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# Adult Stem and Progenitor Cells

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**Abstract** The discovery of adult stem cells in most adult tissues is the basis of a number of clinical studies that are carried out, with therapeutic use of hematopoietic stem cells as a prime example. Intense scientific debate is still ongoing as to whether adult stem cells may have a greater plasticity than previously thought. Although cells with some features of embryonic stem cells that, among others, express Oct4, Nanog and SSEA1 are isolated from fresh tissue, it is not clear if the greater differentiation potential is acquired during cell culture. Moreover, adult more pluripotent cells do not have all pluripotent characteristics typical for embryonic stem cells. Recently, some elegant studies were published in which adult cells could be completely reprogrammed to embryonic stem cell-like cells by overexpression of some key transcription factors for pluripotency (Oct4, Sox2, Klf4 and c-Myc). It will be interesting for the future to investigate the exact mechanisms underlying this reprogramming and whether similar transcription factor pathways are present and/or can be activated in adult more pluripotent stem cells.

**Keywords** Adult stem cell, Plasticity, Pluripotency

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**Abbreviations**

| | |
|---|---|
| BM | Bone marrow |
| BMSC | Bone marrow stem cell |
| EB | Embryoid body |
| ESC | Embryonic stem cell |
| HSC | Hematopoeietic stem cell |
| iPS | Induced pluripotent stem cell |
| MAPC | Multipotent adult progenitor cell |
| MEF | Mouse embryonic fibroblast |
| MSC | Mesenchymal stem cell |

## 1 Stem Cells: General Concepts

Over the last decade, stem cell research has made significant strides due to important new discoveries both in the embryonic and the adult stem cell field. Stem cells are the most primitive, unspecialized cells in embryonic, fetal or adult tissues. Due to lack of definitive markers, they are generally defined based on three functional properties. First, unlike most specialized tissue-specific cells, stem cells that do not express tissue-specific transcripts, proteins or functions, have the capacity to replicate themselves clonally for many times through symmetrical cell divisions and both daughter stem cells continue to be identical to the unspecialized parent stem cell. Alternatively, in asymmetric stem cell divisions, one of the two daughter cells is identical to the parent stem cell. This proliferating capacity is called long-term self-renewal.

Second, unspecialized stem cells can give rise to specialized cells in general via asymmetric divisions, where one of the two daughter cells undergoes lineage commitment and differentiation under influence of signals inside and outside the cell (cell-intrinsic and -extrinsic factors). The potency of a stem cell is defined based on the number of different specialized cells that can be generated. The zygote and early blastomeres are *totipotent* stem cells that make up a full organism including extraembryonic lineages. A *pluripotent* stem cell can generate all cells of the three germ layers (endodermal, mesodermal and ectodermal layer) as well as the germline, but not the extraembryonic trophoblast. Pluripotent stem cells are present in the inner cell mass of the blastocyst, and can be isolated and cultured in vitro, cells referred to as embryonic stem cells [1, 2]. The more restricted *multipotent* stem cells only give rise to cells of a specific tissue and are often named after the tissue from which they are derived. For example, neural stem cells are self-renewing cells that can differentiate into the two major cell types of the nervous system; neurons and glia. Most of the stem cells from adult tissues are multipotent [3]. Spermatogonial stem cells are an example for *unipotent* adult stem cells, as they can only generate sperm cells [4]. It is well-known that BM, intestine and lung have stem cell populations. Other organs that were thought to be "post-mitotic" and unable to regenerate now have also been shown to contain stem cell populations, including the brain [3], the heart [5] and the kidney [6]. Adult stem cells are essential for continuously

renewing tissues such as the BM, blood and intestine, and play an important role in recovery from injury in tissues.

Third, stem cells and their progeny are able to reconstitute functionally a given tissue upon transplantation in vivo. The best characterized adult stem cell for transplantation with proven therapeutic efficacy is without doubt the hematopoietic stem cell [7]. Transplantation of undifferentiated embryonic stem cells mostly results in the formation of teratomas, tumors composed of cells of the three germ layers [2]. This proves their true pluripotency, but suggests that ESC-based therapies will only be possible with purified, differentiated cell populations.

## 2 Functional Characteristics of Adult BM-Derived Stem Cells

The BM was for many years regarded as the main source of hematopoietic stem cells. Non-hematopoietic stem cells, such as mesenchymal stem cells and endothelial progenitor cells, can also be isolated from the BM compartment. This reflects the complexity of this organ, in which several cell populations cohabit. Intriguingly, during the last 7 years, new, more pluripotent cell populations have been isolated from the BM by several investigators using different experimental strategies. However, it is important to know that there are various ways to prove the true pluripotency of cells. In vitro induced differentiation and analysis of cell-type specific markers is the easiest and most accessible method of analyzing tri-lineage differentiation capacity of cells. However, expression of some more or less tissue specific transcripts or proteins does not prove that the presumed differentiated cells have acquired the same properties of their in vivo counterparts. In general, expression of lineage specific transcripts and proteins can only be seen as a first step to demonstrate lineage specification/differentiation, but demonstrating that the differentiated cells acquired functional characteristics in vitro and more importantly in vivo is required.

As described above, ESC are considered pluripotent. This designation can be demonstrated using different assays. For instance, ESC have the capacity to form embryoid bodies, three-dimensional aggregates that closely resemble the core structure of a post-implantation embryo where spontaneous differentiation into cells of the three germ layers is seen. This is generally regarded as a typical characteristic of pluripotent cells. However, this does not demonstrate that the differentiated cells are functionally equivalent to cells found in tissues of the three germlayers. Likewise, teratomas generated from subcutaneously transplanted ESC do not prove that ESC can promote normal development. The ultimate proof that ESC are pluripotent, i.e., can generate cells of all organs and tissues, can only be obtained by injection of ESC in blastocyst and generation of germ-line competent chimeric mice. The most stringent test for pluripotency is tetraploid complementation: test cells are injected into 4n blastocysts and somatic lineages are only composed of the injected cells, since 4n host cells only form extraembryonic cell types as placental trophoblast [8].

Similar levels of proof for the presence of a classical multipotent stem cell, namely the hematopoietic stem cells (HSC), exist. HSC are characterized by the presence of certain cell surface proteins and transcripts, which is insufficient to demonstrate that the cells in question are indeed functional HSC. HSC can be induced to differentiate in vitro in most, if not all, of the cells in the hematopoietic system. However, no in vitro assay has been developed that can definitively prove that HSC were present. The only method that conclusively demonstrates that cells have HSC characteristics is transplantation and subsequent reconstitution of the hematopoietic system in a lethally irradiated recipient in which no endogenous hematopoietic cells remain.

In this review, we will give an overview of the BM-derived cell populations (Table 1) keeping the remarks listed above for demonstration of cell differentiation and potency of stem cells in mind.

## *2.1 Hematopoietic Stem Cells*

Hematopoietic stem cells (HSC) were first defined in the early 1960s as a population of clonogenic BM cells with the ability to generate myeloerythroid colonies in the spleens of lethally irradiated hosts [9, 10] and reconstitution of all blood cell lineages after injection into secondary hosts [11]. HSC are by far the most extensively studied stem cells, and knowledge gained from these studies has allowed their use in clinical applications for the treatment of hematological disorders and malignancies. HSC can be harvested from BM, peripheral blood or umbilical cord blood. HSC are capable of long-term self-renewal in vivo, and sit atop a hierarchy of progenitors that become progressively restricted to initially multiple and subsequent single blood lineages. Differentiation into fully specialized blood cells of the lymphoid (T,B and natural killer cells) and myeloid lineages (granulocytes (neutrophils, eosinophils and basophils), monocytes-macrophages, erythrocytes, megakaryocytes and mast cells) goes via stepwise differentiation through intermediate, proliferating cell populations that become progressively more restricted in their differentiation potential, which is accompanied by decreased proliferative potential.

More than 40 years of research has yielded great insight into the identity of HSC, but it should be kept in mind that, despite the many studies, many aspects of HSC biology remain to be identified and that, for instance, the HSC from human origin still cannot be isolated to homogeneity. Enrichment for HSC occurs by combining selection based on specific cell surface markers that are expressed on HSC and elimination of cells expressing cell surface markers present on differentiated cells. In the mouse, HSC are enriched as "LSK" cells (lineage negative cells that are Sca1$^{+}$ and c-Kit$^{+}$) or by using antibodies against the SLAM family (CD150$^{+}$, CD244$^{-}$ and CD48$^{-}$) [12, 13]. The expression pattern of surface antigens on HSC differs between species and some markers change depending on the activation state of the cells: mouse HSC are CD34$^{low/-}$, Sca-1$^{+}$, Thy1$^{+/low}$, CD38$^{+}$, c-Kit$^{+}$, Flt3$^{-}$, lin$^{-}$ and human HSC are CD34$^{+}$, CD59$^{+}$, Thy1$^{+}$, CD38$^{low/-}$, c-Kit$^{-/low}$, lin$^{-}$ (Table 1).

**Table 1** Overview of BM-derived stem cell populations

| Celltype | Derived from | Cultured in | Potency in vitro | potency in vivo | Surface markers | Transcription factors for pluripotency |
|---|---|---|---|---|---|---|
| mHSC | Peripheral blood, bone marrow | Co-culture with stromal feeders | Hematopoietic cells | Hematopoietic cells | $Lin^-$, $Sca^+$, $c\text{-}Kit^+$, $CD150^+$, $CD34^{low/-}$, $Thy1^{+/low}$, $CD38^+$, $Flt3^-$ | / |
| hHSC | Peripheral blood, bone marrow, umbilical cord blood | Co-culture with stromal feeders | Hematopoietic cells | Hematopoietic cells | $CD34^+$, $CD59^+$, $Thy1^+$, $CD38^{low/-}$, $c\text{-}Kit^{-/low}$, $Lin^-$ | / |
| mMSC | Bone marrow, almost every tissue | 10% FBS | Mesenchymal cells: osteocytes, adipocytes, chondrocytes | Mesenchymal tissue: bone, fat, cartilage | $CD10^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD49a\text{-}f^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD106^+$, $CD140b^+$, $CD166^+$,$SSEA1^+$ | / |
| hMSC | Bone marrow, almost every tissue | 10% FBS | Mesenchymal cells | Mesenchymal tissue | $CD10^+$, $CD13^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD140b^+$, $CD146^+$, $C\ D271^+$, $CD340^+$, $CD349^+$, $W8B2^+$, $SSEA4^+$, $Stro\text{-}1^+$ | / |
| mMAPC | Bone marrow, muscle, brain | 2% FBS, LIF, EGF, PDGF-BB | Mesoderm endoderm neuroectoderm | Low frequency chimeric mice: endothelium, neural cells, hepatocytes, intestinal epithelium, retina, kidney, lung, hematopoietic cells | $CD45^-$, $CD34^-$, MHC II, MHC $I^{Low}$, $SSEA1^{+l}$, $cKit^+$, $CD9^+$ | $Oct4^+$,$Sox2^-$, $Nanog^-$ |

(continued)

**Table 1** (continued)

| Celltype | Derived from | Cultured in | potency in vitro | Potency in vivo | Surface markers | Transcription factors for pluripotency |
|---|---|---|---|---|---|---|
| rMAPC | Bone marrow | 2% FBS, LIF-EGF-PDGF-BB | Mesoderm<br>Endoderm<br>Neuroectoderm | | $CD45^-$, $CD34^-$, MHC II, MHC $I^{low}$, $CD31^+$ | $Oct4^+$,$Sox2^-$, $Nanog^-$ |
| hMAPC | Bone marrow | 2% FBS, EGF, PDGF-BB | Mesodermal<br>Endodermal | Endothelial | $CD45^-$, MHC $II^-$, MHC $I^{low}$, $CD44^{low}$ | $Oct4^-$, $Sox2^-$ |
| hMIAMI | Bone marrow | 2% FBS | Mesenchymal cells<br>Neuroectoderm<br>Pancreatic cells | Not described | $SSEA4^+$, $CD45^-$, $CD34^-$ | $Oct4^+$, $Rex1^+$ |
| hBMSC | Bone marrow | 17% FBS | Endothelium<br>Hepatocytes<br>Neuroectoderm | Myocardium regeneration | $CD45^-$, MHC $I/II^-$, c-$Kit^{low}$,$CD90^{low}$, $CD105^{low}$ | $Oct4^-$ |
| hUSSC | Umbilical cord blood | Myelocult medium or 30% FCS | Mesenchymal<br>Hematopoietic<br>Immature hepatocytes<br><br>Neuroectoderm: dopamin ergic neurons | Chondrocytes<br>Neural-like cells<br>Hepatocytes<br>Cardiomyocytes<br>Hematopoeitic cells | $CD45^-$, c-$Kit^-$, HLA-$DR^-$, $CD10^{low}$, $Flk1^{low}$ | $Oct4^-$ |
| hAFS | Amniotic fluid | 15% ES-FBS, 18% Chang B and 2% Chang C | Mesenchymal<br>Endothelial<br>Neuroectoderm<br>Hepatocytes | Bone<br>Neuroectoderm | $cKit^+$, MHC-$I^+$, MHC-$II^{low}$, $CD45^-$, $CD34^-$, $CD133^-$, $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$ and $SSEA4^+$ | $Oct4^+$ |

| hMASC | Liver, heart, bone marrow | Mesencult medium and subsequent 2% FBS, PDGF-BB, EGF | Neuroectoderm<br>Mesenchymal<br>Hepatocytes | Not described | $CD13^+$, $CD49b^+$, $CD90^+$, $CD73^{low}$, $CD44^{low}$, $HLA\text{-}ABC^{low}$, $CD29^{low}$, $CD105^{low}$, $KDR^{low}$, $CD49a^{low}$, $CD117^-$, $CD34^-$,$CD133^-$, $CD45^-$, $CD14^-$, $CD38^-$ | $Oct\text{-}4^+$, $Nanog^+$,$Sox2^+$ |
|---|---|---|---|---|---|---|
| m-maGSC | Neonatal and adult testis | ($Stra\text{-}8^+$ sorted) derived with GDNF, expansion on MEFs with LIF | Trilineage differentiation from embryoid bodies | Teratoma formation germline competent chimeric mice | $SSEA1^+$ | $Oct\text{-}4^+$, $Nanog^+$,$Sox2^+$ |
| m-GPR125-MASC | Testis | ($GPR125^+$ sorted) derived with GDNF on testicular stroma, expansion on MEFs with LIF | Trilineage differentiation from embryoid bodies | Teratoma formation<br>Chimeric mice | Not described | $Oct\text{-}4^+$, $Nanog^+$,$Sox2^+$ |
| hVSEL | Umbilical cord blood | Not expanded | Not described | Not described | $CXCR4^+$, $AC133^+$, $CD34^+$, $Lin^-$, $CD45^-$ | $Oct\text{-}4^+$, $Nanog^+$ |
| mVSEL | Bone marrow<br>Mobilized peripheral blood | Co-cultures for differentiation (BM) and expansion (C2C12) | Neuroectoderm pancreatic cells cardiomyocytes | Myocytes<br>Capillaries | $CXCR4^+$, $SSEA1^+$, $Sca\text{-}1^+$, $CD45^-$, $Lin^-$, $HLA\text{-}DR^-$, $MHC\text{-}I^-$, $CD90^-$, $CD29^-$, $CD105^-$ | $Oct\text{-}4^+$, $Nanog^+$ |
| mPre-MSC | Bone marrow | Mesencult medium and mMAPC medium after sort | Mesenchymal cells<br>Hepatocytes<br>Endothelial cells<br>Astrocytes | Hematopoietic cells | $SSEA1^+$ | $Oct\text{-}4^+$, $Nanog^+$, $Rex1^+$ |

Flow cytometry sorting using a combination of the KLS or SLAM phenotype with additional cell surface markers, can enrich HSC to near homogeneity in the mouse [12, 14]. It has been estimated that HSC represent only about 1 out of every 100,000 cells in mouse BM. However, in large part due to the absence of good in vivo reconstitution assays from human HSC, the phenotype of human HSC is yet to be fully determined.

During embryologic development, HSC are derived from the ventral mesoderm [15]. A first wave of blood production in mammals occurs in the yolk sac. During this primitive hematopoiesis, mainly red blood cells are generated that help in oxygenating all the growing tissues of the developing embryo. A second wave of hematopoiesis occurs in an area surrounding the dorsal aorta termed the aorta-gonad mesonephros (AGM) region. It is believed that the cells originating in the AGM region subsequently populate the fetal liver, later the fetal thymus, spleen and finally the BM.

The HSC niche, defined as a specialized microenvironment in different tissues capable of housing and maintaining hematopoiesis, is starting to be characterized [12]. In postnatal animals, where HSC are chiefly present in the BM, individual HSC occupy facultative niches scattered over BM sinusoids (specialized blood vessels that allow cells to pass in and out the circulation) and near the vast endosteal surface (interface of bone and marrow) of the trabecular bone [16]. It is not clear, however, whether these two sites represent separate niches or if perivascular, endothelial cells and endosteal osteoblasts/osteoclasts collaborate in a common niche. HSC constantly circulate from one BM compartment to another (for instance from femur to tibia). It has been hypothesized that recirculation of HSC between one facultative niche may be required for the maintenance of the HSC phenotype. Alternatively, this apparent recirculation between possible different niches may simply reflect the passage of HSC through some of these locations during their migration. The spleen and liver, where HSC are present during fetal live, contain only a few HSC under normal conditions. However, in certain hematopoietic malignancies or other stresses, hematopoiesis can be re-established in these organs, demonstrating that facultative niches that support the long-term maintenance of HSC and hematopoiesis can be re-activated in these organs.

CXCL12, previously termed SDF1, and angiopoietin-1 are some of the factors that regulate HSC maintenance and that are produced by multiple cell types within the HSC niche, including osteoblasts, perivascular and endosteal cells in different regions of the BM [17]. The BM microenvironment is a complex system wherein several factors work together in inducing differentiation or maintenance of HSC self-renewal. Despite the many years of investigation, no single cytokine responsible for HSC self-renewal has been identified. As a result, HSC can only be maintained in vitro with a supportive cellular microenvironment of mixed or cloned stromal cells [18]. Several groups have evaluated the expressed gene profile of different stromal feeders that support HSC in vitro. However, this has not yet yielded sufficient information to allow one to develop a culture system wherein HSC can be maintained or expanded in the absence of feeders but solely supplemented with defined proteins generated by such feeders. In fact, the study of hematopoietic

niches in vivo has demonstrated that cell–cell based signals, such as for instance the Notch pathway, play a significant role in maintaining HSC undifferentiated. Morphogens such as bone morphogenetic proteins, hedgehogs and Wnts, commonly thought of as factors that govern defined steps in development, are a second class of factors that play a major role in HSC self-renewal [19]. Finally, cell intrinsic factors like the activation of specific transcription factors, such as the homeobox genes Hox-A4 and Hox-A3, are also known to govern self-renewal of HSC [20].

Because the hematopoietic system is so well-studied, it can serve as a model system to be applied to define the phenotype and function of other adult stem cells. Prospective isolation of (subsets of) cells and subsequent analysis in well-defined cell culture systems or after transplantation as has been done for HSC is crucial for the characterization of all stem cells. Only this approach will provide insight in the phenotype and developmental potential of other stem cells.

## *2.2 Mesenchymal Stem Cells*

Next to HSC, the BM harbors a second stem cell population that was discovered by the groundbreaking work of Friedenstein in the early 1970s [21–23]. He placed the whole BM into tissue culture flasks, removed the non-adherent cells and characterized the spindle-like adherent colony-forming fibroblast-like cells as rapidly growing cells that can be differentiated by various factors to osteocytes, chondrocytes and adipocytes. Subsequent studies confirmed these findings and demonstrated that these colony forming fibroblasts (CFU-F), as Friedenstein termed them, can, at the clonal level, differentiate to multiple connective tissue types. These cells were then renamed mesenchymal stem cells or marrow stromal cells (MSC). There is no consensus yet concerning their phenotypic and functional characteristics, as preparations of cells generated through adherence and culture differ among species and laboratories.

No single marker or combination of markers is known that can unequivocally identify MSC neither in vitro nor in vivo and there are no quantitative assays to assess the presence of MSC in a given cell population. Currently, MSC are defined by a combination of morphologic, phenotypic and functional properties [24]. Human and rodent MSC are enriched by their preferential ability to adhere to culture plastic. It is hence unavoidable that hematopoietic cells such as macrophages, and endothelial cells or smooth muscle cells, which also adhere to plastic, “contaminate” the cultures. Further enrichment of MSC is obtained by repeated passaging of the mixed cell population, by plating cells at low densities, by exposure to potassium thiocyanate that selectively kills macrophages and other hematopoietic cell types, or by negative selection to exclude hematopoietic cells (CD45, Glycophorin-A) with commercially available columns, flow cytometry sorting or immunomagnetic selection [25–27]. Because culture methods that differ between different investigators, likely select or expand different cell types or sub-populations, the phenotypic

expression of culture expanded MSC varies. Thus, several cell surface antigens have been described that would identify cultured MSC, including CD10, CD13, CD29, CD44, CD49a–f, CD63, CD90, CD105, CD106, CD140b and SB-10 (antibody against CD166) [28, 29]. Cultured MSC do not express antigens found on endothelial (progenitor) cells (CD31), although CD105 is found on EPC, and hematopoietic cells (CD45, CD3, CD14, CD11b, CD19, CD38 and CD66b). Number, differentiation potential and maximal life span of MSC declines with age [30]. The frequency of colony forming fibroblasts from the BM is low but can be enriched 100-fold by positive selection with the Stro-1 antibody, as described by Simmons et al. [31]. After this initial paper, various other surface markers have been used for positive selection of MSC, such as Sca-1, SH3/SH4 (antibodies against CD73), SH2 (antibody against CD105), SSEA1/4, MCAM/CD146, GD2, STRO-1 (binds to tissue nonspecific alkaline phosphatase) and CD271 (low-affinity nerve growth factor receptor) (Table 1) [17, 32–36].

To demonstrate multipotency of MSC, one needs to demonstrate that clonally isolated and expanded MSC differentiate to alizarin red positive osteoblasts, oil-red O-positive adipocytes, and alcian blue positive chondrocytes. However, many studies have used non-clonal isolations, which cannot prove multilineage differentiation at the single cell level. It should also be noted that these in vitro assays correlate poorly with in vivo differentiation assays [37]. In contrast to in vivo studies with HSC, no in vivo assays to assess self-renewal and differentiation properties of freshly isolated or culture-expanded MSC at the clonal level have been developed. In vivo analysis of MSC multipotency is mostly carried out by heterotopic transplantation and only approximately 10% of clonal MSC are able to form bone, stroma, and marrow adipocytes. Although some in vitro studies have suggested that MSC can also differentiate into other mesodermal cell types, such as skeletal and cardiac muscle or endothelial cells, this has not been proven at the clonal level after heterotopic transplantation [38]. Assaying self-renewal of MSCs in vitro is based on sustained growth in culture and on the retention of differentiation properties after multiple population doublings. However, after 20–40 population doublings, depending on the isolate, MSC senesce due to progressive telomere shortening [39]. Long-term expansion of human and mouse MSC induces cell transformations and, after transplantation of these cells in immuno-compromised mice, sarcomas are formed [40, 41]. Demonstration of self-renewal of MSC in vitro and in situ relies on persistent expression of cell surface markers thought to identify primitive MSC. In addition, as little is known about the normal physiological role, the exact tissue location and the development of MSC in vivo, identification of primitive MSC markers is crucial.

Recently, Sacchetti et al. [17] identified MCAM/CD146 as an in situ MSC marker in human BM. MCAM marks self-renewing adventitial reticular cells, a stromal cell type in the subendothelial layer of BM sinusoids. MCAM is also expressed on circulating endothelial progenitors [42] and pericytes [43], an elusive cell type originally defined by its morphology and close contact to endothelial cells in the microvasculature of every connective tissue. In this regard, BM adventitial reticular cells might function as pericytes in the BM sinusoids. Moreover, pericytes

can differentiate into osteoblasts, chondrocytes, adipocytes, smooth muscle cells, a property shared with MSC. As MSC are not only found in the BM but are present in nearly every organ [44], it has been hypothesized that pericytes and MSCs are one and the same cell, which was highlighted in a study by Covas et al. [43]. Further studies will be needed to define fully the differences and similarities in phenotype and differentiation ability between MSC and pericytes derived from different tissues. Lineage tracing studies have suggested that the first wave of MSC may be derived from Sox-1 + neuroepithelium and not from mesoderm, but that during subsequent steps in organogenesis such neuroepitelium-derived MSC are replaced by MSC from multiple developmental origins [45]. These lineage tracing studies have also suggested that some CFU-Fs in BM are derived from neuroepithelium and neural crest precursors. Many groups have described unexpected differentiation of MSC into neural cells [46], cardiomyocytes [47] and pneumocytes [48]. Although some of these studies may have used not foolproof methods to prove such unexpected differentiation [49], the varied developmental origin of MSC may in part account for these results.

In bone marrow, stromal cells serve two functions: providing a supportive microenvironment for HSC and development/maintenance of the sinusoidal network. These properties together with the ability to form mesenchymal structures like bone and cartilage, resulted currently in a large number of clinical trials with BM-derived cells for organ repair and for tissue engineering applications to treat congenital diseases as Osteogenesis Imperfecta, methachromatic leukodystrophy. It has been shown that MSC aid in engraftment of hematopoietic stem cells, they have an immunosuppressive effect in graft vs host disease and could be beneficial in osteoarthritis and cardiac ischemia [50]. These easily generated, maintained and expanded MSC can in addition be used as vehicles for growth factors or drug delivery.

## *2.3 Adult Stem Cells with a Greater Potency*

Since the late 1990s, several reports described surprising properties of adult stem cells that questioned long-held dogmas that, during development, pluripotent cells were specified to ectoderm, endoderm and mesoderm, and that all adult multipotent stem cells hence belonged to a single germlayer and even more a specific tissue, giving rise only to cells of the tissue they reside in. However, a number of reports described that freshly isolated blood or BM derived cells transplanted in recipient animals were able to differentiate into – aside from the expected hematopoietic lineage, and also into various cell types from endodermal (endocrine pancreas, liver, bile ducts) [51–54], ectodermal (epidermis and neural cells) [55, 56] and mesodermal (endothelium, skeletal and cardiac muscle) origin [57–59]. In most of these experiments whole BM populations were utilized. However, a number of studies also evaluated purified HSC and found that their progeny apparently differentiated into liver, lung, gastrointestinal, and skin epithelium [60, 61]. A number of

subsequent papers confirmed these initial unexpected observations, whereas other studies suggested that the degree of lineage switch that occurs in vivo is minimal or non-existent [62, 63]. It should be noted that most studies claiming a possible lineage switch, based this conclusion chiefly on the acquisition of phenotypic characteristics of the new cell type, not acquisition of functional characteristics, nor evidence for real repopulation of an organ different than the hematopoietic system in vivo. One notable exception was the study by Lagasse et al. [61], demonstrating that grafting of as few as 50 wild-type KLS cells in mice with a fatal liver disorder due to a genetic mutation in the FAH gene could correct the liver disease. Subsequent studies have, however, shown that this rescue was not a cell autonomous effect of the HSC, but was due to the fusion between macrophages derived from the HSC and hepatocytes with introduction of a normal copy of the FAH gene in host hepatocytes [64]. Interestingly, the fusion resulted in the partial effacement of the hematopoietic gene program, which is consistent with a cellular reprogramming event. Aside from fusion resulting in an apparent lineage switch of adult cells, transplantation of heterogeneous cell populations comprising two different stem cells, each of which gives rise to the expected tissue, but not to another tissue, may explain at least some of the apparent stem cell plasticity [65–67]. Obviously technical difficulties in proving lineage switch, including false positive immuno-histological assessments, as well as technical difficulties with the use of sex chromosomes to identify donor and host cells, among others, may explain part of the discrepancies between different studies evaluating this phenomenon. However, another possibility is that trans- or de- and re-differentiation can occur. A final possibility is that more pluripotent, less lineage restricted stem cells persist postnatally and are part of the cells that are injected.

A second series of studies also suggests greater potency of adult cells. In these studies, BM cells [68], spermatogonial stem cells [69] or neurospheres [70] are cultured in vitro, and are subsequently shown to have greater differentiation potency. Clarke et al. [71] isolated neurospheres from Rosa mice, cultured the spheres in vitro, and subsequently injected the spheres in the blastocyst. Of the offspring at E11, 12% were partial chimeras in which neurospheres contributed to the CNS, heart, liver, intestine. However no life chimeric offspring was generated and this study could not be replicated by others [72].

In 2002, our group published the isolation via culture of cells we termed multipotent adult progenitor cells or MAPC from mouse and rat BM. We demonstrated that these cells could be expanded without telomere shortening, and could at the clonal level differentiate into mesodermal (endothelium, smooth muscle cells, skeletal muscle and osteoblasts), neuroectodermal and endodermal (hepatocytes) cells [68, 73–77]. Aside from the presence of transcripts and proteins consistent with the specific cell types, functional attribution of the differentiated mesodermal, hepatic and neuroectodermal cells was demonstrated in vitro. Cells were first isolated from human [78] and rodent BM [68], as well as from newborn rodent brain and muscle tissue [79]. The major difference in culture conditions is the need for LIF to isolate and maintain the rodent cells, but not the human cells. Subsequently, MAPC were also isolated from swine [80], and like the human cells, these do not

require addition of LIF to the culture. Since the initial description of the isolation methods, improvements have been made to the culture system, where isolations and cell maintenance are now done at 5% $O_2$. Mouse, rat and human MAPC do not express CD45 and other more mature hematopoietic cell surface antigens, are MHC Class II negative and express low levels of MHC-class I. Mouse MAPC described in 2002 express low levels of SSEA1, whereas those isolated under hypoxic conditions are SSEA1 negative. Recent mouse MAPC isolates are also c-Kit, EpCam, VLA-6 and CD9 positive, but CD34 negative, whereas rat clones are CD31 positive and human clones are $CD44^{low}$. The MAPC population described in 2002 contributed to many somatic tissues after mouse blastocyst injection, although the degree of contribution was low in most chimeric mice and no germ-line transmission was detected [68]. More recent isolates contribute less than 1–5% to E12 mouse embryos, and no significant contribution to life offspring has been detected. Upon transplantation into sub-lethally irradiated NOD-SCID mice, murine MAPC engraft and differentiate to hematopoietic cells, that upon secondary transfer can rescue the hematopoietic system; and in a limited fashion to epithelium of liver, lung and gut [68, 81]. Moreover, undifferentiated human and mouse MAPC contribute to endothelium, smooth muscle and skeletal muscle when grafted in an ischemic limb model, where they also improve limb function via the secretion of trophic factors [74]. A similar trophic effect has also been noted for mouse and swine MAPC grafted in an acute myocardial infarct model [82, 83].

Comparative transcriptome analysis of MAPC, MSC and ESC showed that MAPC cluster closer to ESC and are significantly different from MSC and MSC-like cells (cells isolated under MAPC conditions that do not express detectable levels of Oct4) [84]. The rodent MAPC gene signature was remarkable for the finding that a number of early endodermal transcription factors are expressed, whereas MSC only express mesoderm specific transcripts. Rat and mouse MAPC express Oct-4, a gene known to maintain pluripotency in ESC, at levels between 5% and 20% of murine ESC. Aside from Oct4 and Rex-1, MAPC also express a number of ESC associated genes (Ecats) but they do not express Nanog and Sox2, two other genes known to play a significant role in the maintenance of the pluripotency transcriptional network in ES cells [85, 86]. Of note, when Nanog is suppressed in ESC using shRNA mediated knock-down, a similar expression of endoderm specific transcripts as is seen in MAPC can be detected. Moreover, there is mounting evidence that Nanog expression fluctuates in ESC [87] and lower levels of Nanog may tip the balance towards differentiation rather than staying pluripotent [88]. Although these results suggest that the presence of ESC specific transcripts may be responsible for the greater potency of MAPC than MSC, studies wherein Oct4 is knocked-down will be needed to prove this notion. Gene expression profiling also identified cell surface markers that could be used for prospective isolation of MAPC such as c-Kit and PDGF-R$\alpha$.

Since the isolation of MAPC, multiple groups have reported isolation of more pluripotent stem cells not only from rodent and human BM [33, 89–91], but also from heart, liver [92], umbilical cord blood [93–95], dermis [96], hair follicles [97], amniotic fluid [98] and skeletal muscle [99, 100]. Methods used for isolation of

these cells were in general relatively similar, even though the $O_2$ tension in the incubator chamber varied between 3 and 20% $O_2$, the serum concentration used ranged from 2 to 20%, and in many instances no other growth factors were added apart from serum; cell densities used differed as well. The potential of cells was evaluated by demonstrating acquisition of transcripts and/or proteins of cells from the three germ layers; functional attributes of the differentiated cells in vitro was only assessed in a limited number of studies and in vivo repopulation was seldom tested.

D'Ippolito et al. [89] isolated a population of "pluripotent" cells from BM of people aged 3–72 years, termed marrow-isolated adult multilineage inducible (MIAMI) cells, that differentiate into cells expressing transcripts and proteins found in mesenchymal lineages as well as neural and pancreatic lineages. When maintained at 3% $O_2$, MIAMI cells could be expanded for more than 50 population doublings. MIAMI cells are $SSEA4^+$, $CD45^-$, $CD34^-$, express telomerase and the transcription factors Oct-4 and Rex-1 (Table 1). Yoon et al. [91] reported the isolation of human BM-derived multipotent stem cells (hBMSC) with the capacity to differentiate into cells expressing transcripts and proteins found in cells of the three germ layers (endothelium, hepatocytes and neuroectoderm) in vitro. hBMSCs may also be able to differentiate into cardiomyocytes in vivo. Single cell clones ($CD45^-$, MHC $I/II^-$, c-$Kit^{low}$,$CD90^{low}$, $CD105^{low}$) could be expanded for more than 140 population doublings without loss of telomere length but did not express the transcription factor Oct-4. Kogler et al. [93] isolated similar cells by culturing umbilical cord blood, naming the cells unrestricted somatic stem cells (USSC). USSC are $CD45^-$, c-$Kit^-$, HLA-$DR^-$, $CD10^{low}$ and $Flk1^{low}$, can be expanded for more than 40 population doublings and were shown to differ from MSC based on their immunophenotype, telomere length, mRNA expression and differentiation capacity. USSCs give rise in vitro to mesenchymal cells (osteoblasts, chondroblasts, adipocytes) and cells with protein expression pattern and some functional attributes of neuroectodermal and hepatic cells. USSCs grafted in utero in pre-immune sheep differentiated into chondrocytes, neuron-like cells, and contributed to a low extent to cardiomyocytes and hematopoietic cells. Similar cells were also isolated by de Coppi et al. [98] by culture of human amniotic fluid cells (AFS cells). C-Kit positive cells were selected from human amniocentesis specimens and can be maintained in culture for more than 250 population doublings without karyotypic instabilities and telomere shortening. Clonal lines express Oct4 and are further MHC-$I^+$, MHC-$II^{low}$, $CD45^-$, $CD34^-$, $CD133^-$, $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$ and $SSEA4^+$. In vitro, AFS cells were shown to differentiate into mesenchymal, endothelial, neuronal and hepatic lineages. Finally, Beltrami et al. [92] published that multipotent adult stem cells (MASC) could be isolated by culture of human BM, cardiac and liver derived cells. MASC express Oct4, Nanog and Sox2, expand without telomere shortening for 40 population doublings and at the clonal level differentiate into cells with phenotypic and functional attributes of several mesodermal cell types, hepatic cells and neuroectodermal cells.

Another example wherein lineage restricted stem cells gained greater potency are spermatogonial stem cells, cultured in vitro. In 2004, Kanatsu-Shinohara et al. [69] demonstrated for the first time that when neonatal spermatogonial stem cells

were cultured for 4–7 weeks in vitro in the presence of bFGF, EGF, LIF and GDNF, approximately 3% of the cells generated ESC-like colonies that could be maintained in ESC conditions (on MEF in medium with 15% FCS and LIF). These multipotent germ stem cells (mGS) express Oct4, Nanog and Rex1 and could form teratomas and germ-line chimeric mice. Subsequently Guan et al. [101] showed that culture of highly purified spermatogonial stem cells (Stra8$^{+}$) from adult mouse testis with GDNF and subsequently on MEF with LIF yielded cells that had all attributes of ESC: EB formation, teratoma formation, and germ-line competent contribution to chimeric mice. These cells were termed maGSC, multipotent adult germline stem cells. In 2007, the group of Rafii [102] found that spermatoginal stem cells selected based on the expression of GPR125, cultured on mouse testicular stromal cells with GDNF, generate after 2–3 months GPR125$^{+}$ multipotent adult spermatogonial derived stem cells (GPR-125-MASC), ESC-like cells that can be maintained and expanded in ESC conditions on MEF. GPR-125-MASC express Oct4, Nanog and Sox2 but not other ESC transcripts as Gdf3 and Rex1. GPR-125-MASC form EBs, teratomas and contribute in part to chimeric mice. Hence, these spermatogonial derived multipotent stem cells are the only postnatal derived stem cells with all pluripotency features of ESC.

A third set of studies suggest that cells expressing gene transcripts responsible for the pluripotency of ESC, such as Oct4, Nanog and Sox2, and cell surface antigens found on ESC, such as SSEA1 and SSEA4, may be isolated from fresh BM or umbilical cord blood. Very Small Embryonic-like (VSEL) cells [94] were sorted from human cord blood as well as mouse BM as CXCR4$^{+}$, AC133$^{+}$, CD34$^{+}$, Lin$^{-}$, CD45$^{-}$ cells. Murine BM-derived VSEL, express Oct-4, Sox2, Nanog. Cells isolated from human express SSEA-4 and from mouse, SSEA1. Whether human VSEL can be expanded is unknown, although mouse VSEL can be expanded as spheres that maintain Oct4, Nanog and SSEA1 expression in co-cultures over C2C12 cells [103]. For mouse VSEL, differentiation to cells with transcripts consistently found in neuroectoderm, pancreas and cardiomyocytes was shown following co-culture with the respective tissues [90]. VSEL only represent 0.02% of the BM mononuclear cells.

Anjos-Afonso et al. [33] demonstrated that SSEA1$^{+}$ primitive cells can be sorted from murine BM. SSEA1$^{+}$ cells could be detected not only in fresh Lineage negative BM but also in BM cells cultured in MesenCult medium for 1–2 passages. SSEA1$^{+}$ cells sorted from both fresh and cultured BM expressed Oct4, Nanog and Rex-1 transcripts and protein, albeit with levels significantly lower than in ESC. SSEA1$^{+}$ cells represented the majority of quiescent ($G_O$) Lineage negative cells and represent only 0.45–0.97% of the total cells. When SSEA1 + cells were plated in Mesencult medium, expression of Oct4, Nanog and Rex1 was lost. By contrast, when cells were maintained in medium also used by Jiang et al. [68] to maintain MAPC, cells retained Oct4, Nanog and Sox2 expression; in fact, the transcript levels of these three transcription factors increased 100-fold. In no instance was Oct4, Nanog or Sox2 expression found when the cultures were initiated with SSEA1 negative cells. One of the clonal cell populations generated under these conditions differentiated in vitro to mesenchymal cell types as well as

astrocyte-, endothelial- and hepatocyte-like cells. When grafted intra-femoral, differentiation to osteocytes, adipocytes, cartilage, endothelium as well as hematopoietic cells was noted. As described for MAPC, expression of for instance Sox17$^+$, PDGF-R$\alpha^+$, and c-Kit$^+$ was found, but in contrast to MAPC a number of mesodermal transcription factors, such as Brachyury$^+$, VE-Cad$^+$, and GATA2$^+$, were also expressed.

The relationship between the different adult "more multipotent" stem cells derived from non-germline tissues is not clear. Despite differences in cell surface phenotype, they are likely all related cell populations. Collaborative studies wherein the relationship between these cells can be assessed using transcriptome and perhaps proteome analysis, as well as by using standardized differentiation studies in vitro and in vivo, will be needed to define the relationship.

## 3 General Conclusions: Multipotent or Pluripotent Adult Stem Cells?

It is well-known that Oct4, together with Sox2 and Nanog, is the major transcription factor that allows maintenance of pluripotency of ESC and loss of Oct4 results in loss of pluripotency [104]. In freshly isolated adult somatic cells, high levels of Oct4 can be detected in germline cells and downregulation of Oct4 leads to apoptosis [105]. A pluripotent state can also be induced in adult somatic cells by forced expression of Oct4 together with Sox2, Klf4 and c-Myc in mouse and human cells [106–108] or in combination with Sox2, LIN28 or Nanog in human cells [109]. These induced pluripotent stem cells (iPS) are almost indistinguishable from ESC. Of the genes that need to be introduced to generate iPS cells, Oct4 and Sox2 are the two key transcription factors that allow de-differentiation of differentiated cells to an iPS state [110, 111].

The finding that Oct4 is also detected in some adult somatic cells and in cancer cells, raises the question whether Oct4 can be used as a marker for pluripotency in adult cells [112]. Although some studies have used immunohistochemistry on histological sections to demonstrate that Oct4 positive cells exist in vivo, this notion should be viewed with care as recent studies have shown that false positive staining is possible. In addition, care should be taken when evaluating Oct4 transcripts in isolated cell populations from human origin because many Oct4 and Nanog pseudogenes exist which are expressed in normal somatic cells and poorly designed primers cannot distinguish between pseudogenes and the specific gene [113]. A recent study evaluated whether Oct4 has a physiological role in postnatal life, employing a conditional Oct4 knockout mouse. This study demonstrated that Oct4 is dispensable for both self-renewal and maintenance of somatic cells from several tissues including intestinal epithelium, BM, skin, brain and liver [114]. In addition, this group also used a second genetically modified mouse, wherein IRES-eGFP was knocked-in behind the fifth exon of Oct4. ESC from these animals are eGFP positive; however, analysis of different somatic tissues did not identify eGFP positive cells. This may

at first sight be inconsistent with the studies from Kucia et al. [90] and Anjos-Afonso et al. [33], who isolated SSEA1/4 positive cells that express Oct4 transcripts and proteins. However, Oct4 levels found in BM derived SSEA1 + cells in the Anjos-Afonso et al. studies were >100-fold lower than in ESC, and the sensitivity of Oct4-IRES-GFP may be too low to yield GFP positive cells in different tissues. Whether low level expression of Oct4 has physiologically relevance is obviously not known. As we hinted earlier in the chapter, Oct4 expression in somatic cells has in general been reported in cultured cells. As most studies demonstrating greater potency of spermatogonial stem cells, that already express Oct4 in vivo, or adult somatic stem cells, coinciding with presence of Oct4, have demonstrated this potential only in cells cultured ex vivo, the possibility exists that the acquisition of potency and/or Oct4 expression may be a culture-induced phenomenon. Alternatively, the possibility exists that during development primitive cells, perhaps pre-gastrulation stage cells, are left in different tissues, which can be enriched by ex vivo culture.

Of note, in the first publication describing iPS cells, the iPS cell lines were not completely reprogrammed to ESC, as shown by gene expression profiling and methylation studies. This first generation of iPS cells formed embryoid bodies and teratomas but could not give rise to postnatal chimeric mice [106]. When reprogramming was allowed to proceed for a longer period of time, iPS cells were highly similar to ESC, again documented by gene expression and DNA methylation [115, 116]. The latter cells form chimeric mice with germ-line transmission [111, 115, 117]. As has also been shown for epiblast cells, that may be slightly more differentiated compared with ESC, the identification of iPS cells that are reprogrammed to an almost ESC state and iPS cells that are reprogrammed to a state indistinguishable from ESC suggests that there are several stages in pluripotency. It is hence possible that the populations of cells isolated from somatic cell cultures may have different degrees of pluripotency, either inherent to the cells that are selected by the culture, or induced by the culture method. Detailed gene expression, epigenetic and genetic studies will be necessary to determine whether transcription factor pathways essential in pluripotent cells like ES and iPS are also present or can be further induced in adult somatic cells with more pluripotent characteristics like MAPC, USSC, MIAMI cells. Regardless of their origin, adult more pluripotent cells may be very valuable as a model for de-,re- and transdifferentiation and form a source of stem cells for cell therapies and drug screening.

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