LUND UNIVERSITY

Biology of Human Primary Bone Marrow Mesenchymal Stromal Stem Cells

Ghazanfari, Roshanak

2017

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Ghazanfari, R. (2017). *Biology of Human Primary Bone Marrow Mesenchymal Stromal Stem Cells*. [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Lund University: Faculty of Medicine.

Total number of authors:
1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Biology of Human Primary Bone Marrow Mesenchymal Stromal Stem Cells

Biology of Human Primary Bone Marrow Mesenchymal Stromal Stem Cells

Roshanak Ghazanfari

LUND UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended on March 3, 2017, at 09.00 in Segerfalksalen, BMC, Lund.

Faculty opponent
Prof. Dirk Strunk

Paracelsus Medical University

Salzburg, Austria

Organization
LUND UNIVERSITY
Division of Molecular Hematology
Department of Laboratory Medicine

Author: Roshanak Ghazanfari

Document name: Doctoral Dissertation

Date of disputation: March 3, 2017

Sponsoring organization

Title and subtitle: Biology of Human Primary Bone Marrow Mesenchymal Stromal Stem Cells

Abstract

In addition to hematopoietic stem cells (HSCs), human bone marrow (BM) contains a population of non-hematopoietic mesenchymal stromal stem/progenitor cells (MSCs) that can generate cells of the skeletal lineages *in vivo*. Furthermore, human BM-MSCs and their descendants are essential constituents of hematopoietic microenvironment and play pivotal roles in supporting and maintaining hematopoiesis. Despite intensive research on BM-MSCs, our knowledge about the precise phenotypic signature and functional properties of primary MSCs is still limited. In the current thesis we therefore aimed to address these issues by employing a variety of approaches. Using gene expression array analysis we have identified a new phenotype for prospective isolation of human BM-MSCs with thus fur unmet precision. Low/negative expression of CD140a on lin$^-$/CD45$^-$/CD271$^+$ cells marked a highly enriched BM stromal population, possessing all typical *in vitro* and *in vivo* BM-MSC properties. Single-cell gene expression (Fluidigm) analysis revealed homogeneity of this population in terms of the expression of selected MSC-relevant genes. Next, to evaluate stem cell properties of this putative stromal stem cell population, we utilized CFU-F assay as well as the recently developed mesensphere assay, which enables MSC amplification while preserving their immature phenotype. With both assays, we have documented clonogenicity and *in vitro* self-renewal capacity of CD271$^+$/CD140a$^{low/-}$ cells, however, *in vivo* self-renewal was observed only when cells were propagated as mesenspheres. Finally, to identify the underlying cause for the observed functional differences between adherent and non-adherent cultured BM-MSCs and to compare with primary BM-MSCs, we performed comparative gene array analysis to elucidate possible differential gene expression signatures. Several distinct clusters of genes were identified with different expression patterns between primary and cultured cells, and also between adherent and non-adherent MSCs. Cluster analysis enabled the identification of a number of potentially important MSC regulatory genes as candidate markers for future studies.

Taken together, we provided evidence that lin$^-$/CD45$^-$/CD271$^+$/CD140$^{low/-}$ cells present a rare homogeneous population of BM-MSCs that demonstrated all essential stroma cell properties, including hematopoietic support function, and – importantly - fulfilled stringent stem cell criteria, i.e. *in vitro* and *in vivo* self-renewal and differentiation. Furthermore, we have identified profound gene expression differences between primary, adherent and non-adherent BM-MSCs. This research contributes to a greater understanding the biology of human BM-MSCs and the mechanisms that regulate their different properties.

Key words: Mesenchymal stem cells, Bone marrow stromal cells, Stem cell properties

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language: English

ISSN and key title: 1652-8220

ISBN: 978-91-7619-411-9

Recipient's notes

Number of pages

Price

Security classification

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature _____Date _____

Biology of Human Primary Bone Marrow Mesenchymal Stromal Stem Cells

Roshanak Ghazanfari

Coverphoto by: Bengt Mattsson

Copyright: Roshanak Ghazanfari

Faculty of Medicine | Department of Laboratory Medicine
Lund University, Faculty of Medicine, Doctoral Dissertation Series 2017:30

ISBN 978-91-7619-411-9
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2017

To my beloved family

Contents

Articles included in this thesis 11
Selected abbreviations 12
Abstract 13
Populärvetenskaplig sammanfattning 15
Introduction 19
Mesenchymal stem cells (MSCs) 19
History and nomenclature 19
Human MSCs in different tissues 22
Ontogeny of MSCs 23
Human primary MSC surface markers 24
Multipotency of human MSCs 26
MSCs Self-renewal 28
Immunomodulatory effects of MSCs 28
MSCs as skeletal stem cells 29
MSCs and their descendants as Niche components 30
The niche concept 30
Anatomical structure of bone marrow niche 32
Endosteal niche 32
Vascular niche 33
Accessory cells of niche 35
Bone marrow niche in homeostasis 36
Aims of the thesis 39
Methods 41
Fluorescence activated cell sorting (FACS) 41
CFU-F assay 41
Mesensphere assay 42
In vitro differentiation assays 42
In vivo transplantation 43
Single cell Real-time qPCR (Fluidigm) 44

Microarray Analysis 44
Ethical considerations 45

Summary of results 47

Paper I 47
Low/negative expression of CD140a identifies the candidate primary mesenchymal stromal cells in adult human bone marrow

Paper II 49
Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion

Paper III 51
Human non-hematopoietic CD271^{pos}/CD140a$^{low/neg}$ bone marrow stroma cells fulfill stringent stem cell criteria in serial transplantations

Paper IV 53
Comparative gene expression analysis of primary human bone marrow mesenchymal stromal cells and their in vitro descendants (generated in adherent and sphere cultures)

Discussion 55

Characterization of human primary BM-MSCs 56

BMSCs and their progeny in the hematopoietic niche 58

Are BM-MSCs stem cells? 59

Sphere assay 61

Concluding remarks 62

Acknowledgments 65

References 69

Articles included in this thesis

I. *Low/Negative Expression of PDGFR-α Identifies the Candidate Primary Mesenchymal Stromal Cells in Adult Human Bone Marrow*
Hongzhe Li, **Roshanak Ghazanfari**, Dimitra Zacharaki, Nicholas Ditzel, Joan Isern, Marja Ekblom, Simón Méndez-Ferrer, Moustapha Kassem and Stefan Scheding*
Stem Cell Reports, 2014, 3 (6):965-74.

II. *Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion*
Joan Isern, Beatriz Martín-Antonio, **Roshanak Ghazanfari**, Ana M. Martín, Juan A. López, Raquel del Toro, Abel Sánchez-Aguilera, Lorena Arranz, Daniel Martín-Pérez, María Suárez-Lledó, Pedro Marín, Melissa Van Pel, Willem E.Fibbe, Jesús Vázquez, Stefan Scheding, Álvaro Urbano-Ispizúa and Simón Méndez-Ferrer*
Cell Reports, 2013, 3(5):1714-24.

III. *Human non-hematopoietic CD271^{pos}/CD140a$^{low/neg}$ bone marrow stroma cells fulfill stringent stem cell criteria in serial transplantations*
Roshanak Ghazanfari, Hongzhe Li, Dimitra Zacharaki, Hooi Ching Lim and Stefan Scheding*
Stem Cells and Development, 2016, 25 (21): 1652-1658

IV. *Comparative gene expression analysis of primary human bone marrow mesenchymal stromal cells and their in vitro descendants (generated in adherent and sphere cultures)*
Roshanak Ghazanfari, Dimitra Zacharaki, Hongzhe Li, Hooi Ching Lim, Shamit Soneji and Stefan Scheding*
Manuscript

Selected abbreviations

AGM	aorta-gonad-mesonephros
AT-MSC	adipose tissue mesenchymal stem cell
BM	bone marrow
BM-MNC	bone marrow mononuclear cell
BM-MSC	bone marrow mesenchymal stem cell
CAR	CXCL12-abundant reticular
CEE	chicken embryo extract
CFU-F	colony forming unit-fibroblast
CXCL12	C-X-C motif chemokine ligand 12
ESC	embryonic stem cell
FACS	fluorescence activated cell sorting
G-CSF	granulocyte colony stimulating factor
HSC	hematopoietic stem cell
IL-6	interleukin 6
IL-7	interleukin 7
ISCT	international society for cellular therapy
MSC	mesenchymal stem cell
Ocn	osteocalcin
Osx	osterix
SCF	stem cell factor
TGF-β	transforming growth factor beta

Abstract

In addition to hematopoietic stem cells (HSCs), human bone marrow (BM) contains a population of non-hematopoietic mesenchymal stromal stem/progenitor cells (MSCs) that can generate cells of the skeletal lineages *in vivo*. Furthermore, human BM-MSCs and their descendants are essential constituents of hematopoietic microenvironment and play pivotal roles in supporting and maintaining hematopoiesis. Despite intensive research on BM-MSCs, our knowledge about the precise phenotypic signature and functional properties of primary MSCs is still limited. In the current thesis we therefore aimed to address these issues by employing a variety of approaches. Using gene expression array analysis we have identified a new phenotype for prospective isolation of human BM-MSCs with thus fur unmet precision. Low/negative expression of CD140a on $lin^-/CD45^-/CD271^+$ cells marked a highly enriched BM stromal population, possessing all typical *in vitro* and *in vivo* BM-MSC properties. Single-cell gene expression (Fluidigm) analysis revealed homogeneity of this population in terms of the expression of selected MSC-relevant genes. Next, to evaluate stem cell properties of this putative stromal stem cell population, we utilized CFU-F assay as well as the recently developed mesensphere assay, which enables MSC amplification while preserving their immature phenotype. With both assays, we have documented clonogenicity and *in vitro* self-renewal capacity of $CD271^+/CD140a^{low/-}$ cells, however, *in vivo* self-renewal was observed only when cells were propagated as mesenspheres. Finally, to identify the

underlying cause for the observed functional differences between adherent and non-adherent cultured BM-MSCs and to compare with primary BM-MSCs, we performed comparative gene array analysis to elucidate possible differential gene expression signatures. Several distinct clusters of genes were identified with different expression patterns between primary and cultured cells, and also between adherent and non-adherent MSCs. Cluster analysis enabled the identification of a number of potentially important MSC regulatory genes as candidate markers for future studies.

Taken together, we provided evidence that $lin^{-}/CD45^{-}/CD271^{+}/CD140^{low/-}$ cells present a rare homogeneous population of BM-MSCs that demonstrated all essential stroma cell properties, including hematopoietic support function, and – importantly - fulfilled stringent stem cell criteria, i.e. *in vitro* and *in vivo* self-renewal and differentiation. Furthermore, we have identified profound gene expression differences between primary, adherent and non-adherent BM-MSCs. This research contributes to a greater understanding the biology of human BM-MSCs and the mechanisms that regulate their different properties.

Populärvetenskaplig sammanfattning

Mesenkymala stamceller (MSC) är en typ av stamceller som kan isoleras från olika vävnader i kroppen hos en vuxen människa, inkluderat benmärgen. I den humana benmärgen finns det två typer av stamceller, det vill säga MSC och hematopoetiska stamceller. De hematopoetiska stamcellerna ansvarar för den dagliga produktionen av blodceller. Benmärgens MSC å andra sidan, kan mogna ut och bilda andra typer av celler som benceller, fettceller och fibroblaster. Benmärgens MSC bildar även mikromiljön som har till uppgift att stödja och bevara blodbildning. Under de senaste årtiondena har MSC studerats ingående, men fortfarande finns det obesvarade frågor så som; hur kan MSC urskiljas från andra celler i benmärgen, eller vilka är deras exakta funktioner och egenskaper i kroppen.

Målet med avhandlingen har därför varit att försöka besvara dessa frågor genom olika infallsvinklar. I den första studien har vi identifierat en ny fenotyp som gör det möjligt för oss att isolera en relativt homogen, det vill säga en renare population av benmärgens MSC. Renheten hos en population utvärderas genom deras förmåga att bilda kolonier då de odlas på plast. Celler med denna förmåga kallas colony-forming unit fibroblast (CFU-F). Celler med fenotypen som vi har funnit i denna studie har den högsta kapaciteten att bilda CFU-F som hittills har rapporterats. Vi har även transplanterat in dessa celler under huden på immundefekta möss och kunde då se att cellerna bildade ben, fett och benmärgs-bindväv, vilket visar på deras differentieringsförmåga in vivo (förmågan att mogna ut till andra celltyper).

Det traditionella sättet att odla MSC är på en plastyta in vitro. Cellerna ska fästa på plasten och i närvaron av rätt odlingsmedium delar sig cellerna enkelt. Ett nytt sätt att odla MSC på är att använda sig av en icke vidhäftande metod. Denna metod innebär att man förhindrar cellerna att fästa på plasten och istället bildar cellerna små sfärer som flyter omkring i odlingsmediumet. I våra studier har vi visat att odling av MSC i sfärer kan ha vissa fördelar jämfört med den konventionella metoden. MSC som har isolerats baserat på den fenotyp som identifierats och som odlats som sfärer, har visat sig vara mer omogna celler. När vi transplanterade dessa sfärer under huden på immundefekta möss såg vi ett ökat antal av sfärer efter primära och sekundära transplantat. Detta betyder att cellerna har en självförnyande kapacitet vilket innebär att de har förmågan att bilda exakta kopior av sig själva. MSC som odlats med hjälp av den traditionella metoden hade däremot inte denna förmåga. Vi har även kunnat visa att sfärerna kunde bilda ben, fett och bindväv då vi genomförde en serie transplantationer (s.k. serial transplantations), vilket indikerar att de har differentierings förmåga in vivo. Vi har alltså kunnat visa att MSC från benmärgen har förmåga till självförnyelse och differentiering in vivo. Detta är två essentiella egenskaper som en stamcell ska besitta, och detta har ingen tidigare kunnat visa hos MSC isolerade från human benmärg.

Baserat på tidigare fynd, det vill säga de olika funktionella egenskaperna hos MSC odlade med den traditionella odlingsmetoden jämfört med MSC odlade som sfärer, bestämde vi oss för att analysera dem i detalj på en molekylär nivå. Detta gjorde vi genom att använda en metod som heter microarray. Med denna metod fann vi skillnader i genuttryck mellan MSC som odlats på det traditionella sättet, MSC som odlats i sfärer och MSC som inte odlats alls. Vi

har identifierat flera gener som kan vara viktiga för regleringen av MSC och dessa är därför kandidatgener för framtida studier.

Sammanfattningsvis bidrar studierna i denna avhandling till att förbättra den biologiska kunskapen om MSC. Vi har identifierat en fenotyp som gör det enklare att isolera humana MSC från benmärgen och som ger en renare MSC-population. Vi har också visat att dessa celler uppfyller stamcellskriterierna, det vill säga förmågan till självförnyelse och att mogna ut till andra celltyper. Denna information är synnerligen viktig för framtida studier där man vill designa reparationsstrategier och metoder för att ersätta stroma stamceller i benmärgen.

Introduction

Bone marrow mesenchymal stem cells (BM-MSCs) are a class of adult stem cells which can give rise *in vivo* to skeletal lineages including bone, adipose tissue and stroma, and therefore they generate a number of niche candidate cells which through direct and indirect mechanisms play central roles in the support and maintenance of hematopoiesis.

In the current thesis we aimed to address some open key questions regarding the biological features of human BM-MSCs, including their phenotype and stem cell properties.

Mesenchymal stem cells (MSCs)

History and nomenclature

The earliest description of bone marrow (BM) fibroblast cultures dates back to 1910 when Carrel and Burrows cultured bone marrow cells derived from dogs, cats and frogs and observed the growth of elongated spindle-shaped cells which were later named fibroblasts[1]. Berman *et al.*[2] adapted the culture methods for human cell culture and established long-term culture of rapidly growing human bone marrow fibroblast-like cells. Years later, the existence of a mesenchymal stem cell population in the BM was reported in a study showing that heterotopic implantation of BM can generate ectopic bone and

marrow[3]. Similar observations were made by Friedenstein and colleagues in a series of breakthrough seminal studies. They assigned the formation of ectopic BM to a rare subpopulation of bone marrow cells that were detectable from the remaining cells by their plastic adherence property. Importantly, they proposed the existence of precursor cells in the BM by showing their ability to form single cell-derived fibroblastic colonies (the colony-forming unit fibroblasts, CFU-Fs) [4-6]. They named these cells as osteogenic stem cells or bone marrow stromal stem cells[7, 8].

Later studies towards characterization of CFU-Fs showed that there was a linear relationship between the number of seeded cells and the number of fibroblast colonies generated, suggesting the clonality of CFU-Fs[9, 10] and this was supported by time lapse photography and chromosome analysis of CFU-Fs derived from mixed male and female plated cells[4, 6]. They described the CFU-F colonies as the aggregation of more than 40 fibroblast-like cells and reported their frequency as 1 per 10^5 marrow cells[9]. In the absence of cell sorting technology based on the cell immunological phenotype, bone marrow stromal cells were purified from bone marrow aspirates by their plastic adherent property, thus cell culture was the main method to separate the stromal cells from hematopoietic cells. Later, the term “mesenchymal stem cells” (MSCs) was coined to address adult bone marrow progenitor cells with capacity to differentiate into skeletal lineages[11]. Mesenchymal stem cells gained vast popularity over the last two decades as they were introduced as a type of adult stem cells with a broad range of differentiation potential that make them an attractive candidate for tissue repair and cell therapy. However, during the recent years some confusion has been created regarding the terminology, characteristics and function of these cells.

In the field of MSC biology, two different definitions of mesenchymal stem cells have been introduced. The first one defines MSCs as a class of adult stem cells with self-renewal and multi-lineage differentiation potential, found in different tissues and supply a reservoir of newly generated cells for replacing old cells and play critical role in the tissue homeostasis (**Fig.1**). In the second view, which has been widely applied in the field, MSCs are not necessarily "stem cells". They are culture-expanded cells that can be derived basically from any type of connective tissues and have gained popularity as a cell therapy tool for a number of diseases based on their immunomodulatory and anti-inflammatory effects but not any possible stem cell function[12].

The "stem cell" term applied in the second view was challenged when heterogeneity of culture-expanded bone marrow-derived cells was suggested[13]. This resulted in the International Society for Cellular Therapy's (ISCT) consent to use the term "multipotent mesenchymal stromal cells" for the fibroblast-like plastic adherent cells for which stem cell activity had not been demonstrated yet, and restrict the term "mesenchymal stem cells" only for those cells that meet stringent stem cell criteria[14]. One year later, ISCT defined human MSCs as plastic-adherent cells that express the surface markers CD90, CD73 and CD105 and lack the expression of CD34, CD45, CD14, CD19 and HLA-DR surface antigens. Additionally, MSCs are required have *in vitro* differentiation potential towards adipocytes, osteoblasts and chondroblasts[15].

The focus of current thesis is the rare population of non-hematopoietic mesenchymal stromal stem cells in human bone marrow, i.e. primary cells with *in vivo* multilineage differentiation capacities towards skeletal lineages according to the first definition[12].

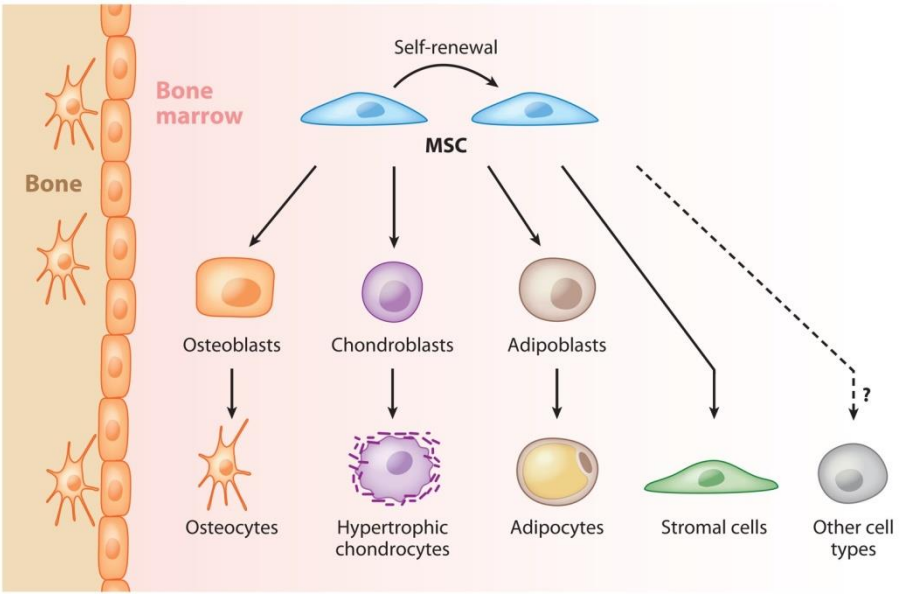

Figure 1. In vivo differentiation potential of BM-MSCs. MSCs residing in the BM have self-renewal capacity to generate identical cells, as well as differentiation potential to produce skeletal lineage cells including osteoblasts, chondroblasts and adipoblasts (The graph was taken from Frenette et al., 2013 with permission of the publisher).

Human MSCs in different tissues

Bone marrow is the most common and the best studied source for isolation of MSCs, however MSCs derived from other tissues such as fat, peripheral blood, synovial membrane, lung, heart, amnion membrane and umbilical cord have been the subject of a number of different studies[16]. MSCs isolated from these sources show similarities and differences with BM-MSCs in regard to their phenotype, clonogenic potential and differentiation capacity. For instance, similar to BM-MSCs, primary adipose tissue MSCs (AT-MSCs) are

positive for CD90[17], CD146[18] CD271[19] and negative for CD31 and CD45[17]. In contrast to BM-MSCs, AT-MSCs are positive for CD34[19] and negative for CD105[17]. Compared to BM-MSCs, AT-MSCs have a higher proliferation rate[20] but similar CFU-F frequency and tri-lineage differentiation potential[17, 21]. However, recent *in vivo* and epigenetic studies have shown that BM-MSCs and AT-MSCs have distinct *in vivo* differentiation potential and molecular signatures[22, 23], highlighting the need of rigorous studies to better characterize MSCs derived from different sources and to identify the exact phenotype and function of their primary MSC.

Ontogeny of MSCs

During the development period, the embryo is formed by pluripotent embryonic stem cells (ESCs) derived from the inner cell mass of blastocyst that generate all the various tissues in the body[24]. Later in life, another class of stem cells, called multipotent adult or somatic stem cells, can be found in different tissues which are more restricted in their potency compare to ESCs and which have maintenance and repairing roles in the tissues that they reside[25, 26].

One of the best-described multipotent adult stem cell types is the hematopoietic stem cell (HSCs), residing in the bone marrow and efficiently providing all the blood cell types throughout the life of the organism[27]. Hematopoiesis takes place within the context of the hematopoietic stroma with the assistance of different bone marrow stromal cells consisting of mesenchymal stem cells and their descendants. Despite the significant progress in the field of MSC biology, several aspects including their ontogeny and developmental origin with respect to their function remain

uncertain. In both mouse and human embryos MSCs have been found in main hematopoietic sites during the development including in aorta-gonad-mesonephros (AGM) region, fetal liver and bone marrow[28-30]. Most of the information about the anatomical mapping of MSCs during the development has been gained through lineage tracing studies in transgenic mouse models and due to the restrictions of human embryo studies, our knowledge about the origin of human MSCs is still limited. In general, it is believed that both mouse and human MSCs are a derivative of the mesoderm layer[31], however some lineage tracing studies in transgenic mouse models have shown that the early waves of MSC generation during development are developed from $SOX1^{+}$ neuroepithelium and later from neural crest[32, 33].

Additionally, the fact that bone marrow MSCs express some of the neural markers such as CD271 and Nestin, as well as their capacity to form mesenspheres, which is similar to neurosphere formation, suggest a possible relationship with the neuroectoderm layer[34]. A more recent study has assigned different functions of mouse MSCs to their distinct developmental origin, i.e., MSCs derived from the mesoderm layer are mainly involved in skeletogenesis through endochondral ossification, while neural crest-derived MSCs are HSC niche-forming cells in the same bones[25]. Although these studies suggest a multiple origin for mouse MSCs, the developmental hierarchy of different MSC derivatives regarding their niche function in postnatal life remains to be clarified.

Human primary MSC surface markers

To study the biological properties and physiological functions of native MSCs in their environment, identification of their phenotype which enables

researchers to isolate them prospectively in the primary state is crucial. Primary MSCs refer to freshly isolated bone marrow stromal cells based on their specific surface marker profile using recently developed sorting technology[35] (**Fig. 2**). On the other hand and as described above, there are cultured MSCs which are isolated based on their plastic adherence capacity and have a typical surface marker profile, as defined by the ISCT[15].

Several positive and negative candidate surface markers have been reported to identify human primary MSCs. Positive markers include STRO-1[36], CD146[37], CD271[38], CD140b[39], CD106[40], CD105[41], SUSD2[42], CD73[43], CD56[44] and CD90[45]. Negative markers consist of CD31[46], CD34[46], CD45[47] and CD44[48]. However, cells expressing these markers have shown a poor enrichment for human BM cells with CFU-F activity, which are considered to reflect the primary bone marrow stromal stem/progenitor cells. CD271 is a marker that can identify all the colony forming cells in human bone marrow and is commonly used for isolation of human primary BM-MSCs[49, 50], however the frequency of CFU-F in $CD271^+$ population is not high.

To purify this population further, we therefore investigated possible novel primary MSC markers and found that low/negative expression of CD140a on $CD271^+$ cells identified a close to pure population of human primary BM-MSCs with the highest stem/progenitor content that had been reported thus far[51].

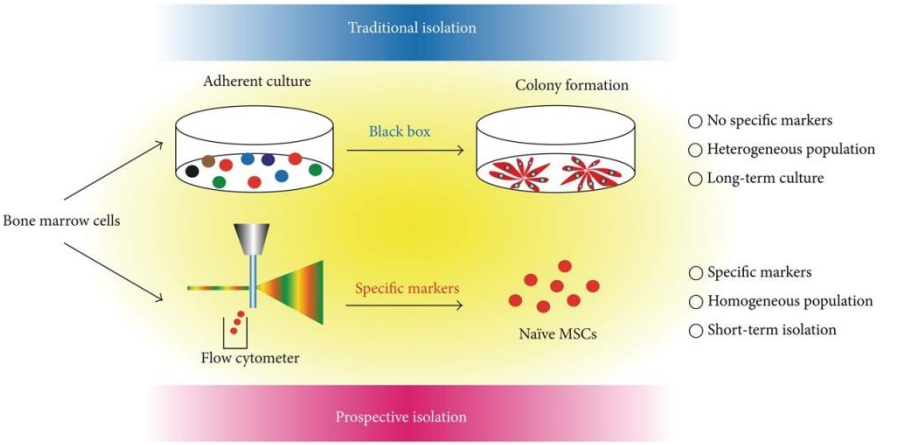

Figure 2. Conventional and prospective MSC isolation methods. In the traditional way, MSCs are isolated based on their plastic-surface adherence property, which results in a heterogeneous population of cultured stromal cells. In the prospective isolation method, MSCs are FACS sorted based on the expression of specific surface markers. This method allows to isolate a more homogenous MSC population (The graph was taken from Mabuchi et al., 2013 with permission of the publisher).

Multipotency of human MSCs

In 1999 Pittenger *et al*. introduced an important characteristic of MSCs. They showed *in vitro* differentiation potential of human MSCs toward three mesodermal lineages, including osteoblasts, adipocytes and chondrocytes following culture in presence of induction media[52]. Later, various studies reported on the capacity of MSCs to differentiate into other cell types such as neuronal-like cells[53] (cells had a typical neuron morphology and expressed neural markers including neuron-specific enolase (NSE) and neurofilament-M (NF-M), hepatocyte-like[54] (cells expressed hepatic proteins including hepatocyte nuclear factor (HNF) and alpha-fetoprotein (AFP) and showed some hepatic functionality) and airway epithelial cells[55] (cells functionally

contributed to apical Cl^- secretion). However, in order to claim that MSC have such a broad differentiation potential it is necessary to demonstrated that differentiated cells exhibit full functionality *in vivo* before one can claim that a certain cell type is produced. Although a number of *in vivo* studies have shown differentiation of human MSCs to skeletal and cardiac muscles[56, 57], others have reported contradictory results[58, 59], suggesting that more investigations are needed to explore *in vivo* differentiation potential of MSCs. Also, it is important to note that *in vitro* multipotency of MSCs has to be tested at clonal level to be able to claim that multiple lineages have been derived from the same progenitor cell. However, the majority of studies have only investigated MSC differentiation in bulk cultures.

One defining feature of any class of stem cells is their *in vivo* multilineage differentiation potential. In the context of BM-MSCs, serial transplantation studies showed that mouse $Nestin^+$ perivascular stromal cells generated the hematopoietic microenvironment *in vivo*[60]. In human fetal bone marrow, CD140a and CD51 marked human $Nestin^+$ mesenchymal stromal cells that were capable of transferring the hematopoietic niche activities *in vivo*[61]. In adult human bone marrow, expression of CD146 identified stromal cells which could generate a heterotopic bone marrow niche following subcutaneous implantation[37]; however the *in vivo* studies presented in this seminal publication were not performed in a serial transplantation setting. To investigate the *in vivo* differentiation potential of human BM-MSCs, we therefore performed serial transplantation assays in one study included in this thesis and we were able to demonstrate that human primary bone marrow $CD271^+/CD140a^{low/-}$ cells fulfilled stringent stem cells criteria, i.e. *in vivo* self-renewal and multilineage differentiation potential[62].

MSC self-renewal

Self-renewal, the process of generating daughter stem cells with preserved undifferentiated state to maintain the stem cell pool, is another major criterion of all postnatal stem cells. In the field of MSC biology, most of the studies evaluating MSC stem cell activity have been limited to *in vitro* experiments and relied on the CFU-F assay as the main evaluation tool. Only few numbers of studies have investigated the *in vivo* self-renewal activity of mouse and human MSCs. Besides the CFU-F assay, a novel non-adherent sphere culture system was introduced into the field as an alternative tool to evaluate the stem cell activity of MSCs[60]. It was shown that mouse Nestin^{+} perivascular stromal cells formed mesenspheres in a modified culture condition adapted from neurosphere assay[63] and exhibited *in vivo* self-renewal activity in serial transplantation experiments[60]. *In vivo* self-renewal capacity of human bone marrow MSCs was not thoroughly investigated before. In this thesis, using the novel mesensphere assay adapted for human cells, we therefore investigated for the first time the self-renewal activity of a pure population of MSCs in a serial transplantation setting[62].

Immunomodulatory effects of MSCs

MSCs have been reported to possess anti-inflammatory and immunoregulatory functions by affecting both the adaptive and innate immune system, and MSC have therefore been applied in a number of clinical trials for treatment of diseases such as graft versus host disease (GVHD), multiple sclerosis (MS), and Crohn's disease[64]. In the innate immune system, MSCs recruit monocytes and macrophages in response to an injury and enhance tissue repair process. In addition, MSCs by secretion of

growth factors such as interleukin 6 (IL-6) and granulocyte-macrophage colony stimulating factor (GM-CSF) facilitate the recruitment of neutrophils to the site of injury and promote their anti-inflammatory function. MSCs modulate adaptive immune responses mainly through suppression of $CD4^+$ and $CD8^+$ T cells, which is mediated by indoleamine 2, 3- dioxygenase (IDO) or nitric oxide (NO) in humans and mice, respectively. Furthermore, MSCs inhibit B cell activity through cell cycle arrest and reduced production of immunoglobulin[65, 66]. In addition, other mechanisms have been proposed and in order to better understand the MSC immunoregulatory properties, more studies are needed to address the underlying procedures.

MSCs as skeletal stem cells

In 2004 Bianco and Robey proposed a new term for BM-MSCs as "skeletal stem cells"[67]. They argued that an adult stem cell is usually described by the cell types that it generates, similar like hematopoietic stem cell. Multipotent BM-MSCs that were introduced by Friedenstein and others have shown *in vivo* differentiation potential limited to skeletal lineages, including osteoblasts, chondroblasts, adipocytes, fibroblasts and reticular cells. Even though different studies have indicated a wider range of differentiation capacity for BM-MSCs, strong experimental evidence is still lacking. Therefore, they suggested until solid proof is provided that shows a clonal stromal cell can generate other cell types than skeletal lineages *in vivo*, the proper term for BM-derived stromal cells would be skeletal stem cells. Skeletal stem cell is a more stringent term that implies proven *in vivo* potential of BM stromal stem cells[67, 68].

MSCs and their descendants as Niche components

The niche concept

HSCs reside in a supportive and highly dynamic microenvironment, the so-called niche, in the BM. During the development, in both mouse and human, hematopoietic niches are found in different sites, including yolk sac, AGM, liver, spleen and bone marrow. After birth, in the normal conditions bone marrow is the major site of hematopoiesis[69]. Under the control of precise and complicated regulatory signals provided by niche, HSCs undergo self-renewal, lineage differentiation, maturation and mobilization[70].

The critical role of the hematopoietic niche is highlighted by the fact that mutations and abnormalities of the non-hematopoietic cells of the BM can cause hematopoietic malignancies[71]. In the initial concept, the niche was first hypothesized as a unit that regulate HSC self-renewal and differentiation[72]. Later studies proved the existence of regulatory niche in the Drosophila Melanogaster gonad[73, 74] as well as other mammalian tissues including brain, skin and intestine[75]. In the BM hematopoietic niche context, the first *in vivo* proof of existence of niche elements with HSC regulatory function was obtained using transgenic mouse models in two different studies[76, 77]. Both studies showed that higher number of osteolineage cells obtained following genetic modifications resulted in higher number of HSCs.

It is worth to note that most of our current knowledge about the hematopoietic niche comes from mouse studies through different transgenic mouse models and *in vivo* lineage trace studies. The BM niche is comprised of multiple cell types and various soluble factors. The main cellular components of the niche include MSCs, osteoblasts, endothelial cells,

perivascular cells, sympathetic nerve fibers and non-myelinating Schwann cells. In addition to the main niche cells, there are several accessory cells, including osteoclasts, adipocytes, megakaryocytes, macrophages, T-regulatory cells and dendritic cells[34, 70, 78] (**Fig. 3**). Different soluble factors as chemokines, cytokines and growth factors are generated by cellular components of the BM. The majority of them which are involved in the regulation of hematopoiesis include C-X-C motif chemokine ligand 12 (CXCL12)[79], stem cell factor (SCF)[80], transforming growth factor beta (TGF-β)[81], granulocyte-colony stimulating factor (G-CSF)[82], thrombopoietin (TPO)[83], angiopoietin-1[84], IL-6[85], interleukin 7 (IL-7)[86, 87], Notch ligands[88] and Wnt ligands[89].

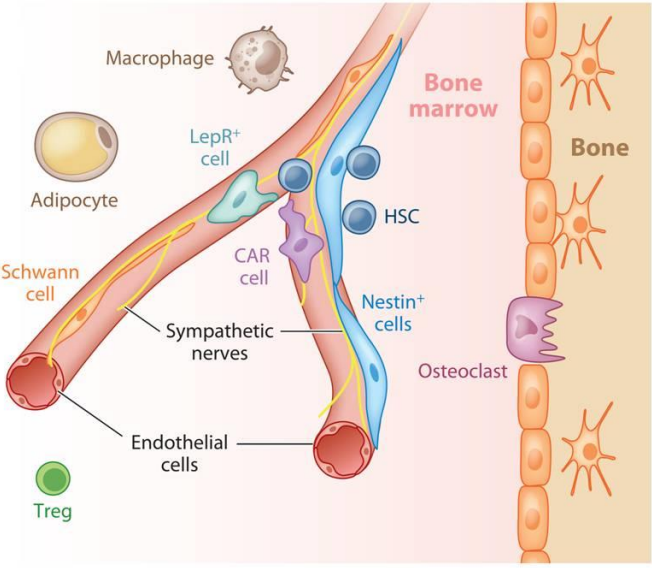

Figure 3. The hematopoietic stem cell niche. The diagram illustrates the most important cellular candidates in bone marrow microenvironment with known functions in regulation of hematopoiesis (The graph was taken from Frenette et al., 2013 with permission of the publisher)

Anatomical structure of the bone marrow niche

Advancements in high-resolution imaging techniques and developed transgenic mouse models have enabled scientist to dissect constituents of bone marrow niche. Bone marrow is a densely vascularized tissue, containing central arteries that branch to small arteries and finally to small arterioles close to the bone surface. Small venous sinusoids arise from endosteal region and move towards the central marrow while making various anastomoses and finally join each other and create the main sinus in the center of bone cavity[90].

Within the BM microenvironment, at least two distinct niches have been proposed: the endosteal (osteoblastic) niche and the perivascular (pericytic arteriolar) niche[70].

Endosteal niche

A minority of HSCs in the BM are localized adjacent to bone surface-lining osteoblasts, indicating the existence of endosteal niche[77]. Different osteolineage cells secrete a number of soluble factors implicated in HSC regulation. G-CSF produced by human mature osteoblasts mediates HSC mobilization[82, 91]. In the mouse, angiopoietin-1, the ligand of the Tie2 receptor, which is mainly expressed by osteoblasts, promotes HSC quiescence and therefore maintains long-term repopulating capacity of HSCs[84]. Osteopontin, a glycoprotein produced by osteoblasts, has a constraining effect on mouse HSC numbers and negatively regulate HSC pool size[92].

In spite of these findings and many other studies indicating the role of osteolineage cells in hematopoiesis, their contribution is under debate. For

instance, global expansion of mouse osteoblasts using a bone anabolic agent makes no impact on hematopoiesis[93] or conversely, deficiency in osteoblasts function in arthritis mouse model has no effect on long-term HSCs[94].

One possible explanation for these controversial findings is the heterogeneity of the osteolineage cells. Mouse osteolineage cells at different stages (early-stage and late-stage) have different impacts on hematopoiesis[95]. Early-stage osteolineage cells produce more CXCL12 and SCF; therefore have a better supporting effect on long-term HSCs compare to more mature osteolineage cells[96]. In addition, early-stage osteolineage cells expressing osterix (Osx^+) play a role on B lymphocyte maturation[79]. More mature osteolineage cells expressing osteocalcin (Ocn^+), on the other hand, only slightly impact B lymphocyte production while significantly affecting T lymphocyte generation[97].

Vascular niche

The predominant localization of HSCs close to the blood vessels motivated the notion that vascular regions may have a crucial role in HSC regulation. Mesenchymal stromal cells that surround blood vessels and express different markers have been suggested to be the main perivascular niche component. In humans, expression of CD271[98] and CD146[37] are reported to define perivascular MSCs with HSC supporting functions. Perivascular mesenchymal stromal cells in different transgenic mice models have been shown to promote HSC maintenance. $Nestin^+$ MSCs showed high levels of expression of HSC regulatory molecules including SCF, CXCL12 and Angiopoietin-1[60]. $CXCL12^+$ perivascular cells named CAR (CXCL12-abundant reticular) cells are adjacent to sinusoids and co-localize with SLAM

signature ($CD150^+CD48^-CD41^-$) HSCs[99] and Leptin receptor positive perivascular cells express SCF, which is essential for HSC maintenance[100]. All of these perivascular cells have MSC characteristics, suggesting that MSCs are a major component of perivascular niche.

Moreover, arterioles that are preferentially located in the endosteal region harbor quiescent HSCs. In mice, these small arterioles are enclosed by $NG2^+$ pericytes and produce high levels of niche factors that are required for HSC maintenance. Depletion of $NG2^+$ pericytes leads to increased cycling of HSCs and decreased long-term repopulating activity of HSCs[101].

Another vascular niche candidate are endothelial cells which release different growth factors and balance the rate of self-renewal versus lineage differentiation of mouse HSCs through AKT signaling pathway[102]. Functional evidence supporting the contribution of endothelial cells in the niche comes from a study showing that deletion of the Gp130 gene in mouse endothelial cells is accompanied by bone marrow hypocellularity and dysfunction[103]. In addition, endothelial cells express different adhesion molecules such as E-selectin which promote mouse HSC proliferation[104] and VCAM-1 that correlates with homing of HSCs[105].

Nerve fibers from the sympathetic nervous system run along the blood vessels in the BM. Non-myelinating Schwann cells, which enclose the sympathetic nerve fibres are reported as a mouse perivascular niche component. They are in direct association with a high proportion of HSCs and activate latent TGF-ß which in turn induce HSC hibernation through inhibition of lipid raft clustering[81]. Furthermore, it has been shown in mice that sympathetic nervous system regulates egression of HSCs from bone marrow through alteration of CXCL12 expression[106]. In addition, circadian

oscillation of physiological trafficking of HSCs into the blood stream is regulated by adrenergic signals delivered by sympathetic nerves into the mouse bone marrow niche[107].

Accessory cells of the niche

Bone-resorbing osteoclasts in concert with bone-forming osteoblasts are responsible for bone remodeling. As a niche component, the role of osteoclasts in HSC regulation is conflicting. It has been reported that their activation may trigger proliferation of mouse HSCs and promote mobilization of HSCs from bone marrow[108]; however another study suggests a dispensable role for osteoclasts in HSC regulation[109].

Bone marrow also contains adipocytes, so-called fatty marrow, which its amount correlates with age. As a niche component they are a negative regulator of hematopoiesis. By production of adiponectin[110], neuropillin-1[111] and lipocalin-2[112] they impair proliferation of hematopoietic progenitors. Administration of adipogenesis antagonist enhances mouse bone marrow engraftment in transplantation settings[113].

Macrophages are another player in the bone marrow microenvironment. In mice, it has been found that following G-CSF administration and HSC mobilization, a population of macrophages associated with the endosteal region was depleted and osteoblast function was suppressed. *In vivo* depletion of macrophages imitated the effect of G-CSF administration, led to loss of osteoblasts and induced HSC mobilization into the blood stream, suggesting the central role of macrophages in HSC maintenance[114]. Moreover, $CD169^{+}$ mouse macrophages are shown to support HSC retention. Depletion of

CD169^{+} macrophages induces down-regulation of HSC retention molecules in MSCs and leads to HSC mobilization[115].

Bone marrow niche in homeostasis

The bone marrow microenvironment plays an important role to maintain the homeostasis condition for hematopoiesis and ensure the daily need of rapid blood cell production. HSC fate decisions and maintaining the balance between self-renewal and differentiation takes place in the bone marrow niche under the influence of extremely complex extrinsic and intrinsic mechanisms.

Asymmetric stem cell division is a crucial phenomenon that maintains the stem cell pool and also generates large number of differentiated cells. In Drosophila, germ stem cell polarity and orientation of mitotic spindle in relation to its contacted niche cell is a critical step in asymmetric stem cell division which is regulated by different cues from the microenvironment[116]. In the human hematopoietic system, asymmetric division of HSCs has been shown *in vitro* by observation of unequal distribution of specific markers between the daughter cells during mitosis[117]. In addition to intrinsic factors, extrinsic signals growth factors and cytokines from the microenvironment regulate HSCs asymmetric division[118, 119].

Mobilizations of HSCs from the bone marrow, their entering into the circulation, and their return and homing to the bone marrow are important physiological processes during homeostasis and following injury that are tightly regulated by niche factors. In fact, HSCs are retained in the BM under the control of restraining factors produced by the niche and a breakdown of maintenance mechanisms is sufficient for mobilization. CXCL12/CXCR4 is

an essential axis in the regulation of HSCs mobilization. The chemokine CXCL12, which is constitutively produced by different niche cells (CAR cells, MSCs, osteolineage cells, etc.), interacts with its receptor CXCR4 expressed on HSCs, and retains the HSCs in the bone marrow. Down-regulation of CXCL12 in mouse niche cells or up-regulation of CXCL12 in peripheral circulation induced by G-CSF or following an injury, leads to HSC mobilization[116, 120]. Another possible retention mechanism in mouse and human bone marrow is interaction of very late antigen 4 (VLA-4), an adhesion receptor expressed by HSCs, with its different ligands such as VCAM-1, osteopontin and fibronectin[121]. Proteolytic cleavage of these adhesion pathways by proteases cause dislodging of HSCs from the bone marrow[121, 122].

Bone marrow homing of circulating HSCs, which is a mirror event of mobilization, is a multistep process that involves similar pathways as the mobilization process. Up-regulation of CXCL12 in different niche cells plays a pivotal role in recruiting HSCs. Furthermore, over-expression of adhesion molecules such as VCAM-1, ICAM-1, E-selectin, and P-selectin by endothelial cells are crucial for HSC homing[120].

Aims of the thesis

BM-MSCs are important cells for not only the skeletal system, but also for hematopoietic stroma and the HSC microenvironment. The exact phenotype of human primary BM-MSCs and their stem cell properties, i.e. self-renewal and differentiation were not well-defined. The overall goal of this thesis was therefore to investigate basic biological features of bona fide human BM-MSC population and in particular, to address the open questions regarding their phenotypical characteristics and functional stem cells properties.

More specifically, the aims were as follows:

- To identify the precise phenotypic profile of human BM-MSCs (Paper I)
- To develop a novel non-adherent culture method ("mesensphere") for the evaluation of human BM-MSC characteristics (Paper II)
- To study functional *in vivo* stem cell properties of prospectively isolated human BM-MSCs (Paper III)
- To investigate the possible different molecular signatures of prospectively-isolated primary BM-MSCs, compared to adherent and non-adherent culture-expanded MSCs in order to identify potential MSC key regulatory genes (Paper IV, manuscript)

Methods

This chapter provides a brief description of the experimental procedures. The reader is referred to more detailed information given in the Materials and Methods of the respective publications included in this thesis.

Fluorescence activated cell sorting (FACS)

Following the incubation in blocking buffer (DPBS, human normal immunoglobulin and fetal bovine serum), BM-MSCs and lineage-depleted bone marrow mononuclear cells (BM-MNCs), respectively were stained with monoclonal antibodies. In the majority of experiments combinations of anti-CD45, CD31, CD71, CD235a, CD271 and CD140a antibodies were used. Sorting gates were set according to the corresponding fluorescence-minus-one (FMO) controls. Cells were sorted on a FACS Aria II or a FACS Aria III cell sorter (BD Biosciences). Dead cells were excluded by 7-amino-actinomycin (7-AAD, Sigma) staining.

CFU-F assay

BM-MNCs were FACS-sorted based on the respective marker combinations and the CFU-F frequency of sorted cells was evaluated by plating the cells at clonal density in standard MSC medium (StemMACS MSC Expansion

Medium, Miltenyi Biotec). At days three and seven the medium was changed. On day 14, cells were fixed with methanol and stained with Crystal Violet. Colonies containing ≥ 40 fibroblast-like cells were defined as a CFU-F[49].

Mesensphere assay

FACS-sorted BM-MNCs were seeded at low density (<1000 cells/cm^2) or single cells in ultra-low adherence plates in presence of sphere growth medium[60]. To prevent cell aggregation, cultures were left untouched for one week and afterwards, the cells were fed twice weekly. Mesenspheres were sub-cultured following enzymatic digestion with 0.25% type I collagenase (StemCell Technologies) and re-plated at clonal density.

In vitro differentiation assays

BM-MSCs derived from adherent and sphere cultures were differentiated towards the adipogenic, osteogenic and chondrogenic lineages. For adipogenic differentiation, cells were cultured for 14 days in AdipoDiff medium (Miltenyi Biotec) and stained with Oil red O (Sigma) following fixation. For osteogenic differentiation, cells were cultured in osteogenesis induction medium [DMEM high glucose/L-glutamine (PAA) containing β-glycerophosphate (Sigma), L-ascorbic acid-2-phosphate (Wako chemicals) and dexamethasone (Sigma)] for 21 days and calcium depositions in the cultures were detected by alizarin red staining (Sigma). Chondrogenic differentiation was induced by culturing cell pellets for 28 days in

chondrogenesis-induction medium [DMEM high glucose/L-glutamine (PAA) containing L-ascorbic acid-2-phosphate, L-proline (Sigma), pyruvic acid sodium salt (Sigma), ITS^+ culture supplement (BD Biosciences) and TGF-β3 (R&D systems)]. Cryosections of fixed pellets were stained with goat anti-human aggrecan (R&D systems) and a corresponding secondary antibody. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, Life Technologies). Sections were analyzed with an Axiovert 200M fluorescence microscope and an AxioCam HRm camera (both from Carl Zeiss).

In vivo transplantation

For primary *in vivo* transplantation assay, FACS-sorted cells were cultured under adherent and/or sphere conditions. After expansion, cells were harvested and loaded overnight on hydroxyapatite/tricalcium phosphate ceramic powder (HA/TCP; TRIOSITE, Zimmer) followed by subcutaneous implantation into 8 week old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice.

To evaluate *in vivo* differentiation capacities, implants were removed after 8 weeks, fixed, decalcified, and paraffin embedded. Sections were either stained with hematoxylin/eosin and analyzed as described [123] or prepared for immunohistochemistry analysis.

To assess *in vivo* self-renewal, implants were removed after 8 weeks and digested in type I collagenase (StemCell Technologies) for 2 hours at 37°C. Harvested cells were stained with specific anti-mouse and anti-human antibodies. After exclusion of dead cells by 7AAD, human sorted cells were plated at clonal density for sphere and CFU-F colony formation. After 10 and 14 days, sphere and CFU-F colonies were counted, respectively.

Single cell Real-time qPCR (Fluidigm)

Single cells from human primary BM were sorted directly into the lysis buffer [low EDTA TE buffer (Teknova), NP-40 (Sigma) and SuperaseIN (Life Technologies)]. cDNA synthesis on single cells was performed using qScript cDNA supermix kit (Quanta Bioscience). Specific Target Amplification (STA) of 48 genes of interest was carried out by TATAA preamp grand master mix kit (TATAA Biocenter) and the final product underwent exonuclease treatment using Exonuclease I kit (New England Biolabs). The samples were mixed with Evagreen supermix-low ROX (BioRad), DNA binding dye and loading reagent (both Fluidigm) and loaded into the 48.48 dynamic array IFC chip and run on BioMark™ system. The data analysis was performed using BioMark Real-Time PCR Analysis and Singular Analysis Toolset software (Fluidigm).

Microarray Analysis

Total RNA from primary, adherent and sphere cultured cells were isolated using the RNeasy plus micro-kit (Qiagen) according to the manufacturer's manual. RNA was subjected to a two-round amplification using the TargetAmp™ 2-Round Biotin-aRNA Amplification Kit (Epicentre, Madison). The amplified RNA was then analyzed for gene expression using Illumina Human HT-12 expression v4 BeadChips (Illumina). Data analysis was performed using the GenomeStudio software. Differentially expressed genes were determined using LIMMA for R[124] using a FDR cutoff of 5%. Heatmaps were created in Genesis[125] using hierarchical clustering using the correlation distance (1-*r*) as a measure of similarity.

Ethical considerations

Human bone marrow samples were aspirated from healthy donors with approval from regional ethical board in Lund (Regionala etikprövningsnämnden i Lund).

All animal procedures were performed in accordance with approval from the regional animal ethical committee (Malmö/Lunds djurförsöksetiska nämnd).

Summary of results

Paper I

Low/negative expression of CD140a identifies the candidate primary mesenchymal stromal cells in adult human bone marrow

Although we and others have previously reported that primary BMSCs were exclusively enriched in $CD271^+$ cell fraction in human bone marrow[49], there is still a considerable number of non-colony forming cells in this population (CFU-F frequency was around 1 in 20). This study therefore aimed to detect new marker(s) that would allow a better characterization of BMSCs. Comparative gene expression profiling between $CD271^+$ and $CD271^-$ cells was performed and in total 215 up-regulated and 97 down-regulated genes in the $CD271^+$ fraction compared to the $CD271^-$ subset were found. Twenty eight out of 215 up-regulated genes were surface markers and except few of them that were previously reported in the context of MSC isolation (CD106, CD140b and CD10), the majority were novel MSC markers including CD151, CD81, CD18, CD140a, TGFBR3, LEPR, IFNGR2 and FGFR3. FACS analysis of these surface markers on $lin^-/CD45^-/CD271^+$ cells was performed and CFU-F content of each sub-population was evaluated. Among all the tested markers, using CD140a enabled to identify a highly enriched population of $lin^-/CD45^-/CD271^+/CD140a^{low/-}$ cells with a CFU-F frequency of 20.8 ± 9.6 per 100 plated cells. Primary $CD271^+/CD140a^{low/-}$ cells co-

expressed MSC typical markers, including CD105, CD90, CD140b and STRO-1 whereas they lacked the expression of CD31 and CD34. Quantitative RT PCR revealed higher expression of pluripotency genes (Nanog, Oct4 and Sox2) in sorted CD140a^{-} cells compared to CD140a^{+} fraction. Moreover, CD140a$^{low/-}$-derived cultured cells showed robust *in vitro* tri-lineage differentiation potential (adipogenic, osteogenic and chondrogenic), and more importantly, *in vivo* they generated bone, adipocytes and hematopoietic stroma when transplanted subcutaneously into NOD-SCID mice. Finally, co-culture experiments showed that CD140a$^{low/-}$-derived cultured cells supported CD34^{+} cells expansion and expanded cells were functionally capable to reconstitute hematopoiesis when injected into immunodeficient mice. Taken together, low/negative expression of CD140a on lin^{-}/CD45^{-}/CD271^{+} cells allowed to identify a close to pure population of the candidate human primary mesenchymal stromal cells, which is an important step towards understanding the physiological role of MSCs *in vivo*.

Paper II

Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion

Based on the results of a previous study that showed Nestin^{+} mouse BM-MSCs contained all the CFU-F activity and were capable to generate self-renewing mesenspheres[60], here we aimed to develop a novel mesensphere assay for studying human BM-MSC properties as non-adherent mesenspheres. In this study we used combination of CD105, a candidate surface marker that co-expressed with a subset of Nestin^{+} cells in bone marrow biopsies, and CD146 which was known to be expressed by human primary BM-MSCs[37], but we have later shown mesenspheres generated from our own phenotype (paper III). Human CD45^{-}/CD31^{-}/CD71^{-}/CD146^{+}/CD105^{+} population contained all the clonogenic and sphere-forming cells and sorted cells had similar efficiency to form CFU-F (3.4% ± 2.1%) and mesenspheres (5.9% ± 2.3%) when seeded at clonal densities. Human mesenspheres had a compact structure containing dense fibroblast-like cells with average diameter of around 200 µm. They kept their surface marker profile over the culture (P=4) and showed robust differentiation capacity into mesodermal lineages following induction, while in undifferentiated state they promoted ex vivo expansion of transplantable CD34^{+} cells through secreted soluble factors in transwell co-culture systems. Secretome analysis of human bone marrow mesenspheres grown in chicken embryo extract (CEE) revealed the existence of different clusters of proteins such as growth factors, calcium binding proteins, extracellular matrix proteins, cadherins, integrins and intermediate filaments. Taken together, these data indicated the capacity of

human BM-MSCs to generate mesenspheres, comparable to the previously characterized mouse mesenspheres. Human mesenspheres conserved their immature phenotype and maintained their ability to support hematopoiesis.

Paper III

Human non-hematopoietic CD271^{pos}/CD140a$^{low/-}$ bone marrow stroma cells fulfill stringent stem cell criteria in serial transplantations

Self-renewal and differentiation are the two key properties of any class of stem cells, however stem cell properties of human adult BM-MSCs had not been demonstrated thoroughly. Based on the results of the prior two papers, in the current study we aimed to investigate *in vitro* and *in vivo* stem cell properties of the lin^{-}/CD45^{-}/CD271^{+}/CD140a$^{low/-}$ bone marrow stromal cell population by employing novel mesensphere and standard CFU-F assays. Single cell Fluidigm analysis of lin^{-}/CD45^{-}/CD271^{+}/CD140a$^{low/-}$ cells was performed to study heterogeneity of this putative stromal cell population. The results demonstrated homogeneous mRNA expression levels of hematopoietic supporting genes (CXCL12 and ANGPT), the majority of MSC associated genes (LEPR, VCAM1, BMP5 and VEGFC) and most of the differentiation genes (ALPL, LPL and PPARG). Additionally, principal component analysis (PCA) showed that CD271^{+}/CD140a$^{low/-}$ cells formed a distinct population compared with non-colony forming CD271^{-} population. Next, we evaluated the *in vitro* clonogenic potential of freshly isolated lin^{-}/CD45^{-}/CD271^{+}/CD140a$^{low/-}$ cells by utilizing standard CFU-F assay and novel non-adherent mesensphere assay and comparable colony frequencies were obtained with both assays (19.3 ± 2 and 17.5 ± 2.3 CFU-F and spheres per 100 plated cells, respectively). In order to test whether both assays identified the same population of clonogenic cells, crossover replating experiments were performed and the results showed comparable capacities of

CFU-Fs and mesenspheres to form secondary and tertiary colony generations. *In vitro* self-renewal as indicated by increasing numbers of CFU-Fs and spheres was observed up to the third generation and decreased thereafter. Furthermore, CFU-Fs and mesenspheres displayed a typical surface marker profile (CD73, CD105, CD90 and HLA-ABC positive; CD31, CD34 and HLA-DR negative) and comparable *in vitro* tri-lineage differentiation potential (osteoblasts, adipocytes and chondrocytes). Next, to study *in vivo* self-renewal and differentiation potential of this putative stromal cell population, CFU-Fs and mesenspheres derived from freshly sorted cells were serially transplanted subcutaneously into NSG mice. After 8 weeks, implants were removed, human cells were FACS-isolated (based on CD90 and CD105 expression) and re-transplanted under CFU-F and mesensphere conditions. CFU-Fs did not show self-renewal, whereas mesenspheres *in vivo* self-renewal was demonstrated by increased numbers of mesenspheres recovered after primary and secondary transplantations (1.16 ± 0.06 and 2.34 ± 0.13 – fold, respectively). Moreover, $lin^{-}/CD45^{-}/CD271^{+}/$ $CD140a^{low/-}$ -derived mesenspheres displayed *in vivo* differentiation capacity into bone, fat and stromal tissues in primary and secondary transplantations. Taken together, our results demonstrated that human $lin^{-}/CD45^{-}/CD271^{+}/CD140a^{low/-}$ cells cultured as non-adherent mesenspheres are capable of *in vivo* self-renewal and differentiation and therefore meet stringent stem cell criteria.

Paper IV

Comparative gene expression analysis of primary human bone marrow mesenchymal stromal cells and their *in vitro* descendants (generated in adherent and sphere cultures)

In paper III, we have provided evidence that mesenspheres, but not adherent cultures preserve the *in vivo* self-renewal capacity of BMSCs in serial transplantations[62]. This important functional difference between the two culture methods prompted us investigate the gene expression profiles of BM-MSCs-derived mesenspheres and adherent cells and compare them with their primary isolated counterparts. Gene expression array analysis of primary $lin^-/CD45^-/CD31^-/CD71^-/CD235a^-/CD271^+$ cells and MSCs derived from these sorted primary cells in adherent and sphere cultures was performed. A considerable number of genes was differentially expressed when comparing adherent and non-adherent MSCs. Moreover, many genes were found to be different in primary cells compared to both adherent and sphere cultured cells, whereas expression of only a fewer number of genes in primary cells was similar to either adherent cultured or sphere cultured MSCs. Interestingly, culturing the cells for longer periods of time (passage 3) did not induce more pronounced changes in gene expression profile of adherent and non-adherent-cultured cells. Next, gene cluster analysis was performed to study possible distinct gene expression patterns in different cultured and primary cells. Among 16 generated clusters, 8 of them showed up-regulated or down-regulated genes in primary cells compared to both types of cultured cells. Therefore these clusters reflected culture-induced gene expression alterations. In other gene clusters, similar gene expression levels in primary

cells and either adherent or sphere-cultured cells were observed. Based on our recent finding that demonstrated the advantage of sphere-cultured MSCs over adherent-cultured cells in preserving stem cell properties, we focused on two of the clusters (clusters 3 and 5) that represented the genes with similar expression levels in primary and sphere-cultured cells, while they were different in adherent-cultured cells. Gene ontology analysis of these two clusters revealed a diverse range of annotated biological functions. In both clusters several transcription factors that have been reported to play a role in MSC regulation were identified, such as FOS, EGR1, ATF4 and NFE2L. These genes can thus be considered as potential candidates for further investigations in order to understand the mechanisms that regulate BM-MSCs properties. Taken together, our data showed a significantly different molecular signature between BM-MSC-derived adherent and non-adherent cells. Furthermore, we observed an immediate change of molecular profile of primary BM-MSCs upon culture. Potential regulators found by cluster analysis are important candidates aiming to uncover gene regulation mechanisms that govern biological properties of human adult BM-MSCs.

Discussion

Human bone marrow mesenchymal stromal cells (BM-MSCs) are a rare population of non-hematopoietic cells which are important constituents of hematopoietic stem cell niche and play a central role in regulation and maintenance of hematopoiesis. During recent years, extensive studies have been done on different aspects of BM-MSC biology. However, still uncertainties remain regarding the phenotypic signature and functional properties of primary isolated human BM-MSCs. Moreover, most of our knowledge about BM-MSC properties has been gained from culture expanded cells which may not reflect the properties of native MSCs and little is known about their characteristics compared to their primary counterparts. Therefore, in the current thesis we aimed to gain new insight into the field of MSC biology by characterizing human primary BM-MSCs and studying their functional properties. Here, we are introducing a new phenotype for the prospective isolation of a close to pure population of human primary BM-MSCs and we provide evidence that this cell population meets stringent stem cell criteria. Furthermore, we demonstrate the molecular signature changes of BM-MSCs which occur early upon culture, which is an important finding and emphasizes the significance of studying the cells in their native primary state.

In this chapter a more general discussion, including the relevance and limitations of the current work in regard to what is known in the field is presented. For a more detailed discussion of the individual data sets the

reader is referred to the discussion sections of the manuscripts included in this thesis.

Characterization of human primary BM-MSCs

Conventional approach for isolation of BM-MSCs relies on the expansion of unfractionated bone marrow mononuclear cells based on their plastic adherence property. The main disadvantages of this approach include non-specificity which allows the expansion of undesired adherent cells in the culture, and more importantly, phenotypic and gene expression profile alterations of the cells upon culture, as it has been reported by us and others[48-50, 126]. Therefore, it is certainly preferable to study prospectively isolated cells based on the expression of selective surface markers when aiming to characterize native MSCs in their physiological state.

To fulfill this aim and considering that the precise phenotypic definition of human BM-MSCs was unknown when we started with our experiments, we applied comparative gene expression analysis to identify a new phenotype, which would allow to effectively purify the human BM-MSC population. Although, several surface molecule antigens have been reported for prospective isolation of human primary BM-MSCs, including CD271[38], CD105[41], CD106[40], CD146[37], Stro-1[36], CD31[46], CD45[47] and CD44[48], none of them is specific for human BM-MSCs. Previously Churchman *et al.*[50] and Tormin *et al.*[49] had shown that all CFU-F colony forming cells in the human BM were exclusively enriched in the non-hematopoietic $CD271^{+}$ fraction, but the CFU-F frequency in this population was only 5% which indicates a high percentage of non-colony forming cells in this cell population. In order to

enrich the colony forming population, combinations of other candidate markers, such as CD140b, CD56 and CD146, with CD271 were reported before[49, 126]. None of these marker combinations could enrich the population significantly, however they could help to discriminate functionally different subsets of MSCs. Using array-based global gene expression analysis we identified CD140a as a candidate surface marker that in combination with CD271 enriched the clonogenic stromal cell population significantly. We demonstrated that low/negative expression of CD140a on $CD271^{+}$ primary stromal cells identified a close to pure population of human BM-MSCs with a 20% frequency of colony forming cells. A similar CFU-F frequency was reported by using combination of CD271 and CD90 markers for isolation of human BM-MSCs[45], however these authors collected the samples from crushed femur heads which has a poor hematopoietic activity in human adults, whereas we isolated the stromal cells from aspiration of iliac crest BM, which is an active hematopoietic site. The percentage of clonogenic cells that we obtained almost matched the ones reported for other types of adult stem cells, like HSCs. In order to better characterize $CD271^{+}/CD140a^{low/-}$ cells and understand a possible heterogeneity of this population, we performed single-cell gene expression analysis (Fluidigm) and studied the expression of a panel of selected MSC-relevant genes. The cells expressed quite homogeneously the majority of genes associated with hematopoietic support and MSC function at single cell level, which was in accordance with the reported data on bulk sorted $CD271^{+}$ stromal cells[50]. The developmental basis for the origin of primary human BM-MSCs is poorly understood. In mice, two sources, including mesoderm and neural crest, have been suggested as the origin of BM-MSCs[25]. To study the developmental origin of the stromal cells, some selective mesodermal and neural crest

markers were tested, however they showed low levels of expression which may be explained by the fact that these genes are mainly master regulators of mesoderm and neural crest specification during the embryonic period and that they may get down-regulated in the adult cells. In this regard, fetal bone marrow MSCs seems to be a more appropriate material to map the developmental origin of bone marrow stromal cells. Identification of a highly enriched population of BM-MSCs was a critical step towards the next studies with the aim of investigating the stem cell characteristics of stromal cells in human adult BM.

BMSCs and their progeny in the hematopoietic niche

Mesenchymal or skeletal stem cells are multipotent cells that are capable to differentiate into skeletal lineages. Osteoblastic cells were the first mesenchymal population that was proposed as a niche cell type based on *in vivo* mouse studies which showed that genetic modification of osteoblasts change the HSC number and influence the hematopoiesis[76, 77]. More recent studies have assigned specific roles for different subtypes of osteoblasts in the regulation of hematopoiesis[79, 97]. Another mesenchymal niche candidate are perivascular cells that are located around blood vessels and are marked by expression of Nestin[60], LEPR[100], Mx-1-Cre[127] in mouse and of CD146[37] and CD271[49] in human bone marrow. In our studies we have demonstrated the capacity of human $CD271^{+}/CD140a^{low/-}$ cells to form bone and stroma *in vivo*, however in situ studies in order to detect the localization of this population in the BM were not performed.

Are BM-MSCs stem cells?

Although human BM-MSCs have been extensively called “stem cells” in the literature, their stem cell properties were not investigated thoroughly. Mendez-Ferrer *et al*[60] proved that mouse Nestin^{+} -derived mesenspheres can self-renew and generate hematopoietic activity *in vivo*, and therefore meet the requirements to be considered as stem cells. In humans, Sacchetti *et al*[37] reported that CD146^{+} -derived CFU-Fs are self-renewing stem cells and capable of generating *in vivo* hematopoietic microenvironment. This was the first study defining the stem cell properties of human adult BM-MSCs. However, we believed that some important points were not addressed in the Sacchetti *et.al.* study[37]. First, they did not perform serial transplantation experiments and all the *in vivo* results were obtained from the primary heterotopic transplantation assay. Additionally, Sacchetti *et al* compared *in vivo* generated colony numbers with pre-expansion culture input CFU-F values, which led to an underestimation of the actually transplanted number of colonies. Later, Pinho *et al*[61] showed that human fetal bone marrow CD51^{+}/CD140a^{+} -derived mesenspheres have self-renewal capacity and are capable to transfer hematopoietic niche activity *in vivo*.

In order to investigate these properties in human adult bone marrow, we therefore investigated the *in vivo* self-renewal and differentiation potential of single-cell- and bulk-sorted CD271^{+}/CD140a$^{low/-}$ BMSCs in serial xenotransplantation experiments to study the stemness properties of this putative BM-MSC population in a rigorous manner. In our work, when we calculated the CFU-F content of culture-expanded cells before transplantation, implantation of adherently cultured CFU-Fs resulted in a more than 100-fold reduction of colony numbers after primary

transplantation, which was in contrast to the Sacchetti *et al* results. This is most probably due to the different approach to calculate the CFU-F content of transplanted cells, as it is mentioned above. In parallel to standard CFU-F assay, we investigated the potential stem cell properties of this population through non-adherent mesensphere assay, which was employed in mouse studies[60] and established in a human study by us[128] and showed to be capable to preserve MSCs in an immature condition. Through sphere assay and serial transplantation experiments, for the first time, we could provide definitive proof that primary human adult BM-MSCs fulfill stem cell criteria, i.e. *in vivo* self-renewal and differentiation capacity, in accordance with what has been demonstrated for mouse and human fetal MSCs using sphere transplantation approach. The obvious functional advantage of non-adherent mesenspheres over adherent cultured cells that we observed in our results encouraged us to compare the molecular signature of these two different types of cultured cells and with primary BM-MSCs, aiming to identify potential regulatory molecules that induce functional differences. Interestingly, we found distinct clusters of genes being differentially expressed between mesenspheres and adherent MSCs. We chose two clusters which had a similar gene expression level between primary cells and mesenspheres and were different in adherent cells, and therefore might contain genes that are involved in preserving stem cell characteristics in mesensphere cultures, for more detailed analysis and identified several potential regulatory molecules relevant to BM-MSC properties. These candidate markers are important clues for future studies aiming to identify regulatory mechanism of different physiological properties of BM-MSCs, such as self-renewal, differentiation, proliferation and hematopoietic support function.

Sphere assay

The sphere-forming assay as a non-adherent culture condition to study stem cells from different sources has been applied widely in stem cell biology. For the first time, this method was used in the neural stem cell field when the sub-ventricular zone (SVZ) of the brain was dissected and primary cells were plated in non-adherent conditions and formed floating spheres, called neurospheres[129]. Later, the sphere assay was used to investigate stem cells derived from different tissues, such as breast[130], prostate[131], pancreas[132] and retina[133], as well as in cancer stem cell research[134]. Although the sphere assay appeared to be a proper retrospective assay to evaluate functional characteristics of stem cells and may provide readout of the stem cell or progenitor cell pool size in the tissue, some limitations of this method which may lead to a misinterpretations of the data should be taken into consideration. The main principle of the sphere assay is clonality, meaning that each sphere has been generated from a single cell. Thus, the plating cell density is the major factor that impacts on clonality[135]. A wide range of cell densities has been suggested by different groups (from 0.2 to 20 cells per microliter), and at least for neurosphere assays the plating density of 1-10 cells per microliter has been suggested as a reliable cell density[136]. In our research, we either generated the spheres from single-cell sorted cells or in case of bulk-sorting, we employed the density that was recommended for mesensphere formation in the literature[60, 61] (<500 cells/cm^2). Besides, the sphere cultures should be left untouched to reduce the chance of cell aggregation and in the optimal condition it should be verified that spheres are formed as a result of cell proliferation and not cell aggregation (e.g. with time-lapse imaging techniques)[135, 136]. Another critical consideration for

sphere assay is that mainly actively dividing cells respond to growth factors and cytokines present in the medium and dormant stem cells which are predominantly in G_0 state may not be detected by this method. Additionally, not only stem cells but also amplifying progenitors can give rise to spheres and therefore, considering of sphere forming assay as a readout of *in vivo* genuine stem cell frequency may be misleading[135]. In our research, since the hierarchy of stromal stem and progenitor cells in the bone marrow and their phenotypic profile is not well-defined yet, at this point we cannot claim that whether our results are showing genuine stem cell or multipotent progenitor activities, or both. Additional studies are needed to map the hierarchy of BM stroma compartment and provide the precise definition of each population and their functional properties, comparable to what has been extensively done in the hematopoietic system.

Concluding remarks

A considerable number of publications about human BM-MSCs can be retrieved from databases, which are mainly performed on culture expanded MSCs and thus indicate some biological features or therapeutic potential of cultured stroma cells. Mainly due to the rarity of human BM stromal cells and limited knowledge about their precise phenotypic definition, only a few numbers of studies have investigated the biological and functional properties of BM-MSCs in their native state (primary or bona fide BMSCs). This thesis presents several studies that provide important information for better understanding of biological characteristics of this native human bone marrow stromal compartment and pave the way for future studies towards revealing

the detailed mechanisms that regulate the different properties of human BM-MSCs.

Acknowledgments

I would like to express my sincere appreciation to all the people who supported and encouraged me throughout the last five years and I believe that without their help and contribution this thesis would not have been possible.

First and foremost, I am very grateful to my main supervisor, **Stefan Scheding**, for giving me the opportunity to join his group and for all the endless support over the period of my PhD studies. Your scientific enthusiasm and passion has been always inspiring. Many thanks for all the constructive discussions and critical feedbacks that have definitely been helpful in my scientific career development.

I would like to thank my co-supervisors, **Jonas Larsson** and **Gunnar Juliusson**. Your valuable feedbacks on my projects during the TAC meeting and half-time review helped me to improve my work.

I would also like to express my gratitude to **Simón Méndez-Ferrer**. I really appreciate you for sharing your great knowledge and all the scientific inputs to my projects. Thanks for letting me to come to your lab and for a very warm welcome in Madrid!

My friends in the lab, I was lucky to have you and I will miss you! **Hongzhe**, you have been a real supportive and kind friend throughout this journey. I have learnt many things from you through our scientific and non-scientific talks☺, thanks for everything! **Dimitra**, we shared our office for almost four years and you have been such a wonderful roommate and friend, thanks for

your kindness and support. **Hooi Ching**, I have had a lot of great moments with you, thanks for all your help inside the lab and organizing enjoyable dinners outside. **Sara**, you have been so kind to me all the time and special thanks for helping me with Swedish popular summary. **Marie**, thanks for taking care of many things in the lab and making everything go smoothly. **Anke** and **Franziska**, thanks for your contribution to nice work environment. I would also like to thank former members of the group, **Arshad** and **Ingbritt**.

I want to specially thank my former supervisor in Tübingen, Germany, **Hans-Jörg Bühring**, for providing me the opportunity to come to your lab and for all your guidance and encouragements. Also, special thanks to **Kavitha** and **Abhishek**, who helped me a lot during the eight months staying in Tübingen.

My friends in B10 (former and present), **Isabelle**, **Tania**, **Francesca**, **Andreas**, **Giedre**, **Tamar, Monni, Daniel**, **Ruimin**, **Cecilia**, **Qianren**, **Jonas** and **Isaac**, thanks for creating such a wonderful work environment. I would also like to thank the past and present administrative staff in B10, **Märta**, **Mats** and **Kicki**, for all your help and a very special thanks to **Claire**, you helped me to organize the dissertation ceremony and also kindly accepted to proof-read my thesis in such a short notice, thank you so much!

I would also like to thank **all the people in B12** who have made a very stimulating and encouraging environment. Special thanks to **Göran Karlsson** and **Eva** for helping me with Fluidigm, **Shamit Soneji** for great help with data analysis, FACS facility members **Zhi** and **Teia** for your endless support, **Ewa Sitnicka** and your former group members for shared meetings and journal clubs in B10, **Alya** for your support and help, **Lilian** for all your

kindness, **Mojgan** for your company and our conversations in Persian! and **Hanna** for always being available to help with administrative issues.

My friends in A12, **Roksana**, **Mehrnaz**, **Xiaojie**, **Shub**, **Abdul**, **Sandra** and **Simon**, thanks for your help and support. Special thanks to **Christine Karlsson**, for helping me to organize my dissertation.

My dear friends outside Sweden, **Jila**, **Behnaz**, **Golsima**, **Nahid**, **Leila**, **Parisa**, **Nazanin**, **Mani**, and **Sheida**, you have always been in touch and around me, it means a lot to me, thanks!

And finally, above all, I would like to express my appreciation to my dear family, my mum **Homa**, my brother **Reza** and my sister **Ghazal**, thanks for your unconditional love and support. You made it possible for me to finish this step in my life. Also thanks to my dear **Haleh**, **Amir**, **Mahmoud**, **Mohsen** and **Ali**, for your great support and encouragements throughout these years.

References

1. Carrel, A. & Burrows, M.T. Cultivation of adult tissues and organs outside of the body. *JAMA* **55**, 1379 (1910).
2. Berman, L., Stulberg, C.S. & Ruddle, F.H. Long-term tissue culture of human bone marrow. I. Report of isolation of a strain of cells resembling epithelial cells from bone marrow of a patient with carcinoma of the lung. *Blood* **10**, 896-911 (1955).
3. Tavassoli, M. & Crosby, W.H. Transplantation of marrow to extramedullary sites. *Science* **161**, 54-56 (1968).
4. Friedenstein, A.J., Chailakhjan, R.K. & Lalykina, K.S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* **3**, 393-403 (1970).
5. Friedenstein, A.J., Chailakhyan, R.K., Latsinik, N.V., Panasyuk, A.F. & Keiliss-Borok, I.V. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation* **17**, 331-340 (1974).
6. Friedenstein, A.J. Precursor cells of mechanocytes. *Int Rev Cytol* **47**, 327-359 (1976).
7. Friedenstein, A.J., Chailakhyan, R.K. & Gerasimov, U.V. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* **20**, 263-272 (1987).
8. Owen, M. & Friedenstein, A.J. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* **136**, 42-60 (1988).
9. Castro-Malaspina, H. *et al.* Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* **56**, 289-301 (1980).
10. Nagao, T., Komatsuda, M., Yamauchi, K. & Arimori, S. Fibroblast colonies in monolayer cultures of human bone marrow. *J Cell Physiol* **108**, 155-161 (1981).
11. Caplan, A.I. Mesenchymal stem cells. *J Orthop Res* **9**, 641-650 (1991).
12. Bianco, P. "Mesenchymal" stem cells. *Annu Rev Cell Dev Biol* **30**, 677-704 (2014).
13. Horwitz, E.M. & Keating, A. Nonhematopoietic mesenchymal stem cells: what are they? *Cytotherapy* **2**, 387-388 (2000).
14. Horwitz, E.M. *et al.* Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* **7**, 393-395 (2005).

15. Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315-317 (2006).
16. Hass, R., Kasper, C., Bohm, S. & Jacobs, R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal* **9**, 12 (2011).
17. Yoshimura, K. *et al.* Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J Cell Physiol* **208**, 64-76 (2006).
18. Zannettino, A.C. *et al.* Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J Cell Physiol* **214**, 413-421 (2008).
19. Quirici, N. *et al.* Anti-L-NGFR and -CD34 monoclonal antibodies identify multipotent mesenchymal stem cells in human adipose tissue. *Stem Cells Dev* **19**, 915-925 (2010).
20. Kern, S., Eichler, H., Stoeve, J., Kluter, H. & Bieback, K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24**, 1294-1301 (2006).
21. Dmitrieva, R.I. *et al.* Bone marrow- and subcutaneous adipose tissue-derived mesenchymal stem cells: differences and similarities. *Cell Cycle* **11**, 377-383 (2012).
22. Reinisch, A. *et al.* Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. *Blood* **125**, 249-260 (2015).
23. de Almeida, D.C. *et al.* Epigenetic Classification of Human Mesenchymal Stromal Cells. *Stem Cell Reports* **6**, 168-175 (2016).
24. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147 (1998).
25. Isern, J. *et al.* The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *Elife* **3**, e03696 (2014).
26. Martinez-Agosto, J.A., Mikkola, H.K., Hartenstein, V. & Banerjee, U. The hematopoietic stem cell and its niche: a comparative view. *Genes Dev* **21**, 3044-3060 (2007).
27. Seita, J. & Weissman, I.L. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* **2**, 640-653 (2010).
28. Mendes, S.C., Robin, C. & Dzierzak, E. Mesenchymal progenitor cells localize within hematopoietic sites throughout ontogeny. *Development* **132**, 1127-1136 (2005).
29. Wang, X.Y. *et al.* Identification of mesenchymal stem cells in aorta-gonad-mesonephros and yolk sac of human embryos. *Blood* **111**, 2436-2443 (2008).
30. Campagnoli, C. *et al.* Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* **98**, 2396-2402 (2001).

31. Dennis, J.E. & Charbord, P. Origin and differentiation of human and murine stroma. *Stem Cells* **20**, 205-214 (2002).
32. Takashima, Y. *et al.* Neuroepithelial cells supply an initial transient wave of MSC differentiation. *Cell* **129**, 1377-1388 (2007).
33. Matsuoka, T. *et al.* Neural crest origins of the neck and shoulder. *Nature* **436**, 347-355 (2005).
34. Frenette, P.S., Pinho, S., Lucas, D. & Scheiermann, C. Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for regenerative medicine. *Annu Rev Immunol* **31**, 285-316 (2013).
35. Li, H., Ghazanfari, R., Zacharaki, D., Lim, H.C. & Scheding, S. Isolation and characterization of primary bone marrow mesenchymal stromal cells. *Ann N Y Acad Sci* **1370**, 109-118 (2016).
36. Simmons, P.J. & Torok-Storb, B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* **78**, 55-62 (1991).
37. Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**, 324-336 (2007).
38. Jones, E.A. *et al.* Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* **46**, 3349-3360 (2002).
39. Buhring, H.J. *et al.* Novel markers for the prospective isolation of human MSC. *Ann N Y Acad Sci* **1106**, 262-271 (2007).
40. Gronthos, S. *et al.* Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* **116**, 1827-1835 (2003).
41. Aslan, H. *et al.* Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105+ cells. *Stem Cells* **24**, 1728-1737 (2006).
42. Sivasubramaniyan, K. *et al.* Prospective isolation of mesenchymal stem cells from human bone marrow using novel antibodies directed against Sushi domain containing 2. *Stem Cells Dev* **22**, 1944-1954 (2013).
43. Veyrat-Masson, R. *et al.* Mesenchymal content of fresh bone marrow: a proposed quality control method for cell therapy. *Br J Haematol* **139**, 312-320 (2007).
44. Battula, V.L. *et al.* Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. *Haematologica* **94**, 173-184 (2009).
45. Mabuchi, Y. *et al.* LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. *Stem Cell Reports* **1**, 152-165 (2013).
46. Liu, L. *et al.* Ex vivo expansion and in vivo infusion of bone marrow-derived Flk-1+CD31-CD34- mesenchymal stem cells: feasibility and safety from monkey to human. *Stem Cells Dev* **15**, 349-357 (2006).
47. Jones, E.A. *et al.* Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal

stromal cells from human bone marrow. *Cytometry B Clin Cytom* **70**, 391-399 (2006).
48. Qian, H., Le Blanc, K. & Sigvardsson, M. Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. *J Biol Chem* **287**, 25795-25807 (2012).
49. Tormin, A. *et al.* CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood* **117**, 5067-5077 (2011).
50. Churchman, S.M. *et al.* Transcriptional profile of native CD271+ multipotential stromal cells: evidence for multiple fates, with prominent osteogenic and Wnt pathway signaling activity. *Arthritis Rheum* **64**, 2632-2643 (2012).
51. Li, H. *et al.* Low/negative expression of PDGFR-alpha identifies the candidate primary mesenchymal stromal cells in adult human bone marrow. *Stem Cell Reports* **3**, 965-974 (2014).
52. Pittenger, M.F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147 (1999).
53. Woodbury, D., Schwarz, E.J., Prockop, D.J. & Black, I.B. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* **61**, 364-370 (2000).
54. Snykers, S., De Kock, J., Rogiers, V. & Vanhaecke, T. In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem Cells* **27**, 577-605 (2009).
55. Wang, G. *et al.* Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. *Proc Natl Acad Sci U S A* **102**, 186-191 (2005).
56. de la Garza-Rodea, A.S. *et al.* Long-term contribution of human bone marrow mesenchymal stromal cells to skeletal muscle regeneration in mice. *Cell Transplant* **20**, 217-231 (2011).
57. Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J. & Kessler, P.D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**, 93-98 (2002).
58. Dixon, J.A. *et al.* Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. *Circulation* **120**, S220-229 (2009).
59. Silva, G.V. *et al.* Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. *Circulation* **111**, 150-156 (2005).
60. Mendez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**, 829-834 (2010).
61. Pinho, S. *et al.* PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med* **210**, 1351-1367 (2013).
62. Ghazanfari, R., Li, H., Zacharaki, D., Lim, H.C. & Scheding, S. Human Non-hematopoietic CD271pos/CD140alow/neg Bone Marrow Stroma Cells

Fulfill Stringent Stem Cell Criteria in Serial Transplantations. *Stem Cells Dev* (2016).
63. Pardal, R., Ortega-Saenz, P., Duran, R. & Lopez-Barneo, J. Glia-like stem cells sustain physiologic neurogenesis in the adult mammalian carotid body. *Cell* **131**, 364-377 (2007).
64. Le Blanc, K. & Mougiakakos, D. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol* **12**, 383-396 (2012).
65. Keating, A. Mesenchymal stromal cells: new directions. *Cell Stem Cell* **10**, 709-716 (2012).
66. Bernardo, M.E. & Fibbe, W.E. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* **13**, 392-402 (2013).
67. Bianco, P. & Robey, P.G. Skeletal stem cells. *Handbook of adult and fetal stem cells, R.P. Lanza*, 415-424 (2004).
68. Bianco, P., Robey, P.G. & Simmons, P.J. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* **2**, 313-319 (2008).
69. Morrison, S.J. & Scadden, D.T. The bone marrow niche for haematopoietic stem cells. *Nature* **505**, 327-334 (2014).
70. Pleyer, L., Valent, P. & Greil, R. Mesenchymal Stem and Progenitor Cells in Normal and Dysplastic Hematopoiesis-Masters of Survival and Clonality? *Int J Mol Sci* **17** (2016).
71. Garcia-Garcia, A., de Castillejo, C.L. & Mendez-Ferrer, S. BMSCs and hematopoiesis. *Immunol Lett* **168**, 129-135 (2015).
72. Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* **4**, 7-25 (1978).
73. Kiger, A.A., White-Cooper, H. & Fuller, M.T. Somatic support cells restrict germline stem cell self-renewal and promote differentiation. *Nature* **407**, 750-754 (2000).
74. Kai, T. & Spradling, A. An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells. *Proc Natl Acad Sci U S A* **100**, 4633-4638 (2003).
75. Jones, D.L. & Wagers, A.J. No place like home: anatomy and function of the stem cell niche. *Nat Rev Mol Cell Biol* **9**, 11-21 (2008).
76. Calvi, L.M. *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-846 (2003).
77. Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836-841 (2003).
78. Mendez-Ferrer, S., Scadden, D.T. & Sanchez-Aguilera, A. Bone marrow stem cells: current and emerging concepts. *Ann N Y Acad Sci* **1335**, 32-44 (2015).
79. Greenbaum, A. *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* **495**, 227-230 (2013).
80. Kent, D. *et al.* Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. *Clin Cancer Res* **14**, 1926-1930 (2008).

81. Yamazaki, S. *et al.* Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* **147**, 1146-1158 (2011).
82. Taichman, R.S. & Emerson, S.G. Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. *J Exp Med* **179**, 1677-1682 (1994).
83. Qian, H. *et al.* Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell* **1**, 671-684 (2007).
84. Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149-161 (2004).
85. Cassese, G. *et al.* Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol* **171**, 1684-1690 (2003).
86. Akashi, K., Kondo, M. & Weissman, I.L. Role of interleukin-7 in T-cell development from hematopoietic stem cells. *Immunol Rev* **165**, 13-28 (1998).
87. Peschon, J.J. *et al.* Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* **180**, 1955-1960 (1994).
88. Maillard, I. *et al.* Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. *Cell Stem Cell* **2**, 356-366 (2008).
89. Fleming, H.E. *et al.* Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell* **2**, 274-283 (2008).
90. Nombela-Arrieta, C. *et al.* Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol* **15**, 533-543 (2013).
91. Lapidot, T. & Petit, I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp Hematol* **30**, 973-981 (2002).
92. Stier, S. *et al.* Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J Exp Med* **201**, 1781-1791 (2005).
93. Lymperi, S. *et al.* Strontium can increase some osteoblasts without increasing hematopoietic stem cells. *Blood* **111**, 1173-1181 (2008).
94. Ma, Y.D. *et al.* Defects in osteoblast function but no changes in long-term repopulating potential of hematopoietic stem cells in a mouse chronic inflammatory arthritis model. *Blood* **114**, 4402-4410 (2009).
95. Yu, V.W. & Scadden, D.T. Hematopoietic Stem Cell and Its Bone Marrow Niche. *Curr Top Dev Biol* **118**, 21-44 (2016).
96. Nakamura, Y. *et al.* Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells. *Blood* **116**, 1422-1432 (2010).
97. Yu, V.W. *et al.* Specific bone cells produce DLL4 to generate thymus-seeding progenitors from bone marrow. *J Exp Med* **212**, 759-774 (2015).

98. Matsuoka, Y. *et al.* Prospectively Isolated Human Bone Marrow Cell-Derived MSCs Support Primitive Human CD34-Negative Hematopoietic Stem Cells. *Stem Cells* **33**, 1554-1565 (2015).
99. Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977-988 (2006).
100. Ding, L., Saunders, T.L., Enikolopov, G. & Morrison, S.J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457-462 (2012).
101. Kunisaki, Y. *et al.* Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* **502**, 637-643 (2013).
102. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* **12**, 1046-1056 (2010).
103. Yao, L., Yokota, T., Xia, L., Kincade, P.W. & McEver, R.P. Bone marrow dysfunction in mice lacking the cytokine receptor gp130 in endothelial cells. *Blood* **106**, 4093-4101 (2005).
104. Winkler, I.G. *et al.* Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med* **18**, 1651-1657 (2012).
105. Lo Celso, C. & Scadden, D.T. The haematopoietic stem cell niche at a glance. *J Cell Sci* **124**, 3529-3535 (2011).
106. Katayama, Y. *et al.* Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* **124**, 407-421 (2006).
107. Mendez-Ferrer, S., Lucas, D., Battista, M. & Frenette, P.S. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* **452**, 442-447 (2008).
108. Kollet, O. *et al.* Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med* **12**, 657-664 (2006).
109. Miyamoto, K. *et al.* Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization. *J Exp Med* **208**, 2175-2181 (2011).
110. Yokota, T. *et al.* Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* **96**, 1723-1732 (2000).
111. Belaid-Choucair, Z. *et al.* Human bone marrow adipocytes block granulopoiesis through neuropilin-1-induced granulocyte colony-stimulating factor inhibition. *Stem Cells* **26**, 1556-1564 (2008).
112. Miharada, K. *et al.* Lipocalin 2-mediated growth suppression is evident in human erythroid and monocyte/macrophage lineage cells. *J Cell Physiol* **215**, 526-537 (2008).
113. Naveiras, O. *et al.* Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* **460**, 259-263 (2009).

114. Winkler, I.G. *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* **116**, 4815-4828 (2010).
115. Chow, A. *et al.* Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med* **208**, 261-271 (2011).
116. Yin, T. & Li, L. The stem cell niches in bone. *J Clin Invest* **116**, 1195-1201 (2006).
117. Beckmann, J., Scheitza, S., Wernet, P., Fischer, J.C. & Giebel, B. Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. *Blood* **109**, 5494-5501 (2007).
118. Zon, L.I. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature* **453**, 306-313 (2008).
119. Takano, H., Ema, H., Sudo, K. & Nakauchi, H. 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 (2004).
120. Suarez-Alvarez, B., Lopez-Vazquez, A. & Lopez-Larrea, C. Mobilization and homing of hematopoietic stem cells. *Adv Exp Med Biol* **741**, 152-170 (2012).
121. Bonig, H. & Papayannopoulou, T. Mobilization of hematopoietic stem/progenitor cells: general principles and molecular mechanisms. *Methods Mol Biol* **904**, 1-14 (2012).
122. Levesque, J.P. *et al.* Characterization of hematopoietic progenitor mobilization in protease-deficient mice. *Blood* **104**, 65-72 (2004).
123. Abdallah, B.M., Ditzel, N. & Kassem, M. Assessment of bone formation capacity using in vivo transplantation assays: procedure and tissue analysis. *Methods Mol Biol* **455**, 89-100 (2008).
124. Ritchie, M.E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).
125. Sturn, A., Quackenbush, J. & Trajanoski, Z. Genesis: cluster analysis of microarray data. *Bioinformatics* **18**, 207-208 (2002).
126. Harichandan, A., Sivasubramaniyan, K. & Buhring, H.J. Prospective isolation and characterization of human bone marrow-derived MSCs. *Adv Biochem Eng Biotechnol* **129**, 1-17 (2013).
127. Park, D. *et al.* Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell* **10**, 259-272 (2012).
128. Isern, J. *et al.* Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion. *Cell Rep* **3**, 1714-1724 (2013).
129. Reynolds, B.A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710 (1992).

130. Dontu, G. *et al.* In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* **17**, 1253-1270 (2003).
131. Lawson, D.A., Xin, L., Lukacs, R.U., Cheng, D. & Witte, O.N. Isolation and functional characterization of murine prostate stem cells. *Proc Natl Acad Sci U S A* **104**, 181-186 (2007).
132. Seaberg, R.M. *et al.* Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat Biotechnol* **22**, 1115-1124 (2004).
133. Tropepe, V. *et al.* Retinal stem cells in the adult mammalian eye. *Science* **287**, 2032-2036 (2000).
134. Hirschhaeuser, F. *et al.* Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* **148**, 3-15 (2010).
135. Pastrana, E., Silva-Vargas, V. & Doetsch, F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell* **8**, 486-498 (2011).
136. Coles-Takabe, B.L. *et al.* Don't look: growing clonal versus nonclonal neural stem cell colonies. *Stem Cells* **26**, 2938-2944 (2008).