Gustav Steinhoff *Editor*

Regenerative Medicine - from Protocol to Patient

2. Stem Cell Science and Technology

Third Edition

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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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Contents

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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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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

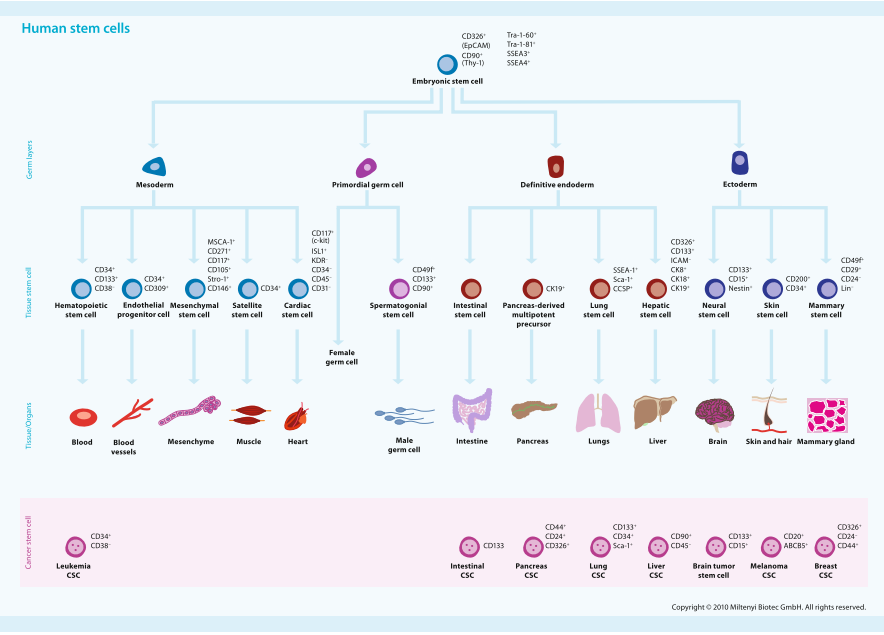

Fig. 1.1 Hierarchical illustration of human stem cells and their cell surface markers

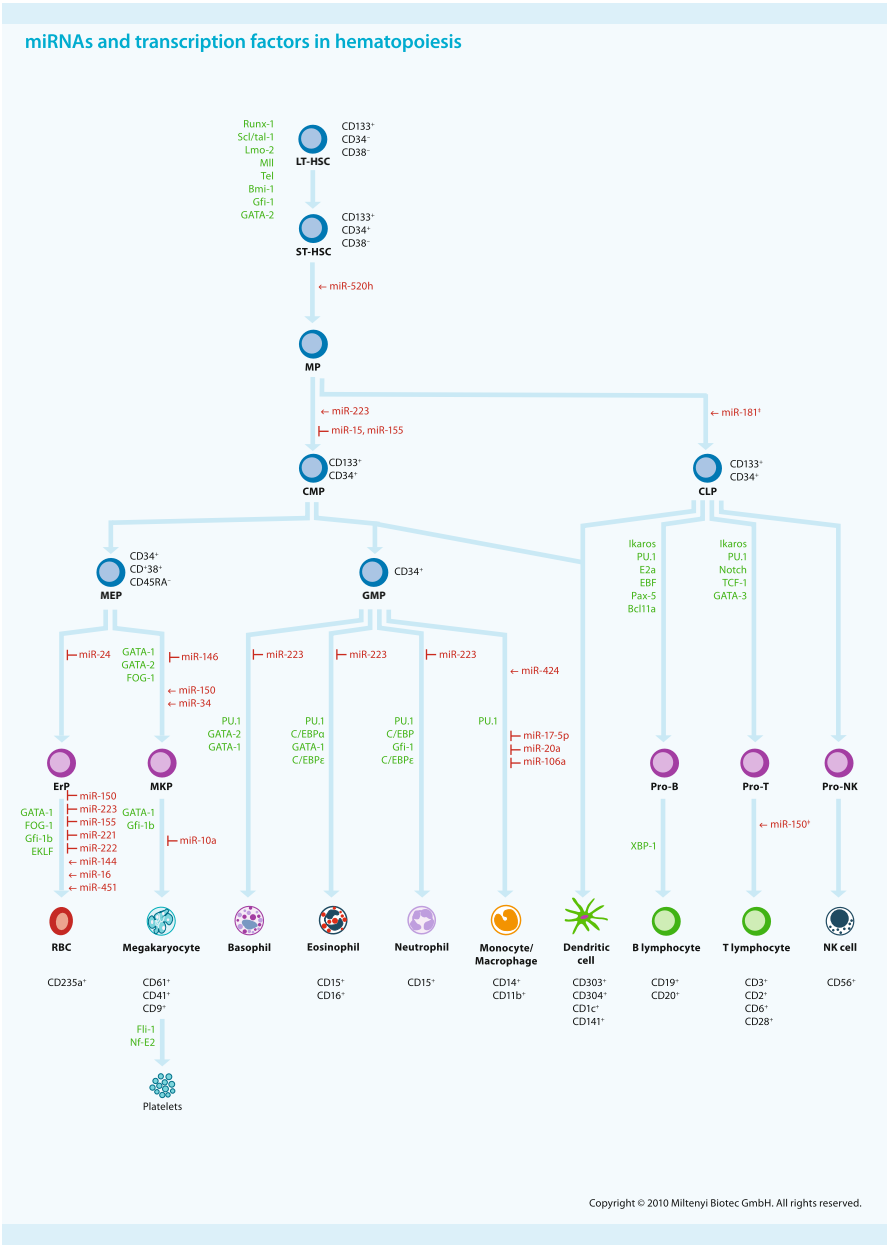

Fig. 1.2 Prominent miRNAs, transcription factors and cell surface markers in hematopoiesis. The miRNAs that regulate the different steps of hematopoiesis are shown in red. The depicted miRNAs were mainly identified in in-vitro assays with human cells. The role of the miRNAs labelled with ‡, e.g. miR-181‡ 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	–	–	–	–