Different facets of aging in human mesenchymal stem cells. Tissue Eng Part B Rev 16:445–453

Walenda T, Bork S, Horn P, Wein F, Saffrich R, Diehlmann A, Eckstein V, Ho AD, Wagner W (2010) Co-culture with mesenchymal stromal cells increases proliferation and maintenance of hematopoietic progenitor cells. J Cell Mol Med 14(1-2):337–350

Wang H, Scott RE (1993) Inhibition of distinct steps in the adipocyte differentiation pathway in 3T3 T mesenchymal stem cells by dimethyl sulphoxide (DMSO). Cell Prolif 26:55–66

Wilson VL, Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220:1055–1057

Wuchter P, Boda-Heggemann J, Straub BK, Grund C, Kuhn C, Krause U, Seckinger A, Peitsch WK, Spring H, Ho AD, Franke WW (2007) Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. Cell Tissue Res 328:499–514

Wuchter P, Saffrich R, Wagner W, Wein F, Schubert M, Eckstein V, Ho AD (2008) Human hematopoietic stem cells and leukemic cells form cadherin-catenin based junctional complexes with mesenchymal stromal cells. Blood (ASH Ann Meet Abstr) 112:1367

Wuchter P, Bieback K, Schrezenmeier H, Bornhäuser M, Mueller L, Boenig H, Wagner W, Meisel R, Pavel P, Tonn T, Lang P, Mueller I, Renner M, Malcherek G, Saffrich R, Buss E, Horn P, Rojewski M, Schmitt A, Ho AD, Sanzenbacher R, Schmitt M (2015) Standardization of GMP-compliant production of bone marrow derived human mesenchymal stromal cells (MSCs) for immunotherapeutic applications. Cytotherapy 17(2):128–139

Ying QL, Nichols J, Evans EP et al (2002) Changing potency by spontaneous fusion. Nature 416:545–548

Young JI, Smith JR (2001) DNA methyltransferase inhibition in normal human fibroblasts induces a p21-dependent cell cycle withdrawal. J Biol Chem 276:19610–19616

Zeddou M, Briquet A, Relic B, Josse C, Malaise MG, Gothot A, Lechanteur C, Beguin Y (2010) The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. Cell Biol Int 34(7):693–701

Zeng L, Rahrmann E, Hu Q et al (2006) Multipotent adult progenitor cells from swine bone marrow. Stem Cells 24:2355–2366

Zhou S, Greenberger JS, Epperly MW, Goff JP, Adler C, Leboff MS, Glowacki J (2008) Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts. Aging Cell 7:335–343

Zimmermann S, Glaser S, Ketteler R, Waller CF, Klingmuller U, Martens UM (2004) Effects of telomerase modulation in human hematopoietic progenitor cells. Stem Cells 22:741–749

Zuk PA, Zhu M, Mizuno H et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228

Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN (2000) Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2(6):477–488

Chapter 12
Musculoskeletal Stem Cells

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Abstract Stem cells can be isolated from various musculoskeletal tissues, including bone marrow, muscle and intervertebral disc. Depending on the cell source, some of these stem cells are clearly assigned as mesenchymal stem cells (MSCs), according to the minimal criteria for MSCs defined by the International Society for Cellular Therapy in 2006, whereas other musculoskeletal stem cells are described in the literature as mesenchymal progenitor cells or MSC-like cells. Criteria to identify MSCs include their adhesion to plastic, their ability to differentiate, and their expression of a set of cell surface markers. MSCs are generally thought to reside in special microenvironments, and to home to the site of inflammation, differentiate into tissue specific cell types, and to secrete trophic factors and exert immunosuppressive effects in response to injury. These characteristics, along with their easy in vitro expansion, make musculoskeletal-derived stem cells an attractive source for different clinical indications. Indeed, these cells have been successfully applied for the treatment of bone and cartilage disorders, diabetes, and improvement of cardiac function. One of the challenges of MSC production is that stem cell populations are a heterogeneous group of cells. Furthermore, lack of alignment between isolation and expansion protocols, morphological changes during monolayer expansion, lack of knowledge of stem cell origin and characteristics and function in their naive environment are additional challenges that need to be faced. This chapter reviews the literature relevant to the different musculoskeletal stem cells, gives information about their derivation and classification, characteristics and properties, and describes isolation, proliferation and differentiation protocols. Finally, the investigation of

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_12

these cells in different clinical trials, the importance of Good Manufacturing Practices (GMP) and future challenges are presented.

Keywords Musculoskeletal stem cell sources • Mesenchymal stem cells • Characterization • Expansion • Differentiation

12.1 Introduction

The musculoskeletal system is essential for structural support, locomotion and movement. It is a multicomponent system composed of muscle, muscle connective tissue, tendon, ligament and bone, innervated by nerves and vascularized by blood vessels (Kardon 2011). It serves as storage for important substrates, is involved in the energy metabolism and a source of various cell types, including mesenchymal stem cells (MSCs).

The most extensively studied MSC source is the bone marrow. Bone marrow-derived MSCs (BM-MSCs) were first described in 1968 by Friedenstein and colleagues, who showed that bone marrow stroma contains cells that have significant proliferative capacity and are able to form bone (Friedenstein et al. 1968). The name MSC was introduced by Caplan and colleagues in 1990 and is based on the MSCs' developmental origin in the mesenchyme (Caplan 1990). More recently, stem and progenitor cells were also isolated from other musculoskeletal tissues, such as from muscle, tendons or synovium (Fig. 12.1).

MSCs are thought to reside in special microenvironments upon tissue regeneration or natural physiological turnover. After tissue injury, it is assumed that MSCs home to the site of inflammation, differentiate into tissue specific cell types, secrete

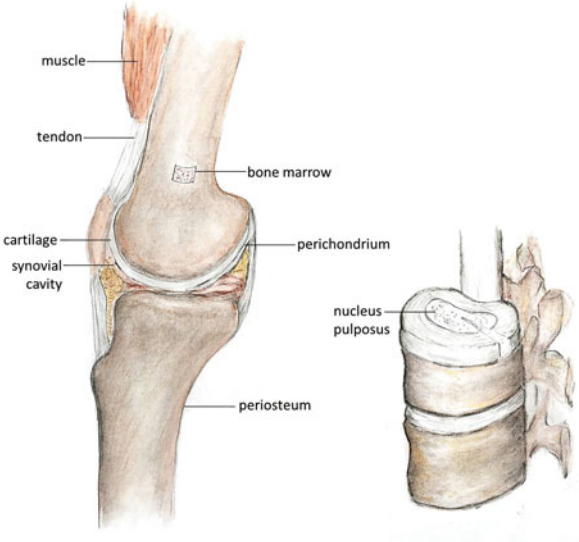

Fig. 12.1 Sources of stem and progenitor cells in the musculoskeletal tissue

trophic factors and exert immunosuppressive effects (Sharma et al. 2014). These properties, along with their accessibility and great expansion potential in vitro make them an attractive cell source for clinical applications. Whatever the tissue source, it needs to be ensured that the MSCs are produced in compliance with Good Manufacturing Practices (GMPs). One of the challenges of MSC production is that stem cell populations are a heterogeneous group of cells, for which no unique markers exist. Criteria to distinguish MSCs from non-MSCs include their adhesion to plastic, their ability to differentiate into the osteogenic, adipogenic and chondrogenic lineages, and their expression of a set of cell surface markers (Dominici et al. 2006). However, the expression profiles of several cell surface markers of in vitro expanded cells compared to freshly isolated cells and those residing in the bone marrow environment, which have been detected in different studies show strong discrepancies, as summarized in a recent review by Bara and colleagues in 2014 (Bara et al. 2014). These differences might result from different culture conditions, donor variation and immunostaining protocols. They point out the need for further alignment of protocols to achieve standardization.

This chapter provides a comprehensive review of the literature relevant to musculoskeletal stem cells. Firstly, the anatomy and physiology of the different musculoskeletal stem cell sources are described. This is followed by information about the MSC's in their natural microenvironment. Next, the characteristics and properties, along with a thorough description of isolation, proliferation and differentiation protocols of the different types of musculoskeletal stem cells are presented. Then a summary on how these properties of MSCs are used for clinical applications and the importance of GMP is highlighted. Finally, the conclusion and potential future development strategies are presented.

12.2 Derivation and Classification

12.2.1 Bone Marrow

Bone has mechanical, protective, but also metabolic functions. It protects the thoracoabdominal organs, serves as home for the bone marrow and functions as the main store and supply of inorganic ions. These include calcium and phosphorus in the form of hydroxyapatite, which in turn impact metabolic processes in the whole body (Hercz 2001). Morphologically, bone is classified into densely packed cortical and loosely organized trabecular bone. Histologically, bone is divided into randomly arranged woven bone, which is chiefly present during bone development and regeneration, and lamellar bone. In lamellar bone, mineralized bone matrix is deposited in concentric layers (lamellae) around a central vascular channel. Both bone types harbor a network of tiny channels pervaded by blood vessels and nerves, allowing for nutrition and cell-to-cell signaling (Buckwalter and Cooper 1987). The principal components of bone are organic matrix comprising type I collagen, proteoglycans and non-collagenous proteins as well as various cell types, including osteoblasts, osteoclasts, osteocytes and osteogenic precursor cells (Oryan et al.

2015). These cells play an important role in bone remodeling, healing and calcium homeostasis (Oryan et al. 2015). The bone marrow, which fills the cavity, harbors blood vessels and is a habitat for haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). As such, it functions as a primary site for blood cell production, including the differentiation of HSCs to all classes of blood cells (Balduino et al. 2005).

Until they are utilized for tissue regeneration or natural physiological turnover, MSCs are thought to reside in special microenvironments. These environments are the endosteal, stromal and perivascular niches (da Silva Meirelles et al. 2008; Mendez-Ferrer et al. 2010; Rasini et al. 2013). Within these niches, MSCs are in direct physical interaction with other cells. In the endosteal niche, MSCs, osteoblasts and HSCs are physically associated and regulate each other's cell behavior and lineage commitment (Calvi et al. 2003; Mendez-Ferrer et al. 2010; Rasini et al. 2013). For example, interaction of cells in the endosteal niche is thought to provide osteoprogenitors and stimulate osteogenesis by the secretion of growth factors and cytokines (Osugi et al. 2012; Bara et al. 2014). In the perivascular niche, the importance of MSCs has been demonstrated by Méndez-Ferrer et al. in 2010, who showed that perivascular MSCs constitute an essential HSC niche component and play a critical role in maintaining a quiescent HSC pool in the bone marrow (Mendez-Ferrer et al. 2010).

12.2.2 Muscle

Skeletal muscle contributes significantly to multiple body functions, including energy metabolism, force and power generation, as well as storage of important substrates, such as amino acids and carbohydrates. It is mainly composed of water (75 %), protein (20 %), and other substances, including inorganic salts, minerals, fat and carbohydrates (5 %). The architecture of skeletal muscle is characterized by a well-described arrangement of myofibers, which are multinucleated and postmitotic and associated connective tissue (Frontera and Ochala 2015).

Several stem cell populations reside within the skeletal muscle. The most common ones are the satellite cells and muscle-derived stem cells (MDSCs). Satellite cells were first described and named in two independent studies by Alexander Mauro and Bernard Katz in 1961, based on their anatomic location between the myofiber plasma and basement membranes (Yablonka-Reuveni and Paterson 2001). In recent years, the expression of a range of specific markers, and in particular of the paired box transcription factor Pax7 is used to identify these cells (Seale et al. 2000). Satellite cells have been mainly recognized as quiescent progenitor cells, which divide and fuse with other progenitors to form myofibers following skeletal muscle injury (Peng and Huard 2004). However, in addition to their ability to give rise to cells of the myogenic lineage, their ability to differentiate into osteoblastic, adipogenic and chondrogenic cell types has been demonstrated in vitro (Asakura et al. 2001).

MDSCs have been first described by Jonny Huard and his colleagues in 2002 (Qu-Petersen et al. 2002). They can be isolated from the myofiber periphery closely

associated with blood vessels (Peault et al. 2007). MDSCs are distinct from satellite cells because they are able to not only differentiate into the myogenic lineage, but also give rise to cells of other cell lineages in vitro and in vivo (Qu-Petersen et al. 2002). Furthermore, they exhibit long-term proliferation ability, capacity to renew themselves and immune-privileged behavior (Qu-Petersen et al. 2002). MDSCs are more heterogeneous when compared to satellite cells, but consistently express the stem cell antigen 1 (Sca-1) and often the cluster of differentiation 34 (CD34) (Qu-Petersen et al. 2002). Their normal physiologic function is unknown to date (Peault et al. 2007).

Other cells found in skeletal muscle are pericytes, which are myogenic precursors that are associated with microvascular walls in human skeletal muscle. Although they have a similar myogenic potential than satellite cells, they are phenotypically distinct from them. Pericytes are located in the periphery of capillaries and microvessels, underneath the basal lamina of muscle fibers and do not express the satellite cell-typical markers, but instead NG2 and ALP (Dellavalle et al. 2007). Their normal physiologic function is assumed to regulate blood flow and control angiogenesis (Peault et al. 2007).

12.2.3 Cartilage and Synovium

Articular cartilage is the highly specialized connective tissue of joints that lacks blood vessels, lymphatics, nerves and has a poor repair capacity following injury (Bornes et al. 2014). In the limb bud, cartilage development begins when condensed mesenchymal stem cells (MSCs), called cartilage anlage, differentiate into chondroprogenitor cells. Chondroprogenitors form mature chondrocytes through a sequential process of proliferation, maturation and hypertrophy representing the distinct zones of the growth plate (Guha et al. 2002; Kronenberg 2003). Multiple signaling factors regulate Sox9, the master transcription factor that drives chondrogenesis (Lefebvre et al. 1997; Akiyama et al. 2002). Sox9 is required for cartilage formation (Bi et al. 1999), and directs hypertrophic maturation of growth plate chondrocytes (Dy et al. 2012). Chondrocytes are the only cell type of articular cartilage, establishing a specialized microenvironment with a dense extracellular matrix (ECM) composed of water, collagens, proteoglycans, and noncollagenous proteins. They are responsible for ECM turnover during cartilage formation and function as a biomechanical loading tissue (Cheng et al. 2014; Jiang and Tuan 2015).

Cartilage/progenitor cells (CSPCs) were the first detected clonal populations in cartilage, capable of differentiating in diverse mesenchymal lineages in dedifferentiated cultured human articular chondrocytes (Barbero et al. 2003). They were also described as mesenchymal progenitors in human articular cartilage expressing CD105 (endoglin) and CD166 (activated leukocyte cell adhesion molecule) markers (Alsalameh et al. 2004). Additional studies identified CSPCs in normal human and osteoarthritic cartilage (Fickert et al. 2004; Williams et al. 2010), and in bovine and equine cartilage samples (Hayes et al. 2008; McCarthy et al. 2012).

The synovium is a thin membrane of specialized mesenchymal tissue lining the spaces of diarthrodial joints, bursae and tendon sheaths, providing a synovial fluid

(SF) filled cavity around the cartilage (Smith 2011). It is composed of two layers: the inner intima, composed of macrophages, fibroblast-like synoviocytes and other cells that secrete synovial fluid, which provides lubrication and nutrition to the articular cartilage. The outer subintima has synoviocytes lying over loose connective tissue with sparse macrophages, lymphocytes, fat cells, and blood vessels, which provide nutrients to the synovial membrane and adjacent avascular cartilage (de Sousa et al. 2014). Synovial tissue has shown regenerative capability, after synovectomy, with removal of part of the synovial tissue in rabbits or horses (Archer et al. 2003; Fan et al. 2009). Isolation of MSCs from human synovial membrane that differentiated into chondrocyte, osteocyte, adipocyte and myogenic lineages was first described by De Bari et al. (2003). Other groups also found stem/progenitor cells in synovium and SF (Jones et al. 2008; Fan et al. 2009; Boeuf and Richter 2010; Krawetz et al. 2012; de Sousa et al. 2014). These SF-MSCs appear phenotypically related to BM-MSCs, though healthy SF-MSCs do not express CD271, or low affinity nerve growth factor receptor. This potentially defines an MSC precursor subpopulation distinct from other tissues, including BM (Boeuf and Richter 2010). In addition, the hyaluronan receptor CD44 has been found to be expressed at higher levels in bovine SF-MSCs compared to BM-MSCs (Jones et al. 2008). This niche of SF-MSCs may contribute to their consistent chondrogenic differentiation potential and highly clonogenic cell populations (Jones and Crawford 2014), while BM-MSCs are more heterogeneous (Pittenger et al. 1999), prompting the suggestion that SF-MSCs are derived from adjacent synovium rather than from BM (Jones et al. 2008). Also SF-MSC numbers appear to increase in the knees of patients with degenerated cartilage and osteoarthritis (Sekiya et al. 2012), and after meniscal injury (Matsukura et al. 2014).

12.2.4 Perichondrium and Periosteum

During long bone development, perichondrium surrounds the cartilage anlagen. Both tissues are derived from mesenchymal stem/progenitor cells (MSPCs) that give rise to chondrocytes from the inner condensation of MSPCs, while peripheral undifferentiated cells form perichondrium (Eames et al. 2003; Colnot et al. 2012). Osteoprogenitors and the residing endothelial cells from the perichondrium, invade calcified hypertrophic cartilage promoting vascular invasion to originate the primary ossification center forming bone and bone marrow (Colnot et al. 2004; Roberts et al. 2015). Therefore, the perichondrium is converted into periosteum tissue, where perichondrial cells become osteoblasts forming mineralized bone collars (Long and Ornitz 2013). This process is highly regulated by signaling pathways and factors (Mackie et al. 2008), including bone morphogenetic protein (BMP), transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF) (Ornitz and Marie 2002; Song et al. 2009), as well as Indian hedgehog (Ihh) and wingless/integrated (Wnt) (Kronenberg 2003; Geetha-Loganathan et al. 2008). These and other specific factors act during the different stages of chondrogenesis: growth plate and

perichondrium formation, osteogenesis, including periosteum and blood vessel invasion, ultimately synchronizing cell differentiation in all of these adjacent tissues (Colnot et al. 2012; Roberts et al. 2015).

Tendons, ligaments and muscles attach to the vascularized and innervated periosteum, which is composed of two layers. The outer fibrous one contains fibroblasts, while the highly cellular inner cambium layer contains osteoprogenitor cells and blood vessels (Allen et al. 2004). These osteoprogenitors give rise to osteoblasts by expressing specific genes, including transcription factors runt-related 2 (Runx2) and osterix (Osx), that promote mature bone-forming cells to differentiate into osteocytes in the bone matrix (Long and Ornitz 2013). Periosteum plays a central role during bone healing and repair (Roberts et al. 2015). In many bone fractures, periosteum-derived cells (PDCs) can differentiate into both chondrogenic and osteogenic lineages, migrate with blood vessels to form a mineralized bone collar, with the periosteum originating from vascularized perichondrium (Maes et al. 2010). In the early stage of callus formation, cartilage and bone appear to derive from periosteum, since bone repair is affected if the periosteum is removed (Zhang et al. 2005). The transcription factor Sox9, driving chondrogenesis during the initiation of healing after bone fracture (Bi et al. 1999; Dy et al. 2012), is expressed in PDCs, but it is not detected in the resting periosteum (Murao et al. 2013). This data suggests that most cells at the fracture site in the soft callus derive from PDCs that differentiate into chondrocytes and osteoblasts. BMP signaling appears essential to initiating chondrogenic/osteogenic differentiation of PDC in bone healing (Yu et al. 2010). In fact, overexpression of its antagonist Noggin, in human PDCs inhibits in vivo bone formation capabilities (Eyckmans et al. 2010). Other signaling pathways/factors, including Notch, Wnt, FGF, and TNF are also involved in bone repair and are currently investigated as potential regulators of differentiation and function of PDCs. It has also been reported that stem/progenitor PDC generate a higher number of colony-forming unit-fibroblasts (CFU-Fs) when compared to bone marrow and adipose tissue MSCs and with significant osteogenic capabilities when implanted into syngeneic rats (Hayashi et al. 2008). Therefore, considering these unique features of the periosteum, strategies for long bone repair promoting the formation of a periosteal-like membrane on its surface for mechanical support of the fracture and expansion of PDC are currently being investigated (Roberts et al. 2015).

12.2.5 Tendon and Ligament

Tendons are the tough yet flexible connective tissue straps that physically bind muscles to skeletal structures. Composed of bundles of collagen that are held together by a layer of loose connective tissue, tendons are well-suited for their role as transducers, enabling the force of a muscle to be exerted at a distance from the muscle itself (Wolfman et al. 1997). Ligaments are composed of the same basic components as tendons, although differences do exist to provide the specific mechanical

properties needed for their tasks. For instance, the collagen fibrils in ligaments are not uniformly parallel oriented, in order to allow for multiaxial loading patterns (Rumian et al. 2007). The primary unit of a tendon or a ligament is the collagen fiber. This extracellular matrix is produced by the tissue cells that lie between the collagen fibers (Kannus 2000). Different tendons and ligaments, similarly to other connective tissues, including cruciate ligaments, periodontal ligaments and patellar and hamstring tendons, have been demonstrated to contain stem/progenitor cells. Since tendons and ligaments are similar in structure, our discussion of tendon tissue-derived skeletal cells in this chapter is equally applicable to ligament-derived skeletal cells. In general, tendon injury arises from acute trauma or inflammation of tendon tissue or surrounding tissues (Aslan et al. 2008). The most common tendon injuries involve the supraspinatus tendon of the rotator cuff, the Achilles tendon, the flexor tendons of the hand, and the anterior cruciate and medial collateral ligaments of the knee (Carpenter and Hankenson 2004). Although there are no accurate figures specifically relating to tendon disorders, studies from primary care show that 16 % of the general population suffer from rotator cuff-related shoulder pain and this rises to 21 % when the statistics shift to elderly hospital and community populations (Urwin et al. 1998). These numbers further increase in the sports community; for example 30–50 % of all sporting injuries involve tendons (Docheva et al. 2015).

Resident cells were isolated from healthy and injured tendons (Lui and Wong 2013). Tendons and ligaments are susceptible to injury from excess mechanical strain experienced during sports or other daily activities (Doral et al. 2010). Although several early studies demonstrated some capacity of tendons to heal intrinsically (Eiken et al. 1975; Furlow 1976), and it is now believed that both intrinsic and extrinsic healing play a synergistic role in tendon regeneration, most probably due to low vascularization and a relatively limited regenerative capacity of the resident cell population. However, the extent of the contribution of each is still not well defined (Cadby et al. 2014). The tendon is surrounded by the paratenon, a loose fibrillar tissue that functions as an elastic sleeve permitting free movement of the tendon against other tissues (Kannus 2000). Under the paratenon, a fine connective tissue sheath, called epitenon, surrounds the entire tendon. The paratenon and the epitenon form together the peritenon (Kannus 2000). Whether the origin of the regenerating cells is internal, the core tendon itself, or external by migration of cells from the surrounding tissue – still remains open. However, cells that resemble mesenchymal stem cells in their phenotype and are multipotential were isolated from both the core tendon and the paratenon (Cadby et al. 2014; Mienaltowski et al. 2014).

12.2.6 Intervertebral Disc

The intervertebral disc (IVD) lies between two vertebral bodies, linking them together. IVDs are the main joints of the spinal column and occupy one-third of its height. Their major role is mechanical, as they constantly transmit loads arising from body weight and muscle activity through the spinal column. They provide flexibility allowing bending, flexion and torsion (Urban and Roberts 2003).

The IVD consists of the gel-like nucleus pulposus (NP) surrounded by the densely fibrous annulus fibrosus (AF). The NP is formed from the embryonic notochord as it segments during fetal development; the surrounding AF is formed from the sclerotome/mesoderm. NP cells produce abundant extracellular matrix, rich in aggrecan and type II collagen. At birth, the NP is populated by morphologically distinct, large vacuolated notochordal cells (NCs) (Roughley 2004). In some vertebrates, these NCs persist throughout most of adult life, whereas in other species, including humans, NCs gradually disappear during maturation (Hunter et al. 2003), eventually becoming undetectable and replaced by a population of smaller round cells – NP cells – believed to differentiate from NCs (McCann et al. 2012).

The economic impact of IVD disease and associated chronic lower back pain in the U.S. is estimated to a total of $50 billion annually (Schafer et al. 2007). Despite decades of research, no fundamental multidisciplinary understanding of the mechanism(s) of IVD degeneration has been gained (Freemont and Hoyland 2007; Zhao et al. 2007). Consequently, clinical therapies are still in the earliest stages of development (Masuda and Lotz 2010). Disc degeneration is characterized by increased breakage of the existing NP matrix due to elevated expression of matrix metalloproteases and inflammatory factors, and altered matrix production. In addition, cell apoptosis and formation of cell clusters, due to accelerated cell replication can lead finally to cell senescence (Le Maitre et al. 2007; Zhao et al. 2007). Finally, the process extends to the AF, as a result of altered loading, and leads to microtrauma and pain (Freemont 2009). All these changes are mediated by disturbances in the function of cells residing in the disc (Risbud et al. 2004; Zhao et al. 2007). The disc, as an organ, possesses a minimal capability for intrinsic regeneration (Zhao et al. 2007). The change in cell population correlates with the initiation of degenerative changes within the disc, suggesting that the loss of NCs may be responsible. Interestingly, animals in which NCs remain throughout the majority of their lifespan, including commonly used experimental animals, such as rabbits, rats and mice do not show signs of degeneration and maintain a more hydrated, proteoglycan-rich matrix than that found in adult human NP tissue (Alini et al. 2008). Supporting this theory, NCs were found to be more metabolically active and to produce more proteoglycans (PGs) than smaller NP cells (Cappello et al. 2006). Besides those two cell types, mesenchymal stem cell-like cells were isolated from the NP (Risbud et al. 2007) and AF of healthy and degenerated discs (Henriksson et al. 2009; Mizrahi et al. 2013), and were shown to be able to differentiate into osteogenic, adipogenic, and chondrogenic lineages in vitro.

12.3 Characteristics and Properties

Since stem and progenitor cell populations are a heterogeneous group of cells for which no unique markers exist, these cells are defined by a large set of cell markers. Some of these markers are restricted to these populations, whereas others overlap with other cell populations. FACS, immunostaining and RT-PCR are the most common methods to characterize stem and progenitor cells on their marker profile.

Depending on the cell host (e.g. murine, human), sub-population of cells (e.g. muscle derived stem cells, satellite cells) and method of detection, the marker profile obtained may vary (Qu-Petersen et al. 2002; Peault et al. 2007). For further characterization, the cells' potential to differentiate into multiple lineages in vitro and in vivo is often used to provide additional evidence for the nature of the stem and progenitor cell, which will be discussed in further detail later in this chapter.

To distinguish bone marrow mesenchymal stem cells (BM-MSCs) from non-MSCs, the International Society for Cellular Therapy recommended in 2006 minimal criteria for MSCs (Dominici et al. 2006). These criteria include the MSC's adhesion to plastic, their ability to differentiate into the osteogenic, adipogenic and chondrogenic lineage, and their expression of a set of cell surface markers. According to their suggestion, MSCs should be >95 % positive for the expression markers CD105, CD73, and CD90 and >95 % negative for CD45, CD34, CD14 or CD11b, CDalpha or CD19, and HLA-DR. Other membrane molecules that are expressed by MSCs include CD44, CD146 and CD106 (Pittenger et al. 1999; Gronthos et al. 2003).

Human and murine satellite cells have been shown to express the phenotypic markers MyoD, Pax7 and Myf5, which are not found in MDSCs or pericytes (Dellavalle et al. 2007; Usas et al. 2011). A characteristic marker of murine MDSCs compared to satellite cells and pericytes is the Stem cell antigen 1 (Sca-1) (Qu-Petersen et al. 2002; Usas et al. 2011). Further evidence that satellite cells and MDSCs are distinct subpopulations has been shown by experiments demonstrating that Pax and Sca-1 positive cells do not co-localize in skeletal muscle (Usas et al. 2011). Human pericytes typically express NG2 proteoglycan, CD146 and PDGFRB, whereas murine pericytes can be characterized by their expression of α-SMA (Dellavalle et al. 2007; Peault et al. 2007).

Multiple cell-related surface markers have been found in cartilage stem/progenitor cells (CSPCs), including CD29 (integrin β 1), CD44 (hyaluronan), CD49e (integrin α 5), CD54 (intercellular adhesion molecule 1), CD73 (5′-nucleotidase), CD90 (Thy 1 membrane glycoprotein), CD105 (endoglin), CD166 (immunoglobulin adhesion molecule), Notch 1 (neurogenic locus notch homologue protein 1), STRO 1 (trypsin-resistant cell surface antigen 1) and others (Jiang and Tuan 2015). However, the origin of CSPCs in cartilage and their role in cartilage repair and regeneration is not completely understood. It has been reported that CSPCs migrate to human cartilage damaged by osteoarthritis (Koelling et al. 2009), and to injured bovine cartilage explants (Seol et al. 2012). This suggests that CSPCs exist in articular cartilage and have a role in repair. However, a definitive panel of biomarkers to trace their origin has not been established.

In the embryonic perichondrium, a population of stem/progenitor cells expressing Nestin (Nes^+) that interact with endothelial ($CD31^+$) and nonendothelial ($CD31^-$) cells, have been identified and shown to increase during endochondral ossification (Ono et al. 2014). For periosteum, there is currently a lack of specific markers allowing the isolation of periosteal progenitor/stem cells (PDCs) as a pure isolate. In fact, cultured PDC have also been shown to contain osteoblast-committed cells

(Chang and Knothe Tate 2012). They express cell surface markers similar to MSCs e.g. CD73, CD90, CD105, CD166 and perivascular ones such as αSMA, CD146, PDGFβ receptor (Roberts et al. 2015).

Recently, skeletal stem cells (SSCs) were identified in mice by lineage tracing studies expressing specific markers, such as CD45-, Ter-119-, AlphaV+, Thy-, 6C3-, CD105- and CD200+, which determined that specific sub-populations derived from these SSCs followed a pattern of endochondral ossification, generating mainly bone or cartilage (Chan et al. 2015). Worthley et al. also found skeletal progenitors by expression of the BMP antagonist Gremlin 1 (Worthley et al. 2015). The Gremlin1+ population was enriched for CD45–, CD31–, Ter119–, CD105+ cells and defined as osteochondroreticular (OCR) stem cells. The OCR population that expresses *Gremlin-1* was shown to have a dominant role during skeletal development and early life, particularly of articular and growth plate cartilage, with only a modest contribution from perisinusoidal MSCs (Worthley et al. 2015). In fact, perisinusoidal MSCs, residing in blood vessel sinusoid-like structures in the outer surface of the BM are the suggested endogenous progenitor source for a major role in skeletal homeostasis and repair in adult life (Bianco et al. 2013b; Worthley et al. 2015). This supports a model where skeletal progenitors are distinct cohorts that do not coincide temporally or spatially with each other (Kassem and Bianco 2015). For example, in developing and growing bones, chondrogenic and osteogenic differentiation occurs at sites distinct from BM and without adipogenesis. Instead, in adults and in aging bone, BM provides osteogenic and adipogenic lineages, but not chondrocytes. This recent groundbreaking work shows that cell-lineage commitment is flexible and that local environment can modulate ultimate cell fate. The findings create a model of two distinct stem cell groups: SSCs or OCR cells and the perisinusoidal MSCs that are each regulated temporally (prenatal, postnatal, adult) and by lineage-specificity (Bianco and Robey 2015).

Mature tendons are normally characterized by low cellular density. Approximately 90–95 % of the cellular content of tendon comprises tendon-specific cell types described in the literature as tenoblasts and tenocytes, the latter being terminally differentiated (Chuen et al. 2004). Tendon cells are able to synthesize all components of the tendon ECM with a peak activity during growth and a gradual decrease during aging (Docheva et al. 2015). Several groups identified Tendon Stem/Progenitor Cells (TSPCs) (Bi et al. 2007) or Tendon-Derived Stem Cells (TDSCs) as they are also referred to (de Mos et al. 2007; Rui et al. 2010). The existence of TSPC was further confirmed in subsequent studies with human, equine, rabbit, rat and mouse tendons (Docheva et al. 2015). Neither the role of developmental origin of those cells, nor their role in maintenance of the tendon tissue is clearly determined. Similarly to other tissues from mesenchymal origin, those skeletal stem cells were characterized in terms of their self-renewal capacity, multipotential differentiation capacity and surface markers Stro-l, CD146, CD90 and CD44 and absence of expression of hematopoietic markers like CD34, CD45, CD31 and CD117 (Bi et al. 2007; de Mos et al. 2007; Rui et al. 2010).

Studies that have assessed the NC phenotype to understand the initial state of NCs prior to their maturation used a porcine model system (Chen et al. 2006). These cells were found to have a reduced gene expression of specific matrix molecules, such as collagen type I and biglycan, and the metalloprotease inhibitor known as tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) compared to mature porcine NP cells. However, no differences in the expression of chondrogenic genes (SOX9, collagen type II and aggrecan) between notochordal and mature porcine NP cells were noted, although these 'notochordal-like' cells did express higher levels of integrins a1 and a6, which are involved in interactions with collagen and laminins during tissue development (Chen et al. 2006). Furthermore, the same group recently demonstrated that laminins may also be used to differentiate between notochordal and mature porcine NP cells within the disc (Chen et al. 2009).

The question of the NP phenotype and specific marker genes is still not fully resolved. The main obstacle was to identify markers that will distinguish between the NP cells to articular chondrocytes that were believed to be very similar in their phenotype. Several studies were done using gene expression profiling to identify the specific and distinctive molecular imprint of the NP cells (Minogue et al. 2010; van den Akker et al. 2014). Recently, a consensus paper was published to address this issue and recommended the following healthy NP phenotypic markers: stabilized expression of HIF-1α, GLUT-1, aggrecan/collagen II ratio>20, Shh, Brachyury, KRT18/19, CA12, and CD24 (Risbud et al. 2014).

12.4 Isolation and Proliferation

For in vitro cultivation, mesenchymal stem cells (MSCs) are typically isolated from the mononuclear layer of the bone marrow in terms of separation by density gradient centrifugation (e.g. with Ficoll™ or Percoll™ density medium) and subsequently by adherence to cell culture plastic (Bara et al. 2014). The resulting cell population is morphologically heterogeneous, containing a major population of large and moderately granular cells, referred to as mature MSCs, and a minor population of small and agranular cells, referred to as recycling stem cells or RS-1 cells (Colter et al. 2000). After short-term cultivation, RS-1 cells give rise to a new population of rapidly growing small and densely granular cells (RS-2). These cells decrease in numbers during passaging until they have disappeared. At this point in time, mature MSCs and RS-1 cells rapidly expand (Colter et al. 2000). Following extended in vitro culture, MSCs lose their multipotency and undergo replicative senescence (Bruder et al. 1997; Digirolamo et al. 1999). In addition to the presence of adherent BM-MSCs, several studies demonstrated the existence of a non-adherent cell population of bone marrow (BM)-MSCs in the mononuclear cell fraction from bone marrow, with a greater proliferation and differentiation potential (Wan et al. 2006; Zhang et al. 2009; Di Maggio et al. 2012). Further research will elucidate, if these cells represent an alternative to adherent BM-MCSs.

To harvest stem cells from muscle biopsies, the tissue is enzymatically digested into its cellular components using mostly collagenase, followed by serial plating of the digested muscle tissue onto collagen-coated flasks (pre-plate technique). This technique allows to separation of committed cells, such as fibroblasts from progenitor cells, since progenitor cells tend to adhere later then the committed cells (Lee et al. 2000; Qu-Petersen et al. 2002; Dellavalle et al. 2007). Muscle derived stem cells (MDSCs) have an extended replicative lifetime and substantial self-renewal capacity comparable to embryonic stem cells (Deasy et al. 2005). MDSCs expanded for 200 population doublings preserved their phenotype and were capable to promote muscle regeneration by serial transplantation into the skeletal muscle of mdx mice, which model Duchenne muscular dystrophy (Deasy et al. 2005).

The isolation of cartilage stem/progenitor cells (CSPCs) from different sources has utilized similar protocols from normal and injured human cartilage (Alsalameh et al. 2004; Ustunel et al. 2008; Seol et al. 2012). The method includes an enzymatic digestion (pronase and collagenase) of human articular cartilage explants, followed by filtration with a cell strainer, plating 4000 cells/ml in serum free media on fibronectin-coated dishes and cultured for a short period of time. Afterwards, the cells are cultured in media supplemented with 10 % FBS for 15 days, until colonies with more than 32 cells can be identified (Dowthwaite et al. 2004). These colonies, defined as CSPCs, are then trypsinized, passaged and characterized by: CFU-F capabilities, multilineage differentiation assays and presence of appropriate cell surface markers.

Skeletal stem cells (SSCs) were, instead, isolated from femoral growth plates of postnatal day 3 mice using mechanical and enzymatic dissociation and analyzed for specific markers (Chan et al. 2015). Worthley et al. also isolated, using enzymatic digestion from the long bone metaphyses adjacent to the growth plate and trabecular bone, skeletal progenitors that were defined as osteochondroreticular (OCR) stem cells (Chan et al. 2015; Worthley et al. 2015).

The isolation of periosteum-derived cells (PDC) is obtained using different methods, such as peeling off the periosteal layer from the bone of young animals (Arnsdorf et al. 2009), enzymatic digest of the tissue diaphyseal bone (van Gastel et al. 2012), or from a femoral bone grafting model where the formed periosteal callus is subjected to an enzymatic digest to obtain periosteal cells (Wang et al. 2011). When cultured, the PDC independent of the isolation method employed, have a fibroblast-like morphology that is maintained during passaging.

The isolation of the tendon-derived stem cells (TDSCs) from human, mouse and rat tendons was done using collagenase type I digestion of the connective tissue and filtering the solution through 70 μm cell strainer, yielding a single cell solution and seeded in vitro, basically selected by plastic adherence (Bi et al. 2007; Rui et al. 2010). The cells were compared to the "gold standard" of musculoskeletal stem cells, bone marrow (BM)-MSCs and found slightly different in their phenotype and differentiation potential. Although tendon stem/progenitor cells (TSPCs) express many of the same markers as BM-MSCs, the expression patterns were not identical (Tan et al. 2012). TSPCs highly express tendon-related factors, such as Scx, TNMD,

Comp and tenascin C (Bi et al. 2007). TDSC exhibited higher clonogenicity, proliferated faster, and expressed higher tenomodulin, scleraxis, collagen 1a1 (Col1A1), decorin, alkaline phosphatase, Col2A1, and biglycan messenger RNA levels than BM-MSC. Surprisingly, TDSCs presented higher proliferative and differentiation potential than BM-MSCs (Tan et al. 2012).

The isolation of the cells from the nucleus pulposus (NP) is usually done by overnight enzymatic digestion with collagenase type I to liberate the cells from the tissue (Blanco et al. 2010). Later on the cells can be cultured in vitro and selected by adherence to get NP cells and NP-derived stem cells (Blanco et al. 2010; Erwin et al. 2013; Mizrahi et al. 2013). Additionally, the notochordal cells can be isolated from the animal discs using the size differences between them and the rest of the cell that populate the disc via 70 μm cell strainer (Korecki et al. 2010).

12.5 Differentiation

The function of mesenchymal stem cells (MSCs) is largely influenced by their surrounding extracellular matrix (ECM) (Streuli 1999). The ECM maintains the tissue architecture, acts as a ligand for cellular adhesion receptors such as integrins, and provides signaling molecules, including growth factors and growth factor-binding proteins, to control cellular behavior (Werb 1997; Streuli 1999). Interaction of cells with the ECM modulates signaling cascades that control cell growth, differentiation, survival and morphogenesis, and therefore changes in the microenvironment are able to affect these processes (Lukashev and Werb 1998). For example, differentiation can be initiated by binding of growth factors, such as TGF-β, to transmembrane serine/threonine kinase receptors that signal via the TGF-β/Smad pathway, which consequently regulates gene expression in the nucleus (Lutz and Knaus 2002). Transcription factors known to be essential to MSC differentiation are runt-related transcription factor2 (Runx2) for osteogenesis, peroxisome proliferator-activated receptor gamma2 (PPARγ2) for adipogenesis and Sry-related high mobility group (HMG) box9 (Sox9) for chondrogenesis (Rosen and Spiegelman 2000; Minguell et al. 2001; Pan et al. 2008). Furthermore, mechanical loading of MSCs seems to affect osteogenic differentiation (Jagodzinski et al. 2004; Mauney et al. 2004). For example, mechanical compression has been shown to induce the expression of type II collagen, Aggrecan and Sox9, suggesting a compressive stress-induced chondrogenesis of MSCs (Takahashi et al. 1998). Another study demonstrated an up-regulation of MMP-13 in response to mechanical loading of MSCs, which was further shown to play a role in osteogenic differentiation of MSCs (Kasper et al. 2007). Stimuli can be transduced via stretch-activated ion channels, cell adhesion molecules (e.g. integrins), and also by guanine nucleotide-binding protein- (G-protein-) coupled receptors and growth factor receptor tyrosine kinases (RTK) (Wang and Thampatty 2006). For example, focal adhesions, comprised of integrins, talin, vinculin and other proteins, connect the ECM to actin

filaments. Intracellular forces are then transmitted through the cytoskeletal network to the nucleus (Jaalouk and Lammerding 2009). Osteogenic signaling pathways activated by external applied forces in MSCs have been shown to include Wnt, Ror2, and Runx2 (Hao et al. 2015).

MSCs have in general the ability to differentiate not only towards the tissue they are originally derived from, but also into other cell lineages. Bone marrow (BM)-MSCs have been shown to differentiate into osteogenic, chondrogenic, adipogenic, and myogenic cells in vivo and in vitro (Pittenger et al. 1999; Caplan 2005). The MSC's ability to give rise to brain astrocytes and neurons has been demonstrated after their transplantation into rodents (Azizi et al. 1998; Brazelton et al. 2000). Furthermore, it was shown that BM-MSCs are able to transdifferentiate into cell types from different germ layers, including endothelial cells, hepatocytes or cardiomyocytes (Lin et al. 2000; Theise et al. 2000; Orlic et al. 2001).

Muscle-derived stem cells (MDSCs) have not only the potential to differentiate into myogenic, but also into other lineages, including osteogenic, chondrogenic, hematopoietic, and neural lineages, which has been demonstrated in several in vitro and in vivo studies (Peault et al. 2007; Usas et al. 2011).

Several groups have reported that cartilage stem/progenitor cells (CSPCs) in culture have self-renewal capabilities and differentiation potential comparable to BM-MSCs, including chondrogenic, osteogenic and adipogenic lineages (Barbero et al. 2003; Alsalameh et al. 2004), with migratory abilities in response to tissue injury (Seol et al. 2012; Jiang and Tuan 2015). However, human limb buds have been shown to contain a population of cartilage progenitor cells (CPCs) specific for chondrogenesis in addition to multipotent cartilage stem cells (CSCs) capable of differentiation into multiple lineages, including chondrocytes (Wu et al. 2013). Skeletal stem cells (SSCs) were shown in in vivo and in vitro studies to be capable of self-renewal, multipotent, and to have the ability to differentiate into lineage-restricted progenitor cells, such as pre-bone, cartilage and stromal progenitors (pre-BCSPs) (Chan et al. 2015). Furthermore, it was shown that a cell, committed to chondrogenic lineages could be induced to generate bone, while by blocking VEGF receptor (creating an avascular environment like cartilage), osteogenic progenitors could shift to a chondrogenic fate. Osteochondroreticular (OCR) cells can self-renew, generate osteoblasts, chondrocytes, reticular marrow stromal cells but not adipocytes, and are apparently used for articular cartilage formation during bone development. They are thought to contribute to early postnatal skeletogenesis remodeling (Worthley et al. 2015). In contrast, perisinusoidal MSCs differentiate in adulthood and especially after bone injury into adipogenic, chondrogenic and osteogenic derived-tissues, contributing to the process of repair and skeletal homeostasis (Bianco et al. 2013b). Under specific conditions, expanded periostum-derived cells (PDCs) can differentiate into osteogenic, chondrogenic and adipogenic lineages in vitro and also form bone, cartilage and hematopoietic marrow upon in vivo transplantation, confirming their multipotent nature (Chang and Knothe Tate 2012; Roberts et al. 2015). It has not been determined yet if the PDC also possess a self-renewal capacity (Bianco et al. 2013b). However, the presence of skeletal stem cells

in the periosteum was demonstrated by single-cell lineage analysis, showing that the periosteum contains cells with mesenchymal multipotency (De Bari et al. 2006).

Since the availability of tendon-derived stem cells (TDSC) is very low, most of the effort – besides showing multi-potential – was directed towards differentiation of those cells towards tenocytes. The roles of cytokines and growth factors in tendon formation and regeneration have been identified (Evans 1999); their overexpression induces tenogenic differentiation and enhances the healing process (Gerich et al. 1996; Evans 1999; Wang et al. 2005; Majewski et al. 2008). Specifically, overexpression of BMP-2 (Martinek et al. 2002; Chen et al. 2008), BMP-12 (also known as GDF-7) (Wang et al. 2005; Majewski et al. 2008; Ma et al. 2009), and IGF-1 (Steinert et al. 2008) have all been shown to induce tenogenesis. Recently, overexpression of tendon-related proteins like Celecoxib (Zhang et al. 2014), Scleraxis (Tan et al. 2014) was shown to be effective in differentiation of TDSC and promote tendon/ligament regeneration. However, many questions regarding the regenerative process in tendon and ligament are still unanswered, and much remains to be done before gene-and-cell therapy meets the enormous need in clinical practice for tendon and ligament regeneration.

Similar to tendons and ligament, the nucleus pulposus (NP)-derived stem cells are as attractive source of stem cells as other skeletal stem cells due to very low availability. However, these cells can be used to investigate the intervertebral disc (IVD) environment and degeneration process (Mern et al. 2014). The natural environment within the disc is very challenging to implanted cells, particularly if they have been sub-cultured in normal laboratory conditions. Several studies showed that musculoskeletal stem cell differentiation towards NP-like phenotype can be achieved by mimicking the NP environment in vitro, namely low glucose and hypoxic conditions (2–3 % oxygen) (Risbud et al. 2004; Li et al. 2013; Mizrahi et al. 2013). Additionally, the differentiation can be induced using TGF-β1 (Morigele et al. 2013; Clarke et al. 2014; Cui et al. 2015) and biomechanical properties of the biomaterials used also mimicking the environment the cells see in the IVD (Cao et al. 2011; Gilchrist et al. 2011).

12.6 Potential Applications for Therapies

Mesenchymal stem cells (MSCs) are thought to exert their therapeutic effects by several mechanisms, including their ability to home to the site of inflammation after tissue injury, capacity of multi-lineage differentiation, secretion of trophic factors, and the lack of immunogenicity and immunosuppressive effects (Sharma et al. 2014). Their immunologic function has been demonstrated to result from secretion of soluble mediators, such as human leucocyte antigen (HLA-G), which seem to prevent the immune response, against MSCs (Sotiropoulou et al. 2006; Nasef et al. 2007). Furthermore, the reduction of inflammation and stimulation of tissue regeneration is thought to be greatly mediated by secretion of trophic factors, including growth factors, cytokines and soluble extracellular matrix (ECM) molecules

(Sharma et al. 2014). These unique biologic properties, which have been demonstrated in preclinical in vivo studies (Otto and Wright 2011), and their accessibility and great expansion potential in vitro make them an attractive cell source for clinical applications. Indeed, MSCs are investigated in more than 200 clinical trials for a variety of therapeutic indications ranging from immunomodulation to tissue repair and regeneration (Sharma et al. 2014). Although tissue sources differ in these trials, more than 50 % of the MSCs that are currently investigated in the clinic are bone marrow (BM)-MSCs, with encouraging results, including the therapy of bone and cartilage disorders, diabetes and cardiovascular diseases (Sharma et al. 2014). In addition to their healing promoting effects, MSCs seem to be well tolerated, since most trials reported a lack of any serious adverse events and tumor formation, as a result of MSC application in humans has been never reported (Otto and Wright 2011).

Autologous skeletal myoblasts (satellite cell progeny) have been proven to improve cardiac function and to meet feasibility and safety criteria for cardiac repair in clinical phase I trials (Joggerst and Hatzopoulos 2009). However, site effects, such as the incidence of arrhythmias have been reported in the first randomized placebo-controlled study of myoblast transplantation (Menasche et al. 2008). Recent studies demonstrated advantages of using muscle-derived stem cells (MDSCs) compared to myoblasts, including a superior engraftment of the MDSCs within the infarcted myocardium and improvement of left ventricular function (Okada et al. 2008). MDSCs were shown to display a superior regenerative capacity relative to satellite cells following transplantation into dystrophic muscle in a murine model of muscular dystrophy (*mdx*) (Qu-Petersen et al. 2002). Furthermore, they are at least partially immune-privileged, as the transplantation of MDSCs resulted in a robust dystrophin expression in mdx mice over 3 months after injection (Qu-Petersen et al. 2002). In pre-clinical and clinical trials, muscle-derived cell transplantation including MDSCs for treatment of urological disorders demonstrated a high efficacy in restoring urethral function (Usas et al. 2011).

Since the capacity for self-repair and biological interventions are limited in cartilage there is a need for improved therapeutic strategies to address disorders such as trauma, fracture, osteoporosis, osteoarthritis and genetic skeletal deformities (Caldwell and Wang 2015). Its structure, avascularity and low cell density with a dense extracellular matrix has been challenging for developing cell therapies using MSCs (Bornes et al. 2014) or regenerative approaches with autologous chondrocyte implantation or autologous chondrocyte implantation (Madeira et al. 2015). Focal cartilage injuries are primarily treated by surgical techniques including bone marrow stimulating interventions, such as microfracture. Mosaicplasty is another approach where osteochondral autografts of mature hyaline cartilage are transplanted to the defects. This latter technique appears to show a superior level of athletic activity compared to microfracture especially at short term follow up (<5 years) (Caldwell and Wang 2015). Cell-based products using autologous chondrocyte implantation (ACI) that are expanded ex vivo, combined with biological scaffolds and collagen hydrogel patches have improved the approach (Benders et al. 2013). However, these therapies have not been successful in restoring the native

articular cartilage structures. Newer cell therapy strategies involving tissue engineering for cartilage repair has been an intense focus in recent years, integrating the use of biomaterial-based scaffolding (natural or synthetic), a cell source (e.g. autologous chondrocytes, BM-MSCs) and differentiation factors, such as TGF-β, IGF-1 and specific FGF family members (Caldwell and Wang 2015). Finally, approaches utilizing human embryonic stem cells differentiated toward articular cartilage are under investigation (Cheng et al. 2014). These allogenic transplants can provide the opportunity for larger-scale production. However, they raise the issue of potential immunological reactions. A similar methodology that would overcome immunological barriers utilizes induced pluripotent stem cells (iPSCs) derived by reprogramming patient cells. The cells could then be differentiated into chondrocyte lineages in vitro (Saitta et al. 2014). Differentiated iPSCs have been used in an in vitro cartilage defect model system for functional cartilage repair (Diekman et al. 2012). This promising approach would utilize patient-derived iPSCs appropriately differentiated and expanded ex vivo before transplanting the autograft back into the patient (Cheng et al. 2014). Overall, developing the regenerative capacity of resident bone and cartilage tissue cells and novel iPSC-based techniques together with tissue engineering will provide exciting new approaches toward treating bone and cartilage disease.

To date, four treatment options have been used to repair or replace damaged tendons: autografts, allografts, xenografts, and synthetic polymers. Implantation of synthetic polymers was very popular in the 1980s, but it frequently led to implant degeneration and failure (Woods et al. 1991). Autografts have produced the most satisfactory long-term results and are referred to as the "gold standard" for treating severe tendon injuries (Poolman et al. 2007). Nevertheless, morbidity at the donor site is often associated with pain, muscle atrophy, and tendonitis, resulting in prolonged rehabilitation periods (Aslan et al. 2008). The risks of disease transmission and infection, the lack of donors, and questionable donor–recipient compatibility pose significant obstacles to the use of allografts to repair or replace damaged tendons. As for xenografts, the potential source for this tissue is still under debate (Stone et al. 2007). In light of the disadvantages of current surgical solutions for tendon repair, the need for gene-modified-cell–mediated tendon tissue engineering is evident.

Mainly due to their low availability, up to date applications of the nucleus pulposus (NP)-derived cells are mostly limited for research of the degeneration of the intervertebral disc (IVD) and aging (Sakai et al. 2012; Mizrahi et al. 2013). However, some attempts were made to use cell isolated from degenerated discs as cell therapy agent in clinic (Meisel et al. 2007; Mochida et al. 2015). Interestingly, unlike other musculoskeletal stem cells, NP-derived cells were found to be very effective in differentiating other stem cells when they are co-cultured with them (Lehmann et al. 2014; Arkesteijn et al. 2015). Additionally, conditioned media, especially the one of notochordal cells, was used to promote NP differentiation of different musculoskeletal stem cells (Korecki et al. 2010; Purmessur et al. 2011), and even induced pluripotent stem cells (Chen et al. 2013).

12.7 Good Manufacturing Practices for MSCs

To be able to use mesenchymal stem cells (MSCs) in clinical trials, their safety, efficacy and reproducibility of MSC production and compliance with Good Manufacturing Practices (GMPs) must be ensured. In Europe, MSCs are somatic cell therapy products, referred to as advanced-therapy medicinal products (ATMPs) and are under European Regulation No. 1394/2007 (Sensebe et al. 2013). As reviewed by Wuchter et al., in Germany, a formal manufacturing license is required, which is granted by the respective local authority of the German federal states. Additional scientific and regulatory support during the approval process as well as clinical trial authorization is provided by the Paul-Ehrlich-Institut (PEI), as overarching Federal Authority for Vaccines and Biomedicines (Bianco et al. 2013a; Wuchter et al. 2015). The production of MSCs in the US must comply with Current Good Tissue Practice requirements, under the Code of Federal Regulations, as described in detail in a review by Sensebe et al. (2013). Complying with GMPs requires precisely defining the production process as well as the multiple criteria required for a quality final product. This includes the environment, staff training and qualification, and controls (Sensebe et al. 2013). Manufacturing issues, which are frequently under discussion during the regulatory authorization process, include the donor selection, choice of culture medium and the advantages and disadvantages of master cell stocks. Measures of quality control include the immunophenotyping by flow cytomerty, testing of immunosuppressive potential of MSCs, tumorigenicity assessment of MSCs and evaluation of cellular senescence (Wuchter et al. 2015). The strategy for quality control testing depends on the cell composition, manufacturing process and indication, and target cell population (Wuchter et al. 2015).

12.8 Conclusion and Future Development

Musculoskeletal tissue-derived mesenchymal stem/ progenitor cells have been isolated from various tissue sources, including bone marrow, muscle, synovium, periosteum, and intervertebral disc. MSCs are generally thought to be responsible for tissue regeneration and homeostasis, although their exact physiological role remains in many of these tissues elusive. Reasons include their low frequency and lack of specific cell surface marker expression (Ren et al. 2012). Musculoskeletal-derived MSCs hold a great therapeutic potential for different clinical indications, including the treatment of bone and cartilage disorders, diabetes, and improvement of cardiac function (Joggerst and Hatzopoulos 2009; Sharma et al. 2014). In addition to their healing promoting effects, which are thought to be a result of their capacity of multi-lineage differentiation, secretion of trophic factors, and lack of immunogenicity and immunosuppressive effects (Sharma et al. 2014), MSCs have several advantages over other stem cell types (e.g. ES cells). This includes their easy expansion

potential in vitro, very low risk of tumor formation and lack of ethical issues (Otto and Wright 2011).

Although musculoskeletal-derived MSCs are widely investigated in clinical trials, with promising results, there are still many obstacles that need to be overcome before MSCs can be used as a regular therapy. One of the major challenges is that much of the knowledge about MSCs is based on in vitro data rather than on data in the MSC's native environment. These cells are known to dramatically adapt their phenotype in response to their environment. For example, monolayer expansion of MSCs has been proven to result in changes in phenotype and cell marker expression. Lack of knowledge about their marker expression in their natural environment makes it difficult to differentiate, if MSC cell populations, identified in different tissues are distinct from each other or are the same cell in a different location, with an adapted phenotype in response to the local environment. Another issue is the lack of standardized isolation and cell expansion protocols. This problem becomes evident when comparing similar trials that resulted in different outcomes. Better understanding of the developmental origin, cell marker expression and cell interactions in their local microenvironment, as well as improvement and alignment of in vitro culture systems, including further standardization of isolation and expansion protocols, will allow the development of more effective therapies. For example, the development of bioreactors that support an expansion and investigation of these cells in a more native environment, might help to choose the right MSC source for treatment of diseases, and to identify dosing and time points. An alternative approach to avoid problems arising from in vitro culture might be the use of the mononuclear fraction of bone marrow instead of using bone marrow (BM)-MSCs, as suggested by Bara et al. (2014). Although this method results in fewer cells, recent approaches with these cells were shown to be safe and efficient, and might therefore be an alternative to in vitro expanded MSCs for some clinical indications.

Acknowledgements We would like to thank Linda Kanim for creating the figure and Khosrowdad Salehi for critical reading of the text.

References

Akiyama H, Chaboissier MC et al (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 16(21):2813–2828

Alini M, Eisenstein SM et al (2008) Are animal models useful for studying human disc disorders/degeneration? Eur Spine J 17(1):2–19

Allen MR, Hock JM et al (2004) Periosteum: biology, regulation, and response to osteoporosis therapies. Bone 35(5):1003–1012

Alsalameh S, Amin R et al (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 50(5):1522–1532

Archer CW, Dowthwaite GP et al (2003) Development of synovial joints. Birth Defects Res Part C, Embryo Today: Rev 69(2):144–155

Arkesteijn IT, Smolders LA et al (2015) Effect of coculturing canine notochordal, nucleus pulposus and mesenchymal stromal cells for intervertebral disc regeneration. Arthritis Res Ther 17(1):60

Arnsdorf EJ, Jones LM et al (2009) The periosteum as a cellular source for functional tissue engineering. Tissue Eng A 15(9):2637–2642

Asakura A, Komaki M et al (2001) Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differ Res Biol Divers 68(4–5):245–253

Aslan H, Kimelman-Bleich N et al (2008) Molecular targets for tendon neoformation. J Clin Invest 118(2):439–444

Azizi SA, Stokes D et al (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats – similarities to astrocyte grafts. Proc Natl Acad Sci U S A 95(7):3908–3913

Balduino A, Hurtado SP et al (2005) Bone marrow subendosteal microenvironment harbours functionally distinct haemosupportive stromal cell populations. Cell Tissue Res 319(2):255–266

Bara JJ, Richards RG et al (2014) Concise review: bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic. Stem Cells 32(7):1713–1723

Barbero A, Ploegert S et al (2003) Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 48(5):1315–1325

Benders KE, van Weeren PR et al (2013) Extracellular matrix scaffolds for cartilage and bone regeneration. Trends Biotechnol 31(3):169–176

Bi W, Deng JM et al (1999) Sox9 is required for cartilage formation. Nat Genet 22(1):85–89

Bi Y, Ehirchiou D et al (2007) Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med 13(10):1219–1227

Bianco P, Robey PG (2015) Skeletal stem cells. Development 142(6):1023–1027

Bianco P, Barker R et al (2013a) Regulation of stem cell therapies under attack in Europe: for whom the bell tolls. EMBO J 32(11):1489–1495

Bianco P, Cao X et al (2013b) The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19(1):35–42

Blanco JF, Graciani IF et al (2010) Isolation and characterization of mesenchymal stromal cells from human degenerated nucleus pulposus: comparison with bone marrow mesenchymal stromal cells from the same subjects. Spine (Phila Pa 1976) 35(26):2259–2265

Boeuf S, Richter W (2010) Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. Stem Cell Res Ther 1(4):31

Bornes TD, Adesida AB et al (2014) Mesenchymal stem cells in the treatment of traumatic articular cartilage defects: a comprehensive review. Arthritis Res Ther 16(5):432

Brazelton TR, Rossi FM et al (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290(5497):1775–1779

Bruder SP, Jaiswal N et al (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64(2):278–294

Buckwalter JA, Cooper RR (1987) Bone structure and function. Instr Course Lect 36:27–48

Cadby JA, Buehler E et al (2014) Differences between the cell populations from the peritenon and the tendon core with regard to their potential implication in tendon repair. PLoS One 9(3):e92474

Caldwell KL, Wang J (2015) Cell-based articular cartilage repair: the link between development and regeneration. Osteoarthr Cartil/OARS Osteoarthr Res Soc 23(3):351–362

Calvi LM, Adams GB et al (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425(6960):841–846

Cao L, Guilak F et al (2011) Three-dimensional finite element modeling of pericellular matrix and cell mechanics in the nucleus pulposus of the intervertebral disk based on in situ morphology. Biomech Model Mechanobiol 10(1):1–10

Caplan AI (1990) Stem cell delivery vehicle. Biomaterials 11:44–46
Caplan AI (2005) Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng 11(7–8):1198–1211
Cappello R, Bird JL et al (2006) Notochordal cell produce and assemble extracellular matrix in a distinct manner, which may be responsible for the maintenance of healthy nucleus pulposus. Spine (Phila Pa 1976) 31(8):873–882; discussion 883
Carpenter JE, Hankenson KD (2004) Animal models of tendon and ligament injuries for tissue engineering applications. Biomaterials 25(9):1715–1722
Chan CK, Seo EY et al (2015) Identification and specification of the mouse skeletal stem cell. Cell 160(1–2):285–298
Chang H, Knothe Tate ML (2012) Concise review: the periosteum: tapping into a reservoir of clinically useful progenitor cells. Stem Cells Transl Med 1(6):480–491
Chen J, Yan W et al (2006) Molecular phenotypes of notochordal cells purified from immature nucleus pulposus. Eur Spine J 15(Suppl 3):S303–S311
Chen YL, Chen PK et al (2008) Periodontal regeneration using ex vivo autologous stem cells engineered to express the BMP-2 gene: an alternative to alveolaplasty. Gene Ther 15(22):1469–1477
Chen J, Jing L et al (2009) Expression of laminin isoforms, receptors, and binding proteins unique to nucleus pulposus cells of immature intervertebral disc. Connect Tissue Res 50(5):294–306
Chen J, Lee EJ et al (2013) Differentiation of mouse induced pluripotent stem cells (iPSCs) into nucleus pulposus-like cells in vitro. PLoS One 8(9):e75548
Cheng A, Hardingham TE et al (2014) Generating cartilage repair from pluripotent stem cells. Tissue Eng B Rev 20(4):257–266
Chuen FS, Chuk CY et al (2004) Immunohistochemical characterization of cells in adult human patellar tendons. J Histochem Cytochem 52(9):1151–1157
Clarke LE, McConnell JC et al (2014) Growth differentiation factor 6 and transforming growth factor-beta differentially mediate mesenchymal stem cell differentiation, composition, and micromechanical properties of nucleus pulposus constructs. Arthritis Res Ther 16(2):R67
Colnot C, Lu C et al (2004) Distinguishing the contributions of the perichondrium, cartilage, and vascular endothelium to skeletal development. Dev Biol 269(1):55–69
Colnot C, Zhang X et al (2012) Current insights on the regenerative potential of the periosteum: molecular, cellular, and endogenous engineering approaches. J Orthop Res: Off Publ Orthop Res Soc 30(12):1869–1878
Colter DC, Class R et al (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci U S A 97(7):3213–3218
Cui X, Liu M et al (2015) Electrospun scaffold containing TGF-beta1 promotes human mesenchymal stem cell differentiation towards a nucleus pulposus-like phenotype under hypoxia. IET Nanobiotechnol 9(2):76–84
da Silva Meirelles L, Caplan AI et al (2008) In search of the in vivo identity of mesenchymal stem cells. Stem Cells 26(9):2287–2299
De Bari C, Dell'Accio F et al (2003) Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol 160(6):909–918
De Bari C, Dell'Accio F et al (2006) Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. Arthritis Rheum 54(4):1209–1221
de Mos M, Koevoet WJ et al (2007) Intrinsic differentiation potential of adolescent human tendon tissue: an in-vitro cell differentiation study. BMC Musculoskelet Disord 8:16
de Sousa EB, Casado PL et al (2014) Synovial fluid and synovial membrane mesenchymal stem cells: latest discoveries and therapeutic perspectives. Stem Cell Res Ther 5(5):112
Deasy BM, Gharaibeh BM et al (2005) Long-term self-renewal of postnatal muscle-derived stem cells. Mol Biol Cell 16(7):3323–3333
Dellavalle A, Sampaolesi M et al (2007) Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol 9(3):255–267

Di Maggio N, Mehrkens A et al (2012) Fibroblast growth factor-2 maintains a niche-dependent population of self-renewing highly potent non-adherent mesenchymal progenitors through FGFR2c. Stem Cells 30(7):1455–1464
Diekman BO, Christoforou N et al (2012) Cartilage tissue engineering using differentiated and purified induced pluripotent stem cells. Proc Natl Acad Sci U S A 109(47):19172–19177
Digirolamo CM, Stokes D et al (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107(2):275–281
Docheva D, Müller SA et al (2015) Biologics for tendon repair. Adv Drug Deliv Rev 84:222–239
Dominici M, Le Blanc K et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317
Doral MN, Bozkurt M et al (2010) Achilles tendon rupture: physiotherapy and endoscopy-assisted surgical treatment of a common sports injury. Open Access J Sports Med 1:233–240
Dowthwaite GP, Bishop JC et al (2004) The surface of articular cartilage contains a progenitor cell population. J Cell Sci 117(Pt 6):889–897
Dy P, Wang W et al (2012) Sox9 directs hypertrophic maturation and blocks osteoblast differentiation of growth plate chondrocytes. Dev Cell 22(3):597–609
Eames BF, de la Fuente L et al (2003) Molecular ontogeny of the skeleton. Birth Defects Res Part C Embryo Today: Rev 69(2):93–101
Eiken O, Lundborg G et al (1975) The role of the digital synovial sheath in tendon grafting. An experimental and clinical study on autologous tendon grafting in the digit. Scand J Plast Reconstr Surg 9(3):182–189
Erwin WM, Islam D et al (2013) Intervertebral disc-derived stem cells: implications for regenerative medicine and neural repair. Spine (Phila Pa 1976) 38(3):211–216
Evans CH (1999) Cytokines and the role they play in the healing of ligaments and tendons. Sports Med 28(2):71–76
Eyckmans J, Roberts SJ et al (2010) A clinically relevant model of osteoinduction: a process requiring calcium phosphate and BMP/Wnt signalling. J Cell Mol Med 14(6B):1845–1856
Fan J, Varshney RR et al (2009) Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration. Tissue Eng B Rev 15(1):75–86
Fickert S, Fiedler J et al (2004) Identification of subpopulations with characteristics of mesenchymal progenitor cells from human osteoarthritic cartilage using triple staining for cell surface markers. Arthritis Res Ther 6(5):R422–R432
Freemont AJ (2009) The cellular pathobiology of the degenerate intervertebral disc and discogenic back pain. Rheumatology (Oxford) 48(1):5–10
Freemont AJ, Hoyland JA (2007) Morphology, mechanisms and pathology of musculoskeletal ageing. J Pathol 211(2):252–259
Friedenstein AJ, Petrakova KV et al (1968) Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 6(2):230–247
Frontera WR, Ochala J (2015) Skeletal muscle: a brief review of structure and function. Calcif Tissue Int 96(3):183–195
Furlow LT Jr (1976) The role of tendon tissues in tendon healing. Plast Reconstr Surg 57(1):39–49
Geetha-Loganathan P, Nimmagadda S et al (2008) Wnt signaling in limb organogenesis. Organogenesis 4(2):109–115
Gerich TG, Kang R et al (1996) Gene transfer to the rabbit patellar tendon: potential for genetic enhancement of tendon and ligament healing. Gene Ther 3(12):1089–1093
Gilchrist CL, Darling EM et al (2011) Extracellular matrix ligand and stiffness modulate immature nucleus pulposus cell-cell interactions. PLoS One 6(11):e27170
Gronthos S, Zannettino AC et al (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 116(Pt 9):1827–1835

Guha U, Gomes WA et al (2002) In vivo evidence that BMP signaling is necessary for apoptosis in the mouse limb. Dev Biol 249(1):108–120
Hao J, Zhang Y et al (2015) Mechanobiology of mesenchymal stem cells: perspective into mechanical induction of MSC fate. Acta Biomater 20:1–9
Hayashi O, Katsube Y et al (2008) Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. Calcif Tissue Int 82(3):238–247
Hayes AJ, Tudor D et al (2008) Chondroitin sulfate sulfation motifs as putative biomarkers for isolation of articular cartilage progenitor cells. J Histochem Cytochem: Off J Histochem Soc 56(2):125–138
Henriksson H, Thornemo M et al (2009) Identification of cell proliferation zones, progenitor cells and a potential stem cell niche in the intervertebral disc region: a study in four species. Spine (Phila Pa 1976) 34(21):2278–2287
Hercz G (2001) Regulation of bone remodeling: impact of novel therapies. Semin Dial 14(1):55–60
Hunter CJ, Matyas JR et al (2003) The notochordal cell in the nucleus pulposus: a review in the context of tissue engineering. Tissue Eng 9(4):667–677
Jaalouk DE, Lammerding J (2009) Mechanotransduction gone awry. Nat Rev Mol Cell Biol 10(1):63–73
Jagodzinski M, Drescher M et al (2004) Effects of cyclic longitudinal mechanical strain and dexamethasone on osteogenic differentiation of human bone marrow stromal cells. Eur Cells Mater 7:35–41; discussion 41
Jiang Y, Tuan RS (2015) Origin and function of cartilage stem/progenitor cells in osteoarthritis. Nat Rev Rheumatol 11(4):206–212
Joggerst SJ, Hatzopoulos AK (2009) Stem cell therapy for cardiac repair: benefits and barriers. Expert Rev Mol Med 11:e20
Jones E, Crawford A (2014) High chondrogenic potential of synovial fluid-derived mesenchymal stromal cells. Cytotherapy 16(11):1595–1596
Jones EA, Crawford A et al (2008) Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level. Arthritis Rheum 58(6):1731–1740
Kannus P (2000) Structure of the tendon connective tissue. Scand J Med Sci Sports 10(6):312–320
Kardon G (2011) Development of the musculoskeletal system: meeting the neighbors. Development 138(14):2855–2859
Kasper G, Glaeser JD et al (2007) Matrix metalloprotease activity is an essential link between mechanical stimulus and mesenchymal stem cell behavior. Stem Cells 25(8):1985–1994
Kassem M, Bianco P (2015) Skeletal stem cells in space and time. Cell 160(1–2):17–19
Koelling S, Kruegel J et al (2009) Migratory chondrogenic progenitor cells from repair tissue during the later stages of human osteoarthritis. Cell Stem Cell 4(4):324–335
Korecki CL, Taboas JM et al (2010) Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype. Stem Cell Res Ther 1(2):18
Krawetz RJ, Wu YE et al (2012) Synovial fluid progenitors expressing CD90+ from normal but not osteoarthritic joints undergo chondrogenic differentiation without micro-mass culture. PLoS One 7(8):e43616
Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423(6937):332–336
Le Maitre CL, Freemont AJ et al (2007) Accelerated cellular senescence in degenerate intervertebral discs: a possible role in the pathogenesis of intervertebral disc degeneration. Arthritis Res Ther 9(3):R45
Lee JY, Qu-Petersen Z et al (2000) Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol 150(5):1085–1100
Lefebvre V, Huang W et al (1997) SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol 17(4):2336–2346

Lehmann TP, Filipiak K et al (2014) Coculture of human nucleus pulposus cells with multipotent mesenchymal stromal cells from human bone marrow reveals formation of tunnelling nanotubes. Mol Med Rep 9(2):574–582
Li H, Tao Y et al (2013) Influence of hypoxia in the intervertebral disc on the biological behaviors of rat adipose- and nucleus pulposus-derived mesenchymal stem cells. Cells Tissues Organs 198(4):266–277
Lin Y, Weisdorf DJ et al (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 105(1):71–77
Long F, Ornitz DM (2013) Development of the endochondral skeleton. Cold Spring Harbor Perspect Biol 5(1):a008334
Lui PP, Wong Y (2013) Higher BMP/Smad sensitivity of tendon-derived stem cells (TDSCs) isolated from the collagenase-induced tendon injury model: possible mechanism for their altered fate in vitro. BMC Musculoskelet Disord 14:248
Lukashev ME, Werb Z (1998) ECM signalling: orchestrating cell behaviour and misbehaviour. Trends Cell Biol 8(11):437–441
Lutz M, Knaus P (2002) Integration of the TGF-beta pathway into the cellular signalling network. Cell Signal 14(12):977–988
Ma Y, Zhang X et al (2009) Effect of bone morphogenetic protein-12 gene transfer on posterior cruciate ligament healing in a rabbit model. Am J Sports Med 37(3):599–609
Mackie EJ, Ahmed YA et al (2008) Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol 40(1):46–62
Madeira C, Santhagunam A et al (2015) Advanced cell therapies for articular cartilage regeneration. Trends Biotechnol 33(1):35–42
Maes C, Kobayashi T et al (2010) Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Dev Cell 19(2):329–344
Majewski M, Betz O et al (2008) Ex vivo adenoviral transfer of bone morphogenetic protein 12 (BMP-12) cDNA improves Achilles tendon healing in a rat model. Gene Ther 15(16):1139–1146
Martinek V, Latterman C et al (2002) Enhancement of tendon-bone integration of anterior cruciate ligament grafts with bone morphogenetic protein-2 gene transfer: a histological and biomechanical study. J Bone Joint Surg Am 84-A(7):1123–1131
Masuda K, Lotz JC (2010) New challenges for intervertebral disc treatment using regenerative medicine. Tissue Eng Part B Rev 16(1):147–158
Matsukura Y, Muneta T et al (2014) Mesenchymal stem cells in synovial fluid increase after meniscus injury. Clin Orthop Relat Res 472(5):1357–1364
Mauney JR, Sjostorm S et al (2004) Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro. Calcif Tissue Int 74(5):458–468
McCann MR, Tamplin OJ et al (2012) Tracing notochord-derived cells using a Noto-cre mouse: implications for intervertebral disc development. Dis Model Mech 5(1):73–82
McCarthy HE, Bara JJ et al (2012) The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. Vet J 192(3):345–351
Meisel HJ, Siodla V et al (2007) Clinical experience in cell-based therapeutics: disc chondrocyte transplantation A treatment for degenerated or damaged intervertebral disc. Biomol Eng 24(1):5–21
Menasche P, Alfieri O et al (2008) The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. Circulation 117(9):1189–1200
Mendez-Ferrer S, Michurina TV et al (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466(7308):829–834
Mern DS, Beierfuss A et al (2014) Enhancing human nucleus pulposus cells for biological treatment approaches of degenerative intervertebral disc diseases: a systematic review. J Tissue Eng Regen Med 8(12):925–936

Mienaltowski MJ, Adams SM et al (2014) Tendon proper- and peritenon-derived progenitor cells have unique tenogenic properties. Stem Cell Res Ther 5(4):86
Minguell JJ, Erices A et al (2001) Mesenchymal stem cells. Exp Biol Med 226(6):507–520
Minogue BM, Richardson SM et al (2010) Characterization of the human nucleus pulposus cell phenotype and evaluation of novel marker gene expression to define adult stem cell differentiation. Arthritis Rheum 62(12):3695–3705
Mizrahi O, Sheyn D et al (2013) Nucleus pulposus degeneration alters properties of resident progenitor cells. Spine J 13(7):803–814
Mochida J, Sakai D et al (2015) Intervertebral disc repair with activated nucleus pulposus cell transplantation: a three-year, prospective clinical study of its safety. Eur Cell Mater 29:202–212; discussion 212
Morigele M, Shao Z et al (2013) TGF-beta1 induces a nucleus pulposus-like phenotype in Notch 1 knockdown rabbit bone marrow mesenchymal stem cells. Cell Biol Int 37(8):820–825
Murao H, Yamamoto K et al (2013) Periosteal cells are a major source of soft callus in bone fracture. J Bone Miner Metab 31(4):390–398
Nasef A, Mathieu N et al (2007) Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. Transplantation 84(2):231–237
Okada M, Payne TR et al (2008) Myogenic endothelial cells purified from human skeletal muscle improve cardiac function after transplantation into infarcted myocardium. J Am Coll Cardiol 52(23):1869–1880
Ono N, Ono W et al (2014) Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. Dev Cell 29(3):330–339
Orlic D, Kajstura J et al (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410(6829):701–705
Ornitz DM, Marie PJ (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev 16(12):1446–1465
Oryan A, Monazzah S et al (2015) Bone injury and fracture healing biology. Biomed Environ Sci: BES 28(1):57–71
Osugi M, Katagiri W et al (2012) Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. Tissue Eng A 18(13–14):1479–1489
Otto WR, Wright NA (2011) Mesenchymal stem cells: from experiment to clinic. Fibrogenesis Tissue Repair 4:20
Pan Q, Yu Y et al (2008) Sox9, a key transcription factor of bone morphogenetic protein-2-induced chondrogenesis, is activated through BMP pathway and a CCAAT box in the proximal promoter. J Cell Physiol 217(1):228–241
Peault B, Rudnicki M et al (2007) Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol Ther: J Am Soc Gene Ther 15(5):867–877
Peng H, Huard J (2004) Muscle-derived stem cells for musculoskeletal tissue regeneration and repair. Transpl Immunol 12(3–4):311–319
Pittenger MF, Mackay AM et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147
Poolman RW, Abouali JA et al (2007) Overlapping systematic reviews of anterior cruciate ligament reconstruction comparing hamstring autograft with bone-patellar tendon-bone autograft: why are they different? J Bone Joint Surg Am 89(7):1542–1552
Purmessur D, Schek RM et al (2011) Notochordal conditioned media from tissue increases proteoglycan accumulation and promotes a healthy nucleus pulposus phenotype in human mesenchymal stem cells. Arthritis Res Ther 13(3):R81
Qu-Petersen Z, Deasy B et al (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol 157(5):851–864
Rasini V, Dominici M et al (2013) Mesenchymal stromal/stem cells markers in the human bone marrow. Cytotherapy 15(3):292–306
Ren G, Chen X et al (2012) Concise review: mesenchymal stem cells and translational medicine: emerging issues. Stem Cells Transl Med 1(1):51–58

Risbud MV, Albert TJ et al (2004) Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy. Spine (Phila Pa 1976) 29(23):2627–2632
Risbud MV, Guttapalli A et al (2007) Evidence for skeletal progenitor cells in the degenerate human intervertebral disc. Spine (Phila Pa 1976) 32(23):2537–2544
Risbud MV, Schoepflin ZR et al (2014) Defining the phenotype of young healthy nucleus pulposus cells: recommendations of the Spine Research Interest Group at the 2014 annual ORS meeting. J Orthop Res
Roberts SJ, van Gastel N et al (2015) Uncovering the periosteum for skeletal regeneration: the stem cell that lies beneath. Bone 70:10–18
Rosen ED, Spiegelman BM (2000) Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol 16:145–171
Roughley PJ (2004) Biology of intervertebral disc aging and degeneration: involvement of the extracellular matrix. Spine (Phila Pa 1976) 29(23):2691–2699
Rui YF, Lui PP et al (2010) Isolation and characterization of multipotent rat tendon-derived stem cells. Tissue Eng Part A 16(5):1549–1558
Rumian AP, Wallace AL et al (2007) Tendons and ligaments are anatomically distinct but overlap in molecular and morphological features – a comparative study in an ovine model. J Orthop Res 25(4):458–464
Saitta B, Passarini J et al (2014) Patient-derived skeletal dysplasia induced pluripotent stem cells display abnormal chondrogenic marker expression and regulation by BMP2 and TGFbeta1. Stem Cells Dev 23(13):1464–1478
Sakai D, Nakamura Y et al (2012) Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. Nat Commun 3:1264
Schafer J, O'Connor D et al (2007) Medicare Evidence Development and Coverage Advisory Committee Meeting on lumbar fusion surgery for treatment of chronic back pain from degenerative disc disease. Spine (Phila Pa 1976) 32(22):2403–2404
Seale P, Sabourin LA et al (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102(6):777–786
Sekiya I, Ojima M et al (2012) Human mesenchymal stem cells in synovial fluid increase in the knee with degenerated cartilage and osteoarthritis. J Orthop Res: Off Publ Orthop Res Soc 30(6):943–949
Sensebe L, Gadelorge M et al (2013) Production of mesenchymal stromal/stem cells according to good manufacturing practices: a review. Stem Cell Res Ther 4(3):66
Seol D, McCabe DJ et al (2012) Chondrogenic progenitor cells respond to cartilage injury. Arthritis Rheum 64(11):3626–3637
Sharma RR, Pollock K et al (2014) Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. Transfusion 54(5):1418–1437
Smith MD (2011) The normal synovium. Open Rheumatol J 5:100–106
Song B, Estrada KD et al (2009) Smad signaling in skeletal development and regeneration. Cytokine Growth Factor Rev 20(5–6):379–388
Sotiropoulou PA, Perez SA et al (2006) Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells 24(1):74–85
Steinert AF, Weber M et al (2008) In situ IGF-1 gene delivery to cells emerging from the injured anterior cruciate ligament. Biomaterials 29(7):904–916
Stone KR, Abdel-Motal UM et al (2007) Replacement of human anterior cruciate ligaments with pig ligaments: a model for anti-non-gal antibody response in long-term xenotransplantation. Transplantation 83(2):211–219
Streuli C (1999) Extracellular matrix remodelling and cellular differentiation. Curr Opin Cell Biol 11(5):634–640
Takahashi I, Nuckolls GH et al (1998) Compressive force promotes sox9, type II collagen and aggrecan and inhibits IL-1beta expression resulting in chondrogenesis in mouse embryonic limb bud mesenchymal cells. J Cell Sci 111(Pt 14):2067–2076

Tan Q, Lui PP et al (2012) Comparison of potentials of stem cells isolated from tendon and bone marrow for musculoskeletal tissue engineering. Tissue Eng Part A 18(7–8):840–851
Tan C, Lui PP et al (2014) Scx-transduced tendon-derived stem cells (tdscs) promoted better tendon repair compared to mock-transduced cells in a rat patellar tendon window injury model. PLoS One 9(5):e97453
Theise ND, Nimmakayalu M et al (2000) Liver from bone marrow in humans. Hepatology 32(1):11–16
Urban JP, Roberts S (2003) Degeneration of the intervertebral disc. Arthritis Res Ther 5(3):120–130
Urwin M, Symmons D et al (1998) Estimating the burden of musculoskeletal disorders in the community: the comparative prevalence of symptoms at different anatomical sites, and the relation to social deprivation. Ann Rheum Dis 57(11):649–655
Usas A, Maciulaitis J et al (2011) Skeletal muscle-derived stem cells: implications for cell-mediated therapies. Medicina 47(9):469–479
Ustunel I, Ozenci AM et al (2008) The immunohistochemical localization of notch receptors and ligands in human articular cartilage, chondroprogenitor culture and ultrastructural characteristics of these progenitor cells. Acta Histochem 110(5):397–407
van den Akker GG, Surtel DA et al (2014) Novel immortal human cell lines reveal subpopulations in the nucleus pulposus. Arthritis Res Ther 16(3):R135
van Gastel N, Torrekens S et al (2012) Engineering vascularized bone: osteogenic and proangiogenic potential of murine periosteal cells. Stem Cells 30(11):2460–2471
Wan C, He Q et al (2006) Nonadherent cell population of human marrow culture is a complementary source of mesenchymal stem cells (MSCs). J Orthop Res: Off Publ Orthop Res Soc 24(1):21–28
Wang JH, Thampatty BP (2006) An introductory review of cell mechanobiology. Biomech Model Mechanobiol 5(1):1–16
Wang QW, Chen ZL et al (2005) Mesenchymal stem cells differentiate into tenocytes by bone morphogenetic protein (BMP) 12 gene transfer. J Biosci Bioeng 100(4):418–422
Wang Q, Huang C et al (2011) Expression of endogenous BMP-2 in periosteal progenitor cells is essential for bone healing. Bone 48(3):524–532
Werb Z (1997) ECM and cell surface proteolysis: regulating cellular ecology. Cell 91(4):439–442
Williams R, Khan IM et al (2010) Identification and clonal characterisation of a progenitor cell sub-population in normal human articular cartilage. PLoS One 5(10):e13246
Wolfman NM, Hattersley G et al (1997) Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family. J Clin Invest 100(2):321–330
Woods GA, Indelicato PA et al (1991) The Gore-Tex anterior cruciate ligament prosthesis. Two versus three year results. Am J Sports Med 19(1):48–55
Worthley DL, Churchill M et al (2015) Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. Cell 160(1–2):269–284
Wu L, Bluguermann C et al (2013) Human developmental chondrogenesis as a basis for engineering chondrocytes from pluripotent stem cells. Stem Cell Rep 1(6):575–589
Wuchter P, Bieback K et al (2015) Standardization of Good Manufacturing Practice-compliant production of bone marrow-derived human mesenchymal stromal cells for immunotherapeutic applications. Cytotherapy 17(2):128–139
Yablonka-Reuveni Z, Paterson BM (2001) MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. J Histochem Cytochem: Off J Histochem Soc 49(4):455–462
Yu YY, Lieu S et al (2010) Immunolocalization of BMPs, BMP antagonists, receptors, and effectors during fracture repair. Bone 46(3):841–851
Zhang X, Xie C et al (2005) Periosteal progenitor cell fate in segmental cortical bone graft transplantations: implications for functional tissue engineering. J Bone Miner Res: Off J Am Soc Bone Miner Res 20(12):2124–2137

Zhang ZL, Tong J et al (2009) Therapeutic potential of non-adherent BM-derived mesenchymal stem cells in tissue regeneration. Bone Marrow Transplant 43(1):69–81
Zhang K, Zhang S et al (2014) Effects of celecoxib on proliferation and tenocytic differentiation of tendon-derived stem cells. Biochem Biophys Res Commun 450(1):762–766
Zhao C-Q, Wang L-M et al (2007) The cell biology of intervertebral disc aging and degeneration. Ageing Res Rev 2(6):247–261

Chapter 13
Pancreas-Derived Multipotent Progenitors

Fang-Xu Jiang and Grant Morahan

Abstract Identification of pancreas-derived multipotent progenitors (PMP) would not only be important in pancreas developmental biology, but also pivotal for generation of insulin-secreting islet β cells. Deficiency of these cells is a feature of Type 1 diabetes and Type 2 diabetes mellitus, a pandemic metabolic disorder. The cure of which would therefore be to replace the lost or deficient β cells, by transplantation of donated islets or *in vitro* differentiated endocrine cells or by regeneration of endogenous islet cells. Thus, endogenous sources that can be directed to becoming insulin-secreting cells are actively sought after. In particular, any cell types in the developing or adult pancreas that may act as PMP would provide an alternative renewable source for endogenous regeneration. In this review, we will summarize the latest progress and knowledge of such PMP, and discuss ways that facilitate the future studies advances in this crucial field.

Keywords Pancreas-derived multipotent progenitors • Regeneration • Self-renewal • Clonogenesis • Differentiation

13.1 Introduction

Diabetes mellitus is a worldwide public health issue and has an increasing prevalence. This metabolic disorder currently affects over 382 million people, and the number is likely to increase to 592 million by 2035 (http://www.idf.org/diabetesatlas). Approximately 10 % of these cases are of type 1 diabetes mellitus (T1D), caused by absolute deficiency of insulin-producing β cells caused by autoimmune destruction. Should autoimmunity to β cells be controlled, a regenerative therapy would be a desirable avenue towards a cure of T1D, either by transplantation of

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_13

donated hormone-secreting islets, or in *vitro* generated functional β cells from pluripotent stem cells (PSC) or by regeneration *in situ* of endogenous β cells. The remaining 90 % of cases are of type 2 diabetes mellitus (T2D), caused by the failure to respond to insulin action by the peripheral tissues, the failure to control the production of glucose by the liver and the failure of islet β cells (Halban et al. 2014). The latter is classically attributed to the increased rate of islet apoptosis (Butler et al. 2007). Recently, substantial evidence has indicated that β-cell failure in T2D may be caused by β cell dedifferentiation (Hanley et al. 2008; Talchai et al. 2012). Thus, restoring function of dedifferentiated β cells would be a new type of regenerative therapy for T2D. With the aim of developing regenerative medicine therapies, research into stem cells and progenitors has recently drawn much attention.

Generally, multipotent progenitors are exchangeable with the concept of stem cells in which pluripotent stem cells are capable of giving rise to all 210 cell-types that make up the body. "Stem cell" is usually used to describe those undifferentiated cells that are capable of both self-renewal and giving rise to specialized functional cells. Arguably, "multipotent progenitors" should have many features of stem cells. Depending on their developmental potential, progenitors could be multipotent, oligopotent or unipotent. Stem cells/multipotent progenitors are of pivotal importance for organ and tissue integrity and for injury and disease repair. Pancreas-derived multipotent progenitors (PMP) would be a rare population residing in the pancreas, and show a powerful potential for regeneration when required although their presence and origin have not yet completely uncovered and are still hotly debated. In this article, we will discuss potential PMP in three major compartments of the pancreas and along the islet lineage developmental pathway. We will also explore future directions of research using these cells.

13.2 Molecular Embryology

The pancreas is derived from the posterior foregut. The endoderm is one of the three primitive germ layers originating from the inner cell mass during gastrulation. The pancreatic endoderm is a thickened epithelium along the dorsal and ventral surfaces. Subsequently, the thickened endoderm epithelia evaginate into the surrounding mesoderm-derived mesenchymal tissue and form dorsal and ventral pancreatic buds. These buds continue to expand, branch and fuse as a result of the gut rotation that brings the buds together. The fused developing pancreas continues to proliferate, differentiate and, ultimately, give rise to the mature organ. The adult pancreas consists of the acinar tissue that secretes digestive enzymes, the islets of Langerhans that secrete hormones responsible for glucose homeostasis, and the ductal tissue that delivers pancreas-derived digestive fluid to the intestine.

Remarkably, PSC can be guided to differentiate to definitive endoderm (DE) cells *in vitro* by applying information from *in vivo* developmental pathways (Fig. 13.1). This can be achieved in the presence of a high concentration of activin A, a member of the transforming growth factor β superfamily. Furthermore,

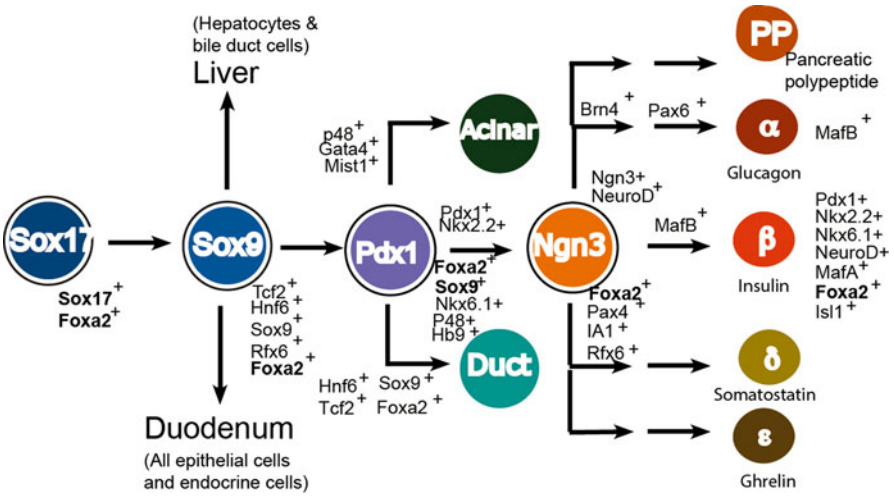

Fig. 13.1 Multiple fate selections allow the development of the pancreas islet lineages. Committed from one of three germ layers (the ectoderm, mesoderm and endoderm) during gastrulation, the definitive endodermal cells (DE) are marked by the expression of Sox17 (the Sry-related HMG box transcription factor 17) and Foxa2 (foxhead homeobox 2a). Along the anterior-posterior axis the DE is divided into foregut (giving rise to the lung, thyroid and oesophagus), posterior foregut [PF, marked by the expression of the transcription factor Hnf4a (hepatocyte nuclear factor 4a) and hindgut (committing the intestine and colon). *In vitro*, retinoid acid would direct the DE cells to PF cells. Largely to the liver and duodenum, a fraction of the PF cells give rise to pancreatic progenitors (PP, marked by the expression of the transcription factor Pdx1). Mostly to the exocrine and ductal tissues, the PP commits to progenitors of the endocrine islet lineages [IP, marked by the expression of Ngn3, as well as NeuroD (neural differentiation 1), IA1 (insulinoma associated 1), Isl1 (Islet 1), Pax6 (paired box factor 6) and Rfx6]. The IP then differentiates into at least five types of islet cells [α, β, δ (somatostatin), PP (pancreatic polypeptide) and ε (ghrelin)]

PSC-derived human expandable DE cells have been reported to self-renew in the presence of a group of growth factors including bone morphogenetic protein 4 (BMP4), fibroblast growth factor 2, vascular endothelial growth factor and epidermal growth factor (EGF) (Cheng et al. 2012). These progenitors can be passaged at least 24 times with a population expansion of hundreds of thousands fold. Although further studies are required, the endodermal progenitors may indeed have an astonishing capacity for expansion and act as pre-PMP.

The sequence of key developmental events in human growth is not identical to that observed during mouse development (Richardson et al. 1997). For example, the dorsal bud can be detected as early as 26 days postcoitum (dpc) which is an equivalent stage to E9.5 mouse embryos, but insulin-positive cells are not visible until 52 dpc, approximately 2 weeks later than the equivalent stage at which they can be detected in mice. The appearance of human insulin-positive cells precedes that of glucagon-positive cells at 8–10 weeks of development (Piper et al. 2004). All islet cells are detectable at the end of the first trimester in humans (Piper et al. 2004), but at later stages (E17.5) in mice (Herrera et al. 1991). Not surprisingly, there are also

differences in gene expression patterns during developmental and disease processes between these species (Fougerousse et al. 2000). More details of human pancreas development can be found in reviews elsewhere (De Krijger et al. 1992; Lukinius et al. 1992; Pan and Brissova 2014; Polak et al. 2000).

13.3 PMP May Be Present in Three Pancreas Compartments

Substantial *in vitro* evidence has indicated that PMP may be present in all three major pancreas compartments of both rodents and humans: the ductal epithelium (Cornelius et al. 1997; Ramiya et al. 2000; Suzuki et al. 2002), acinar tissue and islets of Langerhans (Seaberg et al. 2004; Zulewski et al. 2001). For example, a potential PMP candidate in the developing and adult mouse pancreas was purified by flow cytometry (Suzuki et al. 2004). These cells are defined by expression of the receptor for hepatocyte growth factor, c-Met, and the absence of blood cell surface markers such as CD45, TER119, c-Kit, and Flk-1. The isolated cells can differentiate into multiple pancreatic lineage cells *in vitro* and give rise to pancreatic endocrine and exocrine cells *in vivo* after transplantation (Suzuki et al. 2004). However, the *in vivo* localization and the molecular characteristics of these c-met expressing cells are largely unknown and single-cell clonogenesis has not been established.

13.3.1 Are PMP Present in the Ductal Epithelium?

In vitro and *in vivo* experiments have suggested that PMP are present in the ductal epithelium. Bonner-Weir and colleagues were the first to report that adult human pancreatic ductal epithelial cells gave rise to islet-like clusters and differentiate into insulin-secreting β cells (Bonner-Weir et al. 2000). Ramiya and colleagues reported that *in vitro* generated islet-like structures from mouse PMP were capable of reversing diabetes after transplantation (Ramiya et al. 2000). In cultures of "pancreatic ductal cell aggregates" after purification of human islets, fibroblast-like cells grew out as "pancreatic mesenchymal stem cells" (MSC). The latter were reported to undergo at least 12 passages and expressed a range of bone marrow-derived MSC markers including CD13, CD29, CD44, CD54, CD105, α_6 integrin subunit (also known as CD49f) and Thy1 (also known as CD90). The pancreatic MSC were shown to be capable of generating at least two germ layer cells including endoderm-derived cells, but there was no convincing evidence of pancreatic lineage cells (Seeberger et al. 2006). Using culture conditions suitable for generating neurospheres *ex vivo*, mouse pancreatic ductal cells were shown to give rise to neurosphere-like structures that subsequently differentiated into several types of islet cells, including β cells (Seaberg et al. 2004). However, the molecular signature of these unique cells has not been defined. A limiting feature of all the above studies is their use of mixed cell populations, while most did not demonstrate clonogenesis.

Interestingly, after pancreatic duct ligation (PDL), numerous CK19$^+$ ductal cells can be regenerated and give rise to islet cells (Xu et al. 2008). Their lineage relationship has been independently addressed using the genetic Cre-loxP tracing system. In this system, Cre expression was directed by the promoter of carbonic anhydrase II, a marker of mature ductal cells, resulting in the excision of the stop cassette (Rosa-loxP-stop-loxP-lacZ) in transgenic Rosa26 (R26R) mice. This leads to β-galactosidase activity in cells expressing Cre (in this case, ductal cells). Four weeks later in normal or PDL pancreas, β-galactosidase was detectable in many ducts, patches of acinar cells and 35–40 % of islet cells (Bonner-Weir et al. 2008; Inada et al. 2008). These data provided strong evidence that carbonic anhydrase II-expressing ductal epithelial cells are capable of producing islet cells, at least in mice. It is still unknown, however, whether all or part of the carbonic anhydrase II-expressing cell population has this developmental potential; and whether the differentiation proceeds from PMP or does so as a trans differentiation process from mature ductal cells.

In contrast, when an exon of *Tcf2* (also known as *Hnf1β*, marking pancreatic ductal cells) was replaced with a Cre-containing transgene, genetic lineage tracing showed that the postnatal Tcf2$^+$ cells in these transgenic mice did not differentiate into islet cells, under both normal and after PDL (Solar et al. 2009). However, a complication of this study is that one copy of *Tcf2* was non-functional, resulting in halving the production of Tcf2. Heterozygous *Tcf2* mutant mice and humans display pancreatic agenesis (Haumaitre et al. 2005, 2006), so haploinsufficiency of this gene in tracing experiments may have compromised the differentiation of ductal progenitors into functional islet cells.

Fbw7 (F-box and WD-40 domain protein 7), an ubiquitin ligase, is expressed in embryonic and adult ductal epithelial cells, deletion of which stabilizes the heavily ubiquitinated neurogenin 3 (Ngn3), an endocrine determinant transcription factor, and reprograms the ductal cells to glucagon-producing α, insulin-producing β and somatostatin-producing δ cells (Sancho et al. 2014). This study suggests that pancreatic ductal cells are a latent PMP and Fbw7 is a critical cell-fate regulator. Nevertheless, this report did not describe whether all or a fraction of the ductal cells express Fbw7; the frequency of the reprogramming event, both are critical to assess whether this might become a viable strategy to regenerate islet cells by suppressing the Fbw7 signalling. A fundamental assay on reprogrammed cells to ameliorate diabetes has not been reported.

Interestingly, PDL activates robustly the Wnt signalling pathway and allows in the regenerating ducts the appearance of Lgr5 expression (Huch et al. 2013), a Wnt target which marks actively dividing stem cells such as in the intestine (Barker et al. 2007). Purified ductal Lgr5-expressing cells are also responsive to spondin 1 and form clonal 3D pancreatic organoids within the Matrigel-formed gel that generate ductal as well as endocrine lineages upon transplantation (Huch et al. 2013).

13.3.2 Are PMP Present in Pancreatic Acinar Tissue?

In the transplantation clinic, a large population of acinar cells would be discarded after purification of islets from donated pancreas. Identification of potential value for pancreatic acinar cells has thus drawn significant interest. After co-transplantation with foetal pancreatic cells under the kidney capsule of immunodeficient mice, these acinar cells gave rise to endocrine cells without evidence of β-cell replication or cell fusion (Hao et al. 2006). These experiments suggest the existence of PMP or progenitor cells within the acinar compartment of the adult human pancreas. Consistently, analysis using the Cre/loxP-based tracing system demonstrated that amylase/elastase-expressing exocrine cells were able to give rise to insulin-positive cells in a suspension culture (Minami et al. 2005). However, because a clonal self-renewal assay of these amylase/elastase-expressing cells and their intermediate steps has not been demonstrated, this study may simply reveal that mouse and rat pancreatic exocrine cells are able to trans differentiate into surrogate insulin-expressing cells (Baeyens et al. 2005; Minami et al. 2008). This possibility was supported by a recent study which showed that mouse acinar cells could be directly re-programmed *in vivo* to β-like cells using only three transcription factor genes, namely, *Pdx1* (*pancreas and duodenum homeobox 1*), *Ngn3* and *MafA* (*musculo-aponeurotic fibrosarcoma oncogene family protein A*) (Zhou et al. 2008). Nevertheless, as the donated acinar cells (Hao et al. 2006) were not purified by flow cytometry, the possibility of contamination by ductal or even islet cells cannot be completely ruled out. Similar lineage tracing experiments *in vivo* demonstrate that after 70–80 % pancreatectomy, pre-existing mouse pancreatic acinar cells do not contribute to the regeneration of islet β cells (Desai et al. 2007). These contradictory findings remain to be reconciled.

13.3.3 Are PMP Present in the Islets?

Accumulating evidence suggest that the islets of Langerhans harbour the PMP. Whether distinct nestin$^+$ and hormone$^-$ (insulin, glucagon, somatostatin, pancreatic polypeptide or ghrelin) cells in both rat and human islets are PMP has not yet been established. These nestin$^+$ cells are reported to proliferate in culture extensively (~8 months) and give rise to cells that express liver and acinar pancreas markers, including α-fetoprotein, pancreatic amylase and a ductal/endocrine phenotype with the expression of CK19, neural-specific cell adhesion molecule, insulin, glucagon and PDX1. These nestin$^+$ PMP may therefore participate in the neogenesis of islet endocrine cells (Zulewski et al. 2001), mediated at least partially by glucagon-like peptide-1, an incretin hormone processed from proglucagon (Abraham et al. 2002). It remains however unclear either islet and duct-derived multipotent precursor cells that give rise to neurosphere-like structures and subsequently differentiated into islet β-like cells (Seaberg et al. 2004) or the outgrown fibroblast-like cells that

proliferate readily and give rise *in vitro* to hormone-expressing non-typical islet cell aggregates (Gershengorn et al. 2004) or both are nestin$^+$. Studies on human islets transfected with a rat insulin promoter (RIP)-containing transgene demonstrated that RIP-expressing cells were dedifferentiated to fibroblast-like cells with up to 16 population doublings with undetectable insulin expression (Russ et al. 2008). Nevertheless, *In vivo* studies indicate that Nestin$^+$ cells are mostly restricted in non-endodermal-derived cells (Lardon et al. 2002; Selander and Edlund 2002). A rigorous genetic-based lineage tracing in mice under the control of *Pdx1* or *RIP* also demonstrated that PDX1- or RIP-expressing cells did not contribute significantly to these fibroblast-like cells *in vitro* (Chase et al. 2007). These discrepancies require future clarification.

13.4 Developmental Intermediates as Potential PMP

13.4.1 Sox9-Expressing Pluripotent Progenitors

Protocols for generation of cystic E-cadherin–expressing epithelial colonies were first developed by our group (Fig. 13.2). Dissociated foetal pancreatic cells in the presence of laminin 1,1,1 and BMPs generated cystic colonies containing β cells (Jiang and Harrison 2005a; Jiang et al. 2002). Interestingly, colonies promoted by EGF were spherical but not cystic (Jiang and Harrison 2005b), suggesting they may have different developmental potential. However, we did not determine whether all cystic epithelial colonies stochastically committed to various lineages or only certain fractions of cystic epithelial colonies had defined differentiation potential. Now it seems clear that our cystic colonies were generated from Sox9$^+$ multipotent progenitors (see below).

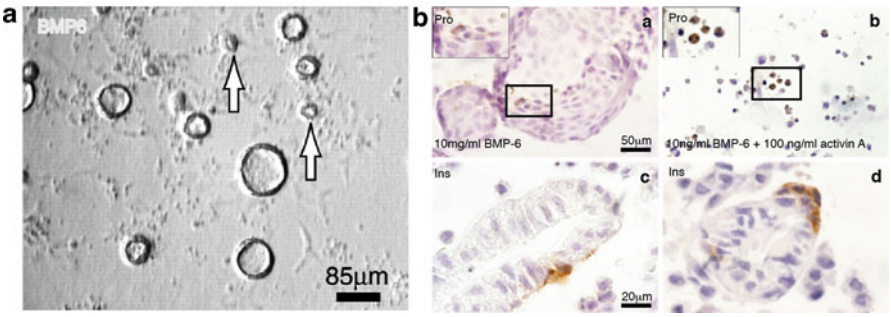

Fig. 13.2 Cystic colony formation from dissociated foetal mouse pancreas cells. (**a**) Phase contrast image showing that BMP6 promotes colony formation. *Open arrows* indicate colonies ≤30 μm. (**b**) Immunocytochemical analyses. (**a**) Proinsulin staining. Fixed colonies were stained with proinsulin antibody (*brown*). (**b**) Activin A antagonizes colony formation. (**c**, **d**) Insulin staining. Histological sections of harvested colonies were stained with anti-insulin antibody (*brown*) (Adapted and modified from Jiang et al. (2002))

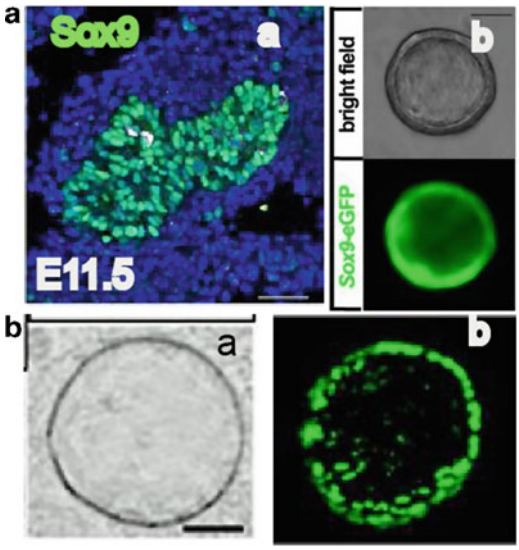

Fig. 13.3 Cystic colonies generated from Sox9-tagged cells. (**a**) Embryonic Sox9$^+$ progenitors in the pancreas capable of generating cystic colonies. (**a**) Sox9 is expressed in most ductal progenitors in E11.5 mouse pancreas. (**b**) Cystic colonies are formed from purified Sox9-eGFP$^+$ progenitors in E11.5 mouse pancreas. (**b**) Purified Sox9$^+$ cells in adult mouse pancreas capable of generating cystic colonies under a phase contrast (**a**) or a fluorescence microscope (**b**) (Adapted and modified from Jin et al. (2013); Sugiyama et al. (2013))

Sox genes are members of the Sry (sex determining region Y) box-related high-mobility group box transcription factor family and are versatile regulators of stem/progenitor cell fate (Sarkar and Hochedlinger 2013). *Sox9* plays pivotal roles in embryonic development of several organs including the pancreas. This critical transcription factor can be detected at E10.5 in the dorsal and ventral pancreatic epithelia (Lynn et al. 2007). Importantly, Sox9-expressing embryonic pancreatic epithelia at E13.5 (Fig. 13.3) are capable of giving rise to all acinar, ductal and islet lineages in the pancreas (Furuyama et al. 2011). However, *Sox9* expression is gradually confined to pancreatic duct cells by E16.5 (Furuyama et al. 2011). Lineage tracing studies demonstrate that *Sox9* is also expressed in other posterior foregut-derived organs including the bile duct, the duodenum and the liver. For example, it is expressed in bile duct cells adjacent to the portal vein from E16.5. *Sox9* also is broadly expressed in the intestinal epithelia at E13.5 but restricted to the crypt from E18.5 (Furuyama et al. 2011). Hence these *Sox9*-expressing cells in the posterior foregut region could be PMP. Consistent with these observations, Sox9-expressing multipotent progenitors purified from E11.5 Sox9-eGFP embryos form expandable cystoid colonies containing endocrine cells in a Matrigel-containing culture system (Sugiyama et al. 2013).

A methylcellulose-based semisolid medium containing Matrigel allows growth of duct-like "ring/dense" colonies from CD133$^+$Sox9$^+$ ductal cells (19 %). With the

addition of the roof plate-specific spondin 1, a Wnt agonist, ring/dense colony-forming cells can be expanded more than 100,000-fold when serially dissociated and replated in the presence of Matrigel (Jin et al. 2013). The $CD133^{+}Sox9^{+}$ cells generate endocrine/acinar colonies in a laminin hydrogel (Jin et al. 2013). Future studies are required to determine whether $CD133^{+}Sox9^{+}$ cells are the same as, or different from, the *Lgr5*-expressing cells. Nevertheless, Sox9-positive duct and centroacinar cells have not been shown to regenerate β cells in several *in vivo* regeneration or trans differentiation experiments such as partial pancreatectomy, cerulean-induced acute pancreatitis, a streptozotocin-diabetes and PDL model, or *in vitro* culture experiments (Furuyama et al. 2011). A similar conclusion is drawn from a conditional lineage tracing study (Kopp et al. 2011). Due to a common developmental origin, a human bile duct progenitor population known as "biliary tree-derived cells" (Wang et al. 2013) may behave as PMP and differentiate towards pancreatic endocrine lineages. However, the purified bile duct progenitor population, detailed molecular profile and developmental potential require further investigations.

13.4.2 Pdx1-Expressing Pancreatic Progenitors

A group of special cells in the thickened DE epithelium along the dorsal and ventral surfaces of the posterior mouse foregut at E9.0–9.5 expresses the gene named *Pdx1*. Pdx1 is a member of the parahox homeobox transcription factor family and is essential for both the expansion of pancreas primordial populations (Jonsson et al. 1994) and the function of adult β cells (Gao et al. 2014; Ohneda et al. 2000).

Genetic lineage tracing experiments demonstrated that Pdx1-expressing ($Pdx1^{+}$) progenitors give rise to acinar, duct and endocrine tissues in the pancreas (Gu et al. 2002). These progenitors are located at the tip of the branching pancreatic tree marked by $Pdx1^{+}Ptf1a^{+}$ (pancreas transcription factor 1a) $Cpa1^{+}$ (carboxypeptidase 1) (Zhou et al. 2007). The $Pdx1^{+}$ cells are able to take up bromodeoxyuridine (BrdU) (Seymour et al. 2007), a thymidine analogue incorporated into DNA during S-phase of the cell cycle, indicating that these cells are proliferative.

Following *in vivo* developmental pathways, PSC can be directed to give rise to $Pdx1^{+}$ cells that are able to proliferate 16-fold in the presence of pancreas-derived mesenchymal cells (Sneddon et al. 2012). To verify this capacity of the $Pdx1^{+}$ cells, independent confirmation of these results will be essential. Caution should be taken because there are as yet no data to demonstrate that these PSC-derived $Pdx1^{+}$ cells are the equivalent of pancreatic $Pdx1^{+}$ progenitors, since *Pdx1* is also expressed in other non-pancreas endoderm-derived tissues (Holland et al. 2005).

In humans, numerous $PDX1^{+}$ cells can be detected easily in the pancreas between 8 and 21 weeks of age (Jeon et al. 2009; Lyttle et al. 2008). The number of $PDX1^{+}$ cells that also express insulin or somatostatin progressively increases during this period of development (Lyttle et al. 2008). An unanswered fundamental question is

whether the PDX1$^+$ cells are generated by self-renewal or commitment from their endodermal progenitors or both.

Identification of a specific marker that allows the purification of PSC-derived Pdx1$^+$ pancreatic progenitors would be valuable. The capacity of Pdx1$^+$ progenitors to proliferate and self-renew *in vitro* needs to be evaluated and established. Recently, reserpine and tetrabenzine, both inhibitors of vesicular monoamine transporter-2, were shown to be able to differentiate PSC-derived Pdx1$^+$ cells into *Ngn3*-expressing cells (Sakano et al. 2014). Nevertheless, caution has to be taken for the use of genetic lineage tracing in PSC differentiation because temporospatial cues are critical for the success of *in vivo* lineage tracing studies [see review by (Jiang and Morahan 2015)]. Furthermore, it would be interesting to address whether all or only a fraction of Pdx1$^+$ progenitors commit along the endocrine pathway, and whether the PSC-derived Ngn3-expressing cells were *bona fide* islet progenitors.

13.4.3 Ngn3-Expressing Islet Progenitors

At around E9.5 in mice, a small group of cells in the thickened posterior foregut DE epithelium begins to express the basic helix-loop-helix transcription factor Ngn3 (Gradwohl et al. 2000; Gu et al. 2002; Xu et al. 2008). These Ngn3$^+$ cells are islet progenitors because they can give rise to all islet lineage cells. A number of observations support the importance of Ngn3 in islet development: islet cells do not develop in *Ngn3* knockout mice (Gradwohl et al. 2000); gene lineage tracing shows that Ngn3$^+$ cells give rise to all pancreatic endocrine cells (Gu et al. 2002); in adult pancreas, purified Ngn3$^+$ cells activated by PDL can, after injection into a foetal pancreas *in vitro*, differentiate into all islet cell types (Xu et al. 2008). Nevertheless, another laboratory reported that PDL allows activation of Ngn3 expression but the Ngn3$^+$ cells were not able to complete the entire β-cell regeneration (Kopp et al. 2011) while a more recent study found that β-cell mass and insulin content were totally unchanged by PDL-induced injury (Rankin et al. 2013). These inconsistencies require careful reconciliation.

Whereas mouse *Ngn3* mRNA expression peaks around E15.5 (Schwitzgebel et al. 2000) (equivalent to week 9 in humans), human *NGN3* expression is low prior to 9 weeks, but from 9 weeks onward, its expression increases sharply and remains high until 17 weeks (Jeon et al. 2009). Although a few studies showed that Ngn3$^+$ cells could proliferate (Jensen et al. 2000; Oliver-Krasinski et al. 2009), the recent genetic clonal assays by "mosaic analysis with double marker" (MADM) demonstrated that Ngn3$^+$ cells are quiescent and give rise to a single islet cell type (Desgraz and Herrera 2009). Consistent with this, a molecular study showed that the expression of Ngn3 inhibits proliferation by inducing *cyclin-dependent kinase inhibitor 1a* (*Cdkn1a*) (Miyatsuka et al. 2011). The apparent contradiction between the earlier and latest studies may arise from different interpretations, and requires future studies to reconcile.

13.4.4 Ghrelin (ε)-Expressing Progenitors?

Ghrelin is a 28-amino acid polypeptide hormone that has been shown to inhibit insulin secretion in mice, rats and humans (Dezaki 2013). Ghrelin-expressing ε cells were originally identified in the stomach. They are detectable in mid-gestation in mouse and human developing pancreas and their number peaks at late gestation or in neonates (Wierup et al. 2014). Adult islets in humans, but no other known species, however contain a substantial number of ε cells (Wierup et al. 2014), suggesting that these cells may play an unidentified role in the function of human islets.

Deletion of the *Arx* gene, encoding an α-cell transcription factor, results in drastically decreased numbers of ε cells (Collombat et al. 2003). In contrast, mice with deletion of *Pax4*, *Pax6* or *Nkx2.2* display significantly increased numbers of ε cells, though this is at the expense of other islet cell types (Hill et al. 2009; Prado et al. 2004). Interestingly, genetic lineage tracing studies demonstrated that ε cells are not terminally differentiated endocrine cells, because in adult mouse pancreas they can give rise to α, PP and, to a lesser extent, β cells (Arnes et al. 2012). These data suggest that ε cells are progenitors of other islet cells. Whether ε cells act as PMP of functional islets remains to be established.

13.4.5 Insulin$^+$ Multipotent Progenitors

Earlier studies demonstrate that insulin-expressing cells in the developing pancreas can give rise to other islet cell types in addition to β cells (Alpert et al. 1988), suggesting that a fraction of insulin-expressing cells are progenitors of other islet cells. Recently, by using genetic lineage tracing techniques, the insulin$^+$ multipotent progenitors (equivalent to PMP herein) in the adult pancreas were derived from the embryonic pancreatic Pdx1$^+$ cells, but not from the neural crest. These PMP-like cells expressed an array of markers typical of islet progenitors and gave rise to all endocrine cells *in vivo*. The PMP are distinct from mature functional cells as they expressed a low level of insulin and a low level or absence of glucose transporter-2 (Smukler et al. 2011). However, given insulin expression starts from Pdx1$^+$ progenitors, through Ngn3$^+$ progenitors to mature β cells (Jiang et al. 2010; Jiang and Morahan 2012), further studies are required to verify whether the insulin$^+$ multipotent progenitors are not only present in adult islets but also in other developing stages.

Human counterparts of the mouse insulin$^+$ multipotent progenitors have also been identified. These cells, isolated from either mice or humans, could develop after transplantation into diabetic mice, and ameliorate their diabetes. Surprisingly, however, these insulin$^+$ multipotent progenitors also gave rise to neural lineage cells (Smukler et al. 2011).

Several pieces of strong evidence demonstrate that islet β cells act as facultative "stem" cells able to reproduce themselves. Dor and colleagues used RIP-driven

reporter genes for genetic tracing of the fate of insulin-secreting cells (Dor et al. 2004). They first revealed that adult mouse pancreatic β cells could be duplicated by RIP-expressing cells within the islets, both physiologically and after partial pancreatectomy. This study assumed that all RIP-expressing cells in adult islets are functional β cells so it did not preclude the presence and action of PMP. Similarly, use of a transgenic model in which the expression of diphtheria toxin was directed by RIP to β cells resulted in apoptosis of 70–80 % of β cells, destruction of islet architecture and eventual diabetes. Withdrawal of diphtheria expression led to a significant regeneration of β-cell mass and a spontaneous normalization of blood glucose levels and islet architecture. Simultaneously, RIP-based lineage tracing analysis indicated that the proliferation of a subset of 20–30 % surviving 'β' cells played a major role in this regeneration and in recovery of euglycemia (Nir et al. 2007). These studies suggest that islet β cells are indeed facultative unipotent stem cells.

Using the sophisticated MADM system in double transgenic mice (designated RIP-CreER; $Rosa26^{GR}/Rosa26^{RG}$) each RIP-expressing clone was composed of 5.1 ± 5.4 cells after 1 month of chase, increasing to 8.2 ± 6.9 cells by 2 months (Brennand et al. 2007). These RIP-expressing clones have been taken as further evidence of regeneration of functional β cells, but this should be balanced in view of fidelity of insulin gene expression, as discussed below. An additional loss-of-function study following knockout of the *Hnf4*α (*hepatocyte nuclear factor 4*α) gene suggested that the β-cell regeneration may involve the Ras/Erk signalling cascade (Gupta et al. 2007) and ultimately be regulated by cycling modulators including cyclin D2 (Georgia and Bhushan 2004). Taken together, further identification and characterization of the so-called self-replicative or dedifferentiative RIP-expressing cells both *in vivo* and *in vitro* is critical, because these cells may hold the key for a regenerative therapy for T1D.

On the other hand, thymidine-based lineage tracing experiments showed that β cells were produced within an islet by rare self-renewing cells with a long replication-refractory period, but both the identity of these unique cells and the length of the replication-refractory period are unknown. The frequency of these self-renewing cells was significantly increased after partial pancreatectomy or during pregnancy (Teta et al. 2007). Further studies should determine the molecular signature and biological potential of these replicating self-renewal cells. Due to obvious ethical and technical issues, similar studies cannot be performed in human islets, but such investigation could at least be repeated in larger mammal islets.

The abovementioned investigations of β-cell duplication as a mechanism of islet regeneration have drawn great attention in recent years because of their potential to develop a regenerative therapy for diabetes. However, a limitation of these studies is whether insulin gene expression (as directed, for example, by RIP-containing transgenes) is a marker exclusively of functional β cells. There is increasing evidence that it is not. First, insulin gene expression starts from the $Pdx1^{+}$ progenitor stage, progressively increasing through $Ngn3^{+}$ progenitors and reaching its plateau in mature islet β cells (Jiang et al. 2010; Jiang and Morahan 2012). Second, the demonstration of insulin^{+} multipotent progenitors (Smukler et al. 2011) precludes insulin as an exclusive marker of functional β cells. Third, insulin protein has been

detected in islet progenitors in both humans and mice. In a dual fluorescence reporter mouse line, a few Ngn3$^+$ cells in the developing pancreas coexpress insulin (Hara et al. 2006). In humans some NGN3$^+$ cells were also detected to coexpress insulin in the foetal pancreas between 10 and 21 weeks (Lyttle et al. 2008).

Taken together, insulin gene expression is not a marker exclusively of functional β cells. It is formally possible β-cell regeneration in adults is derived from both self-duplication of glucose-responsive functional cells and self-renewal and differentiation of PMP. To validate that PMP do exist in the adult islets their self-renewal must be demonstrated at the single cell level, with observations made of the intermediary stages of clonogenesis, and the ability to give rise at least non-β endocrine cells.

13.5 Concluding Remarks

Conclusive evidence of PMP remains to be established. Embryonic Sox9$^+$ progenitors and to a less extent, adult Sox9$^+$ ductal cells satisfy some but not all criteria of PMP. Future goals of the field should be to: define well-agreed general features for PMP; identify specific cell surface markers that can be used to characterize and purify cells that may have PMP potential; establish a simple, effective and reproducible *in vitro* assay to examine self-renewal and differentiation potential of purified cells; and develop *in vivo* functional assays to determine biological function in both experimental animals and humans.

We propose that PMP should be defined with at least the following criteria:

(i) clonogenesis should be demonstrated at the level of single cells sorted by flow cytometry;
(ii) sorted single cells should be tested by *in vitro* self-renewal assays;
(iii) clonogenic cells should give rise *in vitro* to multiple specialized cell lineages; and
(iv) after transplantation, these cells should differentiate *in vivo* into different functional lineages.

Moreover, investigation of PMP should be particularly informed based on two considerations. First, PMP and dedifferentiation or trans differentiation of non-β cell types in the pancreas may provide an alternative source of surrogate β cells. Second, as there is a significant difference in regeneration capacity between rodent and human islets, it may be wise not to extrapolate directly from regeneration data of rodents to humans.

Unlike previous experiments that were only performed *in vivo* or *in vitro* without targeting specific cell types, future PMP work should employ integrated approaches. Identification of cell surface markers and application of flow cytometry would allow fractionation of heterogeneous cell populations for examining *in vitro* their potential for self-renewal and clonogenesis. Single-cell biology (i.e., single-cell transcriptomics and single-cell RNA-seq) (Shapiro et al. 2013; Trapnell et al. 2014) would dissect their developmental pathways and molecular mechanisms and in combination

with genetic tracing examine their lineage contribution and developmental potential *in vivo*. Finally multi-discipline, multi-laboratory and even cross-national collaborations including the participation of the pharmaceutical sector will ultimately generate a depth of understanding of PMP that could allow the development of a regenerative therapy for diabetes, particularly T1D.

Acknowledgements The authors are supported by grants from Telethon Perth Child Health Research Foundation, the Diabetes Research Foundation of Western Australia, the University of Western Australia and the National Health and Medical Research Council Program Grant (53000400).

References

Abraham EJ, Leech CA, Lin JC, Zulewski H, Habener JF (2002) Insulinotropic hormone glucagon-like peptide-1 differentiation of human pancreatic islet-derived progenitor cells into insulin-producing cells. Endocrinology 143:3152–3161

Alpert S, Hanahan D, Teitelman G (1988) Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell 53:295–308

Arnes L, Hill JT, Gross S, Magnuson MA, Sussel L (2012) Ghrelin expression in the mouse pancreas defines a unique multipotent progenitor population. PLoS One 7:e52026

Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L (2005) In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia 48:49–57

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ et al (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449:1003–1007

Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil JJ (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci U S A 97:7999–8004

Bonner-Weir S, Inada A, Yatoh S, Li WC, Aye T, Toschi E, Sharma A (2008) Transdifferentiation of pancreatic ductal cells to endocrine beta-cells. Biochem Soc Trans 36:353–356

Brennand K, Huangfu D, Melton D (2007) All beta cells contribute equally to islet growth and maintenance. PLoS Biol 5:e163

Butler PC, Meier JJ, Butler AE, Bhushan A (2007) The replication of beta cells in normal physiology, in disease and for therapy. Nat Clin Pract Endocrinol Metab 3:758–768

Chase LG, Ulloa-Montoya F, Kidder BL, Verfaillie CM (2007) Islet-derived fibroblast-like cells are not derived via epithelial-mesenchymal transition from Pdx-1 or insulin-positive cells. Diabetes 56:3–7

Cheng X, Ying L, Lu L, Galvao AM, Mills JA, Lin HC, Kotton DN, Shen SS, Nostro MC, Choi JK et al (2012) Self-renewing endodermal progenitor lines generated from human pluripotent stem cells. Cell Stem Cell 10:371–384

Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P (2003) Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes Dev 17:2591–2603

Cornelius JG, Tchernev V, Kao KJ, Peck AB (1997) In vitro-generation of islets in long-term cultures of pluripotent stem cells from adult mouse pancreas. Horm Metab Res 29:271–277

De Krijger RR, Aanstoot HJ, Kranenburg G, Reinhard M, Visser WJ, Bruining GJ (1992) The midgestational human fetal pancreas contains cells coexpressing islet hormones. Dev Biol 153:368–375

Desai BM, Oliver-Krasinski J, De Leon DD, Farzad C, Hong N, Leach SD, Stoffers DA (2007) Preexisting pancreatic acinar cells contribute to acinar cell, but not islet beta cell, regeneration. J Clin Invest 117:971–977

Desgraz R, Herrera PL (2009) Pancreatic neurogenin 3-expressing cells are unipotent islet precursors. Development 136:3567–3574

Dezaki K (2013) Ghrelin function in insulin release and glucose metabolism. Endocr Dev 25:135–143

Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429:41–46

Fougerousse F, Bullen P, Herasse M, Lindsay S, Richard I, Wilson D, Suel L, Durand M, Robson S, Abitbol M et al (2000) Human-mouse differences in the embryonic expression patterns of developmental control genes and disease genes. Hum Mol Genet 9:165–173

Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M et al (2011) Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet 43:34–41

Gao T, McKenna B, Li C, Reichert M, Nguyen J, Singh T, Yang C, Pannikar A, Doliba N, Zhang T et al (2014) Pdx1 maintains beta cell identity and function by repressing an alpha cell program. Cell Metab 19:259–271

Georgia S, Bhushan A (2004) Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. J Clin Invest 114:963–968

Gershengorn MC, Hardikar AA, Wei C, Geras-Raaka E, Marcus-Samuels B, Raaka BM (2004) Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. Science 306:2261–2264

Gradwohl G, Dierich A, LeMeur M, Guillemot F (2000) neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 97:1607–1611

Gu G, Dubauskaite J, Melton DA (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129:2447–2457

Gupta RK, Gao N, Gorski RK, White P, Hardy OT, Rafiq K, Brestelli JE, Chen G, Stoeckert CJ Jr, Kaestner KH (2007) Expansion of adult beta-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. Genes Dev 21:756–769

Halban PA, Polonsky KS, Bowden DW, Hawkins MA, Ling C, Mather KJ, Powers AC, Rhodes CJ, Sussel L, Weir GC (2014) beta-cell failure in type 2 diabetes: postulated mechanisms and prospects for prevention and treatment. J Clin Endocrinol Metab 99:1983–1992

Hanley SC, Pilotte A, Massie B, Rosenberg L (2008) Cellular origins of adult human islet in vitro dedifferentiation. Lab Invest 88:761–772

Hao E, Tyrberg B, Itkin-Ansari P, Lakey JR, Geron I, Monosov EZ, Barcova M, Mercola M, Levine F (2006) Beta-cell differentiation from nonendocrine epithelial cells of the adult human pancreas. Nat Med 12:310–316

Hara M, Dizon RF, Glick BS, Lee CS, Kaestner KH, Piston DW, Bindokas VP (2006) Imaging pancreatic beta-cells in the intact pancreas. Am J Physiol Endocrinol Metab 290:E1041–E1047

Haumaitre C, Barbacci E, Jenny M, Ott MO, Gradwohl G, Cereghini S (2005) Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. Proc Natl Acad Sci U S A 102:1490–1495

Haumaitre C, Fabre M, Cormier S, Baumann C, Delezoide AL, Cereghini S (2006) Severe pancreas hypoplasia and multicystic renal dysplasia in two human fetuses carrying novel HNF1beta/MODY5 mutations. Hum Mol Genet 15:2363–2375

Herrera PL, Huarte J, Sanvito F, Meda P, Orci L, Vassalli JD (1991) Embryogenesis of the murine endocrine pancreas; early expression of pancreatic polypeptide gene. Development 113:1257–1265

Hill JT, Mastracci TL, Vinton C, Doyle ML, Anderson KR, Loomis ZL, Schrunk JM, Minic AD, Prabakar KR, Pugliese A et al (2009) Ghrelin is dispensable for embryonic pancreatic islet development and differentiation. Regul Pept 157:51–56

Holland AM, Gonez LJ, Naselli G, Macdonald RJ, Harrison LC (2005) Conditional expression demonstrates the role of the homeodomain transcription factor Pdx1 in maintenance and regeneration of beta-cells in the adult pancreas. Diabetes 54:2586–2595

Huch M, Bonfanti P, Boj SF, Sato T, Loomans CJ, van de Wetering M, Sojoodi M, Li VS, Schuijers J, Gracanin A et al (2013) Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. EMBO J 32:2708–2721

Inada A, Nienaber C, Katsuta H, Fujitani Y, Levine J, Morita R, Sharma A, Bonner-Weir S (2008) Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci U S A 105:19915–19919

Jensen J, Heller RS, Funder-Nielsen T, Pedersen EE, Lindsell C, Weinmaster G, Madsen OD, Serup P (2000) Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes 49:163–176

Jeon J, Correa-Medina M, Ricordi C, Edlund H, Diez JA (2009) Endocrine cell clustering during human pancreas development. J Histochem Cytochem 57:811–824

Jiang FX, Harrison LC (2005a) Convergence of bone morphogenetic protein and laminin-1 signaling pathways promotes proliferation and colony formation by fetal mouse pancreatic cells. Exp Cell Res 308:114–122

Jiang FX, Harrison LC (2005b) Laminin-1 and epidermal growth factor family members co-stimulate fetal pancreas cell proliferation and colony formation. Differentiation 73:45–49

Jiang FX, Morahan G (2012) Pancreatic stem cells: from possible to probable. Stem Cell Rev 8:647–657

Jiang FX, Morahan G (2015) Directed differentiation of late stage islet lineages remains a knowledge gap in pancreatic endocrine development. J J Bone Stem Res 1:002

Jiang FX, Stanley EG, Gonez LJ, Harrison LC (2002) Bone morphogenetic proteins promote development of fetal pancreas epithelial colonies containing insulin-positive cells. J Cell Sci 115:753–760

Jiang FX, Mehta M, Morahan G (2010) Quantification of insulin gene expression during development of pancreatic islet cells. Pancreas 39:201–208

Jin L, Feng T, Shih HP, Zerda R, Luo A, Hsu J, Mahdavi A, Sander M, Tirrell DA, Riggs AD et al (2013) Colony-forming cells in the adult mouse pancreas are expandable in Matrigel and form endocrine/acinar colonies in laminin hydrogel. Proc Natl Acad Sci U S A 110:3907–3912

Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371:606–609

Kopp JL, Dubois CL, Schaffer AE, Hao E, Shih HP, Seymour PA, Ma J, Sander M (2011) Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. Development 138:653–665

Lardon J, Rooman I, Bouwens L (2002) Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. Histochem Cell Biol 117:535–540

Lukinius A, Ericsson JL, Grimelius L, Korsgren O (1992) Ultrastructural studies of the ontogeny of fetal human and porcine endocrine pancreas, with special reference to colocalization of the four major islet hormones. Dev Biol 153:376–385

Lynn FC, Smith SB, Wilson ME, Yang KY, Nekrep N, German MS (2007) Sox9 coordinates a transcriptional network in pancreatic progenitor cells. Proc Natl Acad Sci U S A 104:10500–10505

Lyttle BM, Li J, Krishnamurthy M, Fellows F, Wheeler MB, Goodyer CG, Wang R (2008) Transcription factor expression in the developing human fetal endocrine pancreas. Diabetologia 51:1169–1180

Minami K, Okuno M, Miyawaki K, Okumachi A, Ishizaki K, Oyama K, Kawaguchi M, Ishizuka N, Iwanaga T, Seino S (2005) Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc Natl Acad Sci U S A 102:15116–15121

Minami K, Okano H, Okumachi A, Seino S (2008) Role of cadherin-mediated cell-cell adhesion in pancreatic exocrine-to-endocrine transdifferentiation. J Biol Chem 283:13753–13761

Miyatsuka T, Kosaka Y, Kim H, German MS (2011) Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a. Proc Natl Acad Sci U S A 108:185–190

Nir T, Melton DA, Dor Y (2007) Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 117:2553–2561

Ohneda K, Mirmira RG, Wang J, Johnson JD, German MS (2000) The homeodomain of PDX-1 mediates multiple protein-protein interactions in the formation of a transcriptional activation complex on the insulin promoter. Mol Cell Biol 20:900–911

Oliver-Krasinski JM, Kasner MT, Yang J, Crutchlow MF, Rustgi AK, Kaestner KH, Stoffers DA (2009) The diabetes gene Pdx1 regulates the transcriptional network of pancreatic endocrine progenitor cells in mice. J Clin Invest 119:1888–1898

Pan FC, Brissova M (2014) Pancreas development in humans. Curr Opin Endocrinol Diabetes Obes 21:77–82

Piper K, Brickwood S, Turnpenny LW, Cameron IT, Ball SG, Wilson DI, Hanley NA (2004) Beta cell differentiation during early human pancreas development. J Endocrinol 181:11–23

Polak M, Bouchareb-Banaei L, Scharfmann R, Czernichow P (2000) Early pattern of differentiation in the human pancreas. Diabetes 49:225–232

Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L (2004) Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proc Natl Acad Sci U S A 101:2924–2929

Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG (2000) Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nat Med 6:278–282

Rankin MM, Wilbur CJ, Rak K, Shields EJ, Granger A, Kushner JA (2013) beta-Cells are not generated in pancreatic duct ligation-induced injury in adult mice. Diabetes 62:1634–1645

Richardson MK, Hanken J, Gooneratne ML, Pieau C, Raynaud A, Selwood L, Wright GM (1997) There is no highly conserved embryonic stage in the vertebrates: implications for current theories of evolution and development. Anat Embryol (Berl) 196:91–106

Russ HA, Bar Y, Ravassard P, Efrat S (2008) In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing. Diabetes 57:1575–1583

Sakano D, Shiraki N, Kikawa K, Yamazoe T, Kataoka M, Umeda K, Araki K, Mao D, Matsumoto S, Nakagata N et al (2014) VMAT2 identified as a regulator of late-stage beta-cell differentiation. Nat Chem Biol 10:141–148

Sancho R, Gruber R, Gu G, Behrens A (2014) Loss of Fbw7 reprograms adult pancreatic ductal cells into alpha, delta, and beta cells. Cell Stem Cell 15:139–153

Sarkar A, Hochedlinger K (2013) The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. Cell Stem Cell 12:15–30

Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development 127:3533–3542

Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22:1115–1124

Seeberger KL, Dufour JM, Shapiro AM, Lakey JR, Rajotte RV, Korbutt GS (2006) Expansion of mesenchymal stem cells from human pancreatic ductal epithelium. Lab Invest 86:141–153

Selander L, Edlund H (2002) Nestin is expressed in mesenchymal and not epithelial cells of the developing mouse pancreas. Mech Dev 113:189–192

Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G, Sander M (2007) SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci U S A 104:1865–1870

Shapiro E, Biezuner T, Linnarsson S (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14:618–630

Smukler SR, Arntfield ME, Razavi R, Bikopoulos G, Karpowicz P, Seaberg R, Dai F, Lee S, Ahrens R, Fraser PE et al (2011) The adult mouse and human pancreas contain rare multipotent stem cells that express insulin. Cell Stem Cell 8:281–293

Sneddon JB, Borowiak M, Melton DA (2012) Self-renewal of embryonic-stem-cell-derived progenitors by organ-matched mesenchyme. Nature 491:765–768

Solar M, Cardalda C, Houbracken I, Martin M, Maestro MA, De Medts N, Xu X, Grau V, Heimberg H, Bouwens L et al (2009) Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. Dev Cell 17:849–860

Sugiyama T, Benitez CM, Ghodasara A, Liu L, McLean GW, Lee J, Blauwkamp TA, Nusse R, Wright CV, Gu G et al (2013) Reconstituting pancreas development from purified progenitor cells reveals genes essential for islet differentiation. Proc Natl Acad Sci U S A 110:12691–12696

Suzuki A, Oyama K, Fukao K, Nakauchi H, Taniguchi H (2002) Establishment of clonal colony-forming assay system for pancreatic stem/progenitor cells. Cell Transplant 11:451–453

Suzuki A, Nakauchi H, Taniguchi H (2004) Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. Diabetes 53:2143–2152

Talchai C, Xuan S, Lin HV, Sussel L, Accili D (2012) Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell 150:1223–1234

Teta M, Rankin MM, Long SY, Stein GM, Kushner JA (2007) Growth and regeneration of adult beta cells does not involve specialized progenitors. Dev Cell 12:817–826

Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen TS, Rinn JL (2014) The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. Nat Biotechnol 32:381–386

Wang Y, Lanzoni G, Carpino G, Cui CB, Dominguez-Bendala J, Wauthier E, Cardinale V, Oikawa T, Pileggi A, Gerber D et al (2013) Biliary tree stem cells, precursors to pancreatic committed progenitors: evidence for possible life-long pancreatic organogenesis. Stem Cells 31:1966–1979

Wierup N, Sundler F, Heller RS (2014) The islet ghrelin cell. J Mol Endocrinol 52:R35–R49

Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, Van De Casteele M, Mellitzer G, Ling Z, Pipeleers D et al (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 132:197–207

Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA (2007) A multipotent progenitor domain guides pancreatic organogenesis. Dev Cell 13:103–114

Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455:627–632

Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50:521–533

Chapter 14
Adipose-Derived Stem/Stromal Cells

Jie Li, Elizabeth C. Martin, and Jeffrey M. Gimble

Abstract The adipose tissue is considered as a multifunctional organ which plays an important role in energy storage and endocrine and immune responses. In addition, it serves as a reservoir for a population identified as adipose-derived stem/stromal cells (ASCs). ASCs have been documented to possess the potential to differentiate toward multiple cell lineages both *in vitro* and *in vivo*. At present, 168 national and international clinical trials involving ASC have been registered according to the U.S. National Institutes of Health of which 38 have been completed. Both pre-clinical and clinical studies have shown the effectiveness of ASCs to treat various diseases. The mechanisms by which ASCs may provide regenerative function include their ability to differentiate into target tissue specific cells, the secretion of factors to recruit and direct host-derived reparative cells, and/or immunomodulatory effects. Thus, due to its abundance, easy availability, and low morbidity during harvest, adipose tissue provides a feasible tissue source of adult stromal/stem cells for regenerative medicine.

Keywords Adipose-derived stem cell • White adipose tissue • Brown adipose tissue adipocyte • Chondrocyte • Osteoblast • Secretome • Preconditioning

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_14

14.1 Introduction

Historically, adipose tissue was considered exclusively as an energy reservoir. More recently, other adipose physiological functions such as metabolic regulation, inflammatory processes and endocrine function have been characterized. Additionally, human adipose tissue has been identified as an abundant and accessible source of multipotent stromal/stem cells. These cells have the potential to differentiate into cells of mesodermal origin including adipocytes, chondrocytes, osteoblasts, and myoblasts, as well as cells of non-mesodermal lineages, such as hepatocytes, pancreatic endocrine cells, neurons, cardiomyocytes, and vascular endothelial cells. The International Fat Applied Technology Society (IFATS) and the International Society for Cellular Therapies (ISCT) reached a consensus to adopt the term "adipose-derived stromal/stem cells" (ASCs) to identify the isolated, plastic-adherent, multipotent cell population obtained from adipose tissue (Bourin et al. 2013). Over the past decade and a half, ASCs have been widely studied in tissue engineering and regenerative medicine as well as cell therapy.

14.2 Classification and Derivation of Adipose

Adipose tissue is comprised of a heterogeneous cell population which includes adipocytes, endothelial cells, pericytes, and pre-adipocytes as well as various immune cells (Lee et al. 2013). Cells resembling mesenchymal stem cells (MSCs; also known as multipotent stromal cells) have been found within the adipose tissue which are now identified as ASCs. The ASCs are isolated by collagenase digestion, mechanical disruption and/or explant culture techniques. Anatomically, there are at least five different categories of adipose tissue: bone marrow, brown, mammary, mechanical, and white (Gimble et al. 2007). The ASCs isolated from different depot can display similar but not necessarily identical biological properties (Baglioni et al. 2012; Shah et al. 2015). Additionally, the biological characteristic of ASCs has been reported to differ between lean and obese as well as between young and old donors (Schipper et al. 2008). In the future, it may be necessary to determine whether a particular kind or depot source of ASCs is more suitable for therapy of a specific disease.

14.2.1 White Adipose Tissue

White adipose tissue (WAT) is recognized as a multifunctional organ displaying, for example, endocrine, energy storage, immunomodulatory, and secretory roles. Two of the most prominent WAT depots are the subcutaneous adipose tissue and the visceral adipose tissue. The WAT serves as an energy reservoir, efficiently storing excess calories in the form of triglyceride. Both hypertrophy and hyperplasia of adipocytes occurs when caloric input exceeds expenditure; in contrast, the stored

triglycerides are metabolized into free fatty acids and glycerol when energy is required (Otto and Lane 2005). The ability of WAT to expand extensively suggests the existence of stem cells and labeling studies indicate that adipose tissue contains long term label retaining cell *in vivo*, consistent with the existence of a stem-like cell (Gawronska-Kozak et al. 2014).

In recent years, liposuction has been employed for body sculpting. According to the American Society for Aesthetic Plastic Surgery Reports, 342,494 liposuction procedures were performed in the United States in 2014, making it among the most commonly performed cosmetic procedures. Up to 3 kg of fat can be acquired from this procedure, however the fat is routinely discarded as medical waste. Alternatively, using collagenase enzyme digestion or mechanical disruption, a stromal vascular fraction (SVF) can be isolated from the fat. After expansion *in vitro*, these cells become more homogeneous for a subset of surface antigen biomarkers and are identified as ASCs. In many respects, the ASCs meet the criteria defining human mesenchymal stem cells (MSCs). The ASC are able to differentiate along multiple lineage, including adipocyte, chondrocytes and osteoblasts. These cells express surface markers associated with the MSC immunophenotype. In comparison to bone marrow stem cells (BMSC), ASCs have a higher colony forming unit (CFU) frequency (Kern et al. 2006) and a higher cell yield per gram tissue (Strem et al. 2005). Therefore, the easy accessibility, abundant source, and limited harvest morbidity make subcutaneous WAT an exciting alternative to bone marrow as a stromal/stem cell source.

14.2.2 Brown Adipose Tissue

In humans, brown adipose tissue (BAT) is mainly located on the back and around the major organs in infants and in the cervical, supraclavicular, and superior mediastinal depots in adults (Cypess et al. 2009; Virtanen et al. 2009). BAT is characterized by an abundance of mitochondria expressing uncoupling protein-1 (UCP1) (Cannon and Nedergaard 2004). UCP1 is found in the inner membrane of brown adipocytes mitochondria where it is responsible for the non-shivering thermogenesis of BAT (Cannon and Nedergaard 2004). It has been well recognized that BAT shares a Myf5+ precursor with skeletal muscle, making BAT cells more closely related to skeletal muscle rather than WAT adipocytes. Recently, a population of Myf5+ precursor were found in interscapular WAT (iWAT) and retroperitoneal WAT (rWAT) (Sanchez-Gurmaches et al. 2012). The ASCs isolated from BAT have been found to express a similar cell surface marker profile and a multilineage potential resembling that of ASCs from WAT (Silva et al. 2014). This may reflect an ability of adipose tissue to transdifferentiate between WAT and BAT. Conversion of WAT to BAT is observed during shivering response/cold exposure, or through the enhanced expression of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) or forkhead box protein C2 (FOXC2), agonists to peroxisome proliferator-activated receptor γ (PPARγ), retinoic acid or nicotine (Stephens et al. 2011). However, the BAT-like cells derived from WAT origin are described

currently as a unique sub-type known as beige/brite cells, which will be discussed in greater detail below. BAT exhibits a lower stromal/stem cell yield per weight of tissue and weaker differentiation ability than WAT-derived ASC (Prunet-Marcassus et al. 2006). By comparing Sca-1$^+$/CD45$^-$/Mac1$^-$ ASCs isolated from murine BAT, WAT, and skeletal muscle, investigators have found that ASCs isolated from BAT serve as constitutively committed brown fat precursors (Schulz et al. 2011). Additionally, CD29$^+$ ASCs isolated from BAT could differentiate into cardiomyocytes with a high efficiency relative to those from WAT (Yamada et al. 2006). When these CD29+ BAT-derived ASCs were transplanted *in vivo* along with chitosan hydrogel, they are able to preserve and repair myocardial tissue following myocardial infarction (Wang et al. 2014a). By injection of BAT-derived ASCs in high fat fed NOD-SCID mice, no change was found in body weight compared to the saline control animals (Silva et al. 2014). These results suggest that the BAT derived ASC hold potential for stem cell therapy and regenerative medicine.

Clinically, BAT tissue has been associated with disease types such as obesity, type 2 diabetes and insulin resistance, and atherosclerosis (Harms and Seale 2013). In mice which have been genetically modified to express less BAT there is an observed increase in weight gain, suggesting that there may be an inverse correlation between BAT depot volumes and obesity. Furthermore enhancing the activity or availability of BAT tissue in mice has correlated with resistance to weight gain and suppression of metabolic diseases such as type 2 diabetes (Harms and Seale 2013). Mice with enhanced BAT activity and presence have an increased sensitivity to insulin. BAT is known to be induced thermogenically as well as to be regulated and enhanced through a subset of genes, FOXC2, phosphatase and tensin homolog (PTEN), CCAAT-enhancer-binding protein β (C/EBPβ), and PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16). Likewise, there are many known inhibitors of BAT including activin receptor type IIB (ActRIIB), activating transcription factor 4 (ATF4), pre-adipocyte factor-1 (Pref-1), eukaryotic initiation factor 4E-binding protein-2 (4E-BP2), forkhead box-containing protein O subfamily 1 (FOXO1), transcriptional intermediary factor-2 (TIF2), and protein kinase Cβ (PKCβ) (Seale 2010).

14.2.3 Additional Adipose Depots

Adipose tissues are present throughout the body. In addition to BAT and WAT, adipose tissue has been found in the appendicular skeleton. The bone marrow fat is involved in thermogenesis, hematopoiesis, energy metabolism, and secretion of adipokines (Gimble et al. 1996a; Hardouin et al. 2014). Mammary adipose tissue acts as a local site for hormone action, the storage of lipids, and growth factor production (Hovey et al. 1999). The adipose depots located in the retro-orbital, buccal, palmar, and plantar regions provide mechanical support (Gimble et al. 2007). Further research will be needed to understand the specific biological properties of ASCs from these depots (Fig. 14.1).

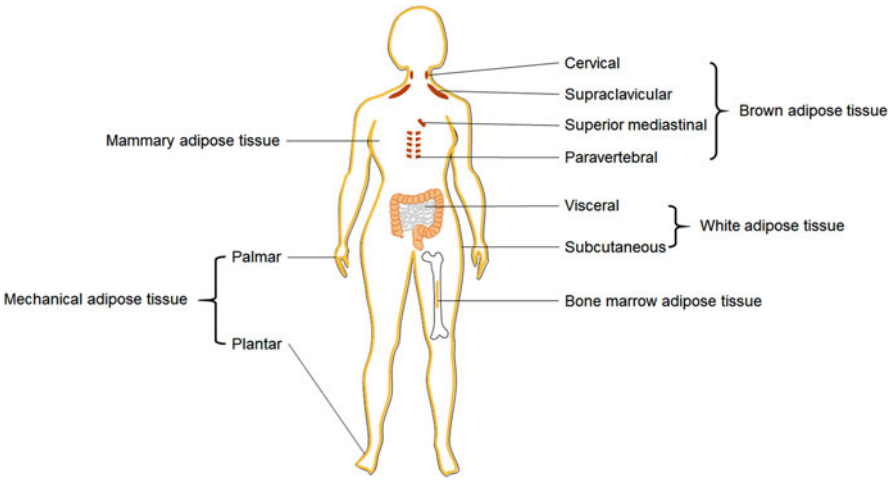

Fig. 14.1 Representative adipose depots

14.2.4 Beige Adipocytes

As stated above, there are instances of brown adipose like cells in WAT and these cells are designated as "beige" or "brite" (brown-in-white) adipocytes (Petrovic et al. 2010). While UCP1 has been used as a definitive biomarker for brown adipocytes, studies using northern analysis and PCR analysis detected UCP1 unexpectedly in WAT (Cousin et al. 1992). After cold exposure or β3-adrenoceptor agonist treatment, the expression of UCP1 increased in WAT in rodents (Cousin et al. 1992). Beige adipocytes were enriched within the inguinal WAT of mice. Beige adipocytes markers including CD137, transmembrane protein 26 (TMEM26), transcription factor T-box 1 (Tbx1) and Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1 (Cited1) have been reported (Wu et al. 2012; Sharp et al. 2012). Early B-cell factor 2 (Ebf2) is selectively expressed in brown and beige adipocytes (Wang et al. 2014b). The Asc-type amino acid transporter 1 (Asc-1), proton assistant amino acid transporter-2 (Pat2), and purinergic receptor P2X, ligand-gated ion channel 5 (P2rx5) have been proposed to serve as distinguishing cell surface markers for white, beige, and brown adipocytes, respectively (Ussar et al. 2014). Studies showed that the beige adipocytes in mice are originally derived from $Myf5^{-}PDGFR\alpha^{+}$ precursor cells (Sanchez-Gurmaches et al. 2012). Meanwhile, white adipocytes can transdifferentiate into beige adipocytes on a second cold stimulation (Rosenwald et al. 2013). In light of these recent studies, the cellular origin of beige, brown and white adipocytes is still under investigation (Bartelt and Heeren 2014; Harms and Seale 2013; Wu et al. 2015).

14.3 Immunophenotype of Adipose-Derived Stem/Stromal Cells

The Mesenchymal and Tissue Stem Cell committee of the ISCT has outlined the minimal criteria for defining the human mesenchymal stem/stromal cells: (1) Plastic-adherent when cultured under standard conditions; (2) The expression of CD105, CD73 and CD90 must $\geq$ 95 %, while, on the other hand, the expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and human leucocyte antigen (HLA) class II must $\leq$ 2 %. (3) The cells must be capable to differentiate into adipocytes, chondroblasts and osteoblasts (Dominici et al. 2006). More recently, the same committee extended its original guidelines by incorporating an evaluation of MSC immunomodulatory function based on mixed lymphocyte reactions and related assays (Krampera et al. 2013).

Similar recommendations from IFATS and ISCT have been proposed for ASCs (Bourin et al. 2013). The ASCs share a great majority of cell surface markers expression with MSCs. The freshly isolated SVF are heterogeneous with expression of low levels of stromal-associated markers such as CD13, CD29, CD44, CD73, CD90, CD105, and CD166. After *in vitro* expansion, high levels of stromal marker expression have been observed in ASCs (Mitchell et al. 2006). The human ASCs are consistently positive for these surface proteins: CD9, CD10, CD13, CD29, CD34, CD44, CD49e, CD54, CD55, CD59, CD90, CD105, CD166 and leucocyte-associated antigens-ABC (HLA-ABC), but negative for CD11a, CD11b, CD11c, CD14, CD18, CD31, CD45, CD49d, CD50, CD56, CD62e, and HLA-DR (Gronthos et al. 2001; Aust et al. 2004). In addition to those markers, studies have reported that ASCs are also positive for CD51, CD71, STRO-1 but negative for CD4, CD8a, CD16, CD41a, CD49f, CD62L, CD62P, CD104, CD106, CD117, CD133, CD243 (Katz et al. 2005; Zuk et al. 2002). However, the expression of CD49b, CD49d, CD61, CD138, and CD140a showed a variable positivity among different donors (Katz et al. 2005). The surface markers expression suggests that ASCs display a similar profile as MSCs.

Studies have documented the immunophenotype change after differentiation. After adipogenic differentiation, the ASCs expression of CD36, CD40, CD146, CD164, and CD271 has been reported to increase while CD49b, CD49c, CD49d, CD71, CD105, and CD166 were decreased. Following osteogenic differentiation, CD164 expression has been found to be up-regulated but CD49a, CD49b, CD49c, CD49d, CD55, CD58, CD105, and CD166 expression was reported as down-regulated (Walmsley et al. 2015).

Efforts have been made to purify the ASCs. Using fluorescence-activated cell sorting (FACS) technique, a subpopulation of Lin−CD29+CD34+Sca-1+CD24+ cells has been sorted out from the mice SVF. These cells are thought to be undifferentiated adipocyte precursor (Rodeheffer et al. 2008). In human, both CD31+ and CD31− SVF are capable of adipogenic differentiation; of these, the CD31− cell population are more robust (Wosnitza et al. 2007). In one study, human CD34− ASCs have been reported to display a stronger adipogenic differentiation capacity

than CD34+ ASCs (Suga et al. 2009). In contrast, CD45−CD31−CD34+ cells sorted from human SVF were reported to represent a subpopulation with an inherent propensity for adipogenesis exceeding that of other subpopulations (Li et al. 2011). Consistent with this observation, the CD34 biomarker has been associated with volume retention in human adipose tissue fat grafts, suggesting that CD34 positivity is a potential biomarker for adipogenic progenitors or stem cells (Philips et al. 2013). Additionally, the CD10 and CD200 expression have been demonstrated to be depot-dependent and are correlated with adipogenic differentiation potential (Ong et al. 2014). Likewise, CD105− ASCs have been found to show stronger adipogenic differentiation ability relative to their CD105+ ASCs counterparts; however the osteogenic and chondrogenic differentiation potential is more robust in CD105+ ASCs than in CD105− ASCs (Jiang et al. 2010). In summary, cells with distinctive lineage differentiation capacity can be sorted by using different combination of surface markers. It remains to be determined if the ASCs contain, in addition, a true stem cell.

14.4 Differentiation of Adipose-Derived Stem/Stromal Cells

As highlighted above, there is evidence from multiple independent laboratories demonstrating that ASCs are multipotent, with adipogenic, osteogenic, and chondrogenic differentiation capability *in vitro* and *in vivo*.

14.4.1 Adipogenic Differentiation

Adipogenic differentiation is one of the hallmark characteristics identifying ASCs. A complex cascade of transcriptional and extracellular signals have been determined to regulate the adipogenic differentiation process (Rosen and MacDougald 2006). The master transcription factors of adipogenesis are PPARγ and the C/EBPs. To study the adipogenic process, ASCs are exposed to a defined adipogenic cocktail which routinely includes dexamethasone (DEX), insulin, and methylisobutylxanthine. Dexamethasone is a synthetic glucocorticoid agonist, which is used to stimulate the glucocorticoid receptor pathway. Insulin acts at the level of the extracellular insulin receptor, initiating a complex cytoplasmic and nuclear response. Methylisobutylxanthine is a cAMP-phosphodiesterase inhibitor which elevates cytoplasmic cAMP levels, thereby activating the cAMP-dependent protein kinase pathway (Ntambi and Young-Cheul 2000). Other adipogenic reagents have been utilized in the adipogenic cocktail such as indomethacin or thiazolidinedione (a ligand for the PPARγ) and triiodothyronine, a ligand for the thyroid receptor, but an adipogenic response can occur in their absence (Gimble et al. 1996b; Hauner et al. 1989). To confirm adipogenic differentiation histochemically, Oil Red O or Nile Red staining is routinely used to detect intracellular lipid accumulation (Yu et al.

2011; Williams et al. 2011). Adipogenic markers such as PPARγ, C/EBPα, lipoprotein lipase, leptin and adipocyte protein 2 can be determined at the mRNA and protein level. The robust adipogenic capacity of ASCs makes it a valuable cell model for human metabolism and obesity studies as well as a resource for regenerative medicine.

14.4.2 Osteogenic Differentiation

To induce osteogenesis in stromal/stem cells, an osteogenic cocktail is used. In general the composition of this cocktail contains β-glycerophosphate (β-GP), DEX, and ascorbate 2-phosphate. The β-GP is hydrolyzed by alkaline phosphatase (ALP) to produce high levels of phosphate which promotes mineral deposition. DEX acts as a ligand for the glucocorticoid receptor and activates *Runt-related transcription factor (Runx2)*/core-binding factor subunit alpha-1 *(CBF-alpha-1)* expression, a transcription factor integral to osteogenesis. In addition DEX also acts to induce activation of the WNT/β-catenin and mitogen-activated protein kinase (MAPK) phosphatase (MKP-1) pathways. Ascorbic acid enhances the formation of the collagenous bone extracellular matrix (Langenbach and Handschel 2013). In addition to extracellular calcium deposition, differentiated osteoblasts have increased ALP activity which can be measured using commercially available kits. Alizarin Red or Von Kossa staining is frequently used to determine the formation of calcium deposits (Bourin et al. 2013). The molecular osteogenic markers such as Runx2, ALP, osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) can be detected via PCR and western blot after osteogenic differentiation. Expression of these genes is temporal where early induced osteogenic genes include Runx2 followed by genes associated with matrix mineralization such as OCN. Due to the abundance and osteogenic capacity of ASCs, the cells has been used for bone regeneration (Lendeckel et al. 2004; Sandor et al. 2014).

14.4.3 Chondrogenic Differentiation

To induce chondrogenesis in ASCs, a chondrogenic cocktail containing ascorbate 2-phosphate, dexamethasone, ITS (insulin, transferrin, and selenium), transforming growth factor beta 3 (TGF-β3) and bone morphogenetic protein 6 (BMP6) is often used (Erickson et al. 2002; Estes et al. 2010). Ascorbic acid serves to promote increased protein and proteoglycan synthesis (Awad et al. 2003). The DEX, acting as a glucocorticoid ligand, is variably used in chondrogenic differentiation, but its effect may be context dependent (Shintani and Hunziker 2011). ITS is suitable as a partial or full substitute for serum in chondrogenic culture (Kisiday et al. 2005). Although TGF-β1 has been used in chondrogenic differentiation, other TGF-β superfamily member such as TGF-β2, TGF-β3, BMP2 and BMP-6 have also been

shown to modulate chondrogenesis (Kramer et al. 2000; Luo et al. 2015; van Osch et al. 1998). For chondrogenesis. ASCs can be cultured as either high-density pellet cultures or in three-dimensional calcium alginate compositions (Izadpanah et al. 2006; Erickson et al. 2002). Histochemically, Alcian Blue, Safranin O and Toluidine Blue staining are used to monitor the accumulation of sulfated proteoglycans. Likewise, immunohistochemistry staining of collagen type I and II has been used to identify active chondrogenesis. Expression of chondrogenic differentiation markers including aggrecan, collagen type I and II, cartilage oligomeric matrix protein/thrombospondin-5 (COMP/TSP5) and Sox9 can be evaluated via PCR and western blot analysis. The chondrogenic potential suggest ASCs is a feasible cell source for cartilage regeneration (Erickson et al. 2002).

14.4.4 Other Lineages Differentiation

Myogenic Differentiation Exposure of ASCs to myogenic medium can induce their differentiation to myocyte lineage pathways (Mizuno et al. 2002). This has suggested the possibility that ASCs can be used in skeletal muscle regeneration in combination with appropriate biomaterials (Kim et al. 2006).

Neurogenic Differentiation ASCs are able to undergo neural differentiation using different combinations of growth factors such as basic fibroblast growth factor (bFGF), glial growth factor-2 (GGF-2) and platelet-derived growth factor (PDGF) and regent like forskolin or retinoic acid (Kingham et al. 2007; Lopatina et al. 2011). The potential usage of ASCs in neural regeneration has also been reported (Lopatina et al. 2011).

Endothelial Differentiation The culture of ASCs using an endothelial differentiation medium which contains vascular endothelial growth factor (VEGF) and bFGF in combination with a Matrigel coating has led to the formation of an *in vitro* vascular-like network. Under these conditions, the ASC were positive for endothelial cell markers including CD31 (PECAM), CD34, and CD144 (VE-cadherin). Based on these findings, ASCs hold the potential therapeutic use in the treatment of ischemic diseases (Cao et al. 2005) and phase I clinical studies to treat ischemia of the lower extremities are now underway (Bura et al. 2014).

Hepatogenic Differentiation Using a two-step differentiation protocol, both BMSCs and ASCs can be differentiated toward hepatocytes. These results suggests that ASCs may serve as an alternative for hepatocyte regeneration (Talens-Visconti et al. 2006).

Tendonogenic Differentiation Growth differentiation factor-5 (GDF-5) treatment has been reported to induce tendonogenic gene expression in rat ASCs (Park et al. 2010a). Furthermore, the use of tendon-derived extracellular matrix can enhance the tenogenic differentiation and may facilitate tendon healing and regeneration (Yang

et al. 2013). Thus, there is the potential to apply ASCs in the treatment of ligamentous defects.

Keratinocyte Like Cells Either by co-culture with human keratinocytes or by exposure to the keratinocyte-derived conditioned media from, ASCs can transdifferentiate into keratinocyte-like cells *in vitro* (Chavez-Munoz et al. 2013). The development of large numbers of keratinocytes would have potential benefit in the treatment of patients with third degree burns covering a large percentage of their body surface area.

14.5 Secretome

The secretome is comprised of the secreted proteins and microRNAs from a cell, tissue, or organ at any given time and under particular growth conditions. The role of the secretome has been noted in light of the reparative effects of transplanted mesenchymal stem cells despite their failure to display long term differentiation and/or engraftment within a damaged tissue. Further mechanistic studies have determined that paracrine factors may be responsible for the enhanced regeneration within the pathologic tissues and organs. Since stromal/stem cells communicate with each other via autocrine and paracrine pathways, it has been hypothesized that such secreted factors play an important role in mediating the multiple biological functions of stromal/stem cells during regeneration, such as cell proliferation, differentiation, apoptosis and signaling.

Numerous studies in the literature have documented that the ASC secretome can exert benefits on angiogenesis, tissue regeneration, wound healing and immunomodulation. The pro-angiogenic effects of the ASC secretome has been correlated with the secretion of angiogenic factors such as VEGF, bFGF, hepatocyte growth factor (HGF), granulocyte colony stimulating factor (G-CSF) and TGF-β (Rehman et al. 2004). The secretome from ASC has been reported to promote endothelial cell survival, proliferation, migration, and vasculogenesis (Merfeld-Clauss et al. 2015). The ASC secretome may promote wound healing by inducing the endothelial cell migration since it exerts this effect on human umbilical vein endothelial cells *in vitro* (Hu et al. 2013). The ASC conditioned media has also been found to increase skin allograft survival (Lee et al. 2014). Likewise, the soluble factors from ASC have shown promise for the treatment of photo-aging (Kim et al. 2009). Additionally, treatment of alopecia patients with adipose-derived stem cell-conditioned medium led to effective hair regeneration (Fukuoka and Suga 2015). The ASC secretome can also alleviate liver damage (Lee et al. 2015b) and enhance the liver regeneration in mice (Lee et al. 2015c). The ASC secretome has been demonstrated to induce bone regeneration in surgically created lesions in a lupine mandibular defect (Linero and Chaparro 2014). The ASC secretome exhibited neuroprotective effects against a pre-clinical stroke mice model (Egashira et al. 2012). Recent studies suggest that the ASC secretome acts mechanistically by inhibiting neuronal cell damage/apop-

tosis, thereby promoting nerve regeneration and repair (Hao et al. 2014). In related studies, ASC have been found to promote peripheral nerve regeneration partly through paracrine secretome effects (Sowa et al. 2012). The ASC conditioned media is able to induce chondrogenesis at a high concentration, due, in part, through TGF-β related signaling pathways (Kim et al. 2010). The ASC conditioned media has been found to promote keratinocyte differentiation via the up-regulation of miR-24 (Seo et al. 2015). While the secretome from ASCs has shown immunomodulatory effects, the cytokines, nitric oxide (NO), and indoleamine 2,3-dioxygenase (IDO) production have been found to vary between different mouse strains (Hashemi et al. 2013). Thus, the ASC secretome has a promising and wide-ranging potential for clinic application and translation.

Nevertheless, while the secretome of ASC has potential benefits to regenerative medicine, the secreted factors themselves are present in relatively low amounts (picogram scale) (Rehman et al. 2004). Efforts have been made to isolate and characterize the components of the secretome by proteomics technologies. Using two-dimensional gel electrophoresis and tandem mass spectrometry, over 80 individual protein have been identified after adipogenic differentiation (Zvonic et al. 2007). Cytokine arrays and liquid chromatography coupled with tandem mass spectrometry were also utilized to analyze the secretome (Kapur and Katz 2013). Future studies will need to develop methods to enrich the relevant proteins or factors in the ASC secretome to insure its utility in regenerative medicine.

14.6 Preconditioning of the Adipose-Derived Stem/Stromal Cells

ASCs therapy has shown promising results in regenerative medicine. However, limited survival has been noted after cell transplantation (Fan et al. 2013; Suga et al. 2014). Cell death is caused by their exposure to a severe microenvironment within the pathologic or reconstructed tissues (Robey et al. 2008). Thus, a variety of preconditioning strategies have reported to enhance the therapeutic effect of MSCs including biological, chemical and physical manipulation (Haider and Ashraf 2010; Sart et al. 2014). These strategies are primarily designed to enhance the pro-angiogenic effects, improve the survival of the ASC and direct cells along a specific lineage pathway.

Biological Preconditioning In this respect, biological macromolecule including growth factors, cytokines, and polysaccharide have been used for preconditioning of ASCs. Erythropoietin preconditioning significantly suppressed ASCs apoptosis (Ercan et al. 2014). Preconditioning with tumor necrosis factor-alpha (TNF-a) increased proliferation, mobilization, and osteogenic differentiation of ASCs (Lu et al. 2013). PDGF-D preconditioned ASCs showed an increase in their proliferation and migration, which further enhanced hair regenerative ability (Hye Kim et al. 2015). Lipopolysaccharide (LPS) pre-conditioning, acting via the toll-like receptor

4 (TLR4), stimulated ASCs to produce a secretome beneficial to hepatic regeneration (Lee et al. 2015a). Betancourt and her colleagues have demonstrated that brief exposure to TLR3 (poly di/DC) or TLR4 (LPS) ligands can promote an anti- or pro-inflammatory phenotype in ASC as well as MSC (Bunnell et al. 2010). Exendin-4 is an antidiabetic polypeptide hormone and its preconditioning is able to prevent ASCs apoptosis (Zhou et al. 2014). Platelet-rich plasma (PRP) contains several growth factors and treating ASCs with PRP is able to promote proliferation and chondrogenic differentiation but inhibit angiogenic factor secretion (Van Pham et al. 2013). These methods may prove of value in future regenerative medical approaches.

Chemical Preconditioning Chemical conditioning of ASCs has included both chemical reagents and drugs. Stromal/stem cells are typically cultured under atmospheric air oxygen tensions of 21 %. However, mesenchymal stem cells *in vivo* reside at lower oxygen tensions of 2 %–8 % (Mohyeldin et al. 2010). Oxygen tension have been found to regulate the differentiation, proliferation and metabolism of ASCs (Wang et al. 2005; Malladi et al. 2006). Extracellular matrix (ECM) protein production by ASCs was decreased by 5 % oxygen tension exposure (Frazier et al. 2013). Insulin-like growth factor binding protein (IGFBP)-1, IGFBP-2, macrophage colony-stimulating factor (M-CSF), M-CSF receptor, platelet-derived growth factor receptor-beta (PDGFR-β), and VEGF secretion in ASC was significantly increased by low oxygen tension exposure (2 %) (Park et al. 2010b). Furthermore, 2 % oxygen tension pre-conditioned ASCs promoted hair growth in mice (Park et al. 2010b) and exhibited increased proliferation and enhanced their wound-healing function (Lee et al. 2009). In addition to oxygen tension manipulation, there are alternative pre-conditioning regimens to consider. Vitamin C preconditioning enhanced the hair growth promoting effect of ASCs (Kim et al. 2014). Studies have found that ascorbate induced expression of HGF in ASCs (Kilroy et al. 2007). Phosphodiesterase-5 (PDE-5) inhibitor sildenafil preconditioning enhanced the therapeutic effect of ASCs for myocardial infarction (Hoke et al. 2012). VEGF secretion was increased by deferoxamine preconditioning, which may enhance their therapeutic efficacy (Liu et al. 2013). Low-dose rapamycin treatment promoted ASCs viability (Fan et al. 2013). Preconditioning ASCs with a mixture of hyaluronic, butyric, and retinoic acids were able to increase the secretion of angiogenic factors (Cavallari et al. 2012). VEGF and HGF secretion was improved and apoptosis was prevented by rotenone and antimycin pretreatment (Carriere et al. 2009). In general, chemical preconditioning primarily improved the pro-angiogenic effects of ASCs.

Physical Preconditioning Physical and mechanical preconditioning of ASCs has also been performed. These approaches including mechanical, sound, electrical, magnetic, light and heat stimulation. Mechanical force has been documented to direct stem cell fate (Estes et al. 2004). Studies found that mechanical properties of three-dimensional biomaterials played an important role for cartilage regeneration (Awad et al. 2004). Research showed that shear stress generated by medium flow

enhanced osteogenic differentiation of ASCs (Frohlich et al. 2010). In addition to mechanical preconditioning, low-intensity ultrasound treatment enhanced cartilage regeneration when BMSCs were seeded into polyglycolic acid following *in vivo* transplantation (Cui et al. 2006). Meanwhile, low-intensity pulsed ultrasound enhanced the osteogenic differentiation of ASCs *in vitro* (Yue et al. 2013). Direct current electrical stimulation of ASCs enhanced the expression of connexin-43 (Cx-43), VEGF, FGF, and thrombomodulin (ThB) (Tandon et al. 2009). In addition to this, electric stimulation enhanced the osteogenic differentiation of ASCs *in vitro* (McCullen et al. 2010). Electromagnetic field preconditioning of ASCs resulted in a significantly better bone regeneration in mouse model (Kang et al. 2012). Low dose of Ultraviolet (UV) B radiation was found to increase the survival, the migration, and secretion of angiogenic factors in ASCs, which further promoted enhanced hair regeneration (Jeong et al. 2013). It has been documented that heat shock preconditioning was able to improve Sca-1+ BMSCs survival (Feng et al. 2014) and enhanced the osteogenic differentiation of BMSC-based cell lines (Norgaard et al. 2006). It will be worthwhile to pursue the effects of physical preconditioning in future *in vitro* and *in vivo* experiments.

14.7 Clinical Applications of Adipose-Derived Stromal/Stem Cells

A number of clinical trials using freshly isolated SVF cells and ASCs are ongoing. Commercial ASCs separation systems are available for clinical usages (Aronowitz and Ellenhorn 2013). By searching on the U.S. National Institutes of Health's website (https://clinicaltrials.gov/), 168 studies were found under the search term of "adipose stem cell" (as of July 1st, 2015). The majority of studies are registered in East Asia (41), Europe (46), and North America (51) (Fig. 14.2).

Soft Tissue The adipose tissue origin of ASC allows its use for adipose tissue reconstruction to be defined unequivocally as "homologous use". Cell assisted lipotransfer (CAL) involves the combination of freshly isolated autologous SVF with the injection of intact lipoaspirate tissue. In early reports on the procedure, the vast majority of the 40 patients receiving CAL for cosmetic purposes were satisfied with the appearance and texture of their augmentation (Yoshimura et al. 2008). At postoperative 12 weeks, patient and doctor surveys confirmed that the results of CAL treatment were significantly better than those grafted with adipose tissue without SVF cells (Lee et al. 2012). It has been reported that CAL techniques have the clinical advantage in reducing the graft resorption at one year (Domenis et al. 2015). Similarly, fat grafts enriched with high dose *ex-vivo* expanded ASCs (20×10^6 cells per mL fat) retained more than 80 % of the initial bolus volume 4 months after the grafting procedure (Kolle et al. 2013). Thus, both SVF and ASCs display potential utility for soft tissue regeneration.

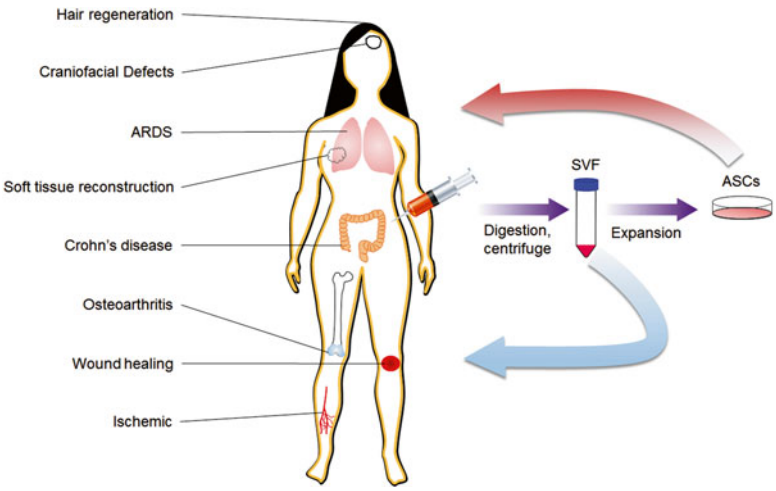

Fig. 14.2 Representative clinical applications of SVF/ASCs

Wound Healing Injection of autologous SVF treated the acute complications of skin necrosis, resulting in much less scarring (Sung et al. 2012). The ASC-based therapy to modulate scar formation has begun to receive particular attention in Korea.

Bone and Cartilage Regeneration Studies suggest that ASCs are an alternative cell source for bone and cartilage regeneration. Scaffolds seeded with autologous ASCs have been used to successfully reconstruct cranio-maxillofacial hard-tissue defects in a series of 13 patients (Sandor et al. 2014). Injection of autologous SVF intra-articularly or peri-articularly was found to improve osteoarthritis symptoms in a total of 1,128 patients after 3–12 months (Michalek et al. 2015). Likewise, injection of ASC into the osteoarthritic knee joint improved function and pain of the knee joint without causing adverse events, and reduced cartilage defects by regeneration of hyaline-like articular cartilage in 18 patients (Jo et al. 2014).

Ischemic As discuss above, ASC secretes a large variety of angiogenic factors which suggested a potential application for ischemic diseases. Intramuscular injection of ASCs was found to improve revascularization and tissue perfusion in ischemic limbs of seven patients (Bura et al. 2014). In a similar study, 10 patients showed variable degrees of recovery by injection of SVF into the edges of ischemic ulcers (Marino et al. 2013).

Crohn's Disease Phase I, II and III clinical trial have documented that both autologous and allogeneic ASCs combined with fibrin glue are an effective and safe treatment for complex perianal fistula (Garcia-Olmo et al. 2005, 2009; de la Portilla et al. 2013; Herreros et al. 2012). ASCs injection combined with thrombin and fibrinogen also showed a complete healing in Crohn's patients (Cho et al. 2013) and

complete closure was well-sustained after 2 years (Cho et al. 2015). Furthermore, such cell therapy did not compromise patient fertility or pregnancy outcomes (Sanz-Baro et al. 2015).

Hair Regeneration Injection of conditioned medium collected from ASCs significantly increased the hair numbers in patients (Fukuoka and Suga 2015; Shin et al. 2015). The secretion of HGF, IGFBPs, M-CSF, PDGF-β, and VEGF might account for the hair follicle promoting effects (Park et al. 2010b; Won et al. 2010).

Others ASCs continue to receive considerable attention from investigators pursuing adult stem cells clinical applications in a variety of disorders. For example, ASCs have been used for the treatment of acute respiratory distress syndrome (Zheng et al. 2014) and a phase I clinical trial used ASCs to serve as carriers for an oncolytic measles virus therapy (Mader et al. 2013). It is likely that many investigators will examine the utility of ASC in multiple disease states; however, it is imperative that a strong mechanistic rationale be presented to regulatory authorities for the use of ASC before initiating any such studies.

14.8 Future Perspectives

Due to the abundance and great potential in stem cell area, ASCs have been received attention from multiple basic science and clinical disciplines. In addition to the direct usage of SVF/ASC/ ASC secretome in multiple disorders, studies have found that ASCs may be an ideal autologous cell source for reprogramming induced pluripotent stem cells (iPS) cells (Sun et al. 2009). ASCs can also serve as an effective therapeutic gene delivery vehicle (Kucerova et al. 2007). There is a growing body of evidence from independent, international laboratories documenting the preclinical and clinical safety and efficacy of ASC therapies. To advance the future clinical translation of ASC, it will be necessary to: (1) establish a Good Manufacturing Practice (GMP) standard for clinical cell isolation; (2) characterize the functional difference of ASC from different depots; (3) evaluate the safety and effectiveness of allogeneic ASC; (4) monitor the behavior of ASCs when they are transplanted *in vivo*, and, last but not least; (5) perform long term follow up on the intended and un-intended consequences in patients receiving ASC and SVF cell therapies.

Disclosure J.M. Gimble is the co-owner, co-founder and Chief Scientific Officer of LaCell LLC, a for-profit biotechnology company focusing on the development of stromal/stem cell research tools and their use for clinical translation.

References

Aronowitz JA, Ellenhorn JD (2013) Adipose stromal vascular fraction isolation: a head-to-head comparison of four commercial cell separation systems. Plastic Reconst Surg 132(6):932e–939e. doi:10.1097/PRS.0b013e3182a80652

Aust L, Devlin B, Foster SJ, Halvorsen YD, Hicok K, du Laney T, Sen A, Willingmyre GD, Gimble JM (2004) Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy 6(1):7–14. doi:10.1080/14653240310004539

Awad HA, Halvorsen YD, Gimble JM, Guilak F (2003) Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. Tissue Eng 9(6):1301–1312. doi:10.1089/10763270360728215

Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 25(16):3211–3222. doi:10.1016/j.biomaterials.2003.10.045

Baglioni S, Cantini G, Poli G, Francalanci M, Squecco R, Di Franco A, Borgogni E, Frontera S, Nesi G, Liotta F, Lucchese M, Perigli G, Francini F, Forti G, Serio M, Luconi M (2012) Functional differences in visceral and subcutaneous fat pads originate from differences in the adipose stem cell. PLoS One 7(5), e36569. doi:10.1371/journal.pone.0036569

Bartelt A, Heeren J (2014) Adipose tissue browning and metabolic health. Nat Rev Endocrinol 10(1):24–36. doi:10.1038/nrendo.2013.204

Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM (2013) Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 15(6):641–648. doi:10.1016/j.jcyt.2013.02.006

Bunnell BA, Betancourt AM, Sullivan DE (2010) New concepts on the immune modulation mediated by mesenchymal stem cells. Stem Cell Res Therapy 1(5):34. doi:10.1186/scrt34

Bura A, Planat-Benard V, Bourin P, Silvestre JS, Gross F, Grolleau JL, Saint-Lebese B, Peyrafitte JA, Fleury S, Gadelorge M, Taurand M, Dupuis-Coronas S, Leobon B, Casteilla L (2014) Phase I trial: the use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia. Cytotherapy 16(2):245–257. doi:10.1016/j.jcyt.2013.11.011

Cannon B, Nedergaard J (2004) Brown adipose tissue: function and physiological significance. Physiol Rev 84(1):277–359. doi:10.1152/physrev.00015.2003

Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC (2005) Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. Biochem Biophys Res Commun 332(2):370–379. doi:10.1016/j.bbrc.2005.04.135

Carriere A, Ebrahimian TG, Dehez S, Auge N, Joffre C, Andre M, Arnal S, Duriez M, Barreau C, Arnaud E, Fernandez Y, Planat-Benard V, Levy B, Penicaud L, Silvestre JS, Casteilla L (2009) Preconditioning by mitochondrial reactive oxygen species improves the proangiogenic potential of adipose-derived cells-based therapy. Arterioscler Thromb Vasc Biol 29(7):1093–1099. doi:10.1161/ATVBAHA.109.188318

Cavallari G, Olivi E, Bianchi F, Neri F, Foroni L, Valente S, La Manna G, Nardo B, Stefoni S, Ventura C (2012) Mesenchymal stem cells and islet cotransplantation in diabetic rats: improved islet graft revascularization and function by human adipose tissue-derived stem cells preconditioned with natural molecules. Cell Transplant 21(12):2771–2781. doi:10.3727/096368912X637046

Chavez-Munoz C, Nguyen KT, Xu W, Hong SJ, Mustoe TA, Galiano RD (2013) Transdifferentiation of adipose-derived stem cells into keratinocyte-like cells: engineering a stratified epidermis. PLoS One 8(12), e80587. doi:10.1371/journal.pone.0080587

Cho YB, Lee WY, Park KJ, Kim M, Yoo HW, Yu CS (2013) Autologous adipose tissue-derived stem cells for the treatment of Crohn's fistula: a phase I clinical study. Cell Transplant 22(2):279–285. doi:10.3727/096368912X656045

Cho YB, Park KJ, Yoon SN, Song KH, do Kim S, Jung SH, Kim M, Jeong HY, Yu CS (2015) Long-term results of adipose-derived stem cell therapy for the treatment of Crohn's fistula. Stem Cells Trans Med 4(5):532–537. doi:10.5966/sctm.2014-0199

Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L, Casteilla L (1992) Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. J Cell Sci 103(Pt 4):931–942

Cui JH, Park K, Park SR, Min BH (2006) Effects of low-intensity ultrasound on chondrogenic differentiation of mesenchymal stem cells embedded in polyglycolic acid: an in vivo study. Tissue Eng 12(1):75–82. doi:10.1089/ten.2006.12.75

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR (2009) Identification and importance of brown adipose tissue in adult humans. N Engl J Med 360(15):1509–1517. doi:10.1056/NEJMoa0810780

de la Portilla F, Alba F, Garcia-Olmo D, Herrerias JM, Gonzalez FX, Galindo A (2013) Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. Int J Color Dis 28(3):313–323. doi:10.1007/s00384-012-1581-9

Domenis R, Lazzaro L, Calabrese S, Mangoni D, Gallelli A, Bourkoula E, Manini I, Bergamin N, Toffoletto B, Beltrami CA, Beltrami AP, Cesselli D, Parodi PC (2015) Adipose tissue derived stem cells: in vitro and in vivo analysis of a standard and three commercially available cell-assisted lipotransfer techniques. Stem Cell Res Therapy 6(1):2. doi:10.1186/scrt536

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(4):315–317. doi:10.1080/14653240600855905

Egashira Y, Sugitani S, Suzuki Y, Mishiro K, Tsuruma K, Shimazawa M, Yoshimura S, Iwama T, Hara H (2012) The conditioned medium of murine and human adipose-derived stem cells exerts neuroprotective effects against experimental stroke model. Brain Res 1461:87–95. doi:10.1016/j.brainres.2012.04.033

Ercan E, Bagla AG, Aksoy A, Gacar G, Unal ZS, Asgun HF, Karaoz E (2014) In vitro protection of adipose tissue-derived mesenchymal stem cells by erythropoietin. Acta Histochem 116(1):117–125. doi:10.1016/j.acthis.2013.06.007

Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F (2002) Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun 290(2):763–769. doi:10.1006/bbrc.2001.6270

Estes BT, Diekman BO, Gimble JM, Guilak F (2010) Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc 5(7):1294–1311. doi:10.1038/nprot.2010.81

Estes BT, Gimble JM, Guilak F (2004) Mechanical signals as regulators of stem cell fate. Curr Top Dev Biol 60:91–126. doi:10.1016/S0070-2153(04)60004-4

Fan W, Cheng K, Qin X, Narsinh KH, Wang S, Hu S, Wang Y, Chen Y, Wu JC, Xiong L, Cao F (2013) mTORC1 and mTORC2 play different roles in the functional survival of transplanted adipose-derived stromal cells in hind limb ischemic mice via regulating inflammation in vivo. Stem Cells 31(1):203–214. doi:10.1002/stem.1265

Feng Y, Huang W, Meng W, Jegga AG, Wang Y, Cai W, Kim HW, Pasha Z, Wen Z, Rao F, Modi RM, Yu X, Ashraf M (2014) Heat shock improves Sca-1+ stem cell survival and directs ischemic cardiomyocytes toward a prosurvival phenotype via exosomal transfer: a critical role for HSF1/miR-34a/HSP70 pathway. Stem Cells 32(2):462–472. doi:10.1002/stem.1571

Frazier TP, Gimble JM, Kheterpal I, Rowan BG (2013) Impact of low oxygen on the secretome of human adipose-derived stromal/stem cell primary cultures. Biochimie 95(12):2286–2296. doi:10.1016/j.biochi.2013.07.011

Frohlich M, Grayson WL, Marolt D, Gimble JM, Kregar-Velikonja N, Vunjak-Novakovic G (2010) Bone grafts engineered from human adipose-derived stem cells in perfusion bioreactor culture. Tissue Eng A 16(1):179–189. doi:10.1089/ten.TEA.2009.0164

Fukuoka H, Suga H (2015) Hair regeneration treatment using adipose-derived stem cell conditioned medium: follow-up with trichograms. Eplasty 15, e10

Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA (2005) A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum 48(7):1416–1423. doi:10.1007/s10350-005-0052-6

Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M (2009) Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. Dis Colon Rectum 52(1):79–86. doi:10.1007/DCR.0b013e3181973487

Gawronska-Kozak B, Staszkiewicz J, Gimble JM, Kirk-Ballard H (2014) Recruitment of fat cell precursors during high fat diet in C57BL/6J mice is fat depot specific. Obesity 22(4):1091–1102. doi:10.1002/oby.20671

Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. Circ Res 100(9):1249–1260. doi:10.1161/01.RES.0000265074.83288.09

Gimble JM, Robinson CE, Wu X, Kelly KA (1996a) The function of adipocytes in the bone marrow stroma: an update. Bone 19(5):421–428

Gimble JM, Robinson CE, Wu X, Kelly KA, Rodriguez BR, Kliewer SA, Lehmann JM, Morris DC (1996b) Peroxisome proliferator-activated receptor-gamma activation by thiazolidinediones induces adipogenesis in bone marrow stromal cells. Mol Pharmacol 50(5):1087–1094

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM (2001) Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 189(1):54–63. doi:10.1002/jcp.1138

Haider H, Ashraf M (2010) Preconditioning and stem cell survival. J Cardiovasc Transl Res 3(2):89–102. doi:10.1007/s12265-009-9161-2

Hao P, Liang Z, Piao H, Ji X, Wang Y, Liu Y, Liu R, Liu J (2014) Conditioned medium of human adipose-derived mesenchymal stem cells mediates protection in neurons following glutamate excitotoxicity by regulating energy metabolism and GAP-43 expression. Metab Brain Dis 29(1):193–205. doi:10.1007/s11011-014-9490-y

Hardouin P, Pansini V, Cortet B (2014) Bone marrow fat. Joint Bone Spine 81(4):313–319. doi:10.1016/j.jbspin.2014.02.013

Harms M, Seale P (2013) Brown and beige fat: development, function and therapeutic potential. Nat Med 19(10):1252–1263. doi:10.1038/nm.3361

Hashemi SM, Hassan ZM, Pourfathollah AA, Soudi S, Shafiee A, Soleimani M (2013) Comparative immunomodulatory properties of adipose-derived mesenchymal stem cells conditioned media from BALB/c, C57BL/6, and DBA mouse strains. J Cell Biochem 114(4):955–965. doi:10.1002/jcb.24437

Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R, Pfeiffer EF (1989) Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J Clin Invest 84(5):1663–1670. doi:10.1172/JCI114345

Herreros MD, Garcia-Arranz M, Guadalajara H, De-La-Quintana P, Garcia-Olmo D, Group FC (2012) Autologous expanded adipose-derived stem cells for the treatment of complex cryptoglandular perianal fistulas: a phase III randomized clinical trial (FATT 1: fistula Advanced Therapy Trial 1) and long-term evaluation. Dis Colon Rectum 55(7):762–772. doi:10.1097/DCR.0b013e318255364a

Hoke NN, Salloum FN, Kass DA, Das A, Kukreja RC (2012) Preconditioning by phosphodiesterase-5 inhibition improves therapeutic efficacy of adipose-derived stem cells following myocardial infarction in mice. Stem Cells 30(2):326–335. doi:10.1002/stem.789

Hovey RC, McFadden TB, Akers RM (1999) Regulation of mammary gland growth and morphogenesis by the mammary fat pad: a species comparison. J Mammary Gland Biol Neoplasia 4(1):53–68

Hu L, Zhao J, Liu J, Gong N, Chen L (2013) Effects of adipose stem cell-conditioned medium on the migration of vascular endothelial cells, fibroblasts and keratinocytes. Exp Therapeutic Med 5(3):701–706. doi:10.3892/etm.2013.887

Hye Kim J, Gyu Park S, Kim WK, Song SU, Sung JH (2015) Functional regulation of adipose-derived stem cells by PDGF-D. Stem Cells 33(2):542–556. doi:10.1002/stem.1865

Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM, Bunnell BA (2006) Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J Cell Biochem 99(5):1285–1297. doi:10.1002/jcb.20904

Jeong YM, Sung YK, Kim WK, Kim JH, Kwack MH, Yoon I, Kim DD, Sung JH (2013) Ultraviolet B preconditioning enhances the hair growth-promoting effects of adipose-derived stem cells via generation of reactive oxygen species. Stem Cells Dev 22(1):158–168. doi:10.1089/scd.2012.0167

Jiang T, Liu W, Lv X, Sun H, Zhang L, Liu Y, Zhang WJ, Cao Y, Zhou G (2010) Potent in vitro chondrogenesis of CD105 enriched human adipose-derived stem cells. Biomaterials 31(13):3564–3571. doi:10.1016/j.biomaterials.2010.01.050

Jo CH, Lee YG, Shin WH, Kim H, Chai JW, Jeong EC, Kim JE, Shim H, Shin JS, Shin IS, Ra JC, Oh S, Yoon KS (2014) Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: a proof-of-concept clinical trial. Stem Cells 32(5):1254–1266. doi:10.1002/stem.1634

Kang KS, Hong JM, Seol YJ, Rhie JW, Jeong YH, Cho DW (2012) Short-term evaluation of electromagnetic field pretreatment of adipose-derived stem cells to improve bone healing. J Tissue Eng Regener Med. doi:10.1002/term.1664

Kapur SK, Katz AJ (2013) Review of the adipose derived stem cell secretome. Biochimie 95(12):2222–2228. doi:10.1016/j.biochi.2013.06.001

Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC (2005) Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. Stem Cells 23(3):412–423. doi:10.1634/stemcells.2004-0021

Kern S, Eichler H, Stoeve J, Kluter H, Bieback K (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24(5):1294–1301. doi:10.1634/stemcells.2005-0342

Kilroy GE, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A, Ludlow JW, Stricker DM, Potiny S, Green P, Halvorsen YD, Cheatham B, Storms RW, Gimble JM (2007) Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. J Cell Physiol 212(3):702–709. doi:10.1002/jcp.21068

Kim BS, Kang KS, Kang SK (2010) Soluble factors from ASCs effectively direct control of chondrogenic fate. Cell Prolif 43(3):249–261. doi:10.1111/j.1365-2184.2010.00680.x

Kim JH, Kim WK, Sung YK, Kwack MH, Song SY, Choi JS, Park SG, Yi T, Lee HJ, Kim DD, Seo HM, Song SU, Sung JH (2014) The molecular mechanism underlying the proliferating and preconditioning effect of vitamin C on adipose-derived stem cells. Stem Cells Dev 23(12):1364–1376. doi:10.1089/scd.2013.0460

Kim M, Choi YS, Yang SH, Hong HN, Cho SW, Cha SM, Pak JH, Kim CW, Kwon SW, Park CJ (2006) Muscle regeneration by adipose tissue-derived adult stem cells attached to injectable PLGA spheres. Biochem Biophys Res Commun 348(2):386–392. doi:10.1016/j.bbrc.2006.07.063

Kim WS, Park BS, Sung JH (2009) Protective role of adipose-derived stem cells and their soluble factors in photoaging. Arch Dermatol Res 301(5):329–336. doi:10.1007/s00403-009-0951-9

Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G (2007) Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Exp Neurol 207(2):267–274. doi:10.1016/j.expneurol.2007.06.029

Kisiday JD, Kurz B, DiMicco MA, Grodzinsky AJ (2005) Evaluation of medium supplemented with insulin-transferrin-selenium for culture of primary bovine calf chondrocytes in three-dimensional hydrogel scaffolds. Tissue Eng 11(1-2):141–151. doi:10.1089/ten.2005.11.141

Kolle SF, Fischer-Nielsen A, Mathiasen AB, Elberg JJ, Oliveri RS, Glovinski PV, Kastrup J, Kirchhoff M, Rasmussen BS, Talman ML, Thomsen C, Dickmeiss E, Drzewiecki KT (2013) Enrichment of autologous fat grafts with ex-vivo expanded adipose tissue-derived stem cells for graft survival: a randomised placebo-controlled trial. Lancet 382(9898):1113–1120. doi:10.1016/S0140-6736(13)61410-5

Kramer J, Hegert C, Guan K, Wobus AM, Muller PK, Rohwedel J (2000) Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. Mech Dev 92(2):193–205

Krampera M, Galipeau J, Shi Y, Tarte K, Sensebe L, Therapy MSCCotISfC (2013) Immunological characterization of multipotent mesenchymal stromal cells--The International Society for Cellular Therapy (ISCT) working proposal. Cytotherapy 15(9):1054–1061. doi:10.1016/j.jcyt.2013.02.010

Kucerova L, Altanerova V, Matuskova M, Tyciakova S, Altaner C (2007) Adipose tissue-derived human mesenchymal stem cells mediated prodrug cancer gene therapy. Cancer Res 67(13):6304–6313. doi:10.1158/0008-5472.CAN-06-4024

Langenbach F, Handschel J (2013) Effects of dexamethasone, ascorbic acid and beta-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Therapy 4(5):117. doi:10.1186/scrt328

Lee EY, Xia Y, Kim WS, Kim MH, Kim TH, Kim KJ, Park BS, Sung JH (2009) Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. Wound Repair Regener 17(4):540–547. doi:10.1111/j.1524-475X.2009.00499.x

Lee MJ, Wu Y, Fried SK (2013) Adipose tissue heterogeneity: implication of depot differences in adipose tissue for obesity complications. Mol Asp Med 34(1):1–11. doi:10.1016/j.mam.2012.10.001

Lee SC, Jeong HJ, Lee SK, Kim SJ (2015a) Lipopolysaccharide preconditioning of adipose-derived stem cells improves liver-regenerating activity of the secretome. Stem Cell Res Therapy 6:75. doi:10.1186/s13287-015-0072-7

Lee SC, Kim JO, Kim SJ (2015b) Secretome from human adipose-derived stem cells protects mouse liver from hepatic ischemia-reperfusion injury. Surgery 157(5):934–943. doi:10.1016/j.surg.2014.12.016

Lee SK, Kim DW, Dhong ES, Park SH, Yoon ES (2012) Facial soft tissue augmentation using autologous fat mixed with stromal vascular fraction. Arch Plastic Surg 39(5):534–539. doi:10.5999/aps.2012.39.5.534

Lee SK, Lee SC, Kim SJ (2015c) A novel cell-free strategy for promoting mouse liver regeneration: utilization of a conditioned medium from adipose-derived stem cells. Hepatol Int 9(2):310–320. doi:10.1007/s12072-014-9599-4

Lee SM, Lee SC, Kim SJ (2014) Contribution of human adipose tissue-derived stem cells and the secretome to the skin allograft survival in mice. J Surg Res 188(1):280–289. doi:10.1016/j.jss.2013.10.063

Lendeckel S, Jodicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP (2004) Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. J Cranio-Maxillo-Facial Surg 32(6):370–373. doi:10.1016/j.jcms.2004.06.002

Li H, Zimmerlin L, Marra KG, Donnenberg VS, Donnenberg AD, Rubin JP (2011) Adipogenic potential of adipose stem cell subpopulations. Plast Reconstr Surg 128(3):663–672. doi:10.1097/PRS.0b013e318221db33

Linero I, Chaparro O (2014) Paracrine effect of mesenchymal stem cells derived from human adipose tissue in bone regeneration. PLoS One 9(9), e107001. doi:10.1371/journal.pone.0107001

Liu GS, Peshavariya HM, Higuchi M, Chan EC, Dusting GJ, Jiang F (2013) Pharmacological priming of adipose-derived stem cells for paracrine VEGF production with deferoxamine. J Tissue Eng Regener Med. doi:10.1002/term.1796

Lopatina T, Kalinina N, Karagyaur M, Stambolsky D, Rubina K, Revischin A, Pavlova G, Parfyonova Y, Tkachuk V (2011) Adipose-derived stem cells stimulate regeneration of peripheral nerves: BDNF secreted by these cells promotes nerve healing and axon growth de novo. PLoS One 6(3), e17899. doi:10.1371/journal.pone.0017899

Lu Z, Wang G, Dunstan CR, Chen Y, Lu WY, Davies B, Zreiqat H (2013) Activation and promotion of adipose stem cells by tumour necrosis factor-alpha preconditioning for bone regeneration. J Cell Physiol 228(8):1737–1744. doi:10.1002/jcp.24330

Luo Z, Jiang L, Xu Y, Li H, Xu W, Wu S, Wang Y, Tang Z, Lv Y, Yang L (2015) Mechano growth factor (MGF) and transforming growth factor (TGF)-beta3 functionalized silk scaffolds enhance articular hyaline cartilage regeneration in rabbit model. Biomaterials 52:463–475. doi:10.1016/j.biomaterials.2015.01.001

Mader EK, Butler G, Dowdy SC, Mariani A, Knutson KL, Federspiel MJ, Russell SJ, Galanis E, Dietz AB, Peng KW (2013) Optimizing patient derived mesenchymal stem cells as virus carriers for a phase I clinical trial in ovarian cancer. J Transl Med 11:20. doi:10.1186/1479-5876-11-20

Malladi P, Xu Y, Chiou M, Giaccia AJ, Longaker MT (2006) Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. Am J Phys Cell Physiol 290(4):C1139–C1146. doi:10.1152/ajpcell.00415.2005

Marino G, Moraci M, Armenia E, Orabona C, Sergio R, De Sena G, Capuozzo V, Barbarisi M, Rosso F, Giordano G, Iovino F, Barbarisi A (2013) Therapy with autologous adipose-derived regenerative cells for the care of chronic ulcer of lower limbs in patients with peripheral arterial disease. J Surg Res 185(1):36–44. doi:10.1016/j.jss.2013.05.024

McCullen SD, McQuilling JP, Grossfeld RM, Lubischer JL, Clarke LI, Loboa EG (2010) Application of low-frequency alternating current electric fields via interdigitated electrodes: effects on cellular viability, cytoplasmic calcium, and osteogenic differentiation of human adipose-derived stem cells. Tissue Eng Part C Methods 16(6):1377–1386. doi:10.1089/ten.TEC.2009.0751

Merfeld-Clauss S, Lupov IP, Lu H, March KL, Traktuev DO (2015) Adipose stromal cell contact with endothelial cells results in loss of complementary vasculogenic activity mediated by induction of Activin A. Stem Cells. doi:10.1002/stem.2074

Michalek J, Moster R, Lukac L, Proefrock K, Petrasovic M, Rybar J, Capkova M, Chaloupka A, Darinskas A, Michalek J Sr, Kristek J, Travnik J, Jabandziev P, Cibulka M, Holek M, Jurik M, Skopalik J, Kristkova Z, Dudasova Z (2015) Autologous adipose tissue-derived stromal vascular fraction cells application in patients with osteoarthritis. Cell Transplant. doi:10.3727/096368915X686760

Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24(2):376–385. doi:10.1634/stemcells.2005-0234

Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH (2002) Myogenic differentiation by human processed lipoaspirate cells. Plast Reconstr Surg 109(1):199–209, discussion 210–191

Mohyeldin A, Garzon-Muvdi T, Quinones-Hinojosa A (2010) Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7(2):150–161. doi:10.1016/j.stem.2010.07.007

Norgaard R, Kassem M, Rattan SI (2006) Heat shock-induced enhancement of osteoblastic differentiation of hTERT-immortalized mesenchymal stem cells. Ann N Y Acad Sci 1067:443–447. doi:10.1196/annals.1354.063

Ntambi JM, Young-Cheul K (2000) Adipocyte differentiation and gene expression. J Nutr 130(12):3122S–3126S

Ong WK, Tan CS, Chan KL, Goesantoso GG, Chan XH, Chan E, Yin J, Yeo CR, Khoo CM, So JB, Shabbir A, Toh SA, Han W, Sugii S (2014) Identification of specific cell-surface markers of adipose-derived stem cells from subcutaneous and visceral fat depots. Stem Cell Rep 2(2):171–179. doi:10.1016/j.stemcr.2014.01.002

Otto TC, Lane MD (2005) Adipose development: from stem cell to adipocyte. Crit Rev Biochem Mol Biol 40(4):229–242. doi:10.1080/10409230591008189

Park A, Hogan MV, Kesturu GS, James R, Balian G, Chhabra AB (2010a) Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendon-specific markers. Tissue Eng A 16(9):2941–2951. doi:10.1089/ten.tea.2009.0710

Park BS, Kim WS, Choi JS, Kim HK, Won JH, Ohkubo F, Fukuoka H (2010b) Hair growth stimulated by conditioned medium of adipose-derived stem cells is enhanced by hypoxia: evidence of increased growth factor secretion. Biomed Res 31(1):27–34

Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J (2010) Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. J Biol Chem 285(10):7153–7164. doi:10.1074/jbc.M109.053942

Philips BJ, Grahovac TL, Valentin JE, Chung CW, Bliley JM, Pfeifer ME, Roy SB, Dreifuss S, Kelmendi-Doko A, Kling RE, Ravuri SK, Marra KG, Donnenberg VS, Donnenberg AD, Rubin JP (2013) Prevalence of endogenous CD34+ adipose stem cells predicts human fat graft retention in a xenograft model. Plast Reconstr Surg 132(4):845–858. doi:10.1097/PRS.0b013e31829fe5b1

Prunet-Marcassus B, Cousin B, Caton D, Andre M, Penicaud L, Casteilla L (2006) From heterogeneity to plasticity in adipose tissues: site-specific differences. Exp Cell Res 312(6):727–736. doi:10.1016/j.yexcr.2005.11.021

Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 109(10):1292–1298. doi:10.1161/01.CIR.0000121425.42966.F1

Robey TE, Saiget MK, Reinecke H, Murry CE (2008) Systems approaches to preventing transplanted cell death in cardiac repair. J Mol Cell Cardiol 45(4):567–581. doi:10.1016/j.yjmcc.2008.03.009

Rodeheffer MS, Birsoy K, Friedman JM (2008) Identification of white adipocyte progenitor cells in vivo. Cell 135(2):240–249. doi:10.1016/j.cell.2008.09.036

Rosen ED, MacDougald OA (2006) Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 7(12):885–896. doi:10.1038/nrm2066

Rosenwald M, Perdikari A, Rulicke T, Wolfrum C (2013) Bi-directional interconversion of brite and white adipocytes. Nat Cell Biol 15(6):659–667. doi:10.1038/ncb2740

Sanchez-Gurmaches J, Hung CM, Sparks CA, Tang Y, Li H, Guertin DA (2012) PTEN loss in the Myf5 lineage redistributes body fat and reveals subsets of white adipocytes that arise from Myf5 precursors. Cell Metab 16(3):348–362. doi:10.1016/j.cmet.2012.08.003

Sandor GK, Numminen J, Wolff J, Thesleff T, Miettinen A, Tuovinen VJ, Mannerstrom B, Patrikoski M, Seppanen R, Miettinen S, Rautiainen M, Ohman J (2014) Adipose stem cells used to reconstruct 13 cases with cranio-maxillofacial hard-tissue defects. Stem Cells Trans Med 3(4):530–540. doi:10.5966/sctm.2013-0173

Sanz-Baro R, Garcia-Arranz M, Guadalajara H, de la Quintana P, Herreros MD, Garcia-Olmo D (2015) First-in-human case study: pregnancy in women with Crohn's perianal fistula treated with adipose-derived stem cells: a safety study. Stem Cells Trans Med 4(6):598–602. doi:10.5966/sctm.2014-0255

Sart S, Ma T, Li Y (2014) Preconditioning stem cells for in vivo delivery. BioRes Open Access 3(4):137–149. doi:10.1089/biores.2014.0012

Schipper BM, Marra KG, Zhang W, Donnenberg AD, Rubin JP (2008) Regional anatomic and age effects on cell function of human adipose-derived stem cells. Ann Plast Surg 60(5):538–544. doi:10.1097/SAP.0b013e3181723bbe

Schulz TJ, Huang TL, Tran TT, Zhang H, Townsend KL, Shadrach JL, Cerletti M, McDougall LE, Giorgadze N, Tchkonia T, Schrier D, Falb D, Kirkland JL, Wagers AJ, Tseng YH (2011) Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. Proc Natl Acad Sci U S A 108(1):143–148. doi:10.1073/pnas.1010929108

Seale P (2010) Transcriptional control of brown adipocyte development and thermogenesis. Int J Obes 34(Suppl 1):S17–S22. doi:10.1038/ijo.2010.178

Seo G, Oh E, Yun M, Lee JY, Bae JS, Joo K, Chae GT, Lee SB (2015) Adipose-derived stem cell conditioned medium accelerates keratinocyte differentiation via the up-regulation of miR-24. Exp Dermatol. doi:10.1111/exd.12754

Shah FS, Li J, Dietrich M, Wu X, Hausmann MG, LeBlanc KA, Wade JW, Gimble JM (2015) Comparison of stromal/stem cells isolated from human Omental and subcutaneous adipose depots: differentiation and immunophenotypic characterization. Cells Tissues Organs. doi:10.1159/000430088

Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, Hu H, Wang L, Pavlova Z, Gilsanz V, Kajimura S (2012) Human BAT possesses molecular signatures that resemble beige/brite cells. PLoS One 7(11), e49452. doi:10.1371/journal.pone.0049452

Shin H, Ryu HH, Kwon O, Park BS, Jo SJ (2015) Clinical use of conditioned media of adipose tissue-derived stem cells in female pattern hair loss: a retrospective case series study. Int J Dermatol 54(6):730–735. doi:10.1111/ijd.12650

Shintani N, Hunziker EB (2011) Differential effects of dexamethasone on the chondrogenesis of mesenchymal stromal cells: influence of microenvironment, tissue origin and growth factor. Eur Cells Mater 22:302–319, discussion 319–320

Silva FJ, Holt DJ, Vargas V, Yockman J, Boudina S, Atkinson D, Grainger DW, Revelo MP, Sherman W, Bull DA, Patel AN (2014) Metabolically active human brown adipose tissue derived stem cells. Stem Cells 32(2):572–581. doi:10.1002/stem.1595

Sowa Y, Imura T, Numajiri T, Nishino K, Fushiki S (2012) Adipose-derived stem cells produce factors enhancing peripheral nerve regeneration: influence of age and anatomic site of origin. Stem Cells Dev 21(11):1852–1862. doi:10.1089/scd.2011.0403

Stephens M, Ludgate M, Rees DA (2011) Brown fat and obesity: the next big thing? Clin Endocrinol 74(6):661–670. doi:10.1111/j.1365-2265.2011.04018.x

Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, Fraser JK, Hedrick MH (2005) Multipotential differentiation of adipose tissue-derived stem cells. Keio J Med 54(3):132–141

Suga H, Glotzbach JP, Sorkin M, Longaker MT, Gurtner GC (2014) Paracrine mechanism of angiogenesis in adipose-derived stem cell transplantation. Ann Plast Surg 72(2):234–241. doi:10.1097/SAP.0b013e318264fd6a

Suga H, Matsumoto D, Eto H, Inoue K, Aoi N, Kato H, Araki J, Yoshimura K (2009) Functional implications of CD34 expression in human adipose-derived stem/progenitor cells. Stem Cells Dev 18(8):1201–1210. doi:10.1089/scd.2009.0003

Sun N, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F, Hu S, Cherry AM, Robbins RC, Longaker MT, Wu JC (2009) Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proc Natl Acad Sci U S A 106(37):15720–15725. doi:10.1073/pnas.0908450106

Sung HM, Suh IS, Lee HB, Tak KS, Moon KM, Jung MS (2012) Case reports of adipose-derived stem cell therapy for nasal skin necrosis after filler injection. Archiv Plastic Surg 39(1):51–54. doi:10.5999/aps.2012.39.1.51

Talens-Visconti R, Bonora A, Jover R, Mirabet V, Carbonell F, Castell JV, Gomez-Lechon MJ (2006) Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. World J Gastroenterol 12(36):5834–5845

Tandon N, Goh B, Marsano A, Chao PH, Montouri-Sorrentino C, Gimble J, Vunjak-Novakovic G (2009) Alignment and elongation of human adipose-derived stem cells in response to direct-current electrical stimulation. In: Conference proceedings: annual international conference of the IEEE engineering in medicine and biology society IEEE engineering in medicine and biology society annual conference 2009:6517–6521. doi:10.1109/IEMBS.2009.5333142

Ussar S, Lee KY, Dankel SN, Boucher J, Haering MF, Kleinridders A, Thomou T, Xue R, Macotela Y, Cypess AM, Tseng YH, Mellgren G, Kahn CR (2014) ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. Sci Trans Med 6(247):247ra103. doi:10.1126/scitranslmed.3008490

van Osch GJ, van der Veen SW, Buma P, Verwoerd-Verhoef HL (1998) Effect of transforming growth factor-beta on proteoglycan synthesis by chondrocytes in relation to differentiation stage and the presence of pericellular matrix. Matrix Biol J Int Soc Matrix Biol 17(6):413–424

Van Pham P, Bui KH, Ngo DQ, Vu NB, Truong NH, Phan NL, Le DM, Duong TD, Nguyen TD, Le VT, Phan NK (2013) Activated platelet-rich plasma improves adipose-derived stem cell transplantation efficiency in injured articular cartilage. Stem Cell Res Therapy 4(4):91. doi:10.1186/scrt277

Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S, Nuutila P (2009) Functional brown adipose tissue in healthy adults. N Engl J Med 360(15):1518–1525. doi:10.1056/NEJMoa0808949

Walmsley GG, Atashroo DA, Maan ZN, Hu MS, Zielins ER, Tsai JM, Duscher D, Paik K, Tevlin R, Marecic O, Wan DC, Gurtner GC, Longaker MT (2015) High-throughput screening of surface marker expression on undifferentiated and differentiated human adipose-derived stromal cells. Tissue Eng A. doi:10.1089/ten.TEA.2015.0039

Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F (2005) Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. J Cell Physiol 204(1):184–191. doi:10.1002/jcp.20324

Wang H, Shi J, Wang Y, Yin Y, Wang L, Liu J, Liu Z, Duan C, Zhu P, Wang C (2014a) Promotion of cardiac differentiation of brown adipose derived stem cells by chitosan hydrogel for repair after myocardial infarction. Biomaterials 35(13):3986–3998. doi:10.1016/j.biomaterials.2014.01.021

Wang W, Kissig M, Rajakumari S, Huang L, Lim HW, Won KJ, Seale P (2014b) Ebf2 is a selective marker of brown and beige adipogenic precursor cells. Proc Natl Acad Sci U S A 111(40):14466–14471. doi:10.1073/pnas.1412685111

Williams KJ, Godke RA, Bondioli KR (2011) Isolation and culture of porcine adipose tissue-derived somatic stem cells. Methods Mol Biol 702:77–86. doi:10.1007/978-1-61737-960-4_7

Won CH, Yoo HG, Kwon OS, Sung MY, Kang YJ, Chung JH, Park BS, Sung JH, Kim WS, Kim KH (2010) Hair growth promoting effects of adipose tissue-derived stem cells. J Dermatol Sci 57(2):134–137. doi:10.1016/j.jdermsci.2009.10.013

Wosnitza M, Hemmrich K, Groger A, Graber S, Pallua N (2007) Plasticity of human adipose stem cells to perform adipogenic and endothelial differentiation. Differ Res Biol Divers 75(1):12–23. doi:10.1111/j.1432-0436.2006.00110.x

Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerback S, Schrauwen P, Spiegelman BM (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell 150(2):366–376. doi:10.1016/j.cell.2012.05.016

Wu J, Jun H, McDermott JR (2015) Formation and activation of thermogenic fat. Trends Genet 31(5):232–238. doi:10.1016/j.tig.2015.03.003

Yamada Y, Wang XD, Yokoyama S, Fukuda N, Takakura N (2006) Cardiac progenitor cells in brown adipose tissue repaired damaged myocardium. Biochem Biophys Res Commun 342(2):662–670. doi:10.1016/j.bbrc.2006.01.181

Yang G, Rothrauff BB, Lin H, Gottardi R, Alexander PG, Tuan RS (2013) Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix. Biomaterials 34(37):9295–9306. doi:10.1016/j.biomaterials.2013.08.054

Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, Harii K (2008) Cell-assisted lipotransfer for cosmetic breast augmentation: supportive use of adipose-derived stem/stromal cells. Aesthet Plast Surg 32(1):48–55. doi:10.1007/s00266-007-9019-4, discussion 56–47

Yu G, Floyd ZE, Wu X, Hebert T, Halvorsen YD, Buehrer BM, Gimble JM (2011) Adipogenic differentiation of adipose-derived stem cells. Methods Mol Biol 702:193–200. doi:10.1007/978-1-61737-960-4_14

Yue Y, Yang X, Wei X, Chen J, Fu N, Fu Y, Ba K, Li G, Yao Y, Liang C, Zhang J, Cai X, Wang M (2013) Osteogenic differentiation of adipose-derived stem cells prompted by low-intensity pulsed ultrasound. Cell Prolif 46(3):320–327. doi:10.1111/cpr.12035

Zheng G, Huang L, Tong H, Shu Q, Hu Y, Ge M, Deng K, Zhang L, Zou B, Cheng B, Xu J (2014) Treatment of acute respiratory distress syndrome with allogeneic adipose-derived mesenchymal stem cells: a randomized, placebo-controlled pilot study. Respir Res 15:39. doi:10.1186/1465-9921-15-39

Zhou H, Yang J, Xin T, Li D, Guo J, Hu S, Zhou S, Zhang T, Zhang Y, Han T, Chen Y (2014) Exendin-4 protects adipose-derived mesenchymal stem cells from apoptosis induced by hydrogen peroxide through the PI3K/Akt-Sfrp2 pathways. Free Radic Biol Med 77:363–375. doi:10.1016/j.freeradbiomed.2014.09.033

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13(12):4279–4295. doi:10.1091/mbc.E02-02-0105

Zvonic S, Lefevre M, Kilroy G, Floyd ZE, DeLany JP, Kheterpal I, Gravois A, Dow R, White A, Wu X, Gimble JM (2007) Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. Mol Cell Proteom 6(1):18–28. doi:10.1074/mcp.M600217-MCP200

Chapter 15
Cell Programming for Future Regenerative Medicine

Frauke Hausburg and Robert David

Abstract Numerous diseases are characterized by malfunction of key cells or their faulty integration within highly complex organ systems. Intense research over the last decade has led to a better understanding of these disorders on the molecular level. This is an indispensable prerequisite for restoring the functionality of the affected organs, representing the first and foremost aim of the rapidly developing field of regenerative medicine. However, adult organs exhibit limited self-renewal capacity and often cannot accomplish a functional restoration on their own. Therefore, new strategies are currently being considered for cell programming based replacement therapies as well as for disease modelling and drug development. Before the discovery that somatic cells could be either reprogrammed to a pluripotent state or directly converted to other somatic cell types, the regenerative field was hampered by ethical concerns connected to human embryonic stem cells as well as by restricted availability of adult stem cells. Meanwhile, several strategies based on the introduction of lineage specific transcription factors, mRNAs, microRNAs and small molecules have opened up new perspectives for safe and efficient generation of induced pluripotent stem cells (iPSCs) as well as various specified somatic cell types. The foremost priority should be the generation of fully functional cells with characteristics as close as possible to their natural counterparts. Furthermore, the production of clinically relevant numbers of healthy cells as well as cells with defined disease patterns could provide an important link between basic research, drug screening and safety testing and ultimately clinical trials. In this article we summarize the remarkable recent successes in cellular reprogramming, which have highly contributed to the great progress of regenerative medicine over the last years.

Keywords iPSC • ESC • Differentiation • Reprogramming • Drug discovery • Disease modeling • Cell transplantation

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_15

15.1 Introduction

The new upcoming field of Regenerative Medicine starts where conventional medicine is no longer satisfactory. Existing medical applications demonstrated the limits of former treatment options. In near future we will have to face an ageing society and its negative health consequences; age-related disorders are becoming visible as well as diseases of affluence in western countries. Physicians and mainly scientists now need to develop new therapeutic options based on the replacement of damaged tissue with patient-specific cell therapy technologies. Human diseases such as neurodegenerative (e.g. Alzheimers or Parkinson disease) or cardiovascular disorders (ischemic heart disease and stroke) and Diabetes Mellitus are consequences of an unusual behavior of resident cells. The adult organs possess only limited self-renewal capacity and therefore lack the potential to restore the original function.

Initial approaches to such novel strategies were based on cell-transplantation using adult stem cells. The first bone-marrow transplantation was performed in 1957 by E. Donnall Thomas between identical twins, the recipient with leukemia. In 1990, the Nobel Prize in Physiology or Medicine was awarded to E. Donnall Thomas together with Joseph E. Murray "for their discoveries concerning organ and cell transplantation in the treatment of human disease" (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1990/). Nowadays, stem cell transplantation therapies in cardiovascular and neurological disorders are concepts progressing toward clinical use (Rosen et al. 2014; Pavo et al. 2014; Matar and Chong 2014); (Feo et al. 2012; Mothe and Tator 2013), but still under controversy discussions regarding their only modest therapeutic outcome (Pavo et al. 2014). Long-term studies with substantial numbers of patients have to clarify all questions addressing safety and efficiency.

Adult stem cells are multipotent and therefore only able to generate cells of a distinct lineage (Fig. 15.1). Therefore, one attractive source for cell replacement therapies and especially for studying key cellular and molecular programs are human pluripotent stem cells (hPSCs). In 1998 the first human embryonic stem cells (hESCs) were obtained, derived from blastocysts (Thomson 1998). In undifferentiated state, they express cell surface markers characteristic for primate ESC, including stage-specific embryonic antigen (SSEA) 3 and 4, tumor rejection antigen 1 (TRA-1-60, TRA-1-81) and alkaline phosphatase, and maintained this *in vitro* for 4–5 months, with the option for unlimited propagation. The human ES cell lines offer the potential to form all three embryonic germ layers (endoderm, mesoderm and ectoderm) (Fig. 15.1) and produce teratomas after injection into severe combined immunodeficient (SCID) mice.

The Human Pluripotent Stem Cell registry (hPSCreg) listed in total 805 cell lines in January 2016 (hESC:685, hiPSC 120) (http://hpscreg.eu/), whereby worldwide most of the hESCs are recorded in the United States (in Europe: Sweden). Beside, over 350 hESC have been approved by the NIH (http://grants.nih.gov/stem_cells/registry/current.htm) and used in several laboratories (Reynolds and Lamba 2014; Atkinson et al. 2013; Barad et al. 2014; Lerou and Daley 2005). Yet, so far only few

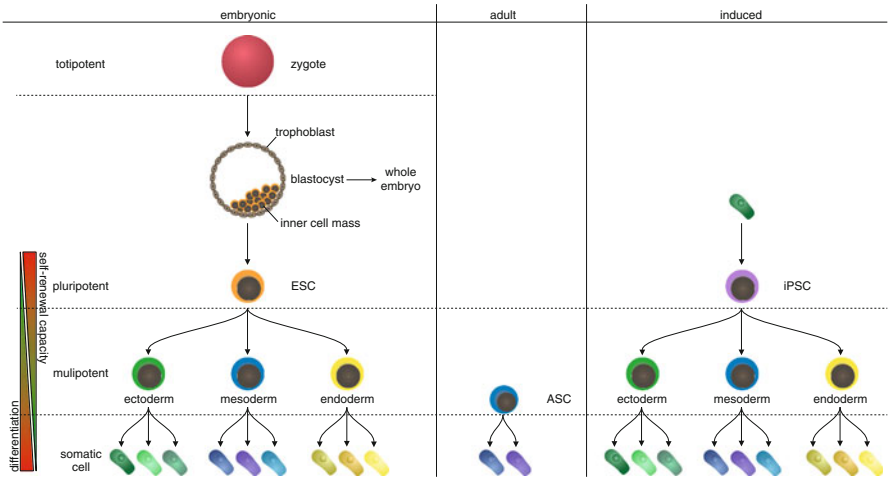

Fig. 15.1 Pathways of embryonic, adult and induced differentiation
Mammalian development begins with the fertilized zygote as a unique totipotent state. The extraction of the inner cell mass is feasible via destruction of the blastocyst, thereby generating *in vitro* cultivable embryonic stem cells (ESCs). Pluripotent ESCs exhibit a high self-renewal capacity and are able to differentiate into all multipotent germ layer progenitors (ectoderm, mesoderm, endoderm), and in consequence into all somatic cells of the mammalian body. In contrast, multipotent adult stem cells (ASCs) are located in the adult body and only capable to generate cells of a specific lineage. By using reprogramming strategies, it is possible to convert fully differentiated somatic cells into induced pluripotent stem cells (iPSC). These iPS cells are characterized by a molecular profile and behavior related to ESCs including a high self-renewal capacity and the ability to differentiate in cell types of all three germ layers

clinical trials have been started with the focus on dry age-related macular degeneration (AMD) and Stargardts macular dystrophy (Schwartz et al. 2012, 2015). However, hESCs bear serious disadvantages for research as well as clinical trials, (i) ethical reasons and (ii) immunological concerns: while ESC have been suggested to be immune privileged and therefore were not expected to cause significant immune rejection, studies with undifferentiated murine ES cells suggested the opposite (Nussbaum et al. 2007; Swijnenburg et al. 2007, 2008). The countries in Europe proceed differently regarding ethical concerns to human embryonic stem cells (http://hpscreg.eu/). Malta, Luxembourg and Ireland for example have currently no legislation. In contrast, in Lithuania, Poland, Germany, Slovakia, Austria and Italy, hESC research or cloning are mostly prohibited or bound to stringent legislations.

To this end, one of the most important and essential findings which could give deeper insights, was the establishment of iPSC technology from Yamanaka's group (Takahashi and Yamanaka 2006; Takahashi et al. 2007) (see below). The idea to dedifferentiate cells back to a pluripotent state was existing since the early 1960s. Gurdon clarified whether the genetic information in the nuclei of one somatic cell is sufficient to generate a whole animal, with studies on tadpoles of *Xenopus laevis* (Gurdon 1962). The technique, somatic cell nuclear transfer (SCNT), describes the

ability to insert a donor nucleus of one somatic cell into an enucleated oocyte with the potential to generate fertile organisms. It took several years until Ian Wilmut raised SCNT to a new level, in which he cloned the first mammalian, the sheep dolly (Wilmut et al. 1997). For their outstanding work, the Nobel Prize in Physiology or Medicine was awarded in 2012 to Sir John B. Gurdon and Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent" (http://www.nobelprize.org/nobel_prizes/medicine/laureates/2012/) (Gurdon 2013; Yamanaka 2013).

A relatively old, but since recent years aspiring method is called direct reprogramming or direct conversion. The basic idea behind it is a cell fate switch from one terminally differentiated somatic cell to another somatic cell of the same germ layer or even across germ layers, without an intermediate pluripotent state. The driving force is the application of key lineage-specific factors, in many cases supported by an intervention of key signaling pathways through adding proteins, microRNAs or small molecules. In this regard, 28 years ago, Weintraub's group reported the first amazing example, that one key transcription factor MyoD is sufficient to convert murine fibroblasts into skeletal muscle cells (Davis et al. 1987). Smooth muscle cells could also be achieved with the overexpression of the single factor Myocardin, via its interaction with serum response factor (SRF) which controls virtually all smooth muscle genes (Wang et al. 2003). Direct conversion with one or more transcription factors has subsequently been realized for various cell types, including insulin$^+$ β-cells (Smith and Zhang 2015; Benraiss et al. 2013; Xu et al. 2015; Heinrich et al. 2015), hepatocytes (Smith and Zhang 2015; Xu et al. 2015), osteoblasts (Ong et al. 2015), hematopoietic lineage cells (Xu et al. 2015; Ong et al. 2015), neurons (Smith and Zhang 2015; Xu et al. 2015; Ong et al. 2015; Ho et al. 2015; Heinrich et al. 2015) and cardiomyocytes (Smith and Zhang 2015; Xu et al. 2015; Chen et al. 2004b; Xu et al. 2015; Ong et al. 2015; Sahara et al. 2015; Budniatzky and Gepstein 2014) (Table 15.2, see below).

Stem cells and their derivatives as well as directly reprogrammed cells show tremendous potential for disease modeling and they represent an important link between basic research and clinical trials. Personalized cell lines from patients with defined disease symptoms gave a valuable insight into the cellular and molecular mechanism which is not possible with animal models.

15.2 iPSC

15.2.1 iPSCs – A Universal Solution

As indicated above, the discovery of induced pluripotent stem cells (iPSCs) now offers great opportunities in regenerative medicine with their benefit to expand disease relevant molecular informations, while avoiding ethical concerns of humane ESCs. In 2006, Takahashi and Yamanaka published the reprogramming of mouse

fibroblasts only via the introduction of the factors octamer-binding transcription factor 3/4 (*Oct3/4*; O), sex determining region Y-box 2 (*Sox2*; S), Krueppel-like factor 4 (*Klf4*, K) and *c-Myc* (M) (Takahashi and Yamanaka 2006). The resulting iPSC highly resemble murine ESCs with respect to their morphological appearance, proliferative potential, as well as teratogenicity. One year later, the same group was able to transfer this approach to human fibroblasts (Takahashi et al. 2007). Simultaneously, Thomson's group reported the successful generation of human iPSC using *OCT4*, *SOX2*, *NANOG* and *LIN28* (Yu et al. 2007). iPSCs have the potential to differentiate into all three germ layers (Fig. 15.1) which made them a great tool for embryonic development as well as differentiation protocols.

Since this, a great number of publications have come out describing optimized generation of iPS cells (Table 15.1), with higher efficiency and lesser risks of genetic alteration (Lister et al. 2011) or integration into the genome. These comprise integrative retro- and lentivirus free introduction of the Yamanaka factors as well as

Table 15.1 Comparison of different integrating and non-integrating approaches for somatic cell reprogramming to iPS cells

Vector type	Cell types	Integration	Factors	Efficiency (%)	Literature
Retroviral	Mouse and human fibroblasts	Yes	OSKM	~0.001–0.1	Takahashi and Yamanaka (2006) and Takahashi et al. (2007)
Lentiviral	Human somatic cells	Yes	OSNL	0.02	Yu et al. (2007)
piggyBac	Human fibroblasts	Yes	OSKM	0.02–0.05	Kaji et al. (2009)
Adenoviral	Human fibroblasts	No	OSKM	~0.001	Zhou and Freed (2009)
Protein	Human fibroblasts	No	OS	0.001	Kim et al. (2009) and Zhou et al. (2009)
Modified mRNA	Human fibroblasts	No	OSKM	~1.4	Warren et al. (2010)
			OSKML + VPA	~4.4	
microRNA	Mouse and human adipose stromal cells and dermal fibroblasts	No	miR-302/miR-367 or miR-200c + miR-302a-d + miR-369-p + miR-369-5p	~0.002–0.1	Miyoshi et al. (2011)
Small-molecules	Mouse fibroblasts	No	VC6TFZ and 2i	0.2	Hou et al. (2013)

Abbreviations: *iPSC* induced pluripotent stem cells, *miR* microRNA, *VC6TFZ* VC6T (valproic acid, CHIR99021, 616452, tranylcypromine), F (forskolin), Z (3-deazaneplanocin), *2i* dual inhibition

piggy-back methods. Many groups constantly make efforts to overcome these problems, in order to create an optimal basis for the translation into the clinic. Non-integrative methods like the introduction of transcription factors as proteins (Kim et al. 2009; Zhou et al. 2009), modified mRNA (Warren et al. 2010) or the use of microRNA (Miyoshi et al. 2011) and small-molecule compounds (Hou et al. 2013) is increasing. The prevention of oncogenes and deletion of tumorsuppressor-genes would be a worthy goal. The aberrant reprogramming of DNA methylation is mainly present in regions proximal to centromeres and telomeres, which display an incomplete reprogramming of non-CG methylation as well as differences in CG methylation and histone modifications (Lister et al. 2011). Furthermore, this reprogramming signature is maintained after differentiation. The epigenetic memory of the cells could also be an advantage for cell replacement therapies (Bar-Nur et al. 2011), since they might be influenced by their cells of origin. To this regard, careful epigenetic screenings based on standardized analyses need to be developed prior to use in large scale clinical trials.

So far, the full mechanism behind iPSC generation is still unclear and the number of articles is infinitesimal. It is assumed that the cellular reprogramming induced by exogenous overexpression of OSKM undergoes a stepwise process (Mah et al. 2011), in which two stages of gene expression ensure a full reprogramming of fibroblasts to induced pluripotent stem cells (Polo et al. 2012). Thereby, the initial burst may come from *c-Myc*/*Klf4* (first wave), which enhances the expression of genes controlling DNA replication and cell division. The epigenetic remodeling (DNA methylation) then takes place after the second wave (*Oct4*/*Sox2*/*Klf4*) (Polo et al. 2012).

However, an international collaboration, termed *Project Grandiose*, achieved a great step forward in understanding the biological properties during iPSC generation. Recent publications (Benevento et al. 2014; Clancy et al. 2014; Hussein et al. 2014; Lee et al. 2014; Tonge et al. 2014) could show that during reprogramming fibroblasts with the Yamanaka factors, different cell types with pluripotency occur, in particular F-class cells and iPSCs. In this regard, they identified the major characteristics arising during reprogramming toward these two different pluripotent cell types in terms of transcriptome, proteome, microRNA profile and epigenome.

15.2.2 iPSCs in the Cardiovascular Field

It was thought that hESCs are an inexhaustible source for basic research of early cardiomyocyte differentiation (Lev et al. 2005; Yang et al. 2008) as well as cell-transplantation procedures (Caspi et al. 2007; Laflamme et al. 2007; Kraehenbuehl et al. 2011; Thompson et al. 2012). Yet, the difficult circumstances to receive patient-specific cells led to the conclusion, that other avenues should be taken for clinically relevant applications. The advent of iPSC technologies has opened up new opportunities for individually tailored treatment of cardiovascular diseases (Moretti et al. 2010; Itzhaki et al. 2011; Lan et al. 2013; Karakikes et al. 2015) and

drug screening (Ebert et al. 2012; Liang et al. 2013a; Mordwinkin et al. 2013; Doherty et al. 2015). The generation of highly pure and mature cardiomyocytes is a demanding task up to now. The first attempts were inefficient and time-consuming, with spontaneously beating areas in approximately 8–25 % of hES-derived cells (Kehat et al. 2001; He et al. 2003) and 1–10 % of hiPS-derived cells (Zhang et al. 2009; Zwi et al. 2009). Two major ideas improved the cardiomyogenic differentiation in both hESCs and hiPSC, (i) influencing signaling pathways, including Activin/Nodal-, BMP-, FGF- and Wnt-mediated signaling cascades (Laflamme et al. 2007; Kattman et al. 2011; Yang et al. 2008; Cai et al. 2013; Chen et al. 2004b; Lian et al. 2012, 2013; Gai et al. 2009; Aikawa et al. 2015), and (ii) monolayer differentiation protocols, unlike embryoid bodies (EBs) (Laflamme et al. 2007; Melkoumian et al. 2010). Recent studies show, however, a large improvement with a 3D sphere culture system for large-scale human pluripotent stem cell production (Otsuji et al. 2014) and cardiac differentiation (Aikawa et al. 2015; Jiang et al. 2015). There are different strategies to overcome the problem of non-pure cell populations resulting from the above mentioned approaches, e.g. physical concepts as well as transgenic selection strategies, even though each of them still bears shortcomings for clinical application. The early physical concepts like mechanical dissection of beating areas (Kehat et al. 2001; Caspi et al. 2007) and Percoll gradient centrifugation (Laflamme et al. 2007) achieve a low degree of purity. Transgenic selection is accomplished by means of genetic manipulation (exogenous expression of fluorescent markers or antibiotic-resistant genes) of the cells which reached a high purification of cardiomyocytes but bears at the same time a disadvantage for clinical use (Huber et al. 2007; van Laake et al. 2010). The most promising enrichment protocols are probably cell sorting strategies via mitochondria-specific fluorescent dyes (Hattori et al. 2010), antibodies against cardiomyocyte-specific markers e.g. SIRPA (signal-reduced protein alpha) (Fujioka et al. 1996; Kharitonenkov et al. 1997; Dubois et al. 2011), EMILIN2 (elastin microfibril interface 2) (van Hoof et al. 2010), VCAM1 (vascular cell adhesion molecule 1) (Uosaki et al. 2011) or sugar metabolism (Tohyama et al. 2013).

If human ESC derived-cells should be transferred to clinical trials, researchers and physicians have to overcome great obstacles (Anderson et al. 2014), even if they demonstrate preliminary results in small (Caspi et al. 2007; Laflamme et al. 2007) and large (Shiba et al. 2012) animal models as well as non-human primates (Chong et al. 2014). The transplantation of hESC-derived cardiomyocytes results in the formation of stable cardiomyocyte graft and thereby in a functional benefit (Caspi et al. 2007) and reduced incidence of both spontaneous and induced ventricular tachycardia (Shiba et al. 2012). Grafts in non-human primates showed regular calcium transients and electromechanical coupling with the host tissue (Chong et al. 2014). With the delivery of one billion hESC-CMs, a substantial amount of the infarcted monkey heart could be re-muscularized. However, the injected cells achieved only an incomplete maturation stage and led to ventricular arrhythmias, albeit, unlike small animal models non-fatal.

In this regard, several groups studied the effect of iPSC derived-CM supporting the way towards the establishment of iPSC in the treatment of heart disease.

The obtained human cardiac progenitors provided a therapeutic benefit in a rodent model of myocardial infarction (Carpenter et al. 2012). The pioneering work has been implemented by (Nelson et al. 2009) the delivery of undifferentiated mouse iPS cells in comparison with parental fibroblasts. iPSC treated mice showed restored post-ischemic contractile performance and ventricular wall thickness. The use of iPSC derived-Flk1^{+} progenitor cells was, however, fundamentally better to minimize tumorgenesis as well as regarding the therapeutic outcome (Mauritz et al. 2011).

Nevertheless, the application of iPSC-derived cardiomyocytes should be reconsidered in the future regarding the suitable area of application. The iPSC technology could help to understand genetic as well as epigenetic mechanisms behind cardiovascular diseases, but the heterogeneity of the differentiating cardiomyocytes and their immature status so far represents major obstacles for clinical use (Zhang et al. 2009; Knollmann 2013; Budniatzky and Gepstein 2014; Sahara et al. 2015).

15.2.3 From Laboratories to Clinical Trials

Nevertheless, the iPS cell technology inspires to new cell replacement therapies, disease models and drug development. The first feasibility analysis for therapeutic use of iPSCs was performed in 2007 by using a humanized sickle cell anemia mouse model (Hanna et al. 2007). It has been shown, that iPS cell derived hematopoietic progenitors generated from autologous skin could cure the disease after transplantation. Yet, isograft transplantation in mice with undifferentiated iPS cells generated immune-rejected teratomas (Zhao et al. 2011). Moreover, most of the teratomas formed were immunogenic accompanied by T-cell infiltration. These findings indicate, as opposed to ESC, abnormal gene expression and T-cell-dependent immune response in syngeneic recipients. However, undifferentiated iPSC would not be used for medical application, which reduces the risk of immune response. The iPSC-based autologous technology with therapeutically relevant differentiated cells could be applied for cell replacement strategies (Araki et al. 2013; Guha et al. 2013). Two independent groups have shown negligible immunogenicity of terminally differentiated mouse cells in vitro and in vivo after their transplantation into syngeneic recipients. This applied to embryoid bodies or cell types comprising all three germ layers (Guha et al. 2013). Tissues derived from ES cells as well as iPS cells show no increase in the expression of the immunogenicity-causing Zg16 and Hormad1 genes (Araki et al. 2013). Due to the high costs and time intensive process during allogeneic transplantation, and patient specific target cells, it will be an important step towards autograft technologies. Takahashi and his group (Morizane et al. 2013) reported, that an autologous transplantation of iPSC-derived neural cells in the brain of a nonhuman primate leads only to a minimal immune response. Furthermore, a high number of dopaminergic neurons survived without immunosuppression.

In 2014, only 8 years after the first publication, the first iPSC-derived human cells were transplanted into a 70-year old Japanese woman (Cyranoski 2014). This pilot safety study for iPSC-based intervention of wet-type AMD involves the trans-

plantation of iPSC-derived retinal pigment epithelium (RPE) cell sheets in patients with exudative (wet-type) age-related macular degeneration (AMD). Safety, immune reaction and tumorigenesis had been tested before in mice and monkey studies (Kamao et al. 2014; Kanemura et al. 2014).

A widely explored branch for the use of iPS cells in medical application is disease modeling. Until 2013, over 180 reports (summarized and detailed information ((Inoue et al. 2014) support information table S1)) in neurological (71), muscular (6), ocular (7), haematological (23), cardiac (26), metabolic (18), imprinting (2), skin (5) and others (30) fields demonstrate the high reliability compared to the unsafe transmission from animal models to human diseases. But there are still several obstacles to be solved (Kitaoka et al. 2011), e.g. phenotype of late-onset disease or the heterogeneous population in iPS cell-derived cultures. There is a need to overcome these problems to optimize disease modeling, with e.g. (Inoue et al. 2014) protocols for efficient and pure target cell generation, optimal control settings and highly sensitive detection systems. Furthermore, patient cohorts could be tested for responder or non-responders in a drug developmental phase of clinical trials (Garbes et al. 2013; Kondo et al. 2013).

15.3 Reprogramming Cell Fate

An alternative approach to the use of pluripotent cells is the cell fate switch of differentiated somatic cells or lineage progenitors directly to the cells of interest. Successful lineage conversions into mature patient-derived somatic cells bear a great potential for biomedical and pharmaceutical applications. Until now, the generation of large numbers of functional healthy cells and especially cells with defined disease patterns is an important and unmet goal. Direct reprogramming of patient-specific fibroblasts or resident cells could offer a solution to this problem without passing an intermediate and so far not exactly explored pluripotent state. This offers two major advantages, (i) it may reduce the risk of tumorigeneses and inflammation after cell transplantation and (ii) bear the potential of directly converting resident cells in the body without a separate *ex vivo* step.

Recently published reports (Table 15.2) addressing the widespread exploration of new cell sources and strategies for basic research and medical applications are described below.

15.3.1 Insulin⁺ β-Cells

At present, only few concepts have been developed to cure Diabetes Mellitus, a complex metabolic disorder characterized by the loss or dysfunction of pancreatic β-cells (Ashcroft and Rorsman 2012). Therefore, pancreatic β-cells are a key target for cellular reprogramming due to their potential for storage and release of insulin.

Table 15.2 Overview of recently published lineage conversion methods

Vector type	Cell origin	Host	Target cell	*in vitro/in vivo*	Factors	Literature
Insulin$^+$ β-cells						
Adenoviral	Pancreatic exocrine cells	Mouse	β-cells	*in vivo*	Ngn3, Pdx1, MafA	Zhou et al. (2008b)
Adenoviral	SOX9$^+$ hepatocyte	Mouse	β-cells	*in vivo*	Ngn3, Pdx1, MafA	Banga et al. (2012)
Small molecules	Liver epithelial stem-like white blood cells	Rat	β-cells	*in vitro/in vivo*	1. Step: Aza and TSA 2. Step: RA and ITS 3. Step: Nicotinamide	Liu et al. (2013)
Lentiviral	Pancreatic exocrine cells	Human	β-cells	*in vivo*	Activated MAPK and STAT3	Lemper et al. (2015)
Hepatocytes						
Lentiviral	Fibroblasts	Mouse	Hepatocytes	*in vitro/in vivo*	Gata4, Hnf1α, Foxa3, knockdown of p19^{Arf}	Huang et al. (2011)
Lentiviral	Fibroblasts	Human	Hepatocytes	*in vitro/in vivo*	HNF1A, HNF4A, FOXA3, SV40 large T antigen	Huang et al. (2014)
Lentiviral	Embryonic fibroblasts	Human	Hepatocytes	*in vitro/in vivo*	HNF1A, HNF4A, HNF6, CEBPA, ATF5, PROX1, p53-siRNA, C-MYC	Du et al. (2014)
Osteoblasts						
Retroviral	Fibroblasts	Human	Osteoblasts	*in vitro/in vivo*	RUNX2, OSTERIX, OCT4, L-MYC	Yamamoto et al. (2015)
Hematopoietic						
Retroviral	Hematopoietic progenitors	Mouse	Increase lymphoid lineage cells Increase myeloid lineage cells	*in vitro/in vivo*	miR-181 miR-223	Chen et al. (2004a)

Lentiviral	Dermal fibroblasts	Human	Myeloid and erythroid progenitors	*in vitro/in vitro*	OCT4, cytokines	Szabo et al. (2010)
microRNA	$CD34^+$	Human	Increase granulopoiesis	*in vitro*	Overexpression miR-223	Vian et al. (2014)
			Increase erythropoiesis and monocytic/macrophage differentiation		Knockdown miR-223	
Lentiviral	Embryonic and adult fibroblasts	Mouse	Hematopoietic progenitor cells	*in vitro/in vivo*	Erg, Gata2, Lmo2, Runx1c, Scl, $p53^{-/-}$	Batta et al. (2014)
Lentiviral	HUVEC	Human	Hematopoietic multipotent progenitor cells	*in vitro/in vivo*	FOSB, GFI1, RUNX1, SPI1	Sandler et al. (2014)
	hDMEC					
Neuronal						
Lentiviral	Embryonic fibroblasts	Mouse	Dopaminergic neuron	*in vitro*	ASCL1, LMX1A, NURR1	Caiazzo et al. (2011)
	Adult healthy and Parkinson's disease fibroblasts	Human				
Retroviral	Postnatal cortical astroglia	Mouse	GABAergic neuron	*in vitro*	Dlx2 or Ascl1, Dlx2	Heinrich et al. (2010)
Retroviral	NG2	Mouse	GABAergic neuron	*in vivo*	NeuroD1	Guo et al. (2014)
Retroviral	Postnatal cortical astroglia	Mouse	Glutamatergic neuron	*in vitro*	Neurog2	Heinrich et al. (2010)
Retroviral	Postnatal cortical NG2, postnatal cortical astroglia cortical astroglia (HA 1800)	Mouse	Glutamatergic neuron	*in vivo*	NeuroD1	Guo et al. (2014)
		Mouse				
		Human				
Lentiviral	Postnatal fibroblasts	Human	Neural crest cells	*in vitro*	SOX10	Kim et al. (2014)

(continued)

Table 15.2 (continued)

Vector type	Cell origin	Host	Target cell	*in vitro/in vivo*	Factors	Literature
microRNA	Fibroblasts	Human	Excitatory and inhibitory neuron	*in vitro*	miR-9/9*, miR-124, NEUROD2, ASCL1, MYT1L	Yoo et al. (2011)
Lentiviral						
Small molecules	Embryonic and tail-tip fibroblasts urinary cells	Mouse	Neural progenitor cells	*In vitro/in vivo*	VPA, CHIR99021, Repsox	Cheng et al. (2014)
		Human				
Endothelial cells						
Lentiviral	c-kit$^-$ lineage-committed mature ACs	Human	Vascular endothelial cells	*in vitro/in vivo*	ETV2, ERG1, FLI1, TGFβ-inhibitor	Ginsberg et al. (2012)
Lentiviral	Fibroblasts	Human	Vascular endothelial cells	*in vitro/in vivo*	ETV2	Morita et al. (2015)
Small molecules	Adult skin fibroblasts	Human	Dermal microvascular endothelial cells	*in vitro/in vivo*	Poly I:C, EC growth factors	Sayed et al. (2015)
Cardiovascular						
DNA Plasmid	Embryonic stem cells	Mouse	Sinoatrial bodies	*in vitro*	Tbx3	Jung et al. (2014)
					Myh6- promotor-selection	
DNA Plasmid	Embryonic stem cells	Mouse	Ventricular cells	*in vitro*	Nkx2.5	David et al. (2009)
microRNA	Cardiomyocyte progenitor cells	Human	Increase cardiac differentiation at an earlier rate	*in vitro*	miR-1	Sluijter et al. (2010)
					miR-499	
Retroviral	Tail-tip fibroblasts	Mouse	Cardiomyocyte-like cells	*in vitro/in vivo*	Gata4, Mef2c, Tbx5	Ieda et al. (2010), Chen et al. (2012), Qian et al. (2012), Inagawa et al. (2012), and Qian et al. (2013)
Lentiviral	Cardiac fibroblasts					

Retroviral	Adult cardiac fibroblasts tail-tip fibroblasts	Mouse	Cardiomyocyte-like cells	*in vitro/in vivo*	Gata4, Mef2c, Tbx5, Hand2	Song et al. (2012)
Lentiviral Proteins	Dermal fibroblasts	Human	Cardiomyocyte-like cells	*in vitro*	ETS2, MESP1, Activina, BMP2	Islas et al. (2012)
microRNA	Neonatal cardiac and tail-tip fibroblasts	Mouse	Cardiomyocyte-like cells	*in vitro/in vivo*	miR-1, miR-133, miR-208, miR-499, JAK inhibitor I	Jayawardena et al. (2012)
Retroviral Small molecules	Embryonic fibroblasts and tail-tip fibroblasts	Mouse	Beating cell clusters	*in vitro*	Oct4, SB431542, CHIR99021, parnate, forskolin	Wang et al. (2014)
mRNA	EPDCs	Mouse	Endothelial and smooth muscle cells	*in vivo*	VEGF-A	Zangi et al. (2013)

Abbreviations: *ACs* amniotic cells, *Aza* 5-Azacytidin, *EPDCs* epicardium-derived cells, *hDMECs* human dermal microvascular endothelial cells, *HUVEC* human umbilical vein endothelial cells, *ITS* insulin, transferrin, selenite, *miR* microRNA, *NG2* oligodendrocyte precursor cells, *RA* Retinoic acid, *TSA* Trichostatin A, *VPA* valproic acid

Zhou and colleagues (Zhou et al. 2008b) reduced the first screening of 1100 transcription factors to a cocktail of only 3 factors (*Pdx1*, *Ngn3*, *MafA*) sufficient for β-cell generation starting with pancreatic exocrine cells (Zhou et al. 2008b). The overexpression of these factors was also suitable to induce duct-like structures in NOD-SCID mice with diabetes induced via treatment with streptozotocin (Banga et al. 2012). Expression of mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) in adult human exocrine cells from pancreas results in an expression of the pro-endocrine factor neurogenin 3 (*Ngn3*) in 50–80 %. A long-term engraftment (over 200 days) increased the efficiency of reprogramming into insulin-positive cells. A promising treatment for type 1 diabetes mellitus was developed by (Liu et al. 2013) with an efficient chemical protocol, thereby avoiding integration of transcription factors into the genome. This stepwise differentiation from rat liver epithelial stem-like white blood cells is characterized by a pancreatic precursor which could contribute to endocrine cells *in vitro* and *in vivo*, leading to functional insulin-secreting cells.

15.3.2 Hepatocytes

The liver plays the major role of the metabolic system in the body, e.g. storage of glycogen, iron, vitamins and minerals as well as drugs, alcohol or other chemical compounds.

Functional hepatocytes have been achieved using hESCs (Basma et al. 2009) or patient-specific hiPSCs (Sullivan et al. 2010; Si-Tayeb et al. 2010), with yields between 70 and 90 %. A new source for drug discovery and development of cell-based therapies to cure liver diseases are hepatocyte-like cells generated by direct reprogramming.

First steps have been made in 2011 via transduction of *Gata4*, *Hnf1α*, *Foxa3* and knockdown of $p19^{Arf}$ in mouse tail-tip fibroblasts (Huang et al. 2011). Generated these "iHeps" appeared to be only partially differentiated, but rescue almost half of the recipients from death by restoring liver functions of fumaryl-acetoacetate-hydrolase-deficient ($Fah^{-/-}$) mice. Two groups upgraded this basic approach to human induced hepatocytes (hiHeps) (Huang et al. 2014; Du et al. 2014), both suitable for large-scale production. HiHeps displayed functional characteristics of mature hepatocytes including expression and activity of CyP3A4, comparable to primary hepatocytes. Transplanted hiHeps repopulated and rescued around 30 % of $Fah^{-/-}$ mice (Huang et al. 2014) and Tet-uPA/$Rag^{2-/-}/\gamma c^{-/-}$ mice (Du et al. 2014), thus providing a cell source for liver disease models and pharmaceutical applications using direct reprogramming.

15.3.3 Osteoblasts

Mesenchymal stem cell-derived osteoblasts are responsible for the synthesis and mineralization of bone, during initial bone formation as well as later bone remodeling (Neve et al. 2011). Disrupted differentiation and activity leads to osteoporosis and osteoarthritis.

Osteoblasts could be achieved via direct reprogramming of human fibroblasts with the combination of *Oct4* and *L-Myc* following *in vitro* cultivation in osteogenic medium (Yamamoto et al. 2015). An even higher efficiency (~80 %) and maturity of osteocalcin-producing cells can be reached by introducing additional osteoblast-specific transcription factors, Runt-related transcription factor 2 (*Runx2*) and *Osterix* (Yamamoto et al. 2015). The so called dOBs (directly converted osteoblasts) displayed a similar gene expression profile as normal human osteoblasts and served as a cell source for bone repair after transplantation into immunodeficient mice.

Cell-based therapies, either autologous or allogenic transplantation could facilitate bone repair based on bone diseases like rheumatoid arthritis or periodontitis.

15.3.4 Hematopoietic Lineage Cells

A rare population of blood-cell precursors, hematopoietic stem cells (HSCs), is able to self-renew and reconstitute the entire hematopoietic system. Therefore, bone-marrow transplants or direct transfusion of HSCs can be life-saving. Patient-derived hematopoietic stem cells or terminally differentiated blood cells (erythrocytes, platelets and granulocytes) represent a promising therapeutic approach to cure benign and malignant hematologic disorders. So far, pluripotent stem cell-derived HSC yield embryonic-like blood cells, but with a low engraftment efficiency into bone marrow (Sturgeon et al. 2013).

An alternative source for human HSC transplants could be the reprogramming of human endothelial cells (ECs), either umbilical vein ECs (HUVECs) or adult primary dermal microvascular ECs (hDMEC) (Sandler et al. 2014). Sandler and colleagues established a method to culture HUVECs and hDMECs after lentiviral transfection with the transcriptionfactors *FOSB*, *GFI1*, *RUNX1* and *SPI1* to phenocopy the vascular-niche microenvironment of hemogenic cells, a serum-free vascular niche monolayer, to induce outgrowth of hematopoietic colonies. The achieved cells displayed functional and immunophenotypical characteristics of multipotent progenitor cells (MPP). The so called rEC-hMPPs can engraft into primary and secondary recipient mice, producing myeloid (granulocytic/monocytic, erythroid, megakaryotic) and lymphoid progeny. However, it remains unclear whether engrafted rEC-hMPPs can give rise to T-cells. Another approach is the direct reprogramming of mouse fibroblasts toward clonal multilineage hematopoietic progenitors (Batta et al. 2014). Reprogrammed cells efficiently generated erythroid,

megakaryocytic, myeloid and lymphoid lineages following lentiviral transduction of *Erg*, *Gata2*, *Lmo2*, *Runx1*, *Scl* and loss of p53 function. The lentiviral introduction of *OCT4* together with specific cytokine treatments is sufficient to enhance hematopoietic progenitors in human dermal fibroblasts (Szabo et al. 2010). Cells expressing CD45, a pan-leukocte marker gave rise to myeloid and erythroid lineages.

The manipulation of hematopoietic progenitor cells with microRNA gave rise to lineage fate decision to myeloid progenitors (Vian et al. 2014) whereby an introduction of miR-223 increases granulopoiesis and impairs erythroid and monocytic/macrophagic lineages. On the other hand, inhibition of miR-223 impairs granulopoiesis and increases erythroid and monocytic/macrophagic lineages. An increase in lymphoid cells can be achieved by overexpression of miR-181 (Chen et al. 2004a).

15.3.5 Neurons

Minor dysfunctions can be buffered by the large plasticity of the human brain, but main reasons of a wide variety of neurological diseases and psychiatric disorders are the aberrant behavior and function of neurons. Due to its complexity, the central nervous system will require various reprogramming strategies to generate diverse functional cell types.

Parkinson's disease, a degeneration of mesencephalic dopaminergic neurons, could be possibly improved by transplantation of dopaminergic neurons (Lindvall and Björklund 2004). The transplantation of iPSC-derived neurons into fetal mouse brain was successful but led to the development of tumors if not properly controlled (Wernig et al. 2008). Direct reprogramming from mouse and human fibroblasts with achaete-scute homolog 1 (*ASCL1*), nuclear receptor related 1 protein (*NURR1*) and LIM homeobox transcription factor 1 alpha (*LMX1A*) generated functional dopaminergic neurons, so called induced dopaminergic cells (iDA) (Caiazzo et al. 2011). iDA release dopamine and show spontaneous electrical activity.

A second source for direct reprogramming of neurons is the use of present astroglia cells from the cerebral cortex to repair the damaged brain tissue. A forced expression of the dorsal telencephalic fate determinant neurogenin-2 (*Neurog2*) generated synapse-forming glutamergic neurons (Heinrich et al. 2010). In contrast, GABAergic identity could be reached by induction of the ventral telencyphalic fate determinant *Dlx2* (Heinrich et al. 2010). A member of the same basic helix-loop-helix (bHLH) transcription factor family, neurogenic differentiation 1 (*NeuroD1*), directly reprogrammed reactive glia cells from the cortex of stab-injured or Alzheimer's disease model mice into glutamatergic neurons (Guo et al. 2014). In contrast, Oligodendrocyte precursor cells (NG2) were reprogrammed with the single transcription factor NeuroD1 into glutamergic as well as GABAergic neurons (Guo et al. 2014). Neuronal precursor cells, which express the neuronal migration protein doublecortin (DCX) were obtained after retroviral-mediated expression of *Sox2* and *Ascl1* in NG2 cells (Heinrich et al. 2014).

SOX10, in combination with environmental cues including WNT activation, could reprogram human postnatal fibroblasts into induced neural crest cells (iNC) (Kim et al. 2014). In this context, a particular cell surface maker was identified and could be further used to study familial dysautonomia.

Another approach is the introduction of microRNAs which interfere in the SWI/SNF-like BAF chromatin-remodeling complex (Yoo et al. 2011). miR-9/9* and miR-124 in combination with two bHLH family members (*NEUROD2* and *ASCL2*) and myelin transcription factor 1-like (*MYT1L*) convert human fibroblasts with an efficiency of 80 % into excitatory and inhibitory neurons. Thereby, miR-9/9* and miR-124 alone could be able to convert fibroblasts, *NEUROD2* facilitated this process, and *ASCL2* and *MYT1L* enhances the maturation of the converted neurons.

Cheng and colleagues recently reported the first conversion of somatic cells to neuronal progenitor cells (NPCs) without introduction of exogenous factors (Cheng et al. 2014). They established a two-step protocol, with applying a chemical cocktail containing inhibitors of HDACs (VPA), GSK-3 kinases (CHIR99021) and TGF-β pathway (Repsox) under hypoxic conditions. The chemically induced NPCs (ciNPCs) retain some residual fibroblast epigenetic memory but they are highly similar to mouse brain-derived embryonic NPCs (self-renewal, proliferation, multipotency).

15.3.6 Endothelial Cells

Revascularization is possibly the most important prerequisite for the recovery of injured tissue and organs. Previous success with ESC- and iPSC-derived cells is mostly minimal, and especially long-term cultivation is impossible; moreover, they drift into non-vascular lineages (James et al. 2010). One approach is their derivation from human mid-gestation c-kit$^-$ lineage-committed amniotic cells (ACs) via forced expression of ETS (E26 transformation-specific) transcription factor family members, including *ETV2*, *ERG1* and *FLI1* (Ginsberg et al. 2012) which are key TFs regulating vascular development and angiogenesis (Val and Black 2009; Sharrocks 2001). Additional TGFβ-inhibition enhances the specification of induced vascular endothelial cells (iVECs) to a stage comparable to adult endothelial cells (ECs), regarding expression of vascular and non-vascular genes. Similar outcome could be achieved via direct conversion of human fibroblasts, which point out the role of the ETS factor ETV2 as a master regulator sufficient to induce multiple endothelial factors during development, like ERG, FLI1 and TAL1 as well as functional molecules, including EGFL7 and Von-Willebrand factor (Morita et al. 2015).

Another approach, avoiding viral transfection, is based on the manipulation of the innate immune system via using the toll-like receptor 3 agonist Poly I:C (Sayed et al. 2015). Small molecule-mediated induction of endothelial cells is further controlled by endothelial growth factors, and thus represents a feasible tool to transdifferentiate human fibroblasts into induced endothelial cells (iECs) with morphological and histochemical similarities to human dermal microvascular endothelial cells (HMVECs).

15.3.7 Cardiomyocytes

Around one billion cardiomyocytes (Laflamme and Murry 2005) are irreversibly destroyed while an irreversible scar formation is built up after myocardial infarction (MI). Due to the little cardiomyocyte proliferation capacity in adult mammalians, this large amount of cells cannot by replaced after cardiac injury. Consequently, faulty electrical coupling, arrhythmias and incomplete ventricular wall formation lead to a reduction of pump function and finally to heart failure (Hasenfuss 1998). MI as a result of coronary/ischemic heart disease (CHD) has led to 50,000 deaths in Germany in 2013. Accordingly, cardiovascular diseases are the number 1 cause of death worldwide, whereby 7.4 million people died of ischemic heart disease and 6.7 million from stroke which is often associated with the former. This means in total ~25 % of deaths worldwide (WHO 2012). Cardiomyocytes in the human heart possess only a very low turnover rate of 1 % at the age of 25 and decreases to 0.45 % at the age of 75 (Bergmann et al. 2009). Fewer than 50 % of human cardiomyocytes are replaced during a life span of 75 years. Moreover, the number of cardiomyocytes in the left ventricle increases 3.4-fold only from the first year of life until the age of 20 showing more cardiomyocyte cycling and division in young humans (Mollova et al. 2013). Resident cell populations like cardiac progenitor cells (CPCs) or preexisting cardiomyocytes which undergo dedifferentiation represent possible sources for heart regeneration after injury (Garbern and Lee 2013; Mollova et al. 2013; Senyo et al. 2013). At present, there are multiple approaches to support cardiac regeneration, which bear potential for clinical applications in the future. This includes cell transplantation of either autologous or allogenic stem cells or terminally differentiated cells from various origins, as well as stimulation of endogenous cell populations and direct reprogramming.

15.3.7.1 Cardiac Development

The heart is the first organ formed during embryonic development, necessary for nutrient supply and removal of waste. The complex system of heart formation requires the generation of diverse muscle and nonmuscle cell-types, including cardiomyocytes of left and right atria as well as left and right ventricles, conduction system, pacemaker, vascular smooth muscles, endo- and epicardial cells (Sahara et al. 2015; Vincent and Buckingham 2010; Xin et al. 2013) (Fig. 15.2). Cardiovascular fate-determinant is the bHLH transcription factor MesP1, which marks the early cardiovascular progenitor (David et al. 2008, 2011). The process is mainly regulated by multipotent cardiac progenitor cells (Musunuru et al. 2010) and mainly structured by two origins, the first (FHF) and second (SHF) heart fields. It was currently reported, that, apart from this, avian pacemaker cells in the sino-atrial (SA) node arise from a so-called tertiary (THF) heart field (Bressan et al. 2013). The first heart field progenitor cells are marked by the expression of Nkx2.5 (Brade et al. 2013) and Hcn4 (Liang et al. 2013b; Später et al. 2013) and their derivatives give rise to myocardium of the left ventricle as well as a minor part of the right ventricle, the right

and left atria and most cell complexes of the conduction system (CS), like the atrioventricular (AV) node and the ventricular CS (Buckingham et al. 2005) (Vincent and Buckingham 2010). Multipotent embryonic Isl1$^+$/Nkx2.5$^+$/Flk1$^+$ progenitor cells of the SHF give rise to myocardium of the right and left atria, the right ventricle and the outflow tract, as well as vascular smooth muscles and the endocardium of the heart (Moretti et al. 2006; Kattman et al. 2006; Misfeldt et al. 2009). Furthermore, epicardial progenitor cells give rise to cardiac fibroblasts, vascular smooth muscle cells and also atrial and venous endothelial cells (Vincent and Buckingham 2010; Schlueter and Brand 2012; Katz et al. 2012) (Zhou et al. 2008a) (Fig. 15.2).

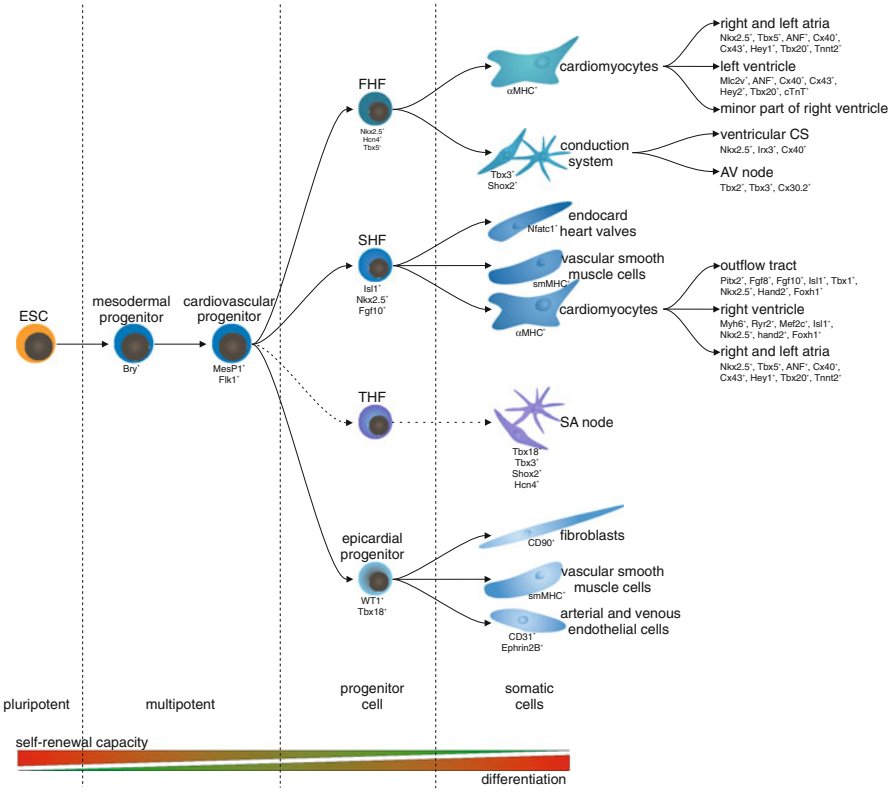

Fig. 15.2 Cardiovascular cell differentiation during development
Cells of the cardiovascular lineage arise from multipotent mesodermal Bra$^+$ progenitor cells. The specific early cardiovascular progenitor cell population is marked by MesP1 and Flk1 and gives rise to four subpopulations. The first heart field (FHF) differentiates into cardiomyocytes of the right and left atria, the left ventricle and the minor part of the right ventricle, and the conduction system (CS), including ventricular CS and atrioventricular (AV) node. The second heart field (SHF) gives rise to cardiomyocytes of the right and left atria, the right ventricle and the outflow tract, vascular smooth muscle cells and the endocard of the heart. Pacemaker cells of the sinoatrial (SA) node are derivatives of a tertiary heart field (THF) progenitor cell population. Epicardium-derived progenitor cells (EPDCs) generate fibroblasts, vascular smooth muscles cells as well as arterial and venous endothelial cells

15.3.7.2 Reprogramming Strategies for Cardiovascular Lineages

The major challenges ahead are the generation of mature and pure cardiac subtypes of all aforementioned cell types required for heart reconstruction. One milestone in this regard is the forward programming of murine ESCs with the nodel cell inducer *Tbx3* (T-box transcription factor Tbx3) plus an additional Myh6-promotor-based antibiotic selection step (Jung et al. 2014; Rimmbach et al. 2015). The so-called iSABs (induced sinoatrial bodies) are over >80 % pure physiologically and pharmacologically functional pacemaker cells with a comparable beating frequency to normal murine SA nodal cells. One disadvantage of this approach is still the genetic alteration through DNA introduction; however, so far no other concept has yet achieved related results. Based on this method, several cardiac subtypes could be achieved by varying the TF used for forced exogenous overexpression of lineage specify. We have demonstrated that expression of *Nkx2.5* (David et al. 2009) similar to *MesP1* (David et al. 2008) is sufficient to enhance cardiogenesis in mESCs, whereby Nkx2.5 induces a large proportion of ventricular cells and MesP1 resulted in early intermediate cell types (Figure 15.3).

Another forward reprogramming strategy is based on cardiomyocyte progenitor cells (Sluijter et al. 2010). Overexpression of miR-1 and miR-499 enhanced differentiation into cardiomyocytes via repression of histone deacetylase 4 or Sox6. The results have been confirmed by (Hosoda et al. 2011) reporting a positive effect, whereby the miR-499 driven improved cardiac performance occurred through repression of Sox6 and Rod1.

The use of embryonic stem cells is still under controversial discussion – therefore researchers made great efforts to overcome this problem and focused on a particular resident cell type, cardiac fibroblasts. So far, there is no single master regulator discovered to switch the cell fate of fully differentiated somatic cells towards cardiomyocyte lineage. To induce "working" myocardium several factor combinations turned out to generate partly differentiated cells. The first successful strategy was based on the transcription-factor-combination "GMT" containing *Gata4* (transcription factor Gata4), *Mef2c* (Myocyte-specific enhancer factor 2c) and *Tbx5* (T-box transcription factor Tbx5), (Ieda et al. 2010). Each of these single factors plays an important role during early heart development (Srivastava 2006); (Olson 2006; Zhao et al. 2008) promoting cardiomyocyte differentiation (Bruneau et al. 2001; Ghosh et al. 2009); (Maitra et al. 2009). Since Ieda's work, various groups have converted different fibroblasts (mouse tail-tip or cardiac fibroblasts) to induced cardiomyocyte (iCM)-like cells with different outcomes based on their markergene expression analysis leading to 30 % $cTnT^+$ (Ieda et al. 2010), 10–15 % iCM (Qian et al. 2012), 3 % αMHC^+ (Inagawa et al. 2012), 20 % αMHC^+ (Qian et al. 2013). Recently published data demonstrated the influence of the protein expression levels of GMT in a certain ratio (Wang et al. 2015). A higher protein level of Mef2c with lower levels of Gata4 and Tbx5 enhanced the reprogramming efficiency and quality of iCM. However, Chen and colleagues (Chen et al. 2012) found, that overexpression of GMT is inefficient in TTFs and CFs regarding molecular and electrophysiological phenotypes of mature cardiomyocytes (0 % αMHC^+, 0 % $Nkx2.5^+$, 35 % $cTnT^+$) (Chen et al. 2012) (Figure 15.3).

Therefore, the parallel investigation of other modification strategies is increasingly becoming important. One solution might be the combination of the early key cardiac transcription factor *MesP1* with v-ets erythroblastosis virus E26 oncogene homolog 2 (*ETS2*), with addition of ActivinA and BMP2 (Islas et al. 2012) to influence signaling pathways during cardiogenesis. While the role of ETS2 in cardiac differentiation is still unclear, in this combination fibroblasts could be reprogrammed to immature cardiomyocytes expressing the core cardiac factors Nkx2.5, Isl1, Tbx20, the mesodermal signaling molecules BMP2, TDGF1 as well as muscle markers Myl2, Myh6. A third solution could be omitting transcription factor overexpression by delivery of cardiac specific microRNAs. The combination of miR-1, −133, −208 and −499 with the application of a chemical Janus kinase inhibitor successfully achieved a cell fate switch towards a cardiomyogenic phenotype with 28 % αMHC$^+$ cells *in vitro* (Jayawardena et al. 2012; Jayawardena et al. 2014) (Figure 15.3). A fourth way and a promising step forward reducing genetic manipulation is the application of small molecules. Wang and colleagues efficiently convert fibroblasts only with one transcription factor, *Oct4*, without an intermediate pluripotent state by adding a defined small-molecule cocktail (Wang et al. 2014). The small-molecule cocktail contains an ALK4/5/7 inhibitor (SB431542), GSK3 inhibitor (CHIR99021), LSD1/KDM1 inhibitor (parnate) and an adenylyl cyclase activator (forskolin) to induce the reprogramming efficiency, including modulators of epigenetic enzymes, signaling pathways, metabolism and transcription. In these proof-of-principle experiments, spontaneously contracting cardiomyocytes with a ventricular phenotype could be achieved.

Nevertheless, most of these reports yielded only immature cardiomyocytes within an inhomogeneous population of working myocardium and cells of the conduction system. Therefore, it is of great interest to fully understand the mechanisms underlying reprogramming strategies to enhance purity, safety and efficiency. The latter has already been improved considerably via application of either GMT (Qian et al. 2012) or GMTH (Song et al. 2012) *in vivo*, indicating that the heart microenvironment is required to fully reprogram somatic cells with forced expression of TFs. This also points out that the transference of direct reprogramming to resident cells is an accessible goal.

Paracrine factors play a major role in promoting cardiac repair and regeneration after MI (Loffredo et al. 2011; Zhou et al. 2011; Malliaras et al. 2013). Likewise, induced heart function through enhancing vascularization is of great interest. (Caprioli et al. 2011) and could be reached by manipulation of epicardial progenitor cells (Zangi et al. 2013). Intramyocardal injection of synthetic modified mRNA (mmRNA) encoding vascular endothelial growth factor-A (*VEGF-A*) initially activates the endogenous progenitor cell pool, thereby improving myocardial function and long-term survival after MI. WT1$^+$ EPDCs are stimulated in the affected tissue following infarction; the effect could be enhanced with exogenous expression of VEGF-A which leads to mobilization of EPDCs into the myocardial layer and enhances their differentiation toward endothelial and smooth muscle cells (Zangi et al. 2013) (Fig. 15.3).

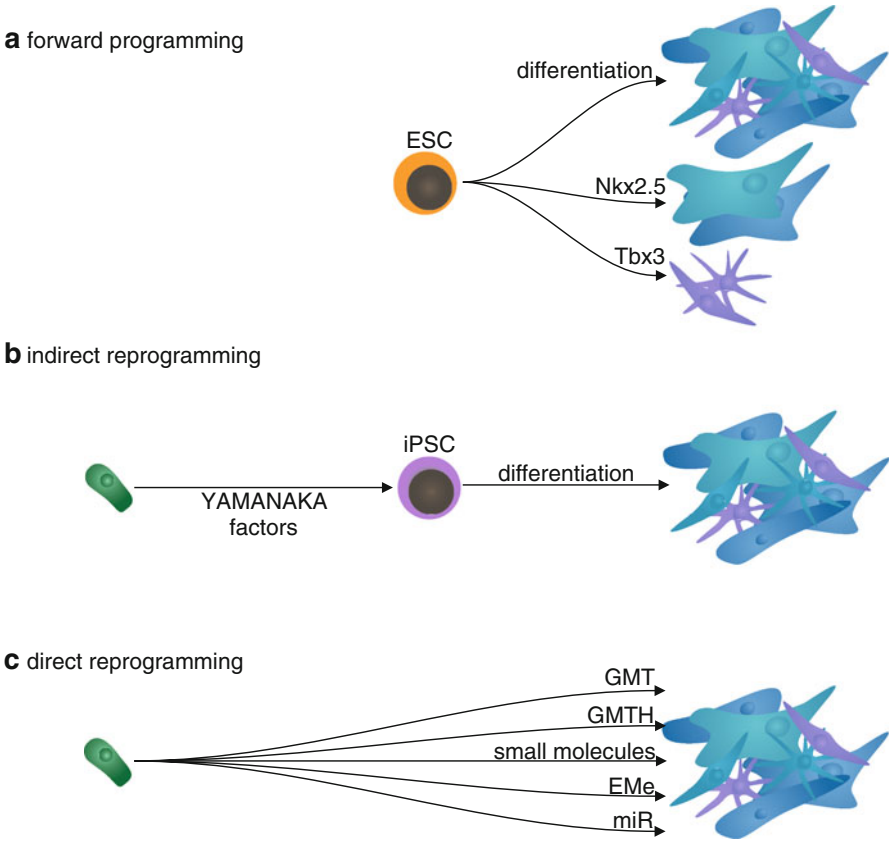

Fig. 15.3 Cell programming strategies in the cardiovascular field
Reprogramming strategies are based on the introduction of lineage specific transcription factors (TFs), small molecules or post-translational modifications. Three major methods have emerged during the last few years: (**a**) forward programming describes differentiation protocols with embryonic stem cells (ESCs) as a starting point. Forced expression of TFs enhances the percentage of distinct cardiac subtypes. (**b**) indirect reprogramming includes a first programming step of a fully differentiated somatic cells with the so-called YAMANAKA factors (OSKM) to induced pluripotent stem cells (iPSCs). iPSCs could then be differentiated to cardiovascular cells. (**c**) direct reprogramming is characterized by the direct conversion of one somatic cell type into another somatic cell type of the same germ layer or even across germ layers. So far, cardiomyocyte-like cells with certain cardiac marker expression characteristics have been achieved using this approach

15.4 Future Perspectives

As described above, research in the area of regenerative medicine has yielded considerable progress over the last decade, with substantial knowledge gain in cell fate determination. However, numerous issues have to be solved until clinical application.

15.4.1 Delivery Systems

Current objectives on improving the delivery systems are the avoidance of integrating viruses, like retro- or lentiviral systems. Many studies are now using adenoviral vectors, which can be produced in high titers and are not integrating. To minimize systemic spreading in vivo, constructs which directly target cells of interest could be one further step as demonstrated by dendritic cell based immune-therapy using a CD40-targeted adenoviral vector (Williams et al. 2012). This viral vector used to reprogram fibroblasts is not only a delivery system; the viral vector itself increases the epigenetic plasticity of the cells during reprogramming towards iPSCs. The same effect could be simulated by manipulation of the innate immune system to influence trans-differentiation of somatic cells (Cooke et al. 2014; Lee et al. 2012; Sayed et al. 2015), as shown via introduction of a TLR3 agonist in combination with a specific microenvironment to induce endothelial lineage conversion (Sayed et al. 2015).

Until now, best results for iPSC generation could be achieved with the delivery of modified mRNA, either OSKM, OSKML (Warren et al. 2010; Mandal and Rossi 2013) or OLSN (Yakubov et al. 2010). The so-called RNA-induced pluripotent stem cells (RiPSCs) express typical stem cell markers, like SSEA4, OCT4, SOX2, NANOG and hTERT and have multilineage differentiation potential. This method offers many advantages: (i) no risk of genomic integration, mutagenesis or systemic circulation, (ii) rapid protein expression, (iii) controllable stochastic variation as well as (iv) transient expression profile, and could be used for direct *in vivo* applications (Zangi et al. 2013; Kormann et al. 2011). *In-vitro* synthesized mRNAs possess cis-acting structural elements (methylated 5′-cap structure analog an a poly-A-tail) which are important for mRNA splicing, stabilization and transport (Quabius and Krupp 2014), thereby mimicing endogenous mature mRNA. The incorporation of 5-methylcytidine-5′-triphosphate (5mC) and pseudouridine-5′-triphosphate (Ψ) results in a lower immune response in vivo in mammalians (Kormann et al. 2011; Karikó et al. 2012). As we have shown, the introduction of mmRNA results in high protein expression levels in various cell types, including fibroblasts as well as human mesenchymal stem cells (Hausburg et al. 2015), making this method an convincing tool for direct as well as forward programming of slowly proliferating stem cells *in vitro* and for resident cells *in vivo*.

Also non-coding RNAs are regulators of cell fate. Data confirm the comprehensive relevance of several lncRNAs for ESC self-renewal and differentiation (Li and Wang 2015). An interesting fact is the distribution of lncRNAs as they are mainly bound to specific lineages (Derrien et al. 2012). This indicates their role in cell fate determination which is, until now, unclear. In contrast, microRNAs are well characterized and their role in biogenesis pathways via controlling post-transcriptional levels of mRNA is well known (Ong et al. 2015). Both, lncRNAs as well as microRNAs (Chen et al. 2004a; Sluijter et al. 2010; Miyoshi et al. 2011; Jayawardena et al. 2012, 2014; Vian et al. 2014) provide an alternative route to force transcription factor-mediated reprogramming.

Likewise, application of small molecules and cytokines bypass the need of transcription factor introduction. Recent reports (Hou et al. 2013; Liu et al. 2013; Cheng et al. 2014; Sayed et al. 2015) highlighted the possibility to convert cell fate simply by adding small molecules and cytokines, thereby mimicking the microenvironment of the natural cell niche.

15.4.2 Protocol Improvement

It needs to be defined which cell types should be used for reprogramming strategies. Stem cells, whether pluripotent stem cells or adult multipotent stem cells are rare and difficult to culture. Lineage specific progenitor cells or resident fibroblasts have advantages but they are more difficult to reprogram. At present, the cells achieved are largely immature and arise as an inhomogeneous cell population. This could lead to major complications *in vivo*, since the injured organs are based on the connection of several functionally different cell types. The application of any "wrong cell" could lead to disconnection or overstimulation which would may aggravate the situation. Therefore, it is crucial to optimize the analysis of marker expression as well as functional behavior in comparison to the mature cells of interest. With regard to clinical use, 100 % pure sub-types need to be generated in large scale-up systems.

Furthermore, the risk of teratoma formation as well as immune-rejection (Lensch et al. 2007; Zhao et al. 2011) must be excluded. To improve cell transplantation therapy it would be worthwhile to enhance long-term engraftment. The initial cell loss of ~95 % (Lang et al. 2014) and remaining fraction of ~1 % at day 35 (Hong et al. 2013) after intramyocardial injection displays the urgency of new application methods.

15.5 Conclusion

Successful lineage conversion of somatic cells or forced differentiation of PSC into mature, highly pure cell populations bear huge potential for biomedical and pharmaceutical applications. Yet, obtaining fully functional cell types remains a major challenge. This will be of crucial importance for regenerative medicine, as well as provide new approaches to study organ development. Moreover, drug testing to treat metabolic disorders will benefit from this rapidly growing field. Moreover, patient-specific cells may give a deeper insight into molecular and regulative mechanisms underlying the respective diseases.

References

Aikawa N, Suzuki Y, Takaba K (2015) A simple protocol for the myocardial differentiation of human iPS cells. Biol Pharm Bull 38(7):1070–1075

Anderson ME, Goldhaber J, Houser SR, Puceat M, Sussman MA (2014) Embryonic stem cell-derived cardiac myocytes are not ready for human trials. Circ Res 115(3):335–338

Araki R, Uda M, Hoki Y, Sunayama M, Nakamura M, Ando S, Sugiura M, Ideno H, Shimada A, Nifuji A, Abe M (2013) Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. Nature 494(7435):100–104

Ashcroft FM, Rorsman P (2012) Diabetes mellitus and the β cell: the last ten years. Cell 148(6):1160–1171

Atkinson SP, Lako M, Armstrong L (2013) Potential for pharmacological manipulation of human embryonic stem cells. Br J Pharmacol 169(2):269–289

Banga A, Akinci E, Greder LV, Dutton JR, Slack JMW (2012) In vivo reprogramming of Sox9+ cells in the liver to insulin-secreting ducts. Proc Natl Acad Sci U S A 109(38):15336–15341

Barad L, Schick R, Zeevi-Levin N, Itskovitz-Eldor J, Binah O (2014) Human embryonic stem cells vs human induced pluripotent stem cells for cardiac repair. Can J Cardiol 30(11):1279–1287

Bar-Nur O, Russ HA, Efrat S, Benvenisty N (2011) Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell 9(1):17–23

Basma H, Soto-Gutiérrez A, Yannam GR, Liu L, Ito R, Yamamoto T, Ellis E, Carson SD, Sato S, Chen Y, Muirhead D, Navarro-Alvarez N, Wong RJ, Roy-Chowdhury J, Platt JL, Mercer DF, Miller JD, Strom SC, Kobayashi N, Fox IJ (2009) Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. Gastroenterology 136(3):990–999

Batta K, Florkowska M, Kouskoff V, Lacaud G (2014) Direct reprogramming of murine fibroblasts to hematopoietic progenitor cells. Cell Rep 9(5):1871–1884

Benevento M, Tonge PD, Puri MC, Hussein SMI, Cloonan N, Wood DL, Grimmond SM, Nagy A, Munoz J, Heck AJR (2014) Proteome adaptation in cell reprogramming proceeds via distinct transcriptional networks. Nat Commun 5:5613

Benraiss A, Toner MJ, Xu Q, Bruel-Jungerman E, Rogers EH, Wang F, Economides AN, Davidson BL, Kageyama R, Nedergaard M, Goldman SA (2013) Sustained mobilization of endogenous neural progenitors delays disease progression in a transgenic model of Huntington's disease. Cell Stem Cell 12(6):787–799

Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J (2009) Evidence for cardiomyocyte renewal in humans. Science 324(5923):98–102

Brade T, Pane LS, Moretti A, Chien KR, Laugwitz K-L (2013) Embryonic heart progenitors and cardiogenesis. Cold Spring Harb Perspect Med 3(10):a013847

Bressan M, Liu G, Mikawa T (2013) Early mesodermal cues assign avian cardiac pacemaker fate potential in a tertiary heart field. Science 340(6133):744–748

Bruneau BG, Nemer G, Schmitt JP, Charron F, Robitaille L, Caron S, Conner DA, Gessler M, Nemer M, Seidman CE, Seidman JG (2001) A murine model of holt-oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 106(6):709–721

Buckingham M, Meilhac S, Zaffran S (2005) Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet 6(11):826–835

Budniatzky I, Gepstein L (2014) Concise review: reprogramming strategies for cardiovascular regenerative medicine: from induced pluripotent stem cells to direct reprogramming. Stem Cells Transl Med 3(4):448–457

Cai W, Albini S, Wei K, Willems E, Guzzo RM, Tsuda M, Giordani L, Spiering S, Kurian L, Yeo GW, Puri PL, Mercola M (2013) Coordinate Nodal and BMP inhibition directs Baf60c-dependent cardiomyocyte commitment. Genes Dev 27(21):2332–2344

Caiazzo M, Dell'Anno MT, Dvoretskova E, Lazarevic D, Taverna S, Leo D, Sotnikova TD, Menegon A, Roncaglia P, Colciago G, Russo G, Carninci P, Pezzoli G, Gainetdinov RR, Gustincich S, Dityatev A, Broccoli V (2011) Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature 476(7359):224–227

Caprioli A, Koyano-Nakagawa N, Iacovino M, Shi X, Ferdous A, Harvey RP, Olson EN, Kyba M, Garry DJ (2011) Nkx2-5 represses Gata1 gene expression and modulates the cellular fate of cardiac progenitors during embryogenesis. Circulation 123(15):1633–1641

Carpenter L, Carr C, Yang CT, Stuckey DJ, Clarke K, Watt SM (2012) Efficient differentiation of human induced pluripotent stem cells generates cardiac cells that provide protection following myocardial infarction in the rat. Stem Cells Dev 21(6):977–986

Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L (2007) Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol 50(19):1884–1893

Chen C-Z, Li L, Lodish HF, Bartel DP (2004a) MicroRNAs modulate hematopoietic lineage differentiation. Science 303(5654):83–86

Chen S-L, Fang W-W, Ye F, Liu Y-H, Qian J, Shan S-J, Zhang J-J, Chunhua RZ, Liao L-M, Lin S, Sun J-P (2004b) Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 94(1):92–95

Chen JX, Krane M, Deutsch M-A, Wang L, Rav-Acha M, Gregoire S, Engels MC, Rajarajan K, Karra R, Abel ED, Wu JC, Milan D, Wu SM (2012) Inefficient reprogramming of fibroblasts into cardiomyocytes using Gata4, Mef2c, and Tbx5. Circ Res 111(1):50–55

Cheng L, Hu W, Qiu B, Zhao J, Yu Y, Guan W, Wang M, Yang W, Pei G (2014) Generation of neural progenitor cells by chemical cocktails and hypoxia. Cell Res 24(6):665–679

Chong JJH, Yang X, Don CW, Minami E, Liu Y-W, Weyers JJ, Mahoney WM, van Biber B, Cook SM, Palpant NJ, Gantz JA, Fugate JA, Muskheli V, Gough GM, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem H-P, Laflamme MA, Murry CE (2014) Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature 510(7504):273–277

Clancy JL, Patel HR, Hussein SMI, Tonge PD, Cloonan N, Corso AJ, Li M, Lee D-S, Shin J-Y, Wong JJL, Bailey CG, Benevento M, Munoz J, Chuah A, Wood D, Rasko JEJ, Heck AJR, Grimmond SM, Rogers IM, Seo J-S, Wells CA, Puri MC, Nagy A, Preiss T (2014) Small RNA changes en route to distinct cellular states of induced pluripotency. Nat Commun 5:5522

Cooke JP, Sayed N, Lee J, Wong WT (2014) Innate immunity and epigenetic plasticity in cellular reprogramming. Curr Opin Genet Dev 28:89–91

Cyranoski D (2014) Japanese woman is first recipient of next-generation stem cells. Nature News. Nature Publishing Group. http://www.nature.com/news/japanese-woman-is-first-recipient-of-next-generation-stemcells-1.15915

David R, Brenner C, Stieber J, Schwarz F, Brunner S, Vollmer M, Mentele E, Müller-Höcker J, Kitajima S, Lickert H, Rupp R, Franz W-M (2008) MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. Nat Cell Biol 10(3):338–345

David R, Stieber J, Fischer E, Brunner S, Brenner C, Pfeiler S, Schwarz F, Franz W-M (2009) Forward programming of pluripotent stem cells towards distinct cardiovascular cell types. Cardiovasc Res 84(2):263–272

David R, Jarsch VB, Schwarz F, Nathan P, Gegg M, Lickert H, Franz W-M (2011) Induction of MesP1 by Brachyury(T) generates the common multipotent cardiovascular stem cell. Cardiovasc Res 92(1):115–122

Davis RL, Weintraub H, Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell Press 51(6):987–1000

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(3):322–333

de Val S, Black BL (2009) Transcriptional control of endothelial cell development. Dev Cell 16(2):180–195

Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, Lagarde J, Veeravalli L, Ruan X, Ruan Y, Lassmann T, Carninci P, Brown JB, Lipovich L, Gonzalez JM, Thomas M, Davis CA, Shiekhattar R, Gingeras TR, Hubbard TJ, Notredame C, Harrow J, Guigó R (2012) The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res 22(9):1775–1789

Doherty KR, Talbert DR, Trusk PB, Moran DM, Shell SA, Bacus S (2015) Structural and functional screening in human induced-pluripotent stem cell-derived cardiomyocytes accurately identifies cardiotoxicity of multiple drug types. Toxicol Appl Pharmacol 285(1):51–60

Du Y, Wang J, Jia J, Song N, Xiang C, Xu J, Hou Z, Su X, Liu B, Jiang T, Zhao D, Sun Y, Shu J, Guo Q, Yin M, Sun D, Lu S, Shi Y, Deng H (2014) Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem Cell 14(3):394–403

Dubois NC, Craft AM, Sharma P, Elliott DA, Stanley EG, Elefanty AG, Gramolini A, Keller G (2011) SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. Nat Biotechnol 29(11):1011–1018

Ebert AD, Liang P, Wu JC (2012) Induced pluripotent stem cells as a disease modeling and drug screening platform. J Cardiovasc Pharmacol 60(4):408–416

Fujioka Y, Matozaki T, Noguchi T, Iwamatsu A, Yamao T, Takahashi N, Tsuda M, Takada T, Kasuga M (1996) A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. Mol Cell Biol 16(12):6887–6899

Gai H, Leung EL-H, Costantino PD, Aguila JR, Nguyen DM, Fink LM, Ward DC, Ma Y (2009) Generation and characterization of functional cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts. Cell Biol Int 33(11):1184–1193

Garbern JC, Lee RT (2013) Cardiac stem cell therapy and the promise of heart regeneration. Cell Stem Cell 12(6):689–698

Garbes L, Heesen L, Hölker I, Bauer T, Schreml J, Zimmermann K, Thoenes M, Walter M, Dimos J, Peitz M, Brüstle O, Heller R, Wirth B (2013) VPA response in SMA is suppressed by the fatty acid translocase CD36. Hum Mol Genet 22(2):398–407

Ghosh TK, Song FF, Packham EA, Buxton S, Robinson TE, Ronksley J, Self T, Bonser AJ, Brook JD (2009) Physical interaction between TBX5 and MEF2C is required for early heart development. Mol Cell Biol 29(8):2205–2218

Ginsberg M, James D, Ding B-S, Nolan D, Geng F, Butler JM, Schachterle W, Pulijaal VR, Mathew S, Chasen ST, Xiang J, Rosenwaks Z, Shido K, Elemento O, Rabbany SY, Rafii S (2012) Efficient direct reprogramming of mature amniotic cells into endothelial cells by ETS factors and TGFβ suppression. Cell 151(3):559–575

Guha P, Morgan JW, Mostoslavsky G, Rodrigues NP, Boyd AS (2013) Lack of immune response to differentiated cells derived from syngeneic induced pluripotent stem cells. Cell Stem Cell 12(4):407–412

Guo Z, Zhang L, Wu Z, Chen Y, Wang F, Chen G (2014) In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model. Cell Stem Cell 14(2):188–202

Gurdon JB (1962) The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morphol 10(10):622–640

Gurdon JB (2013) The egg and the nucleus: a battle for supremacy (Nobel Lecture). Angew Chem Int Ed Engl 52(52):13890–13899

Hanna J, Wernig M, Markoulaki S, Sun C-W, Meissner A, Cassady JP, Beard C, Brambrink T, Wu L-C, Townes TM, Jaenisch R (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318(5858):1920–1923

Hasenfuss G (1998) Animal models of human cardiovascular disease, heart failure and hypertrophy. Cardiovasc Res 39(1):60–76

Hattori F, Chen H, Yamashita H, Tohyama S, Satoh Y-S, Yuasa S, Li W, Yamakawa H, Tanaka T, Onitsuka T, Shimoji K, Ohno Y, Egashira T, Kaneda R, Murata M, Hidaka K, Morisaki T, Sasaki E, Suzuki T, Sano M, Makino S, Oikawa S, Fukuda K (2010) Nongenetic method for purifying stem cell-derived cardiomyocytes. Nat Methods 7(1):61–66

Hausburg F, Na S, Voronina N, Skorska A, Müller P, Steinhoff G, David R (2015) Defining optimized properties of modified mRNA to enhance virus- and DNA- independent protein expression in adult stem cells and fibroblasts. Cell Physiol Biochem Int J Exp Cell Physiol Biochem Pharmacol 35(4):1360–1371

He J-Q, Ma Y, Lee Y, Thomson JA, Kamp TJ (2003) Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. Circ Res 93(1):32–39

Heinrich C, Blum R, Gascón S, Masserdotti G, Tripathi P, Sánchez R, Tiedt S, Schroeder T, Götz M, Berninger B (2010) Directing astroglia from the cerebral cortex into subtype specific functional neurons. PLoS Biol 8(5):e1000373

Heinrich C, Bergami M, Gascón S, Lepier A, Viganò F, Dimou L, Sutor B, Berninger B, Götz M (2014) Sox2-mediated conversion of NG2 glia into induced neurons in the injured adult cerebral cortex. Stem Cell Rep 3(6):1000–1014

Heinrich C, Spagnoli FM, Berninger B (2015) In vivo reprogramming for tissue repair. Nat Cell Biol 17(3):204–211

Ho S-M, Topol A, Brennand KJ (2015) From \"directed differentiation\" to \"neuronal induction\": modeling neuropsychiatric disease. Biomark Insights 10(Suppl 1):31–41

Hong KU, Li Q-H, Guo Y, Patton NS, Moktar A, Bhatnagar A, Bolli R (2013) A highly sensitive and accurate method to quantify absolute numbers of c-kit+cardiac stem cells following transplantation in mice. Basic Res Cardiol 108(3):346

Hosoda T, Zheng H, Cabral-da-Silva M, Sanada F, Ide-Iwata N, Ogórek B, Ferreira-Martins J, Arranto C, D'Amario D, del Monte F, Urbanek K, D'Alessandro DA, Michler RE, Anversa P, Rota M, Kajstura J, Leri A (2011) Human cardiac stem cell differentiation is regulated by a mircrine mechanism. Circulation 123(12):1287–1296

Hou P, Li Y, Zhang X, Liu C, Guan J, Li H, Zhao T, Ye J, Yang W, Liu K, Ge J, Xu J, Zhang Q, Zhao Y, Deng H (2013) Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341(6146):651–654

Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475(7356):386–389

Huang P, Zhang L, Gao Y, He Z, Yao D, Wu Z, Cen J, Chen X, Liu C, Hu Y, Lai D, Hu Z, Chen L, Zhang Y, Cheng X, Ma X, Pan G, Wang X, Hui L (2014) Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. Cell Stem Cell 14(3):370–384

Huber I, Itzhaki I, Caspi O, Arbel G, Tzukerman M, Gepstein A, Habib M, Yankelson L, Kehat I, Gepstein L (2007) Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. FASEB J 21(10):2551–2563

Hussein SMI, Puri MC, Tonge PD, Benevento M, Corso AJ, Clancy JL, Mosbergen R, Li M, Lee D-S, Cloonan N, Wood DLA, Munoz J, Middleton R, Korn O, Patel HR, White CA, Shin J-Y, Gauthier ME, Lê Cao K-A, Kim J-I, Mar JC, Shakiba N, Ritchie W, Rasko JEJ, Grimmond SM, Zandstra PW, Wells CA, Preiss T, Seo J-S, Heck AJR, Rogers IM, Nagy A (2014) Genome-wide characterization of the routes to pluripotency. Nature 516(7530):198–206

Ieda M, Fu J-D, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 142(3):375–386

Inagawa K, Miyamoto K, Yamakawa H, Muraoka N, Sadahiro T, Umei T, Wada R, Katsumata Y, Kaneda R, Nakade K, Kurihara C, Obata Y, Miyake K, Fukuda K, Ieda M (2012) Induction of

cardiomyocyte-like cells in infarct hearts by gene transfer of Gata4, Mef2c, and Tbx5. Circ Res 111(9):1147–1156

Inoue H, Nagata N, Kurokawa H, Yamanaka S (2014) iPS cells: a game changer for future medicine. EMBO J 33(5):409–417

Islas JF, Liu Y, Weng K-C, Robertson MJ, Zhang S, Prejusa A, Harger J, Tikhomirova D, Chopra M, Iyer D, Mercola M, Oshima RG, Willerson JT, Potaman VN, Schwartz RJ (2012) Transcription factors ETS2 and MESP1 transdifferentiate human dermal fibroblasts into cardiac progenitors. Proc Natl Acad Sci U S A 109(32):13016–13021

Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, Winterstern A, Feldman O, Gepstein A, Arbel G, Hammerman H, Boulos M, Gepstein L (2011) Modelling the long QT syndrome with induced pluripotent stem cells. Nature 471(7337):225–229

James D, Nam H-S, Seandel M, Nolan D, Janovitz T, Tomishima M, Studer L, Lee G, Lyden D, Benezra R, Zaninovic N, Rosenwaks Z, Rabbany SY, Rafii S (2010) Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent. Nat Biotechnol 28(2):161–166

Jayawardena TM, Egemnazarov B, Finch EA, Zhang L, Payne JA, Pandya K, Zhang Z, Rosenberg P, Mirotsou M, Dzau VJ (2012) MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. Circ Res 110(11):1465–1473

Jayawardena T, Mirotsou M, Dzau VJ (2014) Direct reprogramming of cardiac fibroblasts to cardiomyocytes using microRNAs. Methods Mol Biol 1150:263–272

Jiang B, Xiang Z, Ai Z, Wang H, Li Y, Ji W, Li T (2015) Generation of cardiac spheres from primate pluripotent stem cells in a small molecule-based 3D system. Biomaterials 65:103–114

Jung JJ, Husse B, Rimmbach C, Krebs S, Stieber J, Steinhoff G, Dendorfer A, Franz W-M, David R (2014) Programming and isolation of highly pure physiologically and pharmacologically functional sinus-nodal bodies from pluripotent stem cells. Stem Cell Rep 2(5):592–605

Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K (2009) Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458(7239):771–775

Kamao H, Mandai M, Okamoto S, Sakai N, Suga A, Sugita S, Kiryu J, Takahashi M (2014) Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application. Stem Cell Rep 2(2):205–218

Kanemura H, Go MJ, Shikamura M, Nishishita N, Sakai N, Kamao H, Mandai M, Morinaga C, Takahashi M, Kawamata S (2014) Tumorigenicity studies of induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) for the treatment of age-related macular degeneration. PLoS One 9(1):e85336

Karakikes I, Ameen M, Termglinchan V, Wu JC (2015) Human induced pluripotent stem cell-derived cardiomyocytes: insights into molecular, cellular, and functional phenotypes. Circ Res 117(1):80–88

Karikó K, Muramatsu H, Keller JM, Weissman D (2012) Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. Mol Ther 20(5):948–953

Kattman SJ, Huber TL, Keller GM (2006) Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell 11(5):723–732

Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G (2011) Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 8(2):228–240

Katz TC, Singh MK, Degenhardt K, Rivera-Feliciano J, Johnson RL, Epstein JA, Tabin CJ (2012) Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells. Dev Cell 22(3):639–650

Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108(3):407–414

Kharitonenkov A, Chen Z, Sures I, Wang H, Schilling J, Ullrich A (1997) A family of proteins that inhibit signalling through tyrosine kinase receptors. Nature 386(6621):181–186

Kim D, Kim C-H, Moon J-I, Chung Y-G, Chang M-Y, Han B-S, Ko S, Yang E, Cha KY, Lanza R, Kim K-S (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4(6):472–476

Kim YJ, Lim H, Li Z, Oh Y, Kovlyagina I, Choi IY, Dong X, Lee G (2014) Generation of multipotent induced neural crest by direct reprogramming of human postnatal fibroblasts with a single transcription factor. Cell Stem Cell 15(4):497–506

Kitaoka S, Kondoh H, Inoue H (eds) (2011) Induced pluripotent stem cell technology for the study of neurodegenerative diseases, induced stem cells. Nova Science Publishers Inc, New York

Knollmann BC (2013) Induced pluripotent stem cell-derived cardiomyocytes: boutique science or valuable arrhythmia model? Circ Res 112(6):969–976; discussion 976

Kondo T, Asai M, Tsukita K, Kutoku Y, Ohsawa Y, Sunada Y, Imamura K, Egawa N, Yahata N, Okita K, Takahashi K, Asaka I, Aoi T, Watanabe A, Watanabe K, Kadoya C, Nakano R, Watanabe D, Maruyama K, Hori O, Hibino S, Choshi T, Nakahata T, Hioki H, Kaneko T, Naitoh M, Yoshikawa K, Yamawaki S, Suzuki S, Hata R, Ueno S-I, Seki T, Kobayashi K, Toda T, Murakami K, Irie K, Klein WL, Mori H, Asada T, Takahashi R, Iwata N, Yamanaka S, Inoue H (2013) Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Aβ and differential drug responsiveness. Cell Stem Cell 12(4):487–496

Kormann MSD, Hasenpusch G, Aneja MK, Nica G, Flemmer AW, Herber-Jonat S, Huppmann M, Mays LE, Illenyi M, Schams A, Griese M, Bittmann I, Handgretinger R, Hartl D, Rosenecker J, Rudolph C (2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nat Biotechnol 29(2):154–157

Kraehenbuehl TP, Ferreira LS, Hayward AM, Nahrendorf M, van der Vlies AJ, Vasile E, Weissleder R, Langer R, Hubbell JA (2011) Human embryonic stem cell-derived microvascular grafts for cardiac tissue preservation after myocardial infarction. Biomaterials 32(4):1102–1109

Laflamme MA, Murry CE (2005) Regenerating the heart. Nat Biotechnol 23(7):845–856

Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE (2007) Cardiomyocytes derived from human embryonic stem cells in prosurvival factors enhance function of infarcted rat hearts. Nat Biotechnol 25(9):1015–1024

Lan F, Lee AS, Liang P, Sanchez-Freire V, Nguyen PK, Wang L, Han L, Yen M, Wang Y, Sun N, Abilez OJ, Hu S, Ebert AD, Navarrete EG, Simmons CS, Wheeler M, Pruitt B, Lewis R, Yamaguchi Y, Ashley EA, Bers DM, Robbins RC, Longaker MT, Wu JC (2013) Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. Cell Stem Cell 12(1):101–113

Lang C, Lehner S, Todica A, Boening G, Zacherl M, Franz W-M, Krause BJ, Bartenstein P, Hacker M, David R (2014) In-vivo comparison of the acute retention of stem cell derivatives and fibroblasts after intramyocardial transplantation in the mouse model. Eur J Nucl Med Mol Imaging 41(12):2325–2336

Lee J, Sayed N, Hunter A, Au KF, Wong WH, Mocarski ES, Pera RR, Yakubov E, Cooke JP (2012) Activation of innate immunity is required for efficient nuclear reprogramming. Cell 151(3):547–558

Lee D-S, Shin J-Y, Tonge PD, Puri MC, Lee S, Park H, Lee W-C, Hussein SMI, Bleazard T, Yun J-Y, Kim J, Li M, Cloonan N, Wood D, Clancy JL, Mosbergen R, Yi J-H, Yang K-S, Kim H, Rhee H, Wells CA, Preiss T, Grimmond SM, Rogers IM, Nagy A, Seo J-S (2014) An epigenomic roadmap to induced pluripotency reveals DNA methylation as a reprogramming modulator. Nat Commun 5:5619

Lemper M, Leuckx G, Heremans Y, German MS, Heimberg H, Bouwens L, Baeyens L (2015) Reprogramming of human pancreatic exocrine cells to β-like cells. Cell Death Differ 22(7):1117–1130

Lensch MW, Schlaeger TM, Zon LI, Daley GQ (2007) Teratoma formation assays with human embryonic stem cells: a rationale for one type of human-animal chimera. Cell Stem Cell 1(3):253–258

Lerou PH, Daley GQ (2005) Therapeutic potential of embryonic stem cells. Blood Rev 19(6):321–331

Lev S, Kehat I, Gepstein L (2005) Differentiation pathways in human embryonic stem cell-derived cardiomyocytes. Ann N Y Acad Sci 1047:50–65

Li Y-P, Wang Y (2015) Large noncoding RNAs are promising regulators in embryonic stem cells. J Genet Genom = Yi chuan xue bao 42(3):99–105

Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, Raval KK, Zhang J, Kamp TJ, Palecek SP (2012) Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci U S A 109(27):E1848–E1857

Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP (2013) Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. Nat Protoc 8(1):162–175

Liang P, Lan F, Lee AS, Gong T, Sanchez-Freire V, Wang Y, Diecke S, Sallam K, Knowles JW, Wang PJ, Nguyen PK, Bers DM, Robbins RC, Wu JC (2013a) Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. Circulation 127(16):1677–1691

Liang X, Wang G, Lin L, Lowe J, Zhang Q, Bu L, Chen Y, Chen J, Sun Y, Evans SM (2013b) HCN4 dynamically marks the first heart field and conduction system precursors. Circ Res 113(4):399–407

Lindvall O, Björklund A (2004) Cell therapy in Parkinson's disease. NeuroRx 1(4):382–393

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

Liu J, Liu Y, Wang H, Hao H, Han Q, Shen J, Shi J, Li C, Mu Y, Han W (2013) Direct differentiation of hepatic stem-like WB cells into insulin-producing cells using small molecules. Sci Rep 3:1185

Loffredo FS, Steinhauser ML, Gannon J, Lee RT (2011) Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell 8(4):389–398

Mah N, Wang Y, Liao M-C, Prigione A, Jozefczuk J, Lichtner B, Wolfrum K, Haltmeier M, Flöttmann M, Schaefer M, Hahn A, Mrowka R, Klipp E, Andrade-Navarro MA, Adjaye J (2011) Molecular insights into reprogramming-initiation events mediated by the OSKM gene regulatory networ. PLoS One 6(8):e24351

Maitra M, Schluterman MK, Nichols HA, Richardson JA, Lo CW, Srivastava D, Garg V (2009) Interaction of Gata4 and Gata6 with Tbx5 is critical for normal cardiac development. Dev Biol 326(2):368–377

Malliaras K, Zhang Y, Seinfeld J, Galang G, Tseliou E, Cheng K, Sun B, Aminzadeh M, Marbán E (2013) Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart. EMBO Mol Med 5(2):191–209

Mandal PK, Rossi DJ (2013) Reprogramming human fibroblasts to pluripotency using modified mRNA. Nat Protoc 8(3):568–582

Matar AA, Chong JJ (2014) Stem cell therapy for cardiac dysfunction. SpringerPlus 3:440

Mauritz C, Martens A, Rojas SV, Schnick T, Rathert C, Schecker N, Menke S, Glage S, Zweigerdt R, Haverich A, Martin U, Kutschka I (2011) Induced pluripotent stem cell (iPSC)-derived Flk-1 progenitor cells engraft, differentiate, and improve heart function in a mouse model of acute myocardial infarction. Eur Heart J 32(21):2634–2641

Melkoumian Z, Weber JL, Weber DM, Fadeev AG, Zhou Y, Dolley-Sonneville P, Yang J, Qiu L, Priest CA, Shogbon C, Martin AW, Nelson J, West P, Beltzer JP, Pal S, Brandenberger R (2010) Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. Nat Biotechnol 28(6):606–610

Misfeldt AM, Boyle SC, Tompkins KL, Bautch VL, Labosky PA, Baldwin HS (2009) Endocardial cells are a distinct endothelial lineage derived from Flk1+ multipotent cardiovascular progenitors. Dev Biol 333(1):78–89

Miyoshi N, Ishii H, Nagano H, Haraguchi N, Dewi DL, Kano Y, Nishikawa S, Tanemura M, Mimori K, Tanaka F, Saito T, Nishimura J, Takemasa I, Mizushima T, Ikeda M, Yamamoto H, Sekimoto M, Doki Y, Mori M (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8(6):633–638

Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park S-Y, Silberstein LE, Remedios D, Cristobal G, Graham D, Colan S, Kühn B (2013) Cardiomyocyte proliferation contributes to heart growth in young humans. Proc Natl Acad Sci U S A 110(4):1446–1451

Mordwinkin NM, Burridge PW, Wu JC (2013) A review of human pluripotent stem cell-derived cardiomyocytes for high-throughput drug discovery, cardiotoxicity screening, and publication standards. J Cardiovasc Transl Res 6(1):22–30

Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz K-L, Chien KR (2006) Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127(6):1151–1165

Moretti A, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flügel L, Dorn T, Goedel A, Höhnke C, Hofmann F, Seyfarth M, Sinnecker D, Schömig A, Laugwitz K-L (2010) Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N Engl J Med 363(15):1397–1409

Morita R, Suzuki M, Kasahara H, Shimizu N, Shichita T, Sekiya T, Kimura A, Sasaki AI, Yasukawa H, Yoshimura A (2015) ETS transcription factor ETV2 directly converts human fibroblasts into functional endothelial cells. Proc Natl Acad Sci U S A 112(1):160–165

Morizane A, Doi D, Kikuchi T, Okita K, Hotta A, Kawasaki T, Hayashi T, Onoe H, Shiina T, Yamanaka S, Takahashi J (2013) Direct comparison of autologous and allogeneic transplantation of iPSC-derived neural cells in the brain of a non-human primate. Stem Cell Rep 1(4):283–292

Mothe AJ, Tator CH (2013) Review of transplantation of neural stem/progenitor cells for spinal cord injury. Int J Dev Neurosci 31(7):701–713

Musunuru K, Domian IJ, Chien KR (2010) Stem cell models of cardiac development and disease. Annu Rev Cell Dev Biol 26:667–687

Nelson TJ, Martinez-Fernandez A, Yamada S, Perez-Terzic C, Ikeda Y, Terzic A (2009) Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. Circulation 120(5):408–416

Neve A, Corrado A, Cantatore FP (2011) Osteoblast physiology in normal and pathological conditions. Cell Tissue Res 343(2):289–302

Nussbaum J, Minami E, Laflamme MA, Virag JAI, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE (2007) Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 21(7):1345–1357

Olson EN (2006) Gene regulatory networks in the evolution and development of the heart. Science 313(5795):1922–1927

Ong S-G, Lee WH, Kodo K, Wu JC (2015) MicroRNA-mediated regulation of differentiation and trans-differentiation in stem cells". Adv Drug Deliv Rev 88:3–15

Otsuji TG, Bin J, Yoshimura A, Tomura M, Tateyama D, Minami I, Yoshikawa Y, Aiba K, Heuser JE, Nishino T, Hasegawa K, Nakatsuji N (2014) A 3D sphere culture system containing functional polymers for large-scale human pluripotent stem cell production. Stem Cell Rep 2(5):734–745

Pavo N, Charwat S, Nyolczas N, Jakab A, Murlasits Z, Bergler-Klein J, Nikfardjam M, Benedek I, Benedek T, Pavo IJ, Gersh BJ, Huber K, Maurer G, Gyöngyösi M (2014) Cell therapy for human ischemic heart diseases: critical review and summary of the clinical experiences. J Mol Cell Cardiol 75:12–24

Polo JM, Anderssen E, Walsh RM, Schwarz BA, Nefzger CM, Lim SM, Borkent M, Apostolou E, Alaei S, Cloutier J, Bar-Nur O, Cheloufi S, Stadtfeld M, Figueroa ME, Robinton D, Natesan S, Melnick A, Zhu J, Ramaswamy S, Hochedlinger K (2012) A molecular roadmap of reprogramming somatic cells into iPS cells. Cell 151(7):1617–1632

Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, Conway SJ, Fu J-D, Srivastava D (2012) In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. Nature 485(7400):593–598

Qian L, Berry EC, Fu J-D, Ieda M, Srivastava D (2013) Reprogramming of mouse fibroblasts into cardiomyocyte-like cells in vitro. Nat Protoc 8(6):1204–1215

Quabius ES, Krupp G (2015) Synthetic mRNAs for manipulating cellular phenotypes: an overview. New Biotechnol 32(1):229–235

Reynolds J, Lamba DA (2014) Human embryonic stem cell applications for retinal degenerations. Exp Eye Res 123:151–160

Rimmbach C, Jung JJ, David R (2015) Generation of murine cardiac pacemaker cell aggregates based on ES-cell-programming in combination with Myh6-promoter-selection. J Vis Exp 96:e52465

Rosen MR, Myerburg RJ, Francis DP, Cole GD, Marbán E (2014) Translating stem cell research to cardiac disease therapies: pitfalls and prospects for improvement. J Am Coll Cardiol 64(9):922–937

Sahara M, Santoro F, Chien KR (2015) Programming and reprogramming a human heart cell. EMBO J 34(6):710–738

Sandler VM, Lis R, Liu Y, Kedem A, James D, Elemento O, Butler JM, Scandura JM, Rafii S (2014) Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. Nature 511(7509):312–318

Sayed N, Wong WT, Ospino F, Meng S, Lee J, Jha A, Dexheimer P, Aronow BJ, Cooke JP (2015) Transdifferentiation of human fibroblasts to endothelial cells: role of innate immunity. Circulation 131(3):300–309

Schlueter J, Brand T (2012) Epicardial progenitor cells in cardiac development and regeneration. J Cardiovasc Transl Res 5(5):641–653

Schwartz SD, Hubschman J-P, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R (2012) Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 379(9817):713–720

Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, Hubschman J-P, Davis JL, Heilwell G, Spirn M, Maguire J, Gay R, Bateman J, Ostrick RM, Morris D, Vincent M, Anglade E, Priore D, Lucian V, Lanza R (2015) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 385(9967):509–516

Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu T-D, Guerquin-Kern J-L, Lechene CP, Lee RT (2013) Mammalian heart renewal by pre-existing cardiomyocytes. Nature 493(7432):433–436

Sharrocks AD (2001) The ETS-domain transcription factor family. Nat Rev Mol Cell Biol 2(11):827–837

Shiba Y, Fernandes S, Zhu W-Z, Filice D, Muskheli V, Kim J, Palpant NJ, Gantz J, Moyes KW, Reinecke H, van Biber B, Dardas T, Mignone JL, Izawa A, Hanna R, Viswanathan M, Gold JD, Kotlikoff MI, Sarvazyan N, Kay MW, Murry CE, Laflamme MA (2012) Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. Nature 489(7415):322–325

Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51(1):297–305

Sluijter JPG, van Mil A, van Vliet P, Metz CHG, Liu J, Doevendans PA, Goumans M-J (2010) MicroRNA-1 and –499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. Arterioscler Thromb Vasc Biol 30(4):859–868

Smith DK, Zhang C-L (2015) Regeneration through reprogramming adult cell identity in vivo. Am J Pathol 185(10):2619–2628

Song K, Nam Y-J, Luo X, Qi X, Tan W, Huang GN, Acharya A, Smith CL, Tallquist MD, Neilson EG, Hill JA, Bassel-Duby R, Olson EN (2012) Heart repair by reprogramming non-myocytes with cardiac transcription factors. Nature 485(7400):599–604

Später D, Abramczuk MK, Buac K, Zangi L, Stachel MW, Clarke J, Sahara M, Ludwig A, Chien KR (2013) A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells. Nat Cell Biol 15(9):1098–1106

Srivastava D (2006) Making or breaking the heart: from lineage determination to morphogenesis. Cell 126(6):1037–1048

Sturgeon CM, Ditadi A, Clarke RL, Keller G (2013) Defining the path to hematopoietic stem cells. Nat Biotechnol 31(5):416–418

Sullivan GJ, Hay DC, Park I-H, Fletcher J, Hannoun Z, Payne CM, Dalgetty D, Black JR, Ross JA, Samuel K, Wang G, Daley GQ, Lee J-H, Church GM, Forbes SJ, Iredale JP, Wilmut I (2010) Generation of functional human hepatic endoderm from human induced pluripotent stem cells. Hepatology 51(1):329–335

Swijnenburg R-J, Sheikh AY, Robbins RC (2007) Comment on \"Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response\". FASEB J 21(7):1290; author reply 1291

Swijnenburg R-J, Schrepfer S, Govaert JA, Cao F, Ransohoff K, Sheikh AY, Haddad M, Connolly AJ, Davis MM, Robbins RC, Wu JC (2008) Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. Proc Natl Acad Sci U S A 105(35):12991–12996

Szabo E, Rampalli S, Risueño RM, Schnerch A, Mitchell R, Fiebig-Comyn A, Levadoux-Martin M, Bhatia M (2010) Direct conversion of human fibroblasts to multilineage blood progenitors. Nature 468(7323):521–526

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

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(5):861–872

Thompson SA, Burridge PW, Lipke EA, Shamblott M, Zambidis ET, Tung L (2012) Engraftment of human embryonic stem cell derived cardiomyocytes improves conduction in an arrhythmogenic in vitro model. J Mol Cell Cardiol 53(1):15–23

Thomson JA (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147

Tohyama S, Hattori F, Sano M, Hishiki T, Nagahata Y, Matsuura T, Hashimoto H, Suzuki T, Yamashita H, Satoh Y, Egashira T, Seki T, Muraoka N, Yamakawa H, Ohgino Y, Tanaka T, Yoichi M, Yuasa S, Murata M, Suematsu M, Fukuda K (2013) Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. Cell Stem Cell 12(1):127–137

Tonge PD, Corso AJ, Monetti C, Hussein SMI, Puri MC, Michael IP, Li M, Lee D-S, Mar JC, Cloonan N, Wood DL, Gauthier ME, Korn O, Clancy JL, Preiss T, Grimmond SM, Shin J-Y, Seo J-S, Wells CA, Rogers IM, Nagy A (2014) Divergent reprogramming routes lead to alternative stem-cell states. Nature 516(7530):192–197

Uosaki H, Fukushima H, Takeuchi A, Matsuoka S, Nakatsuji N, Yamanaka S, Yamashita JK (2011) Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. PLoS One 6(8):e23657

van Hoof D, Dormeyer W, Braam SR, Passier R, Monshouwer-Kloots J, Ward-van Oostwaard D, Heck AJR, Krijgsveld J, Mummery CL (2010) Identification of cell surface proteins for antibody-based selection of human embryonic stem cell-derived cardiomyocytes. J Proteome Res 9(3):1610–1618

van Laake LW, Qian L, Cheng P, Huang Y, Hsiao EC, Conklin BR, Srivastava D (2010) Reporter-based isolation of induced pluripotent stem cell- and embryonic stem cell-derived cardiac progenitors reveals limited gene expression variance. Circ Res 107(3):340–347

Vian L, Di Carlo M, Pelosi E, Fazi F, Santoro S, Cerio AM, Boe A, Rotilio V, Billi M, Racanicchi S, Testa U, Grignani F, Nervi C (2014) Transcriptional fine-tuning of microRNA-223 levels directs lineage choice of human hematopoietic progenitors. Cell Death Differ 21(2):290–301

Vincent SD, Buckingham ME (2010) How to make a heart. In: Koopman P (ed) Organogenesis in development, vol 90, Current topics in developmental biology. Elsevier, Heidelberg, pp 1–41

Wang Z, Wang D-Z, Pipes GC, Olson EN (2003) Myocardin is a master regulator of smooth muscle gene expression. Proc Natl Acad Sci U S A 100(12):7129–7134

Wang H, Cao N, Spencer CI, Nie B, Ma T, Xu T, Zhang Y, Wang X, Srivastava D, Ding S (2014) Small molecules enable cardiac reprogramming of mouse fibroblasts with a single factor, Oct4. Cell Rep 6(5):951–960

Wang L, Liu Z, Yin C, Asfour H, Chen O, Li Y, Bursac N, Liu J, Qian L (2015) Stoichiometry of Gata4, Mef2c, and Tbx5 influences the efficiency and quality of induced cardiac myocyte reprogramming. Circ Res 116(2):237–244

Warren L, Manos PD, Ahfeldt T, Loh Y-H, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7(5):618–630

Wernig M, Zhao J-P, Pruszak J, Hedlund E, Fu D, Soldner F, Broccoli V, Constantine-Paton M, Isacson O, Jaenisch R (2008) Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci U S A 105(15):5856–5861

World Health Organization (2014) WHO methods and data sources for country – level causes of death 2000–2012. Global health estimates technical paper WHO/HIS/HSI/GHE/2014.7. Department of Health Statistics and Information Systems WHO, Geneva

Williams BJ, Bhatia S, Adams LK, Boling S, Carroll JL, Li X-L, Rogers DL, Korokhov N, Kovesdi I, Pereboev AV, Curiel DT, Mathis JM (2012) Dendritic cell based PSMA immunotherapy for prostate cancer using a CD40-targeted adenovirus vector. PLoS One 7(10):e46981

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810–813

Xin M, Olson EN, Bassel-Duby R (2013) Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol 14(8):529–541

Xu J, Du Y, Deng H (2015) Direct lineage reprogramming: strategies, mechanisms, and applications. Cell Stem Cell 16(2):119–134

Yakubov E, Rechavi G, Rozenblatt S, Givol D (2010) Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. Biochem Biophys Res Commun 394(1):189–193

Yamamoto K, Kishida T, Sato Y, Nishioka K, Ejima A, Fujiwara H, Kubo T, Yamamoto T, Kanamura N, Mazda O (2015) Direct conversion of human fibroblasts into functional osteoblasts by defined factors. Proc Natl Acad Sci U S A 112(19):6152–6157

Yamanaka S (2013) The winding road to pluripotency (Nobel Lecture). Angew Chem Int Ed Engl 52(52):13900–13909

Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM (2008) Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 453(7194):524–528

Yoo AS, Sun AX, Li L, Shcheglovitov A, Portmann T, Li Y, Lee-Messer C, Dolmetsch RE, Tsien RW, Crabtree GR (2011) MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476(7359):228–231

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858):1917–1920

Zangi L, Lui KO, von Gise A, Ma Q, Ebina W, Ptaszek LM, Später D, Xu H, Tabebordbar M, Gorbatov R, Sena B, Nahrendorf M, Briscoe DM, Li RA, Wagers AJ, Rossi DJ, Pu WT, Chien KR (2013) Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. Nat Biotechnol 31(10):898–907

Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ (2009) Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 104(4):e30–e41

Zhao R, Watt AJ, Battle MA, Li J, Bondow BJ, Duncan SA (2008) Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. Dev Biol 317(2):614–619

Zhao T, Zhang Z-N, Rong Z, Xu Y (2011) Immunogenicity of induced pluripotent stem cells. Nature 474(7350):212–215

Zhou W, Freed CR (2009) Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27(11):2667–2674

Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT (2008a) Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454(7200):109–113

Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008b) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455(7213):627–632

Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding S (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4(5):381–384

Zhou B, Honor LB, He H, Ma Q, Oh J-H, Butterfield C, Lin R-Z, Melero-Martin JM, Dolmatova E, Duffy HS, von Gise A, Zhou P, Hu YW, Wang G, Zhang B, Wang L, Hall JL, Moses MA, McGowan FX, Pu WT (2011) Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. J Clin Invest 121(5):1894–1904

Zwi L, Caspi O, Arbel G, Huber I, Gepstein A, Park I-H, Gepstein L (2009) Cardiomyocyte differentiation of human induced pluripotent stem cells. Circulation 120(15):1513–1523

Chapter 16
Parthenogenesis

Suresh Dinkar Kharche and Bipul Kumar Jha

Abstract Parthenogenesis (<*Gr. parthenos* virgin + *genesis* birth) a phenomenon of undoubted biological interest which leads to the production of living young in many types of animals, as well as in plants. The word parthenogenesis came first time in mind of Richard Owen (Owen R. On parthenogenesis or the successive production of procreating individuals from a single ovum. John van Voorst, London, 1849) who used it to define the process of 'procreation without the immediate influence of male'; this includes the various processes such as fission, budding as well as the development of unimpregnated ova. After him different authors have attempted to redefine the term accordingly. Beatty (Beatty RA. Ferti 1**:**413–440. Metz and A. Monroy. Academic Press, New York/London, 1967) has defined the term parthenogenesis as 'the production of an embryo from a female gamete without any genetic contribution from a male gamete', and with or without eventual development into an adult. An individual resulted from the parthenogenesis is variously referred to as 'parthenogenone', 'parthenogene' or 'parthenote'. Phenomenon parthenogenesis differs from gynogenesis; in which the oocyte is stimulated by aspermatozoa to complete the second meiotic division and to undergo further development but the male gamete will not contribute genetically to the developing embryos and from androgenesis; in such situation the egg is also activated by a spermatozoa but only the male genome take part in the subsequent development (Kaufman MH. Early mammalian development: parthenogenetic studies. Cambridge University Press, Cambridge, 1983).

Keywords Parthenogenesis • Parthenotes • Genomic imprinting • Parthenogenetic stem cells • Therapeutic application

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_16

16.1 Natural Parthenogenesis

Natural Parthenogenesis is a form of asexual reproduction in which growth and development of embryos occur without fertilization. Parthenogenesis occurs in many plants, some invertebrate species (including nematodes, water fleas, some scorpions, aphids, some bees, some Phasmida and parasitic wasps), and few vertebrates (such as some fish, amphibians, reptiles and very rarely birds). Some species reproduce exclusively by parthenogenesis (such as the Bdelloid rotifers), while others can switch between sexual reproduction and parthenogenesis. This is called "facultative parthenogenesis", "cyclical parthenogenesis", "heterogamy" or "heterogony". The switch between sexuality and parthenogenesis in such species may be triggered by the season (aphid, some gall wasps), or by a lack of males or by conditions that favors rapid population growth (rotifers and cladocerans like daphnia). The phenomenon of parthenogenesis offers benefit for the lower animals in the sense that it provides rapid population growth in favorable seasons without the need of males. At the same time it becomes costly to them in the sense that it creates low genetic diversity among them therefore, susceptibility to adverse mutations that might occur.

Parthenogenetic development may produce all kinds of ploidy as well as homozygous and heterozygous conditions in organism whether it is haploid, diploid or polyploids. It depends on the routes followed by the egg cells whether meiosis of the egg cell is completed normally or, failing this, which of the stages has been omitted. Alternatively, meiosis may be completely suppressed and the egg developed as a result of mitotic divisions only. The number of chromosome sets in the embryo can further be varied by failure of cell division as a result of DNA synthesis, either before meiosis or afterwards, in what would otherwise have been a cleavage division. This process is known as "endoreduplication".

Parthenogenesis may initiate early embryonic development in mammals. It can occur spontaneously or it may be induced in them. There were many cases of spontaneously occurring cleavage have been reported in mammals such as guinea pigs (Pincus 1936), hamster eggs (Austin 1956) as well as in humans ovary, particularly in atretic follicles (Krafka 1939). Its lack of success in this class poses some fundamental and as yet unresolved problems regarding the significance of fertilization in the physiology of reproduction and embryonic development. This is one of the reasons why parthenogenesis is once again an area of active research.

16.2 Induced Parthenogenesis

Even though parthenogenesis is not natural process in mammals but it can be induced in them experimentally by different means. The pioneering study in this regard was carried out by Pincus and his collaborators on rabbit in 1936. He showed that the extrusion of polar body can be induced *in-vitro* by various means such as; by

contact with sperm suspension, heat treatment, exposure to butyric acid and hypertonic solutions. He also noticed that cleavage rate can be increased by applying cold treatment on unfertilized eggs. Subsequently Pincus and Shapiro (1940) described the effect of cold treatment on unfertilized tubal eggs in- vitro and claimed not only an increased incidence of cleavage but also the production of a living young. There has since been abundant confirmation of the possibility of inducing parthenogenetic development in mammals by experimental procedures but none of the embryos so formed has survived beyond the embryonic period.

But In April 2004, scientists at Tokyo University of Agriculture used parthenogenesis successfully to create a fatherless mouse. Using gene targeting, they were able to manipulate two imprinted loci H19/IGF2 and DLK1/MEG3 to produce bimaternal mice at high frequency and subsequently showed that fatherless mice have enhanced longevity. Induced parthenogenesis in mice and monkeys often results in abnormal development. This is because mammals have imprinted genetic regions, where either the maternal or paternal chromosome is inactivated in the offspring in order for development to proceed normally. A mammal created by parthenogenesis would thus have double doses of maternally imprinted genes and lack paternally imprinted genes, leading to developmental abnormalities. It has been suggested that defects in placental folding or interdigitation are one cause of parthenote abortive development.

16.3 General Procedure of Parthenogenetic Embryo Development in Mammals

Parthenogenesis is not reported as a form of natural reproduction in mammals even though mammalian oocytes, under appropriate stimuli, can undergo to parthenogenetic activation in-vivo or in-vitro which mimics embryonic development in its early phases. The general procedure of parthenogenetic embryo development is almost similar to the *in-vitro* embryo development of fertilized oocytes except the substitution of the step of parthenogenetic activation at the place of fertilization (with different activation agents) which could either be electrical, chemical or other types and thus includes; collection of ovaries, recovery of oocytes, in-vitro maturation of oocytes (IVM), activation of oocytes and in-vitro development of parthenogenetically activated embryos.

16.3.1 Recovery of Oocytes

Oocytes can either be retrieved from slaughter house ovaries or from the live animals by different techniques. Super-ovulation (hormonal treatment) of donors are routinely done to increase large number of ova released by the ovary, Sexually mature

animals (female) were used for obtaining oocytes using subcutaneous injection of FSH for 3 consecutive days followed by an intravenous injection of hCG after the last dose of FSH injection (Foote and Simkin 1993). Oocytes were recovered either by flushing or oviduct dissection at 14–15 h post-hCG treatment. Ham's F-10-based medium supplemented with 0.4 % BSA plus 1 % serum used as the flushing medium. Laparoscopic ovum pick up is one of the best techniques because of less adhesion problems compared to laparotomy or surgical oocytes collection from live animals. The cost of oocytes retrieval from live animal is high due to unpredictable results and low oocytes quantity. Therefore, Slaughter house ovaries are attractive alternative source for oocytes retrieval as it is less expensive and most abundant source of immature oocytes for large scale production of embryos. Follicle puncture, Ovary slicing, puncture and follicle aspiration are most routinely used techniques for recovery of oocytes from slaughter house ovaries. However different views come from different researchers. Aspiration technique is more advantageous than other techniques in terms of speed of aspiration (Nandi et al. 2000; Yadav et al. 2007). Slicing and Puncture techniques are most commonly used for smaller animals having small sized ovaries to obtain good quality and quantity of oocytes. However follicle puncture after aspiration increased the overall rate of oocytes recovery (Bonde et al. 2000).

16.3.2 In-Vitro Maturation of Oocytes

Like In-vivo, both nuclear and ooplasmic maturation is required to ensure parthenogenetic activation and embryo development in vitro. In all mammalian species, nuclear maturation can be achieved when the oocytes are removed from the antral follicles and are cultured in-vitro (Thibault et al. 1987). Nuclear maturation refers to the progression to the metaphase ll. Degree of cumulus cells expansion can be used as a morphological indicator for maturation of oocytes. Thus Maturation of oocytes (developed to the metaphase II stage) was determined by expansion of cumulus cells, an evenly granulated homogenous cytoplasm and extrusion of the first polar body, as visualized under a differential interference contrast microscope. So, it can be said that oocyte with expanded cumulus cells indicates mature and good quality oocytes while a compact cumulus cells characterizes immature oocytes. During In-vitro, when fully grown oocytes are released from their follicles to the culture medium they resume meiosis spontaneously, causes condensation of chromatin and breakdown of germinal vesicle leading to MII and a second artificial arrest in the cycle (Edwards 1965).

The immature oocytes having more than three layers of cumulus cells helps in in-vitro maturation while denuded one fail to fertilize (Crister et al. 1986). IVM medium consist of complex medium such as TCM-199 supplemented with 10–20 % fetal bovine serum (FBS), estrus goat/buffalo serum or super ovulated buffalo serum/goat serum (SBS) which is necessary for high achieving nuclear and cytoplasmic maturation of oocytes. Hormones like Luteinizing hormone

(LH) and Estradiol either alone or in combination has been used to induce higher maturation rates of oocytes ranging from 60 to 80 % (Totey et al. 1991).

Various growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor, insulin like growth factor-1 (IGF-1) in Oocyte Culture Medium (OCM) have shown to increase cumulus expansion, nuclear maturation (80–90 %) and post activation cleavage rates (Gupta et al. 2002; Yadav et al. 2009).

16.3.3 General Mechanism of Oocyte Activation

After IVM, cumulus cells were removed by incubation in hyaluronidase (0.2 % in calcium free PBS with 0.1 % polyvinyl alcohol) at 39 °C for 5 min followed by pipetting for 3–5 min. Cumulus-free and healthy appearing oocytes were selected for the presence of the first polar body and remained in maturation medium until activation. Before activation, oocytes were washed three times in Hepes buffered TCM 199 + 10 % FCS (H199) and then gets activated by various methods.

One common, universal method or activation agents has not been developed for all species because the process is highly specific for each species. Therefore, combination of activation agents is also applied. Similarly concentration as well as incubation time of the activation agents are also species specific and need to be optimized. For example, Cat oocyte activation by electric current or chemical agents (ethanol, calcium ionophore) has been reported, along with the inhibitors of protein synthesis, protein phosphorylation or histone kinases (Kinases PF/MAP; cycloheximide, cytochalasine B) (Gomez et al. 2003). A single electrical stimulation seems to be insufficient to activate domestic cat oocytes, but their exposure to cycloheximide following electrical stimulation improved the efficacy of parthenogenetic development (Karja et al. 2005). Results have indicated that exposure of human oocytes to a calcium ionophore alone, or such exposure followed by a protein synthesis inhibitor, leads to high activation rates but poor parthenote development (Sengoku et al. 2004). However, when exposure to an ionophore was followed by a protein kinase inhibitor, better developmental rates were obtained (Lin et al. 2003). Exogenous hyaluronic acid enhances porcine parthenogenetic embryo development in vitro possibly mediated by CD44 (Toyokawa et al 2005).

Methods used for parthenogenetic activation are divided into two groups according the mechanism used to increase intracellular calcium. One method for obtaining free calcium is by exposure to strontium or ionomycin to release cytoplasmic calcium. A second method is to promote influx of calcium from the extracellular medium by using an electrical stimulus or ethanol exposure (Meo et al. 2004). In addition, disturbance of the signal required to maintain MII can induce parthenogenesis. For example, microinjection of guanosine-54- O-(34-thiotriphosphate), a non-hydrozable analogue of guanosine triphosphate (GTP), induces parthenogenesis in porcine oocytes (Machaty et al. 1999). In the latter method, injected materials usually stimulate a specific intracellular signal transduction pathway. Namely, Mitogen-Activated Protein Kinases (MAPK) and cytostatic factor (CSF), important

factors for MII maintenance, are inactivated and Cyclin B, a subunit of maturation-promoting factor (MPF), is destroyed. Thus, these intracellular events result in inactivation of MPF, an important factor for oocyte maturation and maintenance of MII (Meo et al. 2004). Similarly, treatment with protein synthesis inhibitors, such as cycloheximide and puromycin, disturbs the MII maintenance mechanism and induces parthenogenesis in porcine oocytes (Nussbaum and Prather 1995).

Several methods have been developed for induction of parthenogenetic activation, including calcium ionophore (Funahashi et al. 1994), electrical shock (Kim et al. 1996), $CaCl_2$ (Machaty et al. 1996), Ca-EDTA (Zae Young et al. 2007), protein kinase inhibitors (Mayes et al. 1995, Kharche et al. 2015), G protein stimulation (Machaty et al. 1996), cycloheximide (CH) (Nussbaum and Prather 1995, Pathak et al. 2013), ionomycin (Loi et al. 1998), ultrasound (Sato et al. 2005), ethanol (Loi et al. 1998, Kharche et al. 2013), strontium (Meo et al. 2004) and magnetic field (Max et al. 2007), Spontaneous activation has also been reported in matured bovine oocytes. Besides the above mentioned procedure about parthenogenetic oocytes activation, sperms can also be used for parthenogenetic activation of oocytes (Patrick et al. 2004) which subsequently be developed in to embryos *in vitro*.

In each method, an initial response in the mature oocytes arrested at metaphase II is an incremental increase in intracellular Ca^{2+} concentration, similar to that produced after activation by the penetrating spermatozoon during the fertilization process. Then, the artificially activated oocyte further responds by initiating the cortical reaction and resuming meiosis, followed by second polar body extrusion. Thus, the sequence of events occurring during parthenogenetic activation mimics (PAM) the cascade of intracellular activities produced by the penetrating spermatozoon. Studies of in vitro development in parthenogenetically activated oocytes have found that approximately 20–50 % of porcine parthenogenetic oocytes formed a blastocysts (Lee et al. 2004), as compared to 34 % in the bovine (Campbell et al. 2000) and 50 % in ovine (Loi et al. 1998).

After activation; the oocytes were placed in to the embryo development medium containing DMAP(Di-methyl amino purine) for 4–6 h depending upon the types of oocytes treated after which presumptive embryos gets transferred to the embryo development medium. Elevation of intracellular Ca^{2+} levels with ionomycin followed by inhibition of protein phosphorylation with 6-dimethylaminopurine (6-DMAP) results in efficient oocyte activation and better development of parthenotes, even to the blastocyst stage (Paffoni et al. 2007; Kharche et al. 2015).

16.3.4 Molecular Mechanism of Fertilization or Activation of Oocytes

In the majority of mammals, oocytes are ovulated in metaphase II (MII) and remain in this stage until activated by the fertilizing spermatozoon (fertilization) or by an artificial stimulus (parthenogenetic activation).

Oocytes are arrested in MII due to the persistence of greater amounts of maturation promoting factor (MPF), a heterodimer of p34cdc2 kinase, and cyclin B, and of cytostatic factors (CSF), products of the proto-oncogene c-mos which prevent the degradation of cyclins and are maintained by enzymes of the family of mitogen-activated protein kinase (MAPK) (Dupont 1998). Penetration of the sperm cells into the oocyte promotes alterations in intracellular Ca^{2+} concentrations, which leads to cortical reaction, polyspermy block, and resumption of meiosis, followed by extrusion of the second polar body to the perivitelin space (Küpker et al. 1998). These pulses also provoke maternal mRNA recruitment, pronuclear development and mitotic cleavage (Ducibella et al. 2002).

The sperm cell is the natural activator of the oocyte, rendering it capable of forming pronuclei and developing into a zygote. Sperm penetration induces periodical and transient increases in free intracellular Ca^{2+} concentrations for several hours in mice (Jones and Whittingham 1996), pigs (Sun et al. 1992) and cattle (Tosti et al. 2002). Such calcium oscillations are responsible for the inactivation of MPF by means of the inactivation of MAPK and CSF and cyclin destruction (Dupont 1998). Artificial stimuli electrical, chemical or mechanical elevating the cytoplasmic levels of calcium ions in the same manner as sperm even without penetration of the sperm into the oocyte and can induce activation of the oocytes.

16.3.5 Assessment of Activation Treatments

To assess activation rates, oocytes were cultured in embryo development medium (EDM) for 15–16 h and evaluated for the presence of one or more pronuclei. Oocytes with pronuclei were considered activated. To assess parthenogenetic development, embryos were cultured for 7 days and blastocyst development rates were determined. To assess the quality of the embryos, the mean (±S.D.) cell number in blastocysts can be determined.

At the end of the culture period (day 8), some blastocysts developed parthenogenetically from activated oocytes were assessed for cell number using differential fluorescence staining procedure as described by Thouas et al. (2001). Briefly, blastocysts were first incubated in 500 µl of 1% Triton X-100 and 100 µg/ml propidium iodide for up to 30 s and then immediately transferred into 500 µl of 100 % ethanol with 25 µg/ml bisbenzimide (Hoechst 33258) and stored at 4 °C overnight. Fixed and stained embryos were then mounted on to a glass slide in a drop of glycerol, gently flattened with a cover slip and visualized for cell counting on a fluorescence microscope (excitation filter 460 nm for blue and 560 nm for red). Trophectodermal (TE) cells visualized as blue and the inner cell mass (ICM) as pink to red. Total cell number (TCN) then obtained by accounting both ICM and TE numbers.

16.3.6 In-Vitro Development of Parthenogenetically Activated Embryos

A variety of embryo culture system have been developed for in-vitro embryo production either Fertilized or Parthenogenetically activated. Among these major systems of IVC reported by Walker et al. (1996);

(i) Transfer of embryo to ligated oviduct of surrogate recipient,
(ii) Culture *in-vitro* with somatic cells (oviduct epithelial cells, granulose cells vero cells) in medium like TCM-199.
(iii) Culturing such embryo in simple medium like mSOF (synthetic oviductal fluid), KSOM (Potassium Simplex Optimized Medium), or TCM-199 without any somatic cells.

Co-culture of two or four cell embryos to blastocyst has been more successful with uterine or culturing in medium alone (Prichard et al 1992). Bovine embryos developed successfully in simpler medium without any supportive co culture cells (Shamusddin et al. 1994). Protein free salt solution supplemented energy substrate and amino acids were able to grow bovine embryos to the morula and blastocyst stage. However serum factors were still required for maximal blastocyst development and hatching (Saidel et al. 1991).

Thus far, parthenotes obtained in-vitro have been studied and transferred in the uterus of recipient females in a variety of mammals including mice, sheep, cows, pigs, rabbits and monkeys. These studies evidenced that mouse parthenotes can develop beyond implantation until the forelimb bud stage; rabbit parthenotes until day 11 postactivation while parthenotes from primates have only been shown to reach the implantation stage.

The general procedure of parthenogenetic embryo development is almost similar to the *in-vitro* embryo development of fertilized oocytes except the step of parthenogenetic activation with different activation agents which could either be electrical, chemical or other types and thus includes collection of ovaries, recovery of oocytes, *in-vitro* maturation of oocytes (IVM), activation of oocytes with different activation agents and finally *in-vitro* development of parthenogenetic embryos.

Present methods for parthenogenetic embryo production *in-vitro* depend on the use of oocytes with full meiotic competence, which are present in the ovary in limited numbers. Numerous populations of follicles with growing oocytes that have partially developed meiotic competence cannot be used for these purposes. However, embryos produced from these oocytes could be used for breeding, production of cloned or transgenic animals, or for preservation of endangered breeds. To this end, culture systems for *in-vitro* growth and acquisition of full meiotic competence of mammalian oocytes have been developed (Wu et al. 2001). However, the processes involved in the acquisition of full meiotic competence are not fully understood. Sedmíková et al. (2003) demonstrated that drugs elevating intracellular calcium levels can overcome the meiotic block in oocytes with partially developed meiotic competence and can induce their maturation to the metaphase II stage. To achieve

these results they used cyclopiazonic acid, the inhibitor of calcium-dependent ATPases, which elevates intracellular levels of free calcium ions through the mobilization of intracellular calcium deposits (Mason et al. 1991).

16.4 Haploid, Diploid, and Polyploid Parthenogenesis

Parthenogenetic development may proceed by various routes depending on whether meiosis of the egg cell is completed normally or, failing this, which of the stages has been omitted. Alternatively, meiosis may be completely suppressed and the egg develops as a result of mitotic divisions only. The number of chromosome sets in the embryo can further be varied by failure of cell division as a result of DNA synthesis, either before meiosis or afterwards, in what would otherwise have been a cleavage division. This process is known as "Endoreduplication".

If a diploid oogonium completes the two meiotic divisions and continues development in the absence of nuclear fusion, the resultant embryo will be haploid, at least initially. The somatic cells of such embryos may subsequently become diploid or polyploid.

Diploid or polyploid parthenogenesis can proceed via a number of routes. Diploid eggs may be formed by the suppression of either the first or second polar bodies. Alternatively, polar bodies may be extruded and subsequently fuse with the egg cell. The second meiotic division may be completed without extrusion of the second polar body and the resultant diploid egg cell then undergoes cleavage. It is also possible, however, that the two products of the second meiotic division are separated by immediate cleavage, thus initiating haploid parthenogenesis (Witkowska 1973). If neither polar body is extruded, the resulting egg would be tetraploid, since the primary oocyte contains the duplicated number of chromatids in preparation to undergoing two meiotic divisions.

In a parthenogenetically activated oocyte, the second polar may be extruded and a haploid parthenote develops. If the second meiotic division does not take place, a diploid parthenote is formed (Hagemann et al. 1995). To obtained diploid embryos the matured oocytes were treated with various chemicals viz. Cytochalasin (A, B, C, D), Colcimid, Demecolcine etc. in activation medium, aiming to avoid second polar body extrusion while the oocytes treated without the above said chemicals resulted in haploid embryos. These chemicals are cell permeable that disrupts contractile microfilaments by inhibiting actin polymerization. This, in turn, induces DNA fragmentation, inhibits cell division arresting them at metaphase. The type of parthenote formed depends on the activating stimulus, its intensity, the postovulatory age of an oocyte, and the conditions of activation (osmolarity, pH, and temperature of culture media (King et al. 1988). In cattle spontaneous activation rates of aging oocytes were higher than those of the younger cells. Parthenogenesis was also found to increase significantly following microinjection of spermatozoa into mouse oocytes (Macas et al. 1993).

Irrespective of the protocol for activation followed the resulted parthenogenetic embryos may be haploid, diploid, aneuploid, or polyploidy. Witkowska (1973) carried out chromosome analysis of morula and blastocysts obtained by electric treatment and found all the four types of embryos. based on his findings he concluded that Haploid parthenogenesis will result either if meiosis is completed normally or if the egg cleaves in half, so that one cell contains the egg nucleus and the other cell the nucleus of what should have been the polar body (immediate cleavage). If the nucleus of the polar body is retained in the egg, diploid parthenogenesis will result (Graham 1974).

Although the mechanisms involved in the formation of aneuploid, haplodiploid, and polyploidy embryos have not yet been explained. Now a days optimized protocol for production of desired ploidy have been developed

Diploid parthenotes can be obtained in two main different ways. The most common one consists in combining the activation of metaphase-2 oocytes with exposure to an inhibitor of the extrusion of the second polar body without affecting the formation and movement of pronuclei (Balakier and Tarkowski 1976)**.** Alternatively, a diploid parthenote can be generated by treating the oocyte with cytochalasin D during in-vitro maturation before activation. This drug binds to the positive end of F-actin and blocks further addition of G-actin monomers preventing the extrusion of the first polar body. This protocol leads to the formation of tetraploid oocytes **(**Kubiak et al 1991**).** The diploid status is then re-established at the end of oocyte maturation with the extrusion of the second polar body.

The developmental competencies of all the above kind of parthenotes are influenced by the resulting ploidy as well as genetic information the carry. In some species, the development of both haploid and diploid parthenotes has been reported. But in general the developmental competency of haploid embryos is reduced in compared to diploid condition.

There has since been abundant confirmation of the possibility of inducing parthenogenetic development in mammals by experimental procedures but none of the embryos so formed has survived the embryonic period.

16.5 Genome Imprinting Analysis of Parthenotes and Their Significance

When the mammalian oocyte is fertilized with sperm, it receives the paternal genetic materials. The paternal alleles, like the oocyte alleles, have been subjected to epigenetic modifications during gametogenesis that cause a subset of mammalian genes to be expressed from one of the two parental chromosomes in the embryo. This regulatory mechanism is termed genomic imprinting (Kure-bayashi et al. 2000). Additional epigenetic processes also occur during early development after fertilization (Loi et al. 1998). Thus, the maternal and paternal genomes are not functionally equivalent, which is why both a maternal and a paternal genome are required

for normal mammalian development. Mammalian parthenotes are able to undergo several cycles of cell division after oocyte activation, but never proceed to term, arresting at different stages of development, depending on the species (Kure-bayashi et al. 2000).

Success rates and viability of parthenogenetic embryos appear to be organism dependent. Mouse parthenotes are capable of developing beyond the postimplantation stage *in-vivo* (Surani et al. 1984); porcine parthenotes have developed up to post-activation day 29 (limb bud stage, past the early heart beating stage); rabbit parthenotes until day 10–11 Ozil (1990). Primates (Callithrix jacchus) have only been shown to implant (Marshall et al. 1998). The reason for this arrested development is believed to be due to genetic imprinting. Since all genetic material in parthenotes is of maternal origin, there is no paternal imprinting component and this prevents proper development of extraembryonic tissues whose expression is regulated by the male genome (Surani and Barton 1983).

Uniparental embryos, such as parthenotes or androgenotes, have been used to study imprinting processes as well as the role the paternal genome plays during early embryo development (Latham et al. 2002). Since diploid parthenotes (DPs) and fertilized embryos show similar development, at least to the blastocyst stage, their gene transcription patterns during early developmental processes may not differ markedly. However, there may be some more subtle differences in that fertilized embryos may express Y-chromosome-linked genes and imprinting genes during early development, unlike the DPs. Comparison of the gene expression patterns of the fertilized embryo and the DP parthenote may thus illuminate the role(s) paternal genes play in later embryonic development. Compared to DPs, fewer haploid parthenotes (HPs) cultured *in-vitro* reach the blastocyst stage and those that does have lower cell numbers. The reasons for this limited developmental potential of mammalian HPs are not clear. One possibility is that the lack of genetic component(s) in HPs may increase the duration of the cell cycle and consequently slow their development (Liu et al. 2002). This explanation is supported by the observation that mouse HPs develop *in-vitro* more slowly than DPs during the preimplantation period (Henery and Kaufman 1992). Another possible explanation is that the low DNA content in HPs may not be sufficient to control the gene expression network, which could result in apoptosis (Jeong et al. 2005) or the failure of developmental processes during preimplantation development.

To gain insights into the roles the paternal genome and chromosome number play in pre-implantation development, (Kim et al. 2007) cultured fertilized embryos and diploid and haploid parthenotes (DPs and HPs, respectively), and compared their development and gene expression patterns. The DPs and fertilized embryos did not differ in developmental ability but HPs development was slower and characterized by impaired compaction and blastocoel formation. These results are consistent with previous reports that indicated HPs are developmentally retarded and show slow development in mice (Liu et al. 2002). While it remains unclear why HPs show more limited and slower development, it may be speculated that at least part of the reason may involve the difficulties HPs have in compacting. Compaction during embryonic development involves the formation of tight junctions between outer

cells, which permits selective ion transport and facilitates blastocoel formation (Pratt et al. 1982). Thus, the incomplete compaction of HPs may be responsible, at least in part, for their impaired development to the blastocyst stage. Microarray analysis revealed that fertilized blastocysts expressed several genes at higher levels than DP blastocysts; these included the Y-chromosome-specific gene eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (Eif2s3y) and the imprinting gene U2 small nuclear ribonucleoprotein auxiliary factor 1, related sequence 1 (U2af1-rs1). It is found that when DPs and HPs were both harvested at 44 and 58 h of culture, they differed in the expression of 38 and 665 genes, respectively. When differentially expressed genes in the HPs as compared to the DPs at 58 h after activation were analyzed with regard to their putative molecular function. 176 highly expressed and 158 lower expressed genes were unclassified. Of the remainder, it was found that 12 highly expressed and 9 lower expressed genes were related to cell adhesion/cell junction/cytoskeletal-functions.

Moreover, compared to the 58 h DPs, the 58 h HPs showed lower expression of more nucleic acid-binding proteins, oxidoreductase, transcription factors, selected regulatory molecules, and transferase, and highly expression of more receptors, transcription factors, nucleic acid-binding proteins, kinases, and selected regulatory molecules.

However, when DPs and HPs were harvested at the midpoints of 4-cell stage (44 and 49 h, respectively), no differences in expression was observed. Similarly, when the DPs and HPs were harvested when they became blastocysts (102 and 138 h, respectively), only 15 genes showed disparate expression. These results suggest that while transcripts needed for early development are delayed in HPs, it does progress sufficiently for the generation of the various developmental stages despite the lack of genetic components.

Genomic imprinting, a specific genetic mechanism in mammals, plays important roles in the regulation of fetal growth, development, placental function, and postnatal behavior (Kono et al. 2004). It endows some genes with different “imprints,” which lead to their differential expression in fetuses and/or placenta and regulate the transfer of nutrients to fetus and placenta from the mother (Miyoshi et al. 2006).

The establishment of genomic imprinting is controlled by DNA methylation, histone modifications, noncoding RNA, and specialized chromatin structure; DNA methylation is thought to be a major factor (Yang et al. 2003). Specific DNA methylation in the differentially methylated regions (DMRs) of parental origin allows the discrimination between the maternal and the paternal alleles and leads to monoallelic expression of imprinted genes (Thorvaldsen et al. 2006). Uniparental fetuses, including parthenotes and androgenotes, show disrupted expression of several imprinted genes, such as Snrpn, Peg3, H19, and Gtl2 (Warnecke et al. 1998). Studies in mouse uniparental embryos have revealed that the paternal genome is more important for the development of the extraembryonic tissues, while the maternal genome is more essential for fetal development. These distinctive differences are the result of genomic imprinting (Borghol et al. 2006). Parthenogenetic fetuses die by day 10 of gestation (Lucifero et al. 2002). Likely, the cloned animal fetuses exhibit a high rate of developmental abnormalities due to inefficient epigenetic

reprogramming of the donor nucleus within enucleated oocytes. The aberrant epigenetic modifications caused by inefficient reprogramming severely undermine the developmental potency of cloned embryos (Gebert et al. 2006). But to date, our knowledge about the molecular mechanism of epigenetic reprogramming is still very limited (Kang et al. 2001). Compared to the laborious manipulation of somatic cell nuclear transfer, the mouse parthenogenetic embryo is a most suitable alternative to study the events of methylation imprints. Similar to mouse parthenogenetic embryos, the aborted cloned bovine fetus also exhibits disrupted expression of imprinted genes and aberrant methylation imprints (Li et al. 2004).

16.6 Strategy for Parthenogenetic Animal Development

In April 2004, scientists at Tokyo University of Agriculture used parthenogenesis successfully to create a fatherless mouse. Using gene targeting, they were able to manipulate two imprinted loci H19/IGF2 and DLK1/MEG3 to produce bi-maternal mice at high frequency and subsequently show that fatherless mice have enhanced longevity. Parthenogenetic embryo therefore may be a viable alternative for production of donor cell from superior female animal provided they give birth of offspring. The production of commodo dragon, boney head shark by parthenogenesis give an indication that one or other types of parthenogenesis may lead to production of full term animals in higher animals too.

At present several problems of developmental arrest in parthenogenetic embryos have been identified because of lack of full genomic components unlike IVF derived embryos. It is assumed that if the full genetic component of oocytes can be brought forward to parthenogenetic embryos then a viable animal can be produced through parthenogenesis. The reasons for developmental defect in diploid parthenogenetic embryos are being studied extensively mostly in laboratory animals as well as higher mammals like sheep, goat, cattle etc. It has been observed that several developmental related gene expressions are lacking in different types of parthenogenetic embryos during different stages of embryo development. Therefore, the mammalian parthenogenetic embryos invariably die a month of gestation from imprinted gene defects and placental hypoplasia. There is a limitation for foetal development in the ability to reprogramme imprinted genes by repeated rounds of parthenogenetic embryo transfer. However, the placentas of parthenogenetic embryos can escape epigenetic regulation when developed using nuclear transfer techniques, tetraploid embryo production (aggregation of parthenogenetic embryo blastomere with in-vitro fertilized embryo blastomere followed by in-vitro embryo development) and can support foetal development to full gestation. Parthenogenetic embryos die before day 10 of gestation, mainly from restricted placental development.

Differentiation potential of parthenogenetic embryos can be improved by the re-establishment of parthenogentic tetraploid embryo through this technique. This led us to hypothesize that the chimera of parthenogenetic embryos might generate placentas and grow to full term offspring.

As it is not known which type of chimera of parthenogenetic embryos have competency to produce full term offspring, it is important to study the genetic expression of developmental genes of chimera of parthenogenetic embryos produced by different methods to work out the potential of these embryos to form live offspring.

The animal cloning through production of chimeric parthenogenetic embryo will help in conservation of endangered animal breeds as well as multiplying the few superior germplasm, which will help in conservation of endangered breed of animals.

Induced parthenogenesis in mice and monkeys often results in abnormal development. A mammal created by parthenogenesis would have double doses of maternally imprinted genes and lack paternally imprinted genes, leading to developmental abnormalities. It has been suggested that defects in placental folding or interdigitation are one cause of swine parthenote abortive development. As a consequence, research on human parthenogenesis is focused on the production of embryonic stem cells for use in medical treatment, not as a reproductive strategy.

16.7 Parthenogenetic Research on Humans

On June 26, 2007, International Stem Cell Corporation (ISCC), a California-based stem cell research company, announced that their lead scientist, Dr. Elena Revazova, and her research team were the first to intentionally create human stem cells from unfertilized human eggs using parthenogenesis. The process may offer a way for creating stem cells that are genetically matched to a particular woman for the treatment of degenerative diseases that might affect her. In December 2007, Dr. Revazova and ISCC published an article illustrating a breakthrough in the use of parthenogenesis to produce human stem cells that are homozygous in the HLA region of DNA. These stem cells are called HLA homozygous parthenogenetic human stem cells (hpSC-Hhom) and have unique characteristics that would allow derivatives of these cells to be implanted into millions of people without immune rejection. With proper selection of oocyte donors according to HLA haplotype, it is possible to generate a bank of cell lines whose tissue derivatives, collectively, could be MHC-matched with a significant number of individuals within the human population.

On August 2, 2007, after much independent investigation, it was revealed that discredited South Korean scientist **Hwang Woo-Suk** unknowingly produced the first human embryos resulting from parthenogenesis. Initially, Hwang claimed he and his team had extracted stem cells from cloned human embryos, a result later found to be fabricated. Further examination of the chromosomes of these cells show indicators of parthenogenesis in those extracted stem cells, similar to those found in the mice created by Tokyo scientists in 2004. Although Hwang deceived the world about being the first to create artificially cloned human embryos, he did contribute a major breakthrough to stem cell research by creating human embryos using parthenogenesis. The truth was discovered in 2007, long after the embryos were created

by him and his team in February 2004. This made Hwang the first, unknowingly, to successfully perform the process of parthenogenesis to create a human embryon and, ultimately, a human parthenogenetic stem cell line.

16.8 Potential of Parthenogenetic Embryos in Different Field of Research

Parthenogenesis phenomenon does not occur naturally in mammals, although very rarely spontaneous can take place. Parthenogenesis of the oocyte is essential to a number of oocyte- or embryo-related technologies such as intracytoplasmic sperm injection (ICSI) and cloning by nuclear transfer. Somatic cloning can become one of the methods for saving endangered species of animals (including felids). During their in-vitro development to the blastocyst stage, parthenotes are comparable to embryos and therefore may be useful tools for any research aimed at investigating culture conditions, different treatment options, exposure to chemicals, and many variables of the laboratory routine (Paffoni et al. 2008). Moreover, the analysis of parthenotes is a classical way of identifying and characterizing novel imprinted genes that may contribute to mammalian phenotypes displaying parent of origin effects (Ruddock et al. 2004). The parthenogenetic activation of oocytes represents a valid tool to investigate the comparative roles of paternal and maternal genomes in controlling early embryo development. Furthermore, parthenogenetic activation is relevant to cloning research, because artificial activation of oocytes is an essential component of nuclear transfer protocols (Kim et al. 1996). During preliminary studies, parthenogenetically activated oocytes have been recognized as a suitable model for studying activation efficiency. In this regards, the best criterion for evaluating the methods of activation is the ability of parthenogenetically activated oocytes to undergo pre and even post-implantation development (Lee et al. 2004a, b). Parthenogenic studies provide a possibility to evaluate the efficiency of the activation stimuli in steps used for cloning, at the same time avoiding complicated procedures effecting SCNT outcome. An optimized activation protocol may enhance better or complete reprogramming of the reconstructed embryo, which might in turn increase the chance of success in cloning. Obtention of oocytes activated efficiently and in a simple manner constitutes one of the limiting steps for the success of cloning by nuclear transfer (Kishikawa et al. 1999). Oocyte activation allows cell cycle phase synchronization between the cytoplasm of the oocyte and the transferred nucleus, promoting nuclear reprogramming and maintenance of correct ploidy (reviewed by Campbell 1999; Kharche and Birade 2013). The study of parthenogenetic activation also permits a greater understanding of the mechanisms of spontaneous activation, preventing it in in-vitro fertilization systems, and also allows for the study of genomic imprinting during embryonic development (Solter 1988). Tetraploid embryos have limited developmental potency; usually die during postimplantation, and exhibit defects, in particular in the forebrain and eyes, the vertebral axis and heart (Kaufman

and Webb 1990). Nonetheless, tetraploid embryos can be used as complements for cloning offspring from ES cells (Eggan et al. 2001). In the ES cell-tetraploid aggregates, tetraploid cells are selected to form extraembryonic membranes, contributing to functional placenta (Everett and West 1998). This feature has been used to complement placenta deficiency in development of cloned embryos (Hochedlinger and Jaenisch 2002).

Recently, parthenogenesis has attracted wide attention because of the potential for deriving pluripotent lines. Embryonic stem cells (ESCs), typically derived from the inner cell mass (ICM) of the mammalian blastocyst, are in fact of fundamental value for developmental research and both cell and tissue replacement therapy. However, the development of human ESC based clinical therapies has hitherto been limited because of the ethical dilemma involving the destruction of a human embryo. It is noteworthy that establishment of embryonic stem cells (ESCs) from parthenogenetic embryos has attracted attention as an alternative way to derive pluripotent stem cells for application to cell transplantation therapy or drug screening, since it does not involve the destruction of viable embryos (Revazova et al. 2007). The ability to have embryonic stem cells, without sacrificing conception human embryos, would be valuable for patients who require the replacement of damaged or diseased tissue. Despite its necessity for autologous stem cell therapy, somatic cell nuclear transfer (SCNT) may lead to human cloning and has thus provoked ethical disputes. Replacement of the SCNT with other alternatives is important for avoiding cloning, and parthenogenesis has been considered as one of the alternatives. Due to increasing interests in genomic imprinting, potentiating ES cells and cloning, much attention is again being paid to parthenogenetic and tetraploid development. Although parthenogenetic embryos are defective in postimplantation development, in particular by exhibiting limited development of extra-embryonic membranes, ES cells can be derived from parthenogenetic blastocysts (Allen et al. 1994). Creation of ES cells for therapeutic purposes from normal fertilized embryos can be avoided in this way. For example, pluripotent primate ES cells derived from ICM of parthenogenetic blastocysts can proliferate while maintaining their undifferentiated state, and be induced to differentiate in-vitro into neurons (Cibelli et al. 2002).

Derivation of ES cells from parthenogenetic blastocysts suggests that Oct-4 might also be normally expressed in the ICM of those embryos. Androgenetic embryos preferentially develop into trophoblasts (Barton et al. 1984), and ES cells derived from androgenetic blastocysts, if any, exhibit defects in development (Mann et al. 1990). Examples of the possible uses of parthenotes in many field of research such as in-vitro assays aimed to study some aspects of assisted reproductive technologies (ART), toxicology or stem cell.

Parthenotes can also be an interesting source of stem cells due to the unique advantage of homozygosity, which renders them less immunogenic. The occurrence of a high degree of homozygosity has been evaluated in contrasting ways in the perspective of using these entities as a source of embryonic stem cells. The use of parthenotes as experimental models in human reproduction is at its early days. Future studies will define more in detail its real advantages and limitations. Recent studies have illustrated multiple differentiation potentials of embryonic stem cells

(ESCs), derived from parthenogenetic embryos, to various kinds of cells (all three embryonic germ layers). However, differentiation diversity of the parthenogenetic ESCs (PgESCs) in-vivo remains to be elucidated. Parthenogenetic stem cells are able to get induced to differentiate to the myogenic, osteogenic, adipogenic, and endothelial lineages, and were able to form muscle-like and bony-like tissue in-vivo. Furthermore, parthenogenetic stem cells were able to integrate into injured muscle tissue. Therefore, parthenogenetic stem cells can be successfully isolated and utilized for various tissue engineering applications (Koh et al. 2009).

16.9 Parthenogenetic Stem Cell Development and Their Therapeutic Potential

For the replacement of damaged or diseased tissue, most current strategies for tissue engineering depend upon a sample of autologous cells from the diseased organ of the host (De Filippo et al. 2003). However, in the setting of end-stage organ failure, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologous human cells cannot be adequately expanded from a particular organ for replacement purposes. In these situations, embryonic stem cells are envisioned as a viable source of pluripotent cells from which the desired tissue can be derived. Combining regenerative medicine techniques with this potentially endless source of versatile cells could lead to novel sources of replacement organs that would alleviate the chronic shortage of available donor organs.

The fields of regenerative medicine and tissue engineering seek to relieve the suffering of patients from the consequences of disease and injury by restoring the form and function of their damaged tissue and organs. Many disorders, such as congenital anomalies, cancer, trauma, infection, inflammation, iatrogenic injuries, and other conditions, can lead to organ damage or loss and to the eventual need for reconstruction. The majority of current reconstructive techniques rely on donor tissue for replacement; however, a shortage of donor tissue may limit these types of reconstruction, and usually significant morbidity is associated with the harvest procedure. Particularly in cases when donor tissue is unavailable, embryonic stem cells has been envisioned as a potentially endless source of versatile cells for use in regenerative medicine applications because of their ability to proliferate in a undifferentiated, but pluripotent state (self-renew) and to differentiate into specialized cells from all three embryonic germ layers. Skin and neurons have been formed, indicating ectodermal differentiation (Reubinoff et al. 2001) Blood, cardiac cells, cartilage, endothelial cells, and muscle have been formed, indicating mesodermal differentiation (Levenberg et al. 2002). And pancreatic cells have been formed, indicating endodermal differentiation (Assady et al. 2001). In addition, as further evidence of their pluripotency, embryonic stem cells have formed embryoid bodies, which are cell aggregations that contain all three embryonic germ layers, while in

culture, and have formed teratomas in vivo (Itskovitz-Eldor et al. 2000). Oocytes, the sources of these stem cells, can be harvested with the use of minimally invasive techniques with minimal donor morbidity. In addition, these stem cells have a rapid proliferative rate that would allow adequate expansion of these cells for tissue replacement therapy, and do not require the use of feeder layers for expansion, thereby avoiding the exposure of these cells to xenogenic viruses and proteins. Several attempts to produce viable individuals from parthenogenesis in the past have been unsuccessful (Fukui et al. 1992). While it has recently shown that some parthenogenetic mice can survive into adulthood (Kono et al. 2004), they noted the predominant mortality of these embryos in that 99.4 % of the embryos perished prior to adulthood, with the majority of the deaths occurring during embryonic development. This may help to relieve some of the potential ethical and political concerns with the use of parthenogenetic stem cells in that the parthenogenetic embryos rarely survive beyond the embryo stage, but do survive long enough where the embryonic stem cells can be harvested for tissue engineering purposes.

16.10 Ethical Issues Associated with Parthenogenetic Embryo Research

Embryonic stem cells exhibit two remarkable properties: the ability to proliferate in an undifferentiated, but pluripotent state (self-renew), and the ability to differentiate into many specialized cell types (Brivanlou et al. 2003). However, the destruction of embryos required for embryonic stem cell research has led to limitations in many countries where embryonic stem cell research has been restricted to the use of a limited number of cell lines only (Cowan et al. 2004). Parthenogenesis, the process by which an egg can develop into a blastocyst in the absence of sperm, has previously been identified as a potential source for embryonic stem cells. The ability to have embryonic stem cells, without sacrificing conception human embryos, would be valuable for patients who require the replacement of damaged or diseased tissue. Many studies have described the isolation and expansion of embryonic stem cells from parthenogenesis (Cibelli et al. 2002; Cheng 2008; Mai et al. 2007; Lin et al. 2007; Brevini and Gandolfi 2008; Dighe et al. 2008; Sritanaudomchai et al. 2007; Fang et al. 2006) and characterized these cells for their stem-cell properties and differentiated into multiple lineages. Their survival in various in vivo environments have also been evaluated and found that these methods will lead to eventual scale-up of these techniques to produce the large quantity of cells and tissue required for regenerative medicine applications. Thus, Parthenogenesis-derived stem cells are adapted for tissue engineering applications, as they are capable of differentiation into multiple lineages and can form tissue in-vivo. This alternative source of embryonic stem cells may avoid some of the political and ethical concerns that surround current viable embryonic stem cell techniques. Based on the differences between Parthenotes and embryos the use of parthenogenesis is proposed as an experimental

tool to investigate embryo development which may solve many of the ethical concerns associated with the use of embryos for experimental purposes. Using unfertilized eggs, for research, is commonly judged less controversial than using embryos, as many ethical concerns are avoided. However, Parthenotes are not entirely free from ethical controversy since are considered by some as artificial entities challenging natural rules. These contradictions await resolution in a broad ethical framework (Hipp and Atala 2004).

The induced pluripotent stem cells (ie. iPS) cells do present a possibility of embryonic stem cell research without the need for destruction of embryos. However, iPS cell research is still in its infancy, and it is still unclear whether these cells will be safe for use in humans, or whether the cells can be adequately expanded in large quantities necessary for tissue engineering applications.

References

Allen ND, Barton SC, Hilton K, Norris ML, Surani MA (1994) A functional analysis of imprinting in parthenogenetic embryonic stem cells. Development 120:1473–1482

Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M (2001) Insulin production by Human embryonic stem cells. Diabetes 50(8):1691–1697

Austin CR (1956) Activation of eggs by hypothermia in rats and hamsters. J Exp Biol 33:338–347

Balakier H, Tarkowski AK (1976) Diploid parthenogenetic mouse embryos produced by heat-shock and cytochalasin B. J Embryol Exp Morphol 35:25–39

Barton SC, Surani MA, Norris ML (1984) Role of paternal and maternal genomes in mouse development. Nature 311:374–376

Beatty RA (1967) Parthenogenesis in vertebrates. In Ferti 1:413–440. Metz and A. Monroy. Academic Press, New York/London

Bonde SW, Naquvi SMK, Mittal JP (2000) Comparison of oocyte recovery from surface follicles of sheep and goat ovaries. Int J Anim Sci 15:1–4

Borghol N, Lornage J, Blachere T, SophieGarret A, Lefevre A (2006) Epigenetic status of the H19 locus in human oocytes following in vitro maturation. Genomics 87:417–426

Brevini TAL, Gandolfi F (2008) Parthenotes as a source of embryonic stem cells. Cell Prolif 41(1):20–30

Brivanlou AH, Gage FH, Jaenisch R, Jessell T, Melton D, Rossant J (2003) Stem cells. Setting standards for human embryonic stem cells. Science 300(5621):913–916 (comment)

Campbell KHS (1999) Nuclear equivalence, nuclear transfer, and the cell cycle. Cloning 1:3–15

Campbell KD, Reed WA, White KL (2000) Ability of integrins to mediate fertilization, intracellular calcium release, and parthenogenetic development in bovine oocytes. Biol Reprod 62:1702–1709

Cheng L (2008) More new lines of human parthenogenetic embryonic stem cells. Cell Res 18(2):215–217

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

Cowan CA, Klimanskaya I, McMahon J et al (2004) Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 350(13):1353–1356 (see comment)

Crister ES, Leifried-Rutledge ML, Eyestone WH, Northy DL, First NL (1986) Acquisition of development and competence during maturation *in vitro*. Theriogenology 25:15

De Filippo RE, Yoo JJ, Atala A (2003) Engineering of vaginal tissue in-vivo. Tissue Eng 9(2):301–306

Dighe V, Clepper L, Pedersen D et al (2008) Heterozygous embryonic stem cell lines derived from nonhuman primate parthenotes. Stem Cells 26(3):756–766

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S, Ozil JP (2002) Egg-to-embryo transition is driven by differential responses to Ca2+ oscillation number. Dev Biol 250:280–291

Dupont G (1998) Link between fertilization-induced Ca^{2+} oscillations and relief from metaphase II arrest in mammalian eggs: a model based on calmodulin-dependent kinase II activation. Biophys Chem 72:153–167

Edwards RG (1965) Maturation *in-vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature 208:349–351

Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM III et al (2001) Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci U S A 98:6209–6214

Everett CA, West JD (1998) Evidence for selection against tetraploid cells in tetraploiddiploid mouse chimaeras before the late blastocyst stage. Genet Res 72:225–228

Fang ZF, Gai H, Huang YZ et al (2006) Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos. Exp Cell Res 312(18):3669–3682

Foote RH, Simkin ME (1993) Use of gonadotropic releasing hormone for ovulating the rabbit model. Lab Anim Sci 43:383–385

Fukui Y, Sawai K, Furudate M, Sato N, Iwazumi Y, Ohsaki K (1992) Parthenogenetic development of bovine oocytes treated with ethanol and cytochalasin B after in vitro maturation. Mol Reprod Dev 33(3):357–362

Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN (1994) In vitro development of in vitro matured porcine oocytes following chemical activation or in vitro fertilization. Biol Reprod 50:1072–1077

Gebert C et al (2006) The bovine IGF2 gene is differentially methylated in oocyte and sperm DNA. Genomics 88:222–229

Gomez MC, Jenkins JA, Giraldo A, Harris RF, King A, Dresser BL et al (2003) Nuclear transfer of synchronized African Wild Cat somatic cells into enucleated domestic cat oocytes. Biol Reprod 69:1032–1041

Graham CF (1974) The production of parthenogenetic mammalian embryos and their use in biological research. Biol Rev 49:399–422

Gupta PSP, Nandi S, Ravindranantha BN, Sharma PV (2002) *Invitro* maturation of buffalo oocytes with epidermal growth factors and fibroblast growth factor. Ind J Anim Sci 72:20–23

Hagemann LJ, Hillery-Weinhold FL, Leibfried-Rutledge ML, First NL (1995) Activation of murine oocytes with Ca^{2+} ionophore and cycloheximide. J Exp Zool 271:57–61

Henery CC, Kaufman MH (1992) Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. Mol Reprod Dev 31:258–263

Hipp J, Atala A (2004) Tissue engineering, stem cells, cloning, and parthenogenesis: new paradigms for therapy. J Exp Clin Assist Reprod 1:3

Hochedlinger K, Jaenisch R (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature 415:1035–1038

Itskovitz-Eldor J, Schuldiner M, Karsenti D et al (2000) Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6(2):88–95

Jeong YJ, Cui XS, Kim BK, Kim IH, Kim T, Chung YB, Kim NH (2005) Haploidy influences Bak and Bcl-xL mRNA expression and increases incidence of apoptosis in porcine embryos. Zygote 13:17–21

Jones KT, Whittingham DG (1996) A comparison of sperm- and IP3-induced Ca^{2+} release in activated and aging mouse oocytes. Dev Biol 178:229–237

Küpker W, Diedrich K, Edwards RG (1998) Principles of mammalian fertilization. Hum Reprod 13:20–32
Kang YK et al (2001) Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. J Biol Chem 276:39980–39984
Karja NW, Otoi T, Murakami M, Wongsrikeao P, Budivanto A, Fahrudin M et al (2005) Effect of cycloheximide on in vitro development of electrically activated feline oocytes. J Reprod Dev 51:783–786
Kaufman MH (1983) Early mammalian development: parthenogenetic studies. Cambridge University Press, Cambridge
Kaufman MH, Webb S (1990) Postimplantation development of tetraploid mouse embryos produced by electrofusion. Development 110:1121–1132.
Kharche SD, Birade HS (2013) Parthenogenesis and activation of mammalian oocytes for in-vitro embryo production: a review. Adv Biosci Biotechnol 4:170–182
Kharche SD, Goel AK, Jindal SK, Jha BK, Goel P (2013) Assessment of parthenogenetic embryo production by activation of in vitro matured caprine oocytes with different concentrations of ethanol. Small Rumin Res 111:100–103
Kharche SD, Goel AK, Jindal SK, Goel P, Kouamo J, Saraswat S (2015) Effect of protein phosphorylation inhibitor on production of parthenogenetic caprine embryos. Indian J Anim Sci 85:139–142
Kim N-H, Simerly C, Funahashi H, Schatten G, Day BN (1996) Microtubule organization in porcine oocytes during fertilization and parthenogenesis. Biol Reprod 54:1397–1404
Kim K, Ng K, Rugg-Gunn PJ, Shieh J-H, Kirak O, Jaenisch R, Wakayama T, Moore MA, Pedersen RA, Daley GQ (2007) Recombination signatures distinguish embryonic stem cells derived by parthenogenesis and somatic cell nuclear transfer. Cell Stem Cell 1:346–352
King WA, Xu KP, Sirard MA, Greve T, Leclerc P, Lambert RD, Jacques P (1988) Cytogenetic study of parthenogenetically activated bovine oocytes matured in vivo and in vitro. Gamete Res 20:265–274
Kishikawa H, Wakayama T, Yanagimachi R (1999) Comparison of oocyte-activating agents for mouse cloning. Cloning 1:153–159
Koh CJ, Delo DM, Lee JW, Siddiqui MM, Lanza RP, Soker S, Yoo JJ, Atala A (2009) Parthenogenesis-derived multipotent stem cells adapted for tissue engineering applications. Methods 47:90–97
Kono T et al (2004) Birth of parthenogenetic mice that can develop to adulthood. Nature 428:860–864
Krafka J Jr (1939) Parthenogenetic cleavage in the human ovary. Anat Rec 75:19–21
Kubiak J, Paldi A, Weber M, Maro B (1991) Genetically identical parthenogenetic mouse embryos produced by inhibition of the first meiotic cleavage with cytochalasin D. Development 111:763–769
Kure-bayashi S, Miyake M, Okada K, Kato S (2000) Successful implantation of in vitro-matured, electro-activated oocytes in the pig. Theriogenology 53:1105–1119
Latham KE, Akutsu H, Patel B, Yanagimachi R (2002) Comparison of gene expression during preimplantation development between diploid and haploid mouse embryos. Biol Reprod 67:386–392
Lee JW, Tian XC, Yang X (2004) Optimization of parthenogenetic activation protocol in porcine. Mol Reprod Dev 68:51–57
Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R (2002) Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 99(7):4391–4396
Li JY, Lees-Murdock DJ, Xu GL, Walsh CP (2004) Timing of establishment of paternal methylation imprints in the mouse. Genomics 84:952–960
Lin H, Lei J, Wininger D, Nguyen MT, Khanna R, Hartmann C et al (2003) Multilineage potential of homozygous stem cells derived from metaphase II oocytes. Stem Cells 21:152–161

Lin G, OuYang Q, Zhou X et al (2007) A highly homozygous and parthenogenetic human embryonic stem cell line derived from a one-pronuclear oocyte following in vitro fertilization procedure. Cell Res 17(12):999–1007

Liu L, Trimarchi JR, Keefe DL (2002) Haploidy but not parthenogenetic activation leads to increased incidence of apoptosis in mouse embryos. Biol Reprod 66:204–210

Loi P, Ledda S, Fulka J Jr, Cappai P, Moor RM (1998) Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. Biol Reprod 58(5):1177–1187

Lucifero D, Mertineit C, Clarke HJ, Bestor TH, Trasler JM (2002) Methylation dynamics of imprinted genes in mouse germ cells. Genomics 79:530–538

Macas E, Rosselli M, Imthurn B, Keller PJ (1993) Chromosomal constitution of mouse blastocysts derived from oocytes inseminated by multiple sperm insertion into the perivitelline space. J Assist Reprod Genet 7:468–475

Machaty Z, Funahashi H, Mayes MA, Day BN, Prather RS (1996) Effects of injecting calcium chloride into in vitro-matured porcine oocytes. Biol Reprod 54:316–322

Machaty Z, Rickords LF, Prather RS (1999) Parthenogenetic activation of porcine oocytes after nuclear transfer. Cloning 1:101–109

Mai Q, Yu Y, Li T et al (2007) Derivation of human embryonic stem cell lines from parthenogenetic blastocysts. Cell Res 17(12):1008–1019

Mann JR, Gadi I, Harbison ML, Abbondanzo SJ, Stewart CL (1990) Androgenetic mouse embryonic stem cells are pluripotent and cause skeletal defects in chimeras: implications for genetic imprinting. Cell 62:251–260

Marshall VS, Wilton LJ, Moore HD (1998) Parthenogenetic activation of marmoset (Callithrix jacchus) oocytes and the development of marmoset parthenogenones in vitro and in vivo. Biol Reprod 59:1491–1497

Mason MJ, Garcia-Rodriguez C, Grinstein S (1991) Coupling between intracellular Ca^{2+} stores and Ca^{2+} permeability of the plasma membrane: comparison of effect of thapsigargin, 2,5-di-(tert-butyl)-1,4- hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. J Biol Chem 266:20856–20862

Max A, Grabiec A, Tischner M (2007) Parthenogenetic activation of domestic cat oocytes using ethanol, calcium ionophore, cycloheximide and a magnetic field. Theriogenology 67:795–800

Mayes MA, Stogsdill PL, Prather RS (1995) Parthenogenic activation of pig oocytes by protein kinase inhibition. Biol Reprod 53:270–275

Meo SC, Leal CL, Garcia JM (2004) Activation and early parthenogenesis of bovine oocytes treated with ethanol and strontium. Anim Reprod Sci 81:35–46

Miyoshi N, Barton SC, Kaneda M, Hajkova P, Surani MA (2006) The continuing quest to comprehend genomic imprinting. Cytogenet. Genome Res 113:6–11

Nandi S, Chauhan MS, Plata P (2000) Effect of a corpus luteum in the recovery and developmental potential of buffalo oocytes. Vet Rec 147:580–581

Nussbaum DJ, Prather RS (1995) Differential effects of protein synthesis inhibitors on porcine oocyte activation. Mol Reprod Dev 195:70–75

Owen R (1849) On parthenogenesis or the successive production of procreating individuals from a single ovum. John van Voorst, London

Ozil JP (1990) The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. Development 109:117–127

Paffoni A, Brevini TA, Somigliana E, Restelli L, Gandolfi F, Ragni G (2007) In vitro development of human oocytes after parthenogenetic activation or intracytoplasmic sperm injection. Fertil Steril 87:77–82

Paffoni A, Brevini TAL, Gandolfi F, Ragni G (2008) Parthenogenetic activation: biology and applications in the ART laboratory. Placenta 29:S121–S125

Pathak J, Kharche SD, Goel AK, Jindal SK (2013) A comparative study on parthenogenetic activation and embryo production from in vitro matured caprine oocytes. Small Rumin Res 113:136–140

Patrick CD et al (2004) Population models of sperm-dependent parthenogenesis. J Theor Biol 229:559–572
Pincus G (1936) The eggs of mammals. Macmillan, New York
Pincus G, Shapiro H (1940) Further studies on the parthenogenetic activation of rabbit eggs. Proc Natl Acad Sci U S A 26:163–165
Pratt HP, Ziomek CA, Reeve WJ, Johnson MH (1982) Compaction of the mouse embryo: an analysis of its components. J Embryol Exp Morphol 70:113–132
Prichard JF, Thebodeaux JK, Pool SH, Blakewood EG, Menezo Y, Godke RA (1992) In vitro coculture of early stage caprine embryos with oviductal and uterine epithelial cells. Hum Reprod 7:553
Reubinoff BE, Itsykson P, Turetsky T et al (2001) Neural progenitors from human embryonic stem cells. Nat Biotechnol 19(12):1134–1140 (comment)
Revazova ES, Turovets NA, Kochetkova OD, Kindarova LB, Kuzmichev LN, Janus JD, Pryzhkova MV (2007) Patient-specific stem cell lines derived from human parthenogenetic blastocysts. Cloning Stem Cells 9:432–449
Ruddock NT, Wilson KJ, Cooney MA, Korfiatis NA, Tecirlioglu RT, French AJ (2004) Analysis of imprinted messenger RNA expression during bovine preimplantation development. Biol Reprod 70:1131–1135
Saidel GE, Glass T, Olson SE (1991) Culture of one cell bovine ova to blastocyst in chemically defined media. Biol Reprod 44:88 (Abstr.)
Sato K, Yoshida M, Miyoshi K (2005) Utility of ultrasound stimulation for activation of pig oocytes matured in vitro. Mol Reprod Dev 72:396–403
Sedmíková M, Burdová J, Petr J, Etrych M, Rozinek J, Jílek F (2003) Induction and activation of meiosis and subsequent parthenogenetic development of growing pig oocytes using calcium ionophore A23187. Theriogenology 60:1609–1620
Sengoku K, Takuma N, Miyamato T, Yamauchi T, Ishikawa M (2004) Nuclear dynamics of parthenogenesis of human oocytes: effect of oocyte aging in vitro. Gynecol Obstet Invest 58:155–159
Shamusddin M, Larsoon B, Custfson H, Rodriguez Martinez H (1994) A Serum free cell culture system for development of bovine one cell embryos up to blastocyst stage with improved viability. Theriogenology 41:1033–1043
Solter D (1988) Differential imprinting and expression of maternal and paternal genomes. Annu Rev Genet 22:127–146
Sritanaudomchai H, Pavasuthipaisit K, Kitiyanant Y, Kupradinun P, Mitalipov S, Kusamran T (2007) Characterization and multilineage differentiation of embryonic stem cells derived from a buffalo parthenogenetic embryo. Mol Reprod Dev 74(10):1295–1302
Sun FZ, Hoyland J, Huang X, Mason W, Moor RM (1992) A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. Development 115:947–956
Surani MA, Barton SC, Norris ML (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature 308:548–550
Thibault C, Szollosi O, Gerard M (1987) Mammalian oocytes maturation. Reprod Nutr Dev 27:865–896
Thorvaldsen JL, Fedoriw AM, Nguyen S, Bartolomei MS (2006) Developmental profile of H19 differentially methylated domain (DMD) deletion alleles reveals multiple roles of the DMD in regulating allelic expression and DNA methylation at the imprinted H19/Igf2 locus. Mol Cell Biol 26:1245–1258
Thouas GA, Korfiatis NA, French AJ, Jones GM, Trounson AO (2001) Simplified technique for differential staining of inner cell mass and Trophectoderm cells of mouse and bovine blastocysts. Reprod Bio Med Online 149:25–29 (web paper)
Tosti E, Boni R, Cuomo A (2002) Fertilization and activation currents in bovine oocytes. Reproduction 124:835–846
Totey SM, Singh GPG, Taneja M, Pwshe CH, Talwar G (1991) *Invitro* maturation and fertilization of follicular oocytes from buffalo. Theriogenology 35:284

Toyokawa K, Harayama H, Miyake M (2005) Exogenous hyaluronic acid enhances porcine parthenogenetic embryo development in vitro possibly mediated by CD44. Theriogenology 64:378–392

Walker SK, Hartwich KM, Seamark FR (1996) The production of usually large offspring following embryo manipulation: concept and challenges. Theriogenology 45:111–120

Warnecke PM, Mann JR, Frommer M, Clark SJ (1998) Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted H19 gene. Genomics 51:182–190

Witkowska A (1973) Parthenogenetic development of mouse embryos in vivo. I. Preimplantation development. J Embryol Exp Morphol 30:519–545

Wu J, Emery BR, Carrell DT (2001) In vitro growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. Biol Reprod 64:375–381

Yadav EN, Kharche SD, Goel AK, Jindal SK, Sinha NK, Johari DK (2007) Comparative efficacy of different techniques for oocytes recovery from prepubertal goat ovaries. Indian J Anim Sci 77:988–990

Yadav P, Kharche SD, Goel AK, Jindal SK, Sharma MC (2009) Effect of hormones, EGF and β-mercaptoethanol on in vitro maturation of caprine oocytes. Reprod Fertil Dev 22:337

Yang Y et al (2003) Epigenetic regulation of Igf2/H19 imprinting at CTCF insulator binding sites. J Cell Biochem 90:1038–1055

Zae Young R et al (2007) The parthenogenetic activation of canine oocytes with Ca-EDTA by various culture periods and concentrations. Theriogenology 67:698–703

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6