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Key regulatory genes controlling cell fate decisions of Mesenchymal Stem Cells (MSCs)

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Abstract

Given their biological properties, Mesenchymal Stem Cells (MSCs) represent a potentially precious therapeutic tool for clinical application. However, their optimal use depends on our understanding of the molecular mechanisms governing their expansion and differentiation, still poorly understood.

The kinase receptor Kit and the transcription factor Prep1 are pleiotropic regulators playing key roles in development and differentiation of multiple tissues, including hematopoiesis.

The aim of my study is to investigate whether Kit and Prep1 contribute also to the control of MSCs, particularly in their commitment and differentiation towards the osteogenic and the adipogenic lineages, as MSCs and their progeny, in particular osteoblasts, are essential components of the hematopoietic stem cell niche.

As a first step, the expression profiles at the transcriptional and protein level were analyzed in undifferentiated MSCs, and during *in vitro* osteogenic and adipogenic differentiation. Subsequently, to gain insights into Prep1 function in mesenchymal cells, the effects of its *in vivo* downregulation were investigated by using hypomorphic mice exhibiting low levels of Prep1 product.

The expression studies have shown that Kit and Prep1 are both expressed in undifferentiated MSCs and that their activity is inversely correlated during the adipogenic process. Furthermore, analysis of MSCs derived from a *Kit*/GFP transgenic mouse line indicates that regulatory elements that drive correct kit expression in hematopoietic, germ and cardiac cells

are not sufficient to control kit activity in MSCs. In addition, the functional studies demonstrate that down-regulation of Prep1, while favouring *in vitro* adipogenesis, strongly compromise the osteogenic process, leading cells to apoptosis after osteogenic induction.

Taken together, results indicate that Kit and Prep1 are involved in the regulation of murine MSCs, and provide the first evidence pointing to Prep1 as a crucial player in mesenchymal cell fate decisions.

ABSTRACT ---- **2**

1. STEM CELLS ---- **5**

1.2. ADULT STEM CELLS ---- 9

1.3. STEM CELL NICHE ---- 10

2. MESENCHYMAL STEM CELLS (MSCS) ---- **16**

2.1. MOLECULAR DIFFERENTIATION MECHANISMS ---- 28

2.1.1. Adipogenesis ---- *30*

2.1.2. Osteogenesis ---- *42*

2.2. SIGNALING PATHWAYS GOVERNING MSC OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION ---- 46

2.2.1. Wnt signaling ---- *47*

2.2.2. Hedgehog signaling ---- *50*

2.2.3. NELL-1 signaling ---- *52*

2.2.4. Bone morphogenetic proteins (BMPs) ---- *54*

2.2.4. IGF signaling ---- *57*

3. KIT ---- **60**

4. PREP1 ---- **68**

5. AIM OF THE PROJECT ---- **78**

6. MATERIALS AND METHODS ---- **81**

6.1. MESENCHYMAL STEM CELLS (MSCS) ISOLATION AND EXPANSION ---- 81

6.1.1. Adipogenic differentiation ---- *82*

6.1.2. Osteogenic differentiation ---- *82*

6.1.3. Cytochemical Staining ---- *82*

6.2. RNA EXTRACTION ---- 83

6.3. CDNA SYTNTHESIS ---- 84

6.4. REAL TIME QPCR ---- 84

6.5. PROTEIN EXTRACTION ---- 85

6.6. WESTERN BLOT ---- 86

6.7. FLOW CYTOMETRY ANALYSIS ---- 87

6.8. DETECTION OF APOPTOSIS ---- 87

6.8.1. TUNEL assay: ---- *87*

6.8.2. Annexin V and PI staining ---- *88*

INTRODUCTION

1. Stem Cells

Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut and bone marrow, stem cells regularly divide to replace cells for the normal turn over and repair damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions.

According to their developmental potential, stem cells can be classified in different types. Embryonic stem cells are pluripotent cells as they have the ability to generate all the cells present in the embryo. However they cannot be defined totipotent, like the zygote, as they cannot give rise to extra-embryonic tissues. Adult stem cells have a more limited developmental potential, as they generate mature cells of the tissue in which they reside.1.1. Embryonic Stem (ES) Cells and induced Pluripotent Stem Cells (iPS)

Embryonic stem cells (ESCs) are totipotent cells that can be derived from the inner cell mass (ICM) of a blastocyst during gastrulation. The ICM can be maintained in undifferentiated state, and upon differentiation it gives rise to embryoid bodies in which early embryonic cell lineages develop. ESCs represent a potential source of cells with virtually unlimited self-renewal and differentiation capacity. Since these cells are able to give rise to all of the somatic and germ line cells of the fully developed organism, they are “uncommitted” progenitors of the three

embryonic germ layers: ectoderm, endoderm and mesoderm. The ES cell is the prototype stem cell, as defined by its ability to indefinitely expand, self-renew, and give rise to more specialized progeny cells. Despite the totipotency of ES cells, they cannot be safely transplanted in immunocompromised hosts, because they give rise to teratomas, which are tumors containing multiple tissue types, including fully differentiated structures, such as teeth and hair. Therefore, they are not amenable for therapeutic approaches *in vivo*.

Scientists discovered ways to derive embryonic stem cells from early mouse embryos more than 30 years ago (Evans et al., 1981). The detailed study of the biology of mouse stem cells led to the discovery, in 1998, of a method to derive stem cells from human embryos and grow the cells *in vitro* (hESCs) (Thomson et al., 1998). Six years ago, Takahashi and Yamanaka demonstrated that enforced expression of four key transcription factors, Oct4, Sox2, Klf4, and c-Myc, can reprogram mouse somatic cells such as fibroblasts to pluripotency, and achieve similar developmental potential as ESCs, without the requirement for an embryo (Takahashi and Yamanaka, 2006). They named these new cells ‘‘induced Pluripotent Stem Cells’’ or iPSCs. A year later, several groups, including Yamanaka’s, reported the successful generation of iPSCs from human somatic cells (Park et al., 2008, Takahashi et al., 2007). The expectation that iPSCs will offer the same therapeutic potential as hESCs and the robust and reproducible method of deriving iPSCs have generated hundreds of studies addressing *in vitro* disease modeling and cell therapy strategies in preclinical animal models. iPS cell lines have indeed now been generated from patients of several monoallelic and complex genetic disorders (reviewed in Wu et al., 2011). These developments have brought the field closer to the promises of *in vitro* disease modeling,

disease-specific pharmacological treatment testing, and in some instances individualized cell replacement therapy. Several examples of the differentiation of disease-specific iPSCs into the cell types that are implicated in the disorder's pathogenesis have been reported, and therefore this technology is particularly attractive for the diseases for which animal models are either not available or do not accurately represent the human disease etiology. The technology for generation of induced pluripotent stem cells (iPSCs) has made significant contributions to various scientific fields, and the field of cancer biology is no exception. Although cancer is generally believed to develop through accumulation of multiple genetic mutations, there is increasing evidence that cancer cells also acquire epigenetic abnormalities during development, maintenance, and progression. Because the epigenetic status of somatic cells changes dynamically through reprogramming, iPSC technology can be utilized to actively and globally alter the epigenetic status of differentiated cells. Using this technology, a recent study has revealed that some types of cancer can develop mainly through disruption of the epigenetic status triggered by dedifferentiation (Yamada et al., 2014).

The following question, which is still unanswered, is whether iPSCs can replace ESCs in clinical application and disease modeling. Several analyses indicate that iPSCs share many key properties with ESCs including morphology, pluripotency, self-renewal, and similar gene expression profiles. Comparisons of iPSCs and ESCs have indicated that major features of the ESC epigenome are reproduced in iPSCs, including genome-wide methylation patterns and the establishment of bivalent histone marks at specific *loci* (Guenther et al., 2010, Lister et al., 2011, Meissner, 2010). However, reprogramming assays in mouse cells have shown that differences in gene expression and differentiation potential are

observed specifically in early passage iPSCs and have led to the concept that an ‘‘epigenetic memory’’ of previous fate persists in these cells (Bar-Nur et al., 2011, Kim et al., 2010, Polo et al., 2010, Ghosh et al., 2010, Marchetto et al., 2009). Epigenetic memory has been attributed to the incomplete removal of somatic cell- specific DNA methylation marks at regions in proximity to CpG islands known as ‘‘shores’’ (Kim et al., 2010, Doi et al., 2009). The residual DNA methylation and gene expression pattern of the somatic cell of origin are lost upon serial passaging of the iPSCs, or after treatment with DNA methyltransferase activity inhibitors, suggesting that epigenetic memory contributes to identify cells that are incompletely reprogrammed (Kim et al., 2010, Polo et al., 2010). However, these findings suggest that disease modeling may be influenced by the specific cell type of origin, as iPSCs show distinct cellular and molecular characteristics based on which cell types of origin were utilized. This property may improve the ability to generate specific cell types to be used in cell replacement therapy, such as those cells that are difficult to generate by differentiation from ESCs, including insulin producing pancreatic β cells (Bar-Nur et al., 2011).

In agreement with the epigenetic similarity of the two pluripotent cell types, comparative transcriptome analyses using microarray also indicate that hESCs and hiPSCs are highly alike on a global scale, with gene expression patterns clustering together, and separate from the somatic cells of origin (Plath et al., 2011). However, iPSCs may retain a unique gene expression signature, including that of microRNAs and long noncoding RNAs (Mikkelsen et al., 2008, Chin et al., 2009, Loewer et al., 2010, Stadtfeld et al., 2010, Wilson et al., 2009).

Permanent cell lines of pluripotent ESCs and iPSCs and our increasing ability to direct them into any cell type for therapeutic potential holds

enormous promise for future regenerative medicine. ESCs are considered to be the gold standard of pluripotency, while iPSCs offer the ability to develop cells from any adult individual, which holds the future possibility of curing degenerative diseases using cells or tissue grafts with perfect histocompatibility match.

1.2. Adult Stem Cells

Until recently, scientists primarily worked with two types of stem cells: embryonic stem cells and "adult" stem cells.

Virtually in all adult tissues, discrete populations of stem cells maintain the replacement pool to replenish cells that are lost due to physiological turnover, injury, or disease.

Tissue homeostasis requires the presence of multipotent adult stem cells that are capable of efficient self-renewal and differentiation; some of these have been shown to exist in a dormant, or quiescent, cell cycle state. Such quiescence has been proposed as a fundamental property of hematopoietic stem cells (HSCs) in the adult bone marrow, acting to protect HSCs from functional exhaustion and cellular insults to enable lifelong hematopoietic cell production. Recent studies have demonstrated that HSC quiescence is regulated by a complex network of cell-intrinsic and -extrinsic factors.

Stem cells, which are undifferentiated pluripotent cells capable of extensive proliferation and self-renewal are able to respond to the body needs to expand accordingly to finely tuned mechanisms.

Sometimes they divide after long periods of inactivity, as it is observed in pulmonary tissues, heart and pancreas, for instance. In other organs, such as the gut and the bone marrow, stem cells regularly divide to replace

cells.

Stem cells share the ability to balance the cell fate decision of self-renewal versus differentiation. The mechanisms that regulate cell fate choices have cell autonomous (stem cell intrinsic) and non-cell autonomous (microenvironmental) components. During divisional asymmetry, cell-fate determinants are asymmetrically localized to only one of the two daughter cells, which retain stem-cell fate, while the second daughter cell undergoes differentiation. During environmental asymmetry, after division, one of two identical daughter cells remains in what has been called the "self-renewing niche microenvironment", while the other relocates outside the niche to a different differentiation-promoting microenvironment. The niche also helps to integrate stem cells decisions related to maintaining quiescence versus proliferation, self-renewal versus differentiation, migration versus retention, and cell death versus survival.

1.3. Stem cell niche

The self-renewal and differentiation activity of stem cells is controlled by their surrounding microenvironment, which is known as the stem cell niche. The stem cell niche is still poorly understood, as well as the precise location of the stem cells is not completely unravelled. The most characterized mammalian somatic stem cells are the murine HSCs, which reside in the bone marrow. The bone marrow HSC niche is a complex network. Many studies support the idea that there are at least two HSCs niches: the endosteal niche, in which the HSCs are closely associated with the osteoblasts, and the perivascular niche, where the HSCs are supposedly associated with the sinusoidal endothelium. It is also very

likely that multiple niches exist, and that HSCs are not static, but instead dynamically change their niche location in response to injury or feedback signals (Trumpp et al., 2010). Taking advantage of mice that have received total body irradiation, it has been shown that the niche undergoes a dynamic remodeling process. In addition, it has been shown that some important cellular components of the niches are not restricted to the endosteum or the perivascular niche area, but are part of both environments, raising the possibility that both niches and the location of HSCs may not be as distinct as it is currently assumed. Mesenchymal Stem Cells (MSCs) are among the most important component of the HSCs' niche. Méndez-Ferrer et al., (2010) identified a stromal Nestin-expressing MSC population ($Nestin^+$ MSC) that is closely associated with putative HSCs. $Nestin^+$ MSCs are strictly perivascular and are typically found in more central areas of the marrow, but they are also present near the endosteum, although at lower frequency. $Nestin^+$ MSCs are tightly associated with adrenergic nerve fibers of the sympathetic nervous system (SNS) that regulate HSC mobilization and are responsible for the circadian oscillations in circulating HSC numbers (Katayama et al., 2006; Méndez-Ferrer et al., 2008).

These MSCs express higher levels of HSC maintenance factor transcripts, including stromal cell-derived factor 1 (SDF-1) also known as C-X-C motif chemokine 12 (CXCL12), stem cell factor (SCF), angiopoietin-1 (Ang-1), interleukin-7 (IL-7), vascular cell adhesion molecule 1 (VCAM1), and osteopontin (OPN), as compared to any other stromal cell type including the osteoblasts. Most interestingly, $Nestin^+$ MSCs show several similarities to recently identified mesenchymal adipo-osteogenic progenitors (Sugiyama et al., 2006; Omatsu et al., 2010), discovered in a mouse strain in which GFP is driven by the endogenous CXCL12 *locus*

(Sugiyama et al., 2006; Omatsu et al., 2010). Because of their high CXCL12 expression and their long cellular processes, these cells were named CXCL12-abundant reticular (CAR) cells. The majority of putative HSCs are found in close proximity to CAR cells by immunohistochemistry and, like Nestin^{+} MSCs, CAR cells are predominantly found in the more central areas of the marrow; some being also located near endosteal vessels. Although CAR cells are more abundant than Nestin^{+} MSCs, they are tightly associated with the sinusoidal endothelium and have a similar morphology to vascular pericytes. These data are in agreement with studies in humans, suggesting that virtually all MSC activity is found within the larger pericyte population that associates closely with the vascular system throughout the body (Crisan et al., 2008).

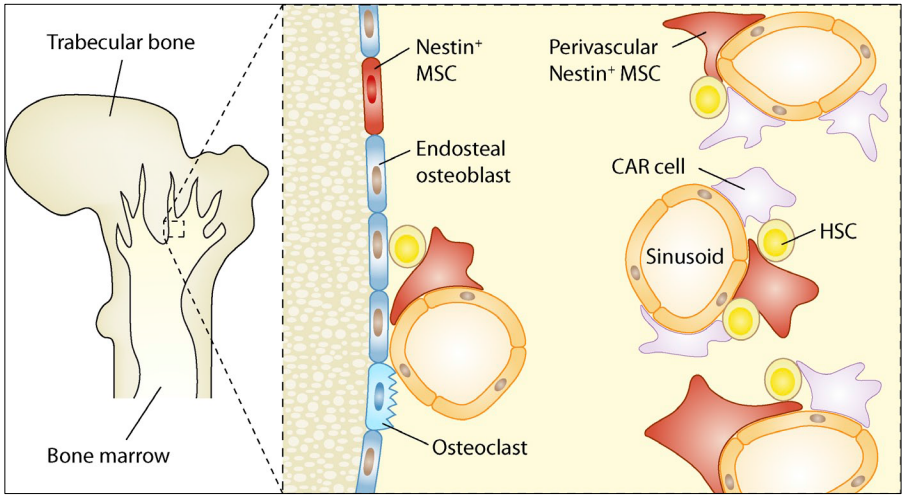

Figure 1. Location of HSC niches in trabecular bone cavities. HSCs are located at the endosteum, which is lined by osteoblasts (OBs) and is remodeled by osteoclasts. OBs promote HSC maintenance. Vascular sinusoids are found close to the endosteum, but more frequently at greater distances. HSCs are also situated nearby sinusoids toward the center of the marrow. Perivascular Nestin^{+} MSCs and the more abundant CAR cells promote HSC maintenance (Ehninger and Trumpp, 2011).

Similar to Nestin^{+} MSCs, CAR cells express HSC maintenance proteins such as CXCL12 and SCF. Induced depletion of CAR cells causes a partial loss of HSC activity associated with a decrease in HSC cycling, suggesting that CAR cells promote HSC cycling and self- renewal. CAR cells and Nestin^{+} MSCs represent two highly overlapping CXCL12-expressing cell populations. In addition CAR cells are bipotent adipo-osteogenic progenitors that show several similarities to Nestin^{+} MSCs. However Nestin^{+} MSCs containing Fibroblast Colony-Forming-Unit activity, are characterized by high self-renewal activity both *in vitro* and *in vivo*, and are capable of multilineage differentiation into bone, cartilage, and fat. It has been postulated that Nestin^{+} MSCs represent a more primitive population compared to CAR cells and may even be a CAR subpopulation (Ehninger and Trumpp, 2011). As it remains unclear whether Nestin^{+} MSCs are homogeneous and whether they all express high levels of CXCL12, it is possible that some Nestin^{+} MSCs may not be CAR cells.

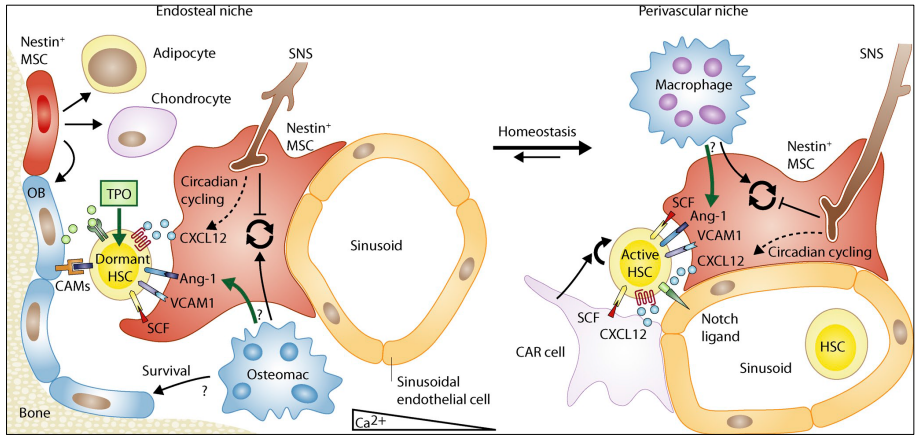

Figure 2. Model illustrating the quiescent endosteal and the active perivascular HSC niche during bone marrow homeostasis. (Ehninger and Trumpp, 2011).

In an attempt to further identify and characterize cellular niche

components, a recent study suggests that Osterix^{+} - rather than mature Osteocalcin^{+} osteoblasts are required for the integrity of the niche (Raaijmakers et al., 2010). However MSCs express much higher levels of some HSC maintenance factors compared with osteoblasts (CXCL12, SCF, IL-7, VCAM1, and OPN) (Méndez-Ferrer et al., 2010).

Another crucial cellular niche component is represented by the osteoblasts (OB). OBs produce factors that are known to be involved in HSC retention and maintenance, including CXCL12, OPN, and N-cadherin, in addition to factors that keep HSCs in a quiescent state, including Angiopoietin-1 (Ang-1), and membrane-bound SCF. OBs are also an exclusive source of thrombopoietin (TPO). TPO signaling via the c-MPL receptor mediates HSC quiescence, a typical feature of the most potent HSCs in steady-state bone marrow. c-MPL$^{-/-}$ mice are born with normal numbers of HSCs, but their frequency progressively declines with age, demonstrating a critical role for OB-derived TPO in adult HSC maintenance *in vivo.*

Lastly, macrophages are a crucial niche component. Loss of monocytes and/or macrophages is associated with mobilization of HSCs out of the bone marrow into the peripheral blood and spleen. This is associated with a 40% reduction in CXCL12 protein in the bone marrow extracellular fluid. CXCL12-mediated activation of the CXCR4 receptor on HSCs is a critical niche retention signal. Macrophages in addition are positive regulators of the OB and Nestin^{+} MSCs that are required to maintain expression of various HSC retention factors, including CXCL12. Importantly macrophages mediate granulocytic-colony stimulating factor (G-CSF)-induced HSC mobilization, which is a common method to release HSCs in the peripheral blood where they can be recovered by apheresis and used in subsequent BM transplantation settings.

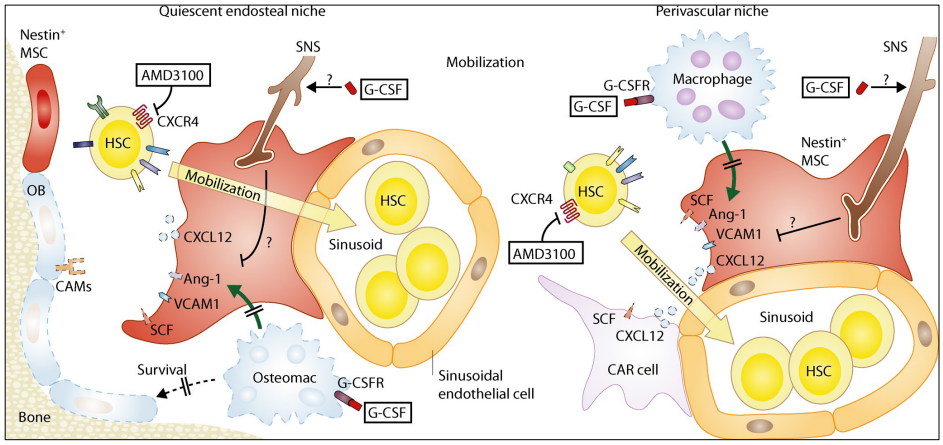

Figure 3. Model illustrating the quiescent endosteal niche and the active perivascular HSC niche after stimulation with G-CSF or depletion of monocytes/macrophages (Ehninger and Trumpp, 2011).

2. Mesenchymal Stem Cells (MSCs)

Any adult tissue harbors tissue-specific stem cells defined as cells that self-renew and retain sufficient proliferative and differentiation potential to be able to repair and/or reconstitute the tissue itself. It is well known that adult bone has an impressive ability to repair; therefore, it is not surprising that the quest to identify and characterize the stem cells responsible for this process is an active field of investigation (Bianco et al., 2008, Caplan, 2007, Kolf et al., 2007, Prockop, 1997). In the early 1970's, the pioneering work of Friedenstein and colleagues demonstrated that the rodent bone marrow had fibroblastoid cells with *in vitro* clonogenic potential (Friedenstein et al., 1970). Friedenstein flushed out the whole bone marrow into plastic culture dishes, and, after discarding the non-adherent cells, isolated spindle-like cells adherent to the plastic, which were heterogeneous in appearance and capable of forming colonies (Colony-Forming Unit Fibroblasts, CFU-F). These cells could also make bone and reconstitute a hematopoietic microenvironment in subcutaneous transplants. Moreover, Friedenstein demonstrated that they could regenerate heterotopic bone tissue in serial transplants, thus providing evidence in support of their self-renewal potential. Over the years, numerous laboratories have confirmed and expanded these findings by showing that cells isolated according to Friedenstein's protocol were also present in the human bone marrow. It was also demonstrated that these cells could be cultured for many passages and differentiated *in vitro* into a variety of cells of the mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts (Bianco et al., 2008, Caplan, 2007, Kolf et al., 2007, Pittenger et al., 1999, Prockop, 1997).

Friedenstein had thus isolated from the bone marrow the cells that Caplan and colleagues renamed as “Mesenchymal Stem Cells” or MSCs (Caplan, 2007). In the adult bone marrow, MSCs are rare and their quantity changes during the lifetime, declining with age, as estimated by CFU-F assay. What controls the number of MSCs in the marrow and why these numbers appear to change with age is not yet known. Little is known about the phenotypic characteristics of *in vivo* MSCs, their developmental origin, their contribution to organogenesis and normal postnatal tissue homeostasis, as well as their precise anatomical localization. While multiple markers expressed in clonogenic stromal cells from human BM have been investigated as potential MSC markers, (Barry et al., 1999; Deschaseaux and Charbord, 2000; Gronthos et al., 1999; Shi and Gronthos, 2003; Simmons and Torok-Storb, 1991; Vogel et al., 2003; Zannettino et al., 2003), none of them is currently used to *in situ* identify MSCs. No evidence of asymmetric cell division, which is considered a property of self-renewing cells (Wu et al., 2008), has been provided yet for MSCs. While the multipotency of a fraction of human skeletal progenitors has been demonstrated (Kuznetsov et al., 1997), their ability to self-renew has not been formally determined for any subset of stromal cells. Furthermore, a faithful assay that would rigorously test for their ability to self-renew *in vivo*, and would thus prove their “true stemness” is still missing. Bianco strongly supports the need to develop *in vivo* assays based on the same rigorous principles used to design HSC bioassays. Stem cell function is indeed exhibited, as shown in the hematopoietic system, by the capacity of a single purified HSC to serially and long term reconstitute multilineage hematopoiesis in lethally irradiated recipient mice. Stringent and rigorous assays are based on three main points: (1) stemness is probed through *in vivo* transplantation experiments; (2)

multipotency can only be probed at the single-cell level; (3) self-renewal means reconstitution of a stem cell population identical in phenotype and function to the one originally explanted. Some of these inherent biological properties of the HSC system are not necessarily present in the MSC system or in other systems in which the investigation of the unique definition of stemness is still in progress. For example, single HSCs can be transplanted *in vivo* via the circulation, and distributed at high efficiency without *ex vivo* culture. On the other hand, sufficient numbers of MSCs, necessary to regenerate a skeletal defect, need to be locally transplanted, and even prospectively isolated; single skeletal progenitors need to be cultured to generate sufficient numbers of cells prior to transplantation.

The capacity to self-renew relates to the rate of tissue turnover. While skin turns over every 30 days, the whole skeleton only turns over three to five times during adulthood. Consequently, self-renewal of stem cells capable of reforming skeletal tissues, in nature, would not be expected to involve the same number of cell divisions as for HSCs or epidermal stem cells.

Therefore, since many questions still need to be properly addressed, it is also questionable if it is appropriate to refer to them as “mesenchymal stem cells” (Dominici et al., 2006; Horwitz et al., 2005). First, the original naming of this class of stem cells as mesenchymal was based on the hypothesis that multiple tissues beyond skeletal lineages could be generated by postnatal MSCs, including skeletal muscle, myocardium, smooth muscle, tendon, etc. (reviewed in Caplan, 2007). However, the non-skeletal potential of single MSCs has not been formally proven *in vivo*, and this issue remains controversial. Second, during prenatal organogenesis bone and skeletal muscle are generated by a system of

distinct progenitors, rather than from a common progenitor. Two opposing descriptions of MSCs exist at this time in cell biology (Bianco et al., 2013), making this field still rather controversial and in need of univocal nomenclature. “Mesenchymal” stem cells are the postnatal, self-renewing, and multipotent stem cells that give rise to all skeletal tissues. This cell is part of a population of clonogenic progenitor cells, which coincide with a specific type of perivascular cell in the mammalian bone marrow. When explanted in culture, these progenitors generate a clonal progeny of transplantable stromal cells. Upon *in vivo* transplantation, these stromal populations generate ossicles, which include bone and bone marrow stroma of donor origin, as well as host-derived hematopoietic tissue and blood vessels within a marrow cavity. The single cell that initiates a clonal population in culture, which in turn can establish a complete organoid *in vivo* (including secondarily transplantable stromal cells) is a stem cell, as it is multipotent and self-renewing (Mendez-Ferrer et al., 2010, Sacchetti et al., 2007). MSCs are skeletal stem cells (Bianco et al., 2006) in that they are found in the skeleton; they are committed to skeletogenesis; they are capable of generating all different skeletal tissues; and they are able to recapitulate initial bone organogenesis *in vivo*. In skeletal physiology, this skeletal stem cell is essential to the growth and lifelong turnover of bone, as well as to its regenerative capacity. In hematopoietic physiology, it is a key player in maintaining hematopoietic stem cells in their niche, and in regulating multiple function of the hematopoietic microenvironment. In the alternative description, *mesenchymal stem cells* are not necessarily stem cells and not necessarily mesenchymal. They can also be *multipotent stromal cells* (Dominici et al., 2006), *mesenchymal stromal cells* (Horwitz et al., 2005), or even *medicinal signaling cells* (Caplan & Correa 2011). There are four

different terms for one invariant acronym (MSC), which contains four different meanings that do not originate from neither a physical *in vivo* entity nor from one defined concept. They describe, instead, a type of cell culturedefined by *in vitro* characteristics (Dominici et al., 2006), which are not even specific to stem cells, or reflective of a precise physiological function. These cultures can be established from virtually every connective tissue. This inconsistency introduced significant uncertainty, reflected in a debate over terminology, criteria, and standards that are in need of precise clarification.

MSCs or MSC-like cells are mainly localized in the bone marrow; however they are also found in tissues such as fat, umbilical cord blood, amniotic fluid, placenta, dental pulp, tendons, synovial membrane and skeletal muscle. However the complete equivalence of such populations has not been formally demonstrated (Rogers and Casper, 2004, Bieback and Kluter, 2007, Xu et al., 2005, Shi and Gronthos, 2003, Tsai et al., 2004, Bi et al., 2007, Igura et al., 2004, De Bari et al., 2001, Crisan et al., 2008). Much effort has been invested both in *in vitro* expanding and phenotypically characterizing these cells, as well as in identifying factors involved in their regulation of proliferation and/or differentiation potential. The major aim being the possibility to transplant them back *in vivo* to repair specific tissues such as bone and cartilage (Tsutsumi, 2001, Kulterer et al., 2007, Pochampally et al., 2004, Hishikawa et al., 2004, Kratchmarova et al., 2005, Song et al., 2006).

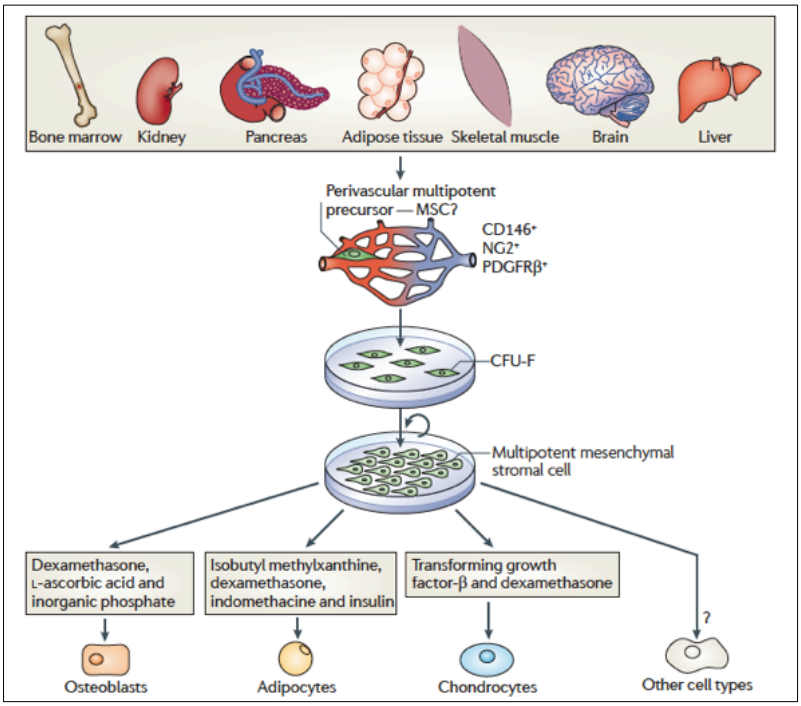

Figure 4. MSCs and multipotent mesenchymal stromal cells. (Nombela-Arrieta et al., 2011)

In our previous study (Picchi et al., 2012) we compared cellular and molecular characteristics of human adult MSCs derived from different body locations, such as bone marrow from iliac crest (Ic-MSCs), sternum (St-MSCs) and vertebrae (V-MSCs), as well as colon (Co-MSCs) and dental pulp (DPSCs). In particular, we investigated whether HOX genes and their TALE (three amino acid loop extension) co-factors provide specific molecular markers for stromal stem cell populations derived from different sources. Our results show that cell populations exhibiting similar immunophenotypes display different *in vitro* growth and differentiation properties, and are characterized by distinct HOX codes and TALE expression profiles. Furthermore, our data strongly suggest that molecular signatures differing only for the expression levels of specific HOX members may reflect differences in stem cell potency. Taken together, our

observations support the view that human MSCs derived from different body sites may not represent equivalent cell sources to regenerate specific tissues/organs. These observations may have important clinical implications, suggesting that successful tissue regeneration may depend on the source of MSCs employed for cell therapy or tissue engineering (Picchi et al., 2012). The notion that bone marrow includes skeletal progenitor (stem) cells, and the notion that BM stroma provides cues for homing, maintenance, proliferation, and maturation of hematopoietic progenitors both derive from classical transplantation experiments. The multipotency of at least a subset of CFU-Fs supports the view that a second type of skeletal stem cell (Bianco and Robey, 2010), stromal (Owen and Friedenstein, 1988), or ‘‘mesenchymal’’ (Caplan, 1991) co-exists with the HSCs in the BM. HSCs would give rise to hematopoietic cell types and to osteoclasts, whereas MSCs would generate CFU-Fs and differentiate *in vitro* into a variety of mesenchymal lineages such as chondrocytes, adipocytes and osteoblasts. Since MSCs represent a distinct population from HSCs, they most probably have different cell surface antigens that would facilitate their purification from the hematopoietic stem cell component. Therefore, the identification of cell-specific cell surface markers for MSCs would be extremely useful. In the early 1980’s, a study from Simmons and colleagues led to the isolation of an antibody known as STRO1, which recognizes a cell surface antigen present in human bone marrow stromal cells. The STRO-1-positive population was highly enriched in clonogenic cells that were able to both generate CFU-Fs and differentiate into multiple mesenchymal lineages *in vitro* (Simmons and Torok-Storb, 1991). The same group reported that the degree of homogeneity of the STRO-1-positive population could be further enhanced by positive selection for VCAM/CD106 (Gronthos et

al., 2003). In absence of specific and unique markers that would allow for a proper *in vivo* identification of MSCs, a histological localization of these cells is virtually impossible to achieve. Nevertheless, as reported by Caplan in 2007, every blood vessel in the body appears to have a mesenchymal cell on the tissue side of endothelial cells of large and small vessels. This endothelial layer is present in every tissue of the body, deriving from each of the germ layers. These vascular-associated mesenchymal cells are referred to as pericytes. When isolated and assayed in culture, these cells exhibit MSC-like characteristics. Conversely, marrow MSCs have markers characteristic of pericytes. Sacchetti and colleagues demonstrated that expression of high levels of CD146, a cell adhesion molecule of the immunoglobulin superfamily expressed in a restricted range of normal cells (Shih, 1999), identifies all *ex vivo* assayable CFU-Fs, and a specific subset of stromal cells *in situ*. Explantable CFU-Fs exhibit the same phenotype as Adventitial Reticular Cells (ARCs), which reside in bone marrow sinusoids next to the endothelial layer, strongly indicating that ARCs are in fact the cells explanted *ex vivo* as CFU-Fs. It has been shown that following transplantation of $CD146^+$ stromal cells, a small subset retain CD146 expression, dynamically associate with developing sinusoids, and eventually regenerate heterotopic human cells with the anatomy and phenotype of ARCs. Moreover, transplantation of cell populations derived from either a limited number of $CD146^+$ CFU-Fs or single $CD146^+$ CFU-Fs results in the re-establishment, in the heterotopic ossicles, of $CD146^+$ CFU-Fs that can be secondarily passaged and directly assayed. These data directly identified a clonogenic, multipotent, self-renewing stem cell in the bone marrow stroma, defining the MSC as a perivascular cell. Thus, the sinusoidal wall appears to act as a niche for

skeletal progenitors, while also acting as the niche for hematopoietic cells. These cells, which reside in the wall of the sinusoidal blood vessels of the bone marrow, are also positive for Ang-1, a critical regulator of vascular remodeling. The findings by Bianco and colleagues represent the first rigorous attempt to histologically localize and phenotypically define MSC-like cells, or at least a subpool of this population. Notably, a paper by Crisan and colleagues suggests that multipotent MSCs with perivascular localization exist in numerous human organs (Crisan et al., 2008).

Considerably less progress has been made in the characterization of the cell surface antigens that are expressed by murine MSCs *in vivo*. Van Vlasselaer and colleagues reported the purification of cells with osteogenic potential from murine bone marrow by two-color cell sorting using anti-Sca1 monoclonal antibody and wheat germ agglutinin (Van Vlasselaer et al., 1994). Simmons' laboratory has identified a bone marrow pool of Sca1 (+) CD45 (-) CD31 (-) cells that appears to be enriched in MSCs/progenitors (Lundberg et al., 2007, Short et al., 2003). CD45, a pan-hematopoietic cell marker, and CD31 (PECAM), a classical marker for endothelial cells, were used in the study to negatively select for hematopoietic and endothelial cells, respectively. Interestingly, it was shown that a subset of CD45 (+) Lin (-) bone marrow cells was able to *in vitro* differentiate into a variety of cell types, including endothelial cells, osteoblasts, muscle cells, and neural cells (Rogers et al., 2007). This finding challenges the specificity of CD45 as a specific marker for hematopoietic cells. It raises the question whether the bone marrow contains pluripotent stem cells capable of generating tissues that embryologically derive from all the three embryonic germ layers. This is an appealing possibility for which, however, only a few pieces of

experimental evidence have been provided so far.

A greater understanding of the biology of MSCs, particularly in their *in vivo* setting, will probably provide important insights into the cellular mechanisms of bone development, hematopoiesis, vasculogenesis and angiogenesis. As mentioned above, investigators have identified efficient protocol to expand MSCs isolated from bone marrow or adipose tissue aspirates, while still maintaining their multipotency (Caplan, 2007, Prockop, 2007). These cells have been transplanted at specific sites in experimental animal models by using appropriate scaffolds to form tissues such as bone and cartilage. However, no human MSC-based technology is currently available. Over the years, it has also become progressively clear that MSCs could be the basis for an extremely powerful "natural system of tissue repair" (Phinney and Prockop, 2007). MSCs can serve, upon exogenous administration, as effective therapeutic agents in a variety of experimental models of tissue injuries (Ortiz et al., 2007, Kunter et al., 2006, Minguell and Erices, 2006, Lee et al., 2006, Phinney and Isakova, 2005). In the vast majority of these studies though, the therapeutic efficacy did not correlate with the efficiency of engraftment, which was generally low (Prockop, 2007). This finding suggests that the ability to repair was very likely due to secretion by MSCs of soluble factors that altered the tissue microenvironment, rather than cell fusion or transdifferentiation of MSCs into the appropriate cell phenotype, (Prockop, 2007). MSCs may thus provide what Caplan and colleagues define as "trophic activity" (Caplan, 2007). MSCs secrete bioactive factors which inhibit scarring and apoptosis and, conversely, stimulate angiogenesis and mitosis of tissue-intrinsic stem or progenitor cells (Caplan, 2007).

Moreover, MSCs are characterized by immunoregulatory properties. In

particular, MSCs have shown strong immunosuppressive effects. In particular, they inhibit T-cell recognition and expansion by inhibiting TNF-alfa and INF-gamma production and, thus increasing IL-10 levels (Beyth et al., 2005). These immunomodulatory effects support the use of allogeneic MSCs as therapeutic agents.

If MSCs represent a natural system for tissue repair, then the next questions are 1) how MSCs are mobilized and 2) how they reach the site of injury. Natural chemo-attractive mechanisms can bring MSCs to the damaged sites and establish a regenerative microenvironment. Chemokines such as SDF1 and its receptor CXCR4 may have an essential role (Chamberlain et al., 2007) in directing MSCs to sites of injury. More recently, it has been reported that the cytokine receptor CCR2 and its intracellular adaptor molecule FROUNT are necessary for homing of bone marrow-derived mesenchymal stem cells to sites of injury (Belema-Bedada et al., 2008). Little is known about how MSCs are mobilized from the bone marrow. It has been reported that MSCs can be observed in the circulating blood and that this circulating pool is dramatically increased by exposure to chronic hypoxia (Rochefort et al., 2006). The age of the individual, the extent of tissue damage and the local and total numbers of MSCs may play a role in their ability to regenerate damaged tissues. However upon direct delivery of MSCs to the injured sites, the regenerative process becomes more efficient.

MSCs can also be grown in biocompatible scaffolds and implanted into different body sites, in order to well integrate them into the newly differentiated tissue (Caplan, 2007).

Much still needs to be done to be able to employ MSCs as therapeutic agents in the future. A necessary precondition for an adequate characterization of the MSC population is the development of a

reproducible and faithful *in vivo* model that would test the ability of MSCs to self-renew, proliferate and differentiate *in vivo*, besides identifying their surface markers. The subcutaneous transplant model first introduced by Friedenstein and colleagues are undoubtedly very useful and informative, but they are not the equivalent of the primary and secondary bone marrow transplants that are classically used in the hematopoietic field to test stemness. Murine intratibial injection of MSCs and characterization of their contribution to bone homeostasis and fracture repair is becoming progressively more popular, (Wang et al., 2003). An important goal, in addition to a proper *in vivo* characterization, would be the identification of pharmacological tools that could be used to expand *in vivo* and/or *ex vivo* the MSC pool. Some attempts in this direction have been already pursued: for example the proteosome inhibitor Velcade has been reported to be able to expand the MSC pool in a murine model *in vivo* (Mukherjee et al., 2008). A successful identification of useful pharmacological tools requires a detailed and systematic analysis of the complex network of signaling pathways and cells that regulate the ability of MSCs to self-renew, proliferate and eventually differentiate. The definition of a possible niche for MSCs and its regulation is another clear priority. The identification of this network is critically important, in order to reach a deeper understanding of the rules that govern the size of the *in vivo* MSC pool, which would eventually favor development of pharmacological interventions. It is likely that a global gene expression profiling approach could be extremely helpful to gain insights into the molecular mechanisms that regulate both the size and the differentiation potential of MSCs (Tsutsumi et al., 2001, Kulterer et al., 2007, Pochampally et al., 2004, Hishikawa et al., 2004, Kratchmarova et al., 2005, Song et al., 2006).

2.1. Molecular Differentiation Mechanisms

Bone is a specialized connective tissue that provides structural support to skeletal muscle, physical protection to vital organs such as the brain and heart, a reserve for minerals such as calcium and phosphate, and a continuous source of hematopoietic stem cells for regeneration of cells of the blood and immune system. Given these vital roles, loss of normal bone structure and function is associated with various diseases, most notably osteoporosis. Throughout life bone is constantly remodeled through the processes of bone formation by osteoblasts, and bone resorption by osteoclasts. Developmentally, osteoclasts are derived from hematopoietic stem cell precursors of the monocyte/macrophage lineage located in the blood and the bone marrow (Teitelbaum et al., 2000), while osteoblasts originate from bone marrow MSCs (Owen et al., 1988). In normal, healthy bone, a balance of bone formation/resorption is achieved in large measure through the coordinated differentiation of these cell types from their stem cell precursors. Bone marrow MSCs can give rise not only to osteoblasts, but also to a range of other cell types including adipocytes, chondrocytes, and myoblasts (Chamberlain et al., 2007). Among these potential fates, differentiation to the osteoblast and adipocyte lineages has particular relevance to the maintenance of normal bone homeostasis. For example, considerable evidence exists to support that a shift in MSC differentiation to favor the adipocyte lineage over the osteoblast lineage can directly contribute to imbalances in bone formation/resorption, and lead to bone loss. This shift of MSC differentiation to the adipocyte lineage may contribute to the progressive increase in adipocyte formation and decrease in osteoblast number that coincides with age-related bone loss (Duque, 2008). In support of this

reciprocal relationship, numerous *in vitro* experiments performed with bone marrow-derived MSCs have demonstrated that factors that induce adipogenesis inhibit osteoblast formation (Beresford et al., 1992, Dorheim et al., 1993) and, likewise, factors that promote osteoblastogenesis inhibit adipocyte formation (Gimble et al., 1995). Furthermore, the majority of conditions associated with bone loss, including aging, glucocorticoid treatment, increased cortisol production, and osteoporosis, also coincide with increased marrow adiposity (as reviewed in Chan and Duque, 2002, Kirkland et al., 2002). Adipocytes may further influence bone remodeling through the secretion of fatty acids and adipokines with paracrine actions that may influence the development and function of stem cells, precursors, as well as mature cell types such as osteoblasts and osteoclasts (Karsenty et al., 2006). Thus, given the close association between adipocyte and osteoblast formation, it is possible to prevent or treat bone loss by inhibiting bone marrow adipogenesis. In bone marrow, the developmental fate of MSCs is largely determined by the expression of specific groups of transcription factors that act as molecular switches to drive the differentiation of uncommitted precursors to a specific lineage. For example, expression of the transcription factors Runx2 and osterix (OSX) are the main determinants of MSC osteoblastogenesis (Ducy et al., 1997, Nakashima et al., 2002). In contrast, the transcription factor PPARγ (Peroxisome Proliferator-Activated Receptor-γ) is the key factor that drives adipogenic differentiation of MSCs (Rosen et al., 2000).

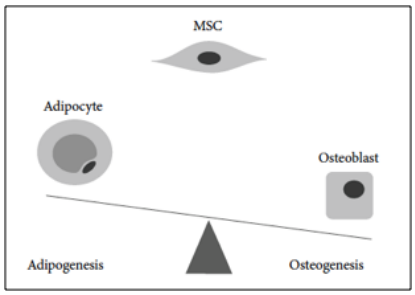

Figure 5. Postulated inverse relationship between osteogenic and adipogenic programming (James, 2013).

2.1.1. Adipogenesis

Sinal's group characterizes adipogenesis in two phases: the determination phase and the terminal differentiation phase (Muruganandan et al., 2009). In the determination phase, multipotent MSCs become committed to the adipocyte lineage, and lose their ability to differentiate into other mesenchymal lineages. During this phase, committed preadipocytes are morphologically indistinguishable from their precursors. Subsequently the terminal differentiation phase, preadipocytes are converted in mature adipocytes that acquire new specific functions, as they can synthesize and transport lipids, secrete adipocyte-specific proteins, and contain the machinery necessary for insulin sensitivity (Rosen et al., 2006). In addition to these two phases, several experimental cellular models of adipogenesis (e.g., the murine preadipocytes cell line 3T3-L1) require a period of mitotic clonal expansion, involving one or two rounds of cell division prior to committing to maturation. In human bone marrow MSCs, clonal expansion is not required for the adipogenic differentiation (Janderova et al., 2003). However, even though the mitotic clonal expansion has not been unequivocally established as a requisite for bone marrow adipogenesis, it is instead clear that some cell cycle proteins that

act to regulate mitosis also act in regulating some aspects of adipocyte differentiation (Tang et al., 2003). Functionally, adipogenesis reflects a fundamental shift in gene expression patterns within uncommitted MSCs that promotes and culminates in the phenotypic properties that define mature adipocytes (Rosen et al., 2000). Adipogenesis is driven by a complex and well-orchestrated signaling cascade involving regulated changes in the expression and/or activity of several key transcription factors, most notably PPARγ and several members of the CCAAT/Enhancer-Binding family of proteins (C/EBPs) (reviewed in Rosen et al., 2000). During *in vitro* adipogenesis, first, there is growth arrest of proliferating preadipocytes, usually achieved in cultured cell lines after contact inhibition. In culture cell models, initial growth arrest is induced by the addition of a pro-differentiative hormonal regimen and it is followed by one or two additional rounds of cell division known as clonal expansion. This process is coincident with the expression of the key transcription factors PPARγ and C/EBPα (Shao and Lazar 1997; Morrison and Farmer 1999). The induction of these two proteins is characterized by a second, permanent period of growth arrest followed by expression of the fully differentiated phenotype. Although growth arrest is virtually a *sine qua non* of the differentiation process, there is some debate about the requirement for clonal expansion *in vivo*. Several studies show that inhibition of cell division in cultured preadipocytic lines clearly blocks their subsequent differentiation. This process of terminal differentiation occurs over several days in cultured cell lines. A second, permanent state of growth arrest occurs following the accumulation of phenotypic markers of the mature adipocyte.

Morphologically, the earliest events of adipogenesis include a rounding up of the fibroblast-like preadipocytes and the expression of specific

transcripts including lipoprotein lipase, and the transient induction of the transcriptional components C/EBPβ and C/EBPδ (MacDougald and Lane 1995; Darlington et al., 1998). These earliest events are followed by the appearance of PPARγ and C/EBPα, which activate *de novo* or enhanced expression of most or all of the genes that characterize the adipocytic phenotype. These genes include glycerophosphate dehydrogenase, fatty acid synthase, acetyl CoA carboxylase, malic enzyme, Glut 4, insulin receptor, and aP2 (adipocyte-selective fatty acid binding protein) (Spiegelman et al., 1993). Throughout this process, lipid droplets begin to appear in the cytoplasm, and over time they become quite large and often fuse into one or a few major droplets. Studies in preadipocytic and fibroblastic cultured cell lines have provided insights into the transcriptional cascade that drives adipogenesis. Three classes of transcription factor have been identified that directly influence fat cell development. These include PPARγ, C/EBPs, and the basic helix–loop–helix family (ADD1/SREBP1c). PPARγ is a member of the nuclear hormone receptor superfamily, and, like many members of this class of transcription factor, PPARγ must heterodimerize with another nuclear hormone receptor (the retinoid X receptor, or RXR) to bind DNA and be transcriptionally active. The first "gain-of-function" experiments that linked PPARγ to fat cell development utilized retrovirally-expressed PPARγ in nonadipogenic, fibroblastic cells. Using the relatively nonspecific ligands available at the time, activation of PPARγ was shown to strongly induce adipogenesis (Tontonoz et al., 1994). The effect of PPARγ activation regards all aspects of the mature fat cell phenotype, including morphological changes, lipid accumulation, and the acquisition of insulin sensitivity. PPARγ is a potent inducer of adipogenesis, as it can indeed promote the transdifferentiation of cultured myoblasts to

adipocytes, particularly when co-expressed with C/EBPα (Hu et al., 1995). PPARγ knock-out mice do not survive past embryonic day 10–10.5, due to a defect in their placental development (Barak et al., 1999; Kubota et al., 1999). This occurs prior to the formation of identifiable fat cells in mice. One approach to specifically pinpoint PPARγ contribution to adipogenesis was to create chimeric mice derived from both wild-type ES cells and cells with a homozygous deletion of PPARγ (Rosen et al., 1999). This strategy allowed quantifying the contribution of PPARγ null cells to adult tissues in healthy animals. PPARγ was found to be required for *in vivo* adipogenesis. Interestingly, animals with only one PPARγ allele exhibited resistance to diet-induced obesity, although this results at least in part from elevated serum leptin levels, and decreased food intake (Kubota et al., 1999; Miles et al., 2000). PPARγ is also required for the differentiation of adipose cells from ES cells (Rosen et al., 1999) or from embryonic fibroblasts *in vitro* (Kubota et al., 1999).

The C/EBP proteins belong to the basic-leucine zipper class of transcription factors. Six isoforms have been described, all of which act as homo- and/or heterodimers formed through a highly conserved bZIP domain (Lekstrom-Himes and Xanthopoulos 1998). Their tissue distribution is not restricted to fat cells, and a role for C/EBP proteins has been demonstrated in the terminal differentiation of granulocytes (Zhang et al., 1997) hepatocytes (Wang et al., 1995; Flodby et al., 1996), and pulmonary cells (Basseres and Levantini et al., 2006). C/EBPs also play an important role in resistance to infection (Yamanaka et al., 1997) and tissue response to injury (Flodby et al., 1996) in addition to transactivating a wide variety of target genes. C/EBPs can be regulated at many levels, including transcriptionally, as measured by mRNA levels in cells. There are many isoforms for these proteins. For example, the 42-kD

C/EBPα isoform is a stronger transcriptional activator than the 30-kD isoform, and the p42/p30 ratio increases during the course of adipogenesis (Lin et al., 1993). The amount of the 20-kD inhibitory isoform of C/EBPβ decreases during adipogenesis relative to the 32-kD active isoform (Bachmeier and Loffler 1997). Post-translational regulation of C/EBPs, particularly changes in phosphorylation, can modify the activity of C/EBP proteins as well. In cultured preadipocytic cell lines that have been induced to differentiate, C/EBPβ and δ mRNA and protein levels rise early and transiently (Cao et al., 1991; Yeh et al., 1995). C/EBPα, on the other hand, is induced later in the differentiation process, slightly preceding the induction of most of the end-product genes of fat cells. The proadipogenic role of C/EBPβ and δ was originally demonstrated in *in vitro* gain-of-function experiments. Ectopic expression of C/EBPβ is sufficient to induce differentiation of 3T3-L1 cells without the addition of hormonal inducers; similar experiments with C/EBPδ reveal that prodifferentiative agents are still required, but adipogenesis is accelerated (Yeh et al., 1995). Ectopic expression of C/EBPβ in NIH-3T3 fibroblasts is permissive for adipogenesis in the presence of hormonal inducers (Wu et al., 1995). Embryonic fibroblasts lacking either C/EBPβ or δ showed slight reductions in adipogenic potential, but cells lacking both C/EBPβ and δ were severely impeded from developing into adipocytes (Tanaka et al., 1997). Mice lacking either C/EBPβ or δ have normal white adipose tissue (WAT), although their brown adipose tissue (BAT) shows reductions in lipid accumulation. Mice that lack both C/EBPβ and δ, however, have a more dramatic phenotype. Approximately 85% of these animals die in the perinatal period of unknown causes; the remaining 15% that survive have sharply reduced BAT and smaller decreases in WAT (Tanaka et al., 1997). Interestingly, the reduction in BAT appears to be a

result of reduced lipid accumulation, whereas the reduction in WAT is reported to involve hypoplasia, despite the few adipocytes that develop show normal size, morphology, and gene expression profiles. The involvement of C/EBPα in adipogenesis is also strongly supported by *in vitro* data. Overexpression of C/EBPα in 3T3-L1 preadipocytes induces their differentiation into mature fat cells (Freytag et al., 1994; Lin and Lane 1992).Animals that carry a homozygous deletion of the C/EBPα gene have dramatically reduced fat accumulation in WAT and BAT pads (Wang et al., 1995). These experiments have led to a model for a transcriptional network in adipogenesis involving the sequential activation of C/EBPs and PPARγ In this model, one major function of C/EBPβ and δ is to induce the expression of PPARγ. This induction is likely to be a direct transcriptional effect through C/EBP binding sites in the PPARγ promoter (Zhu et al., 1995; Fajas et al., 1997). PPARγ, is then responsible for inducing C/EBPα. Evidence for this cascade comes first from the temporal sequence of PPARγ and C/EBPα expression during adipogenesis, as well as from gain-of-function experiments in which ectopic expression of PPARγ or the application of specific PPARγ ligands induces C/EBPα mRNA. Genetic proof of this relationship was obtained from experiments in which cells (embryonic fibroblasts or embryonic stem cells) that are homozygous null for PPARγ were exposed to pro-differentiative signals. These cells do not become adipocytes and they express C/EBPα very poorly, despite normal levels C/EBPβ and δ (Kubota et al., 1999; Rosen et al., 1999). Interestingly, fibroblasts made from C/EBPα$^{-/-}$ embryos have reduced levels of PPARγ and do not form fat readily when exposed to hormonal inducing agents in culture (Wu et al., 1999). When C/EBPα is added back to these cells with a retroviral vector, the expression of PPARγ (and the ability to differentiate) is

restored. This reveals a positive feedback loop within the cascade, in which there is mutually reinforcing expression of PPARγ and C/EBPα; this feature ensures that, once initiated, the cascade will maintain the expression of these critical factors and, therefore, the terminally differentiated state. It is not certain whether the actions of C/EBPs and PPARγ is parallel and reinforcing pathways of adipogenesis, or whether there is really one factor that drives adipogenesis. It is already known that PPARγ can stimulate most, but not all, aspects of adipogenesis in C/EBPα-deficient cells (Wu et al., 1999), hinting to PPARγ as the master regulator of adipogenesis. Fat cells lacking C/EBPα accumulate lipid and express most adipogenic markers, but they have poor insulin sensitivity. This is a result of decreased levels of insulin receptor and one of its primary substrates (IRS-1), as well as an unclear defect in insulin signaling. C/EBPα could cause a lack of proper differentiation, as it is usually needed to completely-terminally differentiated cells.

Coactivator proteins play a key role in gene expression activation, as they unfold the chromatin structures. Once the chromatin has been opened up, transcription factors can bind to DNA and subsequently activate gene expression. The interaction of multiple coactivators with transcription factors in different temporal and spatial contexts provides another possible level of regulation to gene expression. Nuclear hormone receptors such as PPARγ, for example, can interact with coactivators like the p160 family, CBP/p300, and others. These proteins bind to the carboxy-terminal transcriptional activation function-2 (AF-2) domain of nuclear hormone receptors in the presence of ligand binding. Two dominant classes of coactivator complex have been reported to be recruited to nuclear hormone receptors: the p160/CBP/p300 complex and the DRIP/TRAP complex. The first type is represented by SRC-

1/NCoA1, TIF2/GRIP1/NCoA2, and pCIP/ACTR/AIB1 (Leo and Chen 2000). In addition to binding the AF-2 domain of PPARγ, these proteins also interact with CBP/p300 (Yao et al., 1996). CBP/p300 can also interact directly with PPARγ, thus providing potential stability to the complex through multiple contact points (Gelman et al., 1999). The p160 proteins and CBP/p300 both possess intrinsic histone acetyltransferase (HAT) activity, which is necessary to open the chromatin structure to allow full activation of transcription. The second class of coactivators that interact with nuclear receptors in a ligand dependent manner is the DRIP/TRAP/ARC complex (Fondell et al., 1996; Naar et al., 1999; Rachez et al., 1999). Interestingly, DRIP205/TRAP220 shares identity with PBP (PPARγ binding protein). The PBP null mutation is embryonic lethal because of defects in placental development, a phenotype shared also by PPARγ null embryos (Zhu et al., 2000). However, since PPARγ transcriptional activity is only modestly affected in cells lacking PBP, the requirement for this factor in PPARγ-mediated adipogenesis remains to be determined. Two coactivators of PPARγ that are not ligand dependent, but which show considerable biological selectivity, are PPARγ coactivator-1 and -2 (PGC-1,-2). Although it does not appear that PGC-1 has endogenous HAT activity, it does interact through its amino-terminus with p160/CBP/p300, proteins that do possess such activity (Puigserver et al., 1999). One of the most interesting aspects of PGC-1 is its tissue distribution, in that it is expressed in brown but not white fat. Little is known about coactivator complexes interacting with C/EBP family members. Two types of coactivators have been reported to interact with C/EBPβ. One is the chromatin remodeling SWI/SNF complex that is recruited in myeloid cells to activate certain target genes (Kowenz-Leutz and Leutz 1999). The second type is the CBP/p300 coactivator (Mink et

al., 1997). Because it is also a potent activator of PPARγ, it is possible that CBP/p300 is important in the synergistic effects seen between C/EBP proteins and PPARγ.

Other important questions focus on the identification of transcription factors that regulate development of early mesenchymal precursors, before their determination to the adipogenic lineage. Extracellular factors and intracellular signal transduction pathways can influence both *in vitro* and *in vivo* the adipogenic potential. These include the hormones that induce adipogenesis, such as insulin-like growth factor-1 (IGF-1), insulin, growth hormone, glucocorticoids, and thyroid hormone, as well as intracellular pathways involving cAMP and p38 MAP kinase (Gregoire et al., 1998). There are also agents that inhibit fat cell formation, including cytokines such as IL-1 and TNF-α, cell surface proteins such as the preadipocyte-specific protein Pref-1, and intracellular pathways involving MAP kinases such as Erk1, Erk2, and JNK (Gregoire et al., 1998). The link between these factors and the transcriptional factors that regulate adipogenesis remains unclear, although some relationships have become clearer recently. For example, the well-documented inhibition of adipogenesis by MAP kinases can be at least partially explained by the phosphorylation of specific residues of PPARγ (Hu et al., 1995; Adams et al., 1997; Camp and Tafuri 1997; Font de Mora et al., 1997) and RXR (Solomon et al., 1999), which inhibits their activity. Although most studies have been devoted to the role of PPARγ and C/EBP proteins, it is known that a large number of other transcription factors and cofactors are regulated during adipogenesis. These factors may play a role in the expression of subsets of genes within the terminally differentiated adipocyte. Consistent with this idea, recent reports link other transcriptional regulators to the adipogenesis cascade, such as the

Kruppel-like zinc finger transcription factor family members KLF2 (which represses adipogenesis) (Banerjee et al., 2003) and the proadipogenic KLF5 (Oishi et al., 2005) and KLF15 (Mori et al., 2005). Similarly, the zinc finger-containing factor KROX 20 (Chen et al., 2005) has been shown to participate early in the adipogenic cascade. A number of other transcription factors have also been shown to negatively regulate adipogenesis, for example, GATA-2 and -3 (Tong et al., 2000, Tong et al., 2005), the forkhead transcription factors FoxO1 (Nakae et al., 2003) and FoxA2 (Wolfrum et al., 2003), the HMG proteins TCF/Lef (Kennel et al., 2003), and SMAD-3 (Choy et al., 2003). These findings suggest that fat cell development is a more complex process than previously appreciated, requiring the integration of multiple transcriptional regulators to determine differentiation and function of the mature adipocyte. The Ebf family of helix-loop-helix transcription factors plays a significant role in B lymphocyte and neuronal development. The three primary members of this family, Ebf1, 2, and 3, are all expressed in adipocytes, and Ebf1 promotes adipogenesis when overexpressed in NIH 3T3 fibroblasts. Rosen and colleagues performed loss and gain-of-function approaches in multiple adipogenic and nonadipogenic cell types. Results indicate that there is a short but intense increase of Ebf1 expression after addition of the proadipogenic cocktail, followed by a return to a baseline that appears to be maintained for the life of the mature adipocyte. Ebf2 and Ebf3 do not display the same pattern of expression, and although they increase gradually later in development, they never reach peak levels equivalent to that of Ebf1. Moreover, retroviral delivery of shRNA to specifically "knock down" each Ebf isoform in turn has been performed. Differentiation did not proceed in the presence of reduced levels of either Ebf1 or Ebf2, strongly suggesting that these two factors have non-

redundant activities. Specific Ebf3 “knockdown” did not result in changes in lipid accumulation or expression of marker genes like aP2. These experiments establish the importance of Ebf1 and Ebf2 and inform us that these proteins play a role in the process that cannot be compensated by other members of the family. Altogether, these data place Ebf1 within the known transcriptional cascade of adipogenesis and suggest critical roles for Ebf1 and Ebf2. Data suggest that Ebfs promote adipogenesis by inducing expression of C/EBPα and PPARγ, moreover enhancement of endogenous PPARγ and C/EBPα expression in adipocytes transfected with Ebf1 occurs. The evidence that PPAR is a direct target of Ebf1 includes presence of Ebf motifs in the PPARγ promoter. Ebf1 directly binds and activates the C/EBPα promoter, thus exerting positive feedback on C/EBPδ expression. Ebf1 itself is induced by C/EBPβ and δ which binds and activates its promoter. These experiments place Ebf action between C/EBPβ/δ and C/EBPα/PPARγ, and a positive feedback loop involving C/EBPδ, Ebf1, and C/EBPα occurs during adipogenic differentiation (Jimenez et al., 2007).

While there has been significant progress in determining the transcriptional cascade involved in terminal adipocyte differentiation, less is known about early events leading to lineage commitment and cell fate choice. It has been recently discovered that zinc finger protein 423 (Zfp423) is an early actor in adipose determination. A close paralog of Zfp423, Zfp521, acts as a key regulator of adipose commitment and differentiation *in vitro* and *in vivo*. Zfp521 exerts its actions by binding Ebf1, a transcription factor required for the generation of adipocyte progenitors, and inhibiting the expression of Zfp423. Overexpression of Zfp521 in cells greatly inhibits adipogenic potential, whereas RNAi-

mediated knock-down or genetic ablation of Zfp521 enhances differentiation. In addition, Zfp521$^{-/-}$embryos exhibit increased mass of interscapular brown adipose tissue and subcutaneous white adipocytes, a cell autonomous effect. Finally, Ebf1 participates in a negative feedback loop to repress Zfp521 as differentiation proceeds. Because Zfp521 is known to promote bone development, Rosen and colleagues suggest that it acts as a critical switch in the commitment decision between the adipogenic and osteogenic lineages (Kang et al., 2012).

2.1.2. Osteogenesis

Bone formation is a strictly regulated process that takes place during embryonic development, growth, remodelling and fracture repair (Aubin 2001). Bone formation is characterized by a sequence of events starting with the commitment of osteoprogenitor cells and their differentiation into pre-osteoblasts and then into mature osteoblasts whose function is synthesizing the bone matrix that becomes progressively mineralized. Several specific transcription factors are responsible for the commitment of pluripotent mesenchymal cells into the osteoblast cell lineage. One of the most important of these factors is Cbfa1 (core-binding factor α1), a transcription factor belonging to the runt-domain gene family, which plays a critical role in osteoblast differentiation, not being sufficient to support the achievement of the mature osteoblast phenotype (Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997; Lee et al., 1999; Otto et al., 1997). Cbfa1 is highly expressed in osteoblast lineage cells and regulates the expression of various osteoblast-specific genes (Banerjee et al., 1997; Ducy et al., 1997; Ji et al., 1998; Harada et al., 1999; Tsuji et al., 1998); Cbfa1-deficient mice are completely defective in their bone formation ability (Hoshi et al., 1999), because of the maturational arrest of their osteoblasts. Over-expression of Cbfa1 induces non-osteogenic cells to express osteoblast-related genes (Yamaguchi et al., 2000). Another runt-related gene that plays an important role in the commitment of multipotent mesenchymal cells to the osteoblastic lineage, and required for osteoblast differentiation at an early stage is Runx-2, considered the master gene of osteogenesis. Runx-2 is involved in the production of bone matrix proteins (Komori et al., 1997; Otto et al., 1997), as it is able to up-regulate the expression of many bone matrix protein genes, such as type I collagen, osteopontin, bone sialoprotein and osteocalcin (Ducy et al.,

1997; Miyoshi et al., 1991; Ogawa et al., 1993) leading to an increase of immature osteoblasts from pluripotent stem cells; the immature osteoblasts form mature bone (Komori 2010). Runx-2 expression is down-regulated in the late stage of osteoblast maturation, when phenotypically mature osteoblasts form mature bone (Komori 2010). Runx-2-deficient mice are completely lacking in bone formation, because of an absence of osteoblasts (Komori et al., 1997; Otto et al., 1997). Osterix is also an essential transcription factor for osteoblast differentiation at an early stage (Ogawa et al., 1993), whereas it inhibits osteoblast differentiation at later stages (Komori 2003) steoblast commitment, differentiation and growth are controlled by several local and systemic factors that can also act in a paracrine and/or autocrine way and that can regulate the activity of specific transcription factor (Aubin and Liu 1996). They include bone morphogenetic proteins (BMPs; Centrella et al., 1994), hedgehog proteins, cell growth factors (Canalis et al., 1993) such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF), hormones (Cheng et al., 1994), cytokine modulators (Goldring and Goldring 1990), canonical Wingless (Wnt)/β-catenin (Ambrosetti et al., 2008; Hu et al., 2005; Mukherjee and Rotwein 2009) and mechanical physical forces (Baumbach et al., 1984; Buckley et al., 1990). The progressive development of the osteoblast phenotype from a proliferating immature cell to a mature osteoblastic cell synthesizing specific bone proteins is characterized by a definite sequential expression of tissue-specific genes that identifies three distinct periods of osteoblast phenotype development: proliferation, maturation and extracellular matrix synthesis, and matrix mineralization. During the active proliferation phase, osteoblast-committed progenitor cells (pre-osteoblasts) express genes that support proliferation, and several genes encoding extracellular

matrix proteins, such as type I collagen and fibronectin. The precursors that undergo proliferation and differentiate into pre-osteoblasts unable to deposit bone matrix but are still capable of proliferating. In this phase, BMP-2 and BMP-5 play a significant role in increasing alkaline phosphatase activity (ALP), osteocalcin synthesis (Yamaguchi et al., 1991) and parathyroid hormone (PTH9) responsiveness (Kodama et al., 1982; Takuwa et al., 1991). Immediately after growth arrest, a developmental sequence involving the selective expression of specific genes that characterize the differentiated osteoblast phenotype (alkaline phosphatase, osteocalcin) occurs (Collart et al., 1991; Stein et al., 1992). The accumulation of matrix proteins contributes, in part, to the cessation of cell proliferation. The active bone-matrix-secreting osteoblasts are cuboidal cells, with a large Golgi apparatus and an abundant rough endoplasmic reticulum, and are provided with regions of plasma membrane specialized in the trafficking and secretion of vesicles that facilitate the deposition of bone matrix (Anderson 2003). During the post-proliferative phase, which is characterized by the high synthesis of alkaline phosphatase, the extracellular matrix progresses into the mineralization phase in which osteoblasts synthesize several proteins that are associated with the mineralized matrix *in vivo* (Franzen and Heinegard 1985; Hauschka et al., 1989; Whitson et al., 1984), including sialoprotein (Nagata et al., 1991), osteopontin and osteocalcin (Gerstenfeld et al., 1987; Owen et al., 1990). Osteopontin is expressed during the stage of active proliferation (25% of maximal level; Lian and Stein 1995), decreases immediately after the post-proliferative stage and increases again at the onset of mineralization, achieving the greatest level of expression during mineralization. Osteopontin might be involved in the control of the relationship between the cells and extracellular matrix, as

its amino acid sequence containing arg-gly-asp can mediate cell attachment (Oldberg et al., 1986). Osteocalcin is expressed by osteoblasts only in the post-proliferative phase. Osteocalcin is maximally expressed during mineralization *in vivo* (Hauschka et al., 1989) and *in vitro* (Owen et al., 1990). Several studies suggest that osteocalcin is involved in the regulation of mineral deposition and that it acts as a bone matrix signal that promotes osteoblast differentiation and activation (Chenu et al., 1994; DeFranco et al., 1991; Lian et al., 1984; Liggett et al., 1994), confirming that osteocalcin is a marker of mature osteoblasts (Lian et al., 1989, Lian et al., 1991). Osteocalcin synthesis is regulated by various hormones, and growth factors (e.g. TGF-β). The progression of matrix mineralization processes might be responsible for the down-regulation of genes expressed by mature osteoblasts. At the end of the synthesis and mineralization of the extracellular matrix, cellular levels of ALP mRNAs decline (Lian and Stein 1995) and 50–70% of the mature osteoblasts undergo apoptosis, whereas the surviving cells differentiate into lining cells or osteocytes; alternatively they transdifferentiate into cells that deposit chondroid bone (Franz-Odendaal et al., 2006). Lining cells remain on the bone surface, regulate the influx and efflux of mineral ions and retain the ability to re-differentiate into secreting osteoblasts, upon exposure to various stimuli (hormones, mechanical forces; Clarke 2008). Osteocytes are metabolically quiescent osteoblasts embedded in the bone matrix; they communicate with other bone cells through cell processes and function as strain and stress sensors (Lozupone et al., 1996). Bone is constantly undergoing remodelling, a complex process in which osteoblasts play an essential role. Bone remodelling is strictly regulated by several local and systemic stimuli, including bone micro-damage, the reduction or increase of mechanical loading, blood calcium levels,

hormones, cytokines and growth factors. The process of bone remodelling occurs in small groups of cells called basic multicellular units (BMUs), characterized by the coordinated action of osteoclasts and osteoblasts. The life-span of a single BMU is about 6-9 months during which several generations of osteoclasts (average life of about 2 weeks) and osteoblasts (average life of about 3 months) are formed. A bone remodelling cycle consists of four distinct and sequential phases: activation, resorption, reversal and formation.

During the activation phase, osteoclastic precursors are recruited from circulating and bone-marrow mononuclear monocyte-macrophages (Roodman 1999), which differentiate into multinucleated cells, and actively resorbing osteoclasts that begin the resorption process. Osteoclast action is strictly related to their interaction with bone matrix proteins, including osteopontin and bone sialoprotein (Ross et al., 1993), which have been secreted by osteoblasts during the previous cycle of bone formation. When resorption has been completed, the reversal phase starts: the osteoclasts die through apoptosis and osteoblast precursors locally proliferate, differentiate into mature osteoblasts and migrate into the resorption site made by osteoclasts. In the following formative phase, osteoblasts synthesize new unmineralized bone matrix that fills the resorption site and becomes mineralized in the resting phase.

2.2. Signaling pathways governing MSC osteogenic and adipogenic differentiation

The balance between ostegenesis and adipogenesis is regulated by many signaling pathways that converge on the regulation of two main transcription factors: PPARγ and Runx2, generally regarded as the master

regulators of adipogenesis and osteogenesis, respectively. Signaling pathways are generally pro-osteogenic/anti-adipogenic stimuli. These include β-catenin dependent Wnt signaling, Hedgehog signaling (HH), and NELL-1 signaling, as well as bone morphogenic protein) signaling and insulin growth factor signaling, which display both pro-osteogenic and proadipogenic effects. In summary, understanding the factors that govern osteogenic versus adipogenic MSC differentiation has significant implications in diverse areas of human health, from obesity to osteoporosis to regenerative medicine.

2.2.1. Wnt signaling

Wnt signaling has been identified to play an essential role in cell fate determination, proliferation, and differentiation (Kim et al., 2013, Niehrs, 2012).

Dysregulation/hyperactivation of Wnt signaling is associated with numerous diseases such as neurodegeneration (Berwick et al., 2012), gastrointestinal cancers (White et al., 2012), and osteoporosis (Kim et al., 2013). Wnt signaling has demonstrated both proosteogenic and antiadipogenic activities, through both canonical (β-catenin dependent) and non-canonical (β-catenin independent) pathways. The β-catenin dependent pathway starts with the binding of extracellular Wnt ligands to the seven-pass transmembrane frizzled receptors (Frz) expressed at the cell surface (Xavier et al., 2014). This induces complex formation with transmembrane low-density lipoprotein receptor (LRP5/6) coreceptor, as well as intracellular proteins of the disheveled (DSH) family (Pandur et al., 2002). The resulting activation of DSH then functions to inhibit a second, intracellular complex comprised of axin, glycogen synthase kinase 3 (GSK3), and adenomatosis polyposis coli (APC) protein. GSK3

normally phosphorylates β-catenin, promoting its degradation. Wnt stimulation inhibits the Axin/GSK3/APC complex, and β-catenin accumulates rather than being degraded, and levels of nuclear β-catenin increase. Once inside the nucleus, β-catenin heterodimerizes with lymphoid enhancer-binding factor/T cell factor (Pandur et al., 2002). β-catenin dependent Wnt signaling activates gene transcriptional activity, and determines MSC lineage commitment (Etheridge et al., 2004). While the noncanonical Wnt pathway is similar in that it involves extracellular Wnt binding to frizzled receptors (Frz) and DSH, it otherwise diverges to mediate its effects through a β-catenin independent manner (Wang et al., 2004, Davis et al., 2008).

Canonical Wnt signaling has well-established effects on bone mass in both animal models and human patients. LRP5 mutational studies first identified a critical role for Wnt signaling in bone maintenance (Case et al., 2010). LRP5 loss-of-function mutations cause pseudo-glioma syndrome, characterized by a low bone mass phenotype. Conversely, LRP5 gain-of- function mutations result in a high bone mass phenotype (Little et al., 2002, Gong et al., 2001, Boyden et al., 2002). A direct role for β-catenin in regulating osteoblast and osteoclast activity has been repeatedly observed (Chen et al., 2013). For example, in mesenchymal osteoblastic precursors, β-catenin deficiency leads to arrest of osteoblast development at an early stage and consequent embryonic skeletal defects (Chen et al., 2013, Hu et al., 2005, Day et al., 2005, Hill et al., 2005). Moreover, in committed osteoblasts, β-catenin deficiency results in impaired maturation and mineralization (Holmen et al., 2005, Glass et al., 2005). Wnt/β-catenin signaling activity in both mature and osteoblastic precursors leads to reductions in osteoclast activity and bone resorption, as well (Takahashi et al., 2011, Nie et al., 2012). Accordingly, current

clinical applications for osteoporosis target Wnt inhibitors in order to stimulate formation of new bone and inhibit bone resorption. Currently targeted Wnt signaling antagonists include Sclerostin (SOST) and Dickkopf-1 (DKK1) (Gatti et al., 2012). As expected, inhibition of these antagonists, via anti-SOST and anti-DKK1, has been shown to stimulate bone formation and increase bone mineral density, respectively. Phase II clinical trials (for anti-SOST) and preclinical trials (for anti-DKK1) are currently underway (Lim et al., 2012, Papapoulos et al., 2011).

Various members of the Wnt signaling family have been identified to inhibit the early stages of adipogenesis (Laudes et al., 2011). For example, WNT10B has been shown to maintain 3T3-L1 preadipocytes in an undifferentiated state by inhibiting PPARγ and C/EBPα (Ross et al., 2000, Liu et al., 2004, Laudes et al., 2011). Similarly, activation of β-catenin through ectopic expression of Wnt1 also leads to direct suppression of PPARγ and prevention of 3T3-L1 cell adipogenic differentiation (Ross et al., 2000, Liu et al., 2004). Interestingly, this negative inhibition is reciprocal, in fact up-regulation of PPARγ functions inhibit β-catenin signaling (Ross et al., 2000, Liu et al., 2004, Moldes et al., 2003). Conversely, inhibition of Wnt/β signaling through treatment with DKK family proteins positively regulates adipogenesis (Laudes et al., 2011, Ross et al., 2000, Bennett et al., 2002). Additional studies suggest that the canonical ligand Wnt3a has antiadipogenic effects, by inhibiting activation of both PPARγ and C/EBPα (Kawai et al., 2007). However, while PPARγ up-regulation may negatively regulate Wnt/β-catenin signaling, overexpression of PPARγ and/or C/EBPα is not sufficient in rescuing Wnt/β-catenin-mediated inhibition of adipogenesis (Muruganandan et al., 2009, Kawai et al., 2007). In general, Wnt/β-catenin signaling pathway activation follows the inverse pattern between

the induction of MSC osteogenic and adipogenic differentiation. The activation of Wnt/β-catenin, via lithium chloride, for instance, inhibits GSK3b, which generally results in both promoting osteogenesis and suppressing adipogenesis (Li et al., 2008, Galli et al., 2013). Similarly, Wnt10b stimulates *in vivo* osteogenesis by increasing bone mass and by blocking adipogenesis in preadipocytes *in vitro* via stabilization of free cystolic β-catenin (Ross et al., 2000, Bennett et al., 2002, Bennett et al., 2007). Other canonical Wnt ligands, such as Wnt6 and Wnt10a, exhibit similar effects in stimulating osteogenesis and concurrently inhibiting adipogenesis (Cawthorn et al., 2012). Not surprisingly, disruption of Wnt/β-catenin impairs *in vitro* osteogenesis (Holmen et al., 2005, Glass et al., 2005) while increasing adipogenesis both *in vitro* and *in vivo* (Ross et al., 2000, Bennett et al., 2002, Castro et al., 2004). Moreover, inhibitors of the Wnt/β-catenin pathway demonstrate consistency with this inverse relationship between osteo- and adipogenic differentiation. DKK1, for instance, which is secreted by preadipocyte cells, inhibits osteogenesis while promoting adipogenesis *in vitro* (Gustafson et al., 2010). The inverse relationship carries over to the non-canonical branch of Wnt signaling as well. Wnt5a, for instance, has been shown to suppress pro-adipogenic PPARγ transactivation when co-induced with pro-osteogenic Runx2 in MSC (Muruganandan et al., 2009, Takada et al., 2007). Thus, as observed with multiple ligands and inhibitors, Wnt signaling generally exhibit pro-osteogenic and anti-adipogenic effects in both canonical and non-canonical signal transduction pathways.

2.2.2. Hedgehog signaling

Sonic Hedgehog (SHH) and Indian Hedgehog (IHH) are critical during

embryological development. In particular, SHH plays a key role during skeletogenesis, involved in patterning of the axial, appendicular, and facial skeleton (Riddle et al., 1993, Ruat et al., 2012). Closely related to SHH through gene duplication, IHH regulates both chondrogenesis and endochondral bone formation (Bitgood et al., 1995). In fact, disruption of HH signaling results in severe skeletal abnormalities, the most common of which being holoprosencephaly (Nanni et al., 1999). By regulating stem cells fate, SHH is a critical modulator of cell differentiation, and it demonstrates pro-osteogenic and anti-adipogenic properties in multiple MSC types (James et al., 2010). All three HH morphogens (Sonic, Indian and Desert) follow the same, highly conserved HH signaling pathway. First, the insoluble HH polypeptide precursor undergoes conversion into a soluble, multimeric form capable of diffusing across the cell membrane. This is then auto-catalytically processed from a 45 kD to a 19 kD protein, with modifications for a cholesterol moiety at the C-terminal and palmitate at the N-terminal (Simpson et al., 2009). Subsequently, the modified HH morphogen is secreted from the cell via Dispatched, a large transmembrane protein. Then, it binds to the receptor Patched (PTCH), a 12-pass transmembrane protein, on the receiving cell. This binding to PTCH relinquishes Smoothened (SMO), a 7-pass transmembrane protein, from PTCH suppression, thereby allowing activation of the glioblastoma gene products (Gli) family of transcription factors (Gli1-3). Since Gli1 is a target gene of the HH pathway, it is used as a reliable marker for HH signaling activity (Tzameli et al., 2004). The anti-adipogenic potential of HH signaling in MSC has been observed across a variety of adipocyte and multipotent cell lineages. Generally, adipogenesis in MSC, as it relates to HH signaling, occurs as a result of decreased Gli1, Gli2, Gli3, and PTCH expression (Fontaine et al., 2008). Conversely, when the HH pathway is

upregulated via the SMO (Smoothened)-activated inducer of HH signaling (Sinha et al., 2006), there is a significant decrease in adipocyte-specific markers: adipocyte fatty acid binding protein, adipsin, CD36, adiponectin, and leptin. Through the inhibition of adipogenic genes, HH signaling ultimately decreases sensitivity to insulin, which in turn reduces the expression of adipogenic transcription factors, such as C/EBPα and PPARγ (Fontaine et al., 2008). Specifically, HH signaling blocks differentiation of white adipocytes. In addition to its anti-adipogenic properties, HH signaling is well known to stimulate MSC osteogenic differentiation. While the exact mechanism and stage at which HH acts during osteoblastogenesis are not completely understood, both *in vivo* and *in vitro* data suggest that bone formation occurs through a positive feedback loop. That is, HH-induced osteoblastogenesis requires BMP signaling, and together they bring to a synergistic expression of ALP activity (Yuasa et al., 2002). This positive feedback loop is further mediated by Gli2 transcription, which serves to upregulate BMP-2 expression, which in turn activates Gli2 transcription itself (Zhao et al., 2006). SHH- induced differentiation is only observed in the immature mesenchymal cell lines 3H10T1/2 and not in the pre-osteoblastic MC3T3-E1, or the osteoblastic cell lines OS 17/2.8 and ROB-C26 (Yuasa et al., 2002, Spinella-Jaegle et al., 2001). These data suggest that SHH activity may be crucial in stimulating osteoblastogenesis in early stages of cell differentiation. In summary, HH signaling promotes MSC osteogenic differentiation over adipogenic differentiation, primarily via Gli transcriptional factor/s activity.

2.2.3. NELL-1 signaling

NELL-1 overexpression selectively increases differentiation and

mineralization in osteoblasts and is highly specific to the osteochondral lineage (Zhang et al., 2010). Transgenic mice overexpressing NELL-1 show premature cranial suture fusion and bone overgrowth,. This finding suggests a relative osteo-specific effect of NELL-1 signaling. Conversely, down-regulation of NELL-1 resulted in inhibited osteoblastogenesis *in vitro* in primary cultures of fetal rat calvarial cells and MC3T3 cell line cultures (Zhang et al., 2002). Moreover, complete loss of NELL-1 in mice results in significant reduction in the mineralization of calvarial bones and attenuated osteoblastogenesis (Zhang et al., 2012). Thus, NELL-1 has been shown to have a critical role in craniofacial osteogenic differentiation and bone formation (Zhang et al., 2002). NELL-1 has comparable bone regeneration capacity to BMP-2, in both calvarial defect and spinal fusion models. It is directly regulated by the transcription factor Runx2 (Zhang et al., 2011, Zhang et al., 2010, Lu et al, 2007). NELL-1 is preferentially expressed in osteoblasts in levels similar to Runx2, and it is most highly expressed during skeletogenesis (Zhang et al., 2011, Zhang et al., 2010). In *Runx2* deficient mice, overexpression of NELL-1 is not sufficient to rescue mineralization, whereas absence of NELL-1 significantly sRunx2 activity *in vitro* (Zhang et al., 2011). Integrin-β1 was recently identified as the first cell surface receptor for NELL-1 (Shen et al., 2012). Cell surface binding in a preosteoblast cell line required Integrinβ1 expression (Shen et al., 2012). Moreover, siRNA for Integrinβ1 blocked at least some of the cellular effects of NELL-1, including induction of preosteoblast attachment (Shen et al., 2012). NELL-1 is known to promote osteogenesis accompanied by activation of MAPK, canonical Wnt and HH signaling (James et al., 2011, James et al., 2012, Chen et al., 2012). NELL-1 activates both ERK1/2 and JNK1 MAPK pathways in Saos-2 osteosarcoma cell type (Chen et al., 2012).

This activation of MAPK signaling is associated with Runx2 protein phosphorylation (activation) (Chen et al., 2012). In addition, NELL-1 induced MAPK activity is accompanied by activation of phosphate transporters Pit1 and Pit2 to increase preosteoblast mineralization (Cowan et al., 2012). NELL-1 induction of Wnt signaling has been observed in both osteoblastic and osteoclastic cell types and is associated with its pro-osteogenic and antiosteoclastic effects (James et al., 2011). Recent data have shown that NELL-1 also has anti-adipogenic effects, found both in the preadipocyte cell line 3T3-L1 cells, as well as in the primary adipose-derived MSCs (ASC) (James et al., 2011). This was observed both in adipocyte specific gene expression and intracellular lipid accumulation. Recent *in vivo* studies have confirmed the anti-adipogenic effects of NELL-1, in which direct intramedullary injection of NELL-1 reduced intramarrow adipocytes in a senile rat model Kwak et al., 2013). These antiadipogenic effects of NELL-1 in preadipocytes are associated with activation of HH signaling. These effects may be through activation/intersection with MAPK, Wnt, and HH signaling.

2.2.4. Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, are extracellular cytokines originally isolated from bone extract and found to induce ectopic chondrogenesis and osteogenesis (Wozney et al., 1988). BMPs are responsible for numerous cell regulatory processes, including the differentiation and patterning of bone and cartilage (Chen et al., 2004). Over 20 different BMPs have been identified, of which BMP-2, -4, -7, -9, and -13 are most commonly studied in the context of MSC differentiation (Kang et al., 2009, Bragdon et al., 2011). Both recombinant BMP-2 and -

7 are approved by the FDA for bone regeneration in spinal fusion surgery, and they are commonly used for other orthopaedic applications (Freire et al., 2011, Firedlaender et al., 2001). BMPs produce their effects through interaction with two serine-threonine kinase cell surface BMP receptors (BMPRs). Type II BMPRs initiate signaling upon binding to a BMP ligand, following which recruitment, phosphorylation, and activation of type I BMPRs occurs (Chen et al., 2004, Miyazono et al., 2005, Nohe et al., 2004). While there are several different types of BMPRs, only a few are involved in MSC differentiation, including BMPR-IA and BMPR-IB (Chen et al., 1998). Several downstream BMP signaling elements exist, including Smad1/5/8, MAP Kinase, and c-Jun N-terminal kinase (JNK) signaling pathways, which are phosphorylated and thereby activated (Chen et al., 1998, Tzameli et al., 2004, Nishimura et al., 2012). Transcriptional regulation of adipogenic and osteogenic during MSC commitment is regulated by Smad1/5/8 signaling transduction through the Smad-protein (Chen et al., 2004, Miyazono et al., 2005, Nohe et al., 2004). BMP induced adipogenesis involves both Smad1/5/8 and MAPK activation (Hata et al., 2003). BMP induced Smad1/5/8 signaling activates PPARγ via zinc finger transcription factor Schnurri-2 and C/EBPα, which exhibit adipogenic effects (Krishnan et al., 2006, Takagi et al., 2006). Accordingly, a Smad antagonist such as Smad6 reduces both PPARγ signaling and BMP-associated adipogenesis (Hata et al., 2003). Similar to Smad1/5/8 signaling, BMP induced activation of MAPK signaling is associated with PPARγ activation and adipogenic differentiation (Hata et al., 2003). Conversely, disruption of MAPK signaling also inhibits both PPARγ expression and BMP-associated adipogenesis (Hata et al., 2003). Investigators have identified BMP signaling activity at the earliest stages of MSC adipogenesis (Bowers et al., 2007, Bowers et al., 2006). When

MSCs are forced into a preadipocyte cell lineage through exposure to 5-azacytidine, a potent inhibitor of DNA methylation, BMP- 4 expression increases (Bowers et al., 2007, Bowers et al., 2006). Forced expression of BMP-4 in white adipocytes induces a brown adipocyte phenotype, including increased energy expenditure and insulin sensitivity (Qian et al., 2013). Moreover, once MSC have been forced into preadipocyte cells, BMP-4 over-expression is sufficient to induce commitment to adipocyte lineage differentiation (Kang et al., 2009, Bowers et al., 2007, Ahrens et al., 1993). BMP signaling is one of the central signaling pathways involved in the induction of osteogenic differentiation and regulation of bone formation. Multiple murine studies involving genetically modified BMP ligands, BMP receptors, and BMP inhibitors demonstrate a critical role for BMP signaling in bone formation (Mishina et al., 2004, Okamoto et al., 2006, Gazzerro et al., 2007, Gazzerro et al., 2005). For example, transgenic mice with modified BMPR-IA receptors exhibit low bone mass and irregular calcification (Mishina et al., 2004). Inhibitors of BMP signaling, such as Noggin and Gremlin, impair bone formation when overexpressed (Qian et al., 2013, Davis et al., 2007, Zhu et al., 2006). In general, BMP induces osteogenesis utilizing both autocrine and paracrine pathways (Cheng et al., 2003, Suzawa et al., 1999) and it works in conjunction with Osterix through both Runx2-dependent and -- independent pathways. BMP receptor activation in osteogenesis, as well as adipogenesis, involves both Smad1/5/8 and MAPK down-stream signaling activation. While 31 different BMP ligands are identified to date, only some of them actually promote MSC osteogenic differentiation (Ducy et al., 2000). Specifically, BMP-2, -4, -6, - 7, and -9 have been shown to promote osteogenic commitment, as well as terminal osteogenic differentiation in MSC (Kang et al., 2009, Dorman et al., 2012). BMP-2,

the most commonly studied BMP ligand, induces MSC osteogenesis both *in vitro* and *in vivo* (Reid et al., 1982, Varkey et al., 2006, Partirdge et al., 2002, Wegman et al., 2011, Park et al., 2010, Tang et al., 2008, Kempen et al., 2008, Cheng et al., 2001). Furthermore, investigators have found that short-term BMP-2 treatment is both necessary and sufficient for osteogenic commitment in the murine mesenchymal stem cell line C3H10T1/2 (Noel et al., 2004).

The precise factors that govern BMP signaling-induced adipogenesis versus osteogenesis in MSCs are not well understood. Two variables that may determine the effects of BMP on MSC differentiation have been observed: dosage and receptor type. In terms of dosage, lower concentrations of BMP-2 have been shown to favor adipocyte formation, while higher concentrations support osteogenic differentiation in C3H10T1/2 (Wang et al., 1993). However, these dosage effects may be ligand- and cell-type dependent. In terms of receptor type, BMPR-IA induces adipogenic effects, while BMPR-1B induces osteogenic effects. Even though BMP receptor type and dosage are two known variables that affect MSC lineage determination, no global rule applies (Rahman et al., 2012).

2.2.4. IGF signaling

IGF-1 induces its effects through the IGF-I receptor (IGF1R) and IGF-binding proteins (IGFBPs) 1–6 (Kawai et al., 2009). IGF-1 can be found systemically and it is present in most peripheral tissues, including the bone (Giustina et al., 2008, Kawai et al., 2009, Govoni et al., 2012). The functions of IGF- 1 in bone have been well documented. IGF-1 produces its effect by inducing several intracellular signaling pathways. First, IGF-1 binds to the IGF-1 receptor, which intracellularly autophosphorylates

the receptor at the kinase domain. Upon receptor activation, various protein substrates are consequently activated, including insulin receptor substrate-1 (IRS-1), Src homology and collagen protein (SHC) (Kawai et al., 2009). IRS-1 goes on to activate the phospho-inositol 3-kinase (PI3-K), 3-PI-dependent kinase- (PDK-1), and Akt pathways, while SHC is responsible for activating the Ras/Raf/mitogen-activated protein (MAP) kinase pathways (Govoni et al., 2012). IRS-1 produces its effect through interaction with and activation of PI3K, thereby catalyzing the phosphorylation of PIP2 to PIP3. The elevated levels of PIP3 consequently activate PDK-1 and Akt (Peng et al., 2003). Activation of PI3K, PDK-1, and Akt has been shown to be important in skeletal growth (Peng et al., 2003, Ghosh-Choudhury et al., 2002). In fact, knockout Akt1/Akt2 mice demonstrate significantly impaired bone development and skeletal growth (Peng et al., 2003). Meanwhile, SHC, which forms a complex with Grb2 and SOC, is responsible for increasing cell proliferation through activation of the Ras/Raf-1/MAPK pathway. During bone remodeling, IGF-1 is released from the bone matrix to stimulate MSC osteoblastogenesis via activation of mammalian Target Of Rapamycin (mTOR). This allows for the maintenance of both bone structure and mass, both of which were downregulated in mice with knockout of IGF- 1 receptors in pre-osteoblastic cells (Xian et al., 2012). IGF-1 has been found to promote both adipogenic and osteogenic differentiation. For example, IGF-1 induces cell division of adipocyte precursor cells (Wabitsch et al., 1995). In addition, IGF receptors are involved in promoting adipogenesis through induction of advanced glycation end products (AGEs). AGEs activate both NAD(P)H oxidase and Src, which ultimately leads to the phosphorylation/activation of both IGF-1 receptor and Akt downstream in 3T3-L1 preadipocyte cells (Yang

et al., 2013). Further, Akt1/Akt2 knockout mice demonstrate impaired adipogenesis (Peng et al., 2003). In fact, it has been shown that both Akt1 and Akt2 are necessary to induce PPARγ, the key regulator for adipogenesis. Thus, a critical threshold of Akt activity, as regulated by IGF-1, contributes to the maintenance of cell proliferation, growth, and adipogenic differentiation (Peng et al., 2003). In summary, an inverse relationship exists between adipogenic and osteogenic lineage differentiation in MSCs, which is governed by diverse signaling pathways. The understanding of this relationship has far-reaching implications for the understanding of human health and treatment of human disease.

3. Kit

The viral oncogene v-c-Kit was in 1986 identified as the transforming gene of Hardy-Zuckerman 4 feline sarcoma virus (hence its name c-Kit as in kitten) (Besmer et al., 1986). It was discovered that Kit is allelic with the dominant white spotting locus (*W*) of mice (Chabot et al., 1988, Geissler et al., 1988). Mutations in the Steel (*Sl*) locus in mice give rise to a phenotype very much resembling those of mice with loss-of-function mutations in the *W* locus. Therefore it was soon demonstrated that the product of the Steel locus was identical to the ligand for Kit, stem cell factor (SCF) (Copeland et al., 1990, Williams et al., 1990).

Numerous loss-of-function mutations in *W* and the *Sl* loci have been described in mice. These *loci* encode Kit and SCF, respectively. These naturally occurring mutations comprise a spectrum of defects ranging from minor defects in the tyrosine kinase activity of Kit to a complete loss of its kinase activity, resulting in the corresponding degree of severity in the phenotype displayed by these mice. The expression pattern of Kit and SCF during mouse embryogenesis suggests that they are involved in migration of cells of the hematopoietic, germ cell, and melanoblast lineages, as well as in the differentiation and proliferation of these cells (Keshet et al., 1991, Matsui et al., 1990, Or-Urtreger et al., 1990). The numerous loss-of-function mutations in this receptor/ligand system suggests crucial functions in the hematopoietic system, during gametocyte development, pigmentation, intestinal motility, as well as in the nervous system (Keshet et al., 1991, Lev et al., 1994, Russel, 1979). Data from other models also suggests a function in the immune system, including

inflammation (given its expression in both dendritic cells and mast cells) and in the regulation of vasculogenesis (reviewed in Heissig et al., 2003, Metcalfe, 2008, Ray et al., 2010).

Cairns et al., (2003) originally identified the cis-acting elements in the 5' flanking region and the first intron of the *Kit* gene which are essential for its expression in hematopoietic progenitors and primordial germ cells (PGCs). We found six DNase I hypersensitive sites (HS1-HS6) within these genomic regions and developed several mouse lines expressing transgenic green fluorescent protein (GFP) under the control of these regulatory elements. A construct driven by the *Kit* promoter and including all 6 HS sites was found to be highly expressed during mouse development in Kit-expressing cells, including PGCs and hematopoietic progenitors. The *Kit* promoter alone (comprising only HS1) was found to be sufficient to drive low-level GFP expression in PGCs, but unable to function in hematopoietic cells. Hematopoietic expression further required the addition of the intronic HS2 fragment. This intronic fragment also greatly potentiated expression of the reporter gene in PGCs. Thus, elements within the first intron act as an enhancer in both lineages. Optimal hematopoietic expression further required more downstream elements within the first intron, which were instead not required for expression in PGCs (Cairns et al., 2003). The mouse transgenic lines obtained with the constructs containing both the *Kit* promoter region and the first intron were found to express GFP also in postnatal germ cells, while those originating from the construct comprising only the promoter region expressed low levels of GFP only in PGCs, but not in differentiating spermatogonia (Filipponi et al., 2007).

Cerisoli et al., (2009) reported that this transgene is expressed in the large majority of fetal liver and adult bone marrow HSCs. The *Kit/GFP*

transgene appears to be regulated correctly in early Kit^+ hematopoietic progenitors. In fact, over 90% of cells in bone marrow fractions highly enriched in HSC and early progenitor cells, show GFP expression. Furthermore, functional HSC are contained within cell fractions that express the transgene at intermediate levels, and essentially all the HSC capable of long-term reconstitution are in this fraction. In contrast to HSC, multipotent and early erythroyd progenitors (CFU-mix and BFU-E) express Kit/GFP at high levels. The *Kit* gene is active in several types of stem cells, and its defects affect multiple cell lineages (Broudy, 1997) pointing to Kit being a pleiotropic, though cell-type-restricted, molecule. It would, therefore, be particularly relevant to have a mouse model in which the transgene efficiently recapitulates the activity of the endogenous molecule. The currently-used *Kit/GFP* transgene, in addition to being expressed in HSC, in primordial germ cells and subsets of spermatogonia (Cairns et al., 2003, Filipponi et al., 2007), is also active in a population of cardiac stem cells (Messina et al., 2004). Thus, Kit might be regulated by subsets of common transcriptional programs in different stem cells. Finally, transgene expression in HSC/progenitors has enabled monitoring of bone marrow cells homing to muscle and heart following tissue damage (Barile et al., 2011).

Kit is also involved in melanoblasts development, as it is produced in both premigratory and migrating melanocytes. Kit signaling is important at several time points also during melanocyte development, and has independent effects on both migration and survival along the dorsolateral pathway in the embryo (Thomas et al., 2008).

In addition, a recent work found that positivity to Kit marks a subpopulation of human Adipose Stem Cells (ADSCs), which resides in a perivascular location, and shows higher proliferative activity and self-

renewal capacity, higher telomerase activity and expression, higher *in vitro* adipogenic efficiency, a higher capacity for the maintenance of cardiac progenitors, and higher pancreatogenic and hepatogenic efficiency. Moreover, isolation of Kit positive ADSC subpopulations allows for the selection of a more homogeneous subpopulation with increased cardioprotective properties and increased adipogenic and endodermal differentiation potential, providing a useful tool for specific therapies in regenerative medicine applications. (Blazquez-Martinez et al., 2014).

The gene for Kit was cloned and found to be located on chromosome segment 4q11 in humans (D'Auriol et al., 1988, Yarden et al., 1987) and is comprised of 21 exons, spanning more than 34 kb of DNA. The first exon encodes the translational initiation codon and the signal peptide. The remainder of the extracellular part of Kit is encoded by exons 2–9. The transmembrane region is encoded by exon 10, while the remaining exons encode the intracellular part of the receptor. Kit expression can be regulated by transcription factors, such as Myb and Ets-2 in hematopoietic cells. Moreover, Kit has also been reported to be regulated by miRNA (miR-193b in leukemic cells (Gao et al., 2011) and mir-221 in melanoma cells (Igoucheva and Alexeev, 2009). miR-221 and miR-222 have been described as regulators of Kit expression in hematopoietic cells and have also been reported to be potential regulators of Kit expression in gastrointestinal stromal tumors (Felli et al., 2005, Koelz et al., 2011). The receptor tyrosine kinase encoded by the Kit gene is a transmembrane protein with an extracellular domain made up by five immuno-globulin-like domains followed by a single spanning transmembrane region. The phosphorylation sites, necessary for Kit activity, reside in this intracellular region, composed of the juxtamembrane region, and the

kinase domains (tyrosine kinase domain 1 and 2) SCF is a homodimer, and Kit dimerization is driven by its ability to simultaneously interact with two Kit monomers (Lemmon et al., 1997). Furthermore, it was proposed that only the first three Ig-like domains were required for SCF binding. The first three Ig-like domains in Kit have a complementary shape and charge to allow tight binding of SCF, and after binding to each other, no major structural changes occur (Yuzava et al., 2007). Furthermore, in addition to bringing two Kit monomers together into a dimeric complex, ligand binding also induces a conformational change that enables homotypic interactions between Ig-like domains 4 and 5 in two adjacent Kit molecules (Yuzava et al., 2007). There are many signal transduction downstream of Kit, such as the PI3-kinase, Src family kinases, MAPK pathways, and phospholipases. These pathways are integrated into a signaling circuit. Autophosphorylated receptors interact with the Src homology 2 (SH2) domains of the p85 subunit (p85α, p50α, p55α, p85β, and p55γ), resulting in a conformation change in the associated enzymatic p110 subunit (p110α, p110β, and p110δ), which leads to its activation (Klippel et al., 1994). PI3-kinase is activated by SCF through direct binding to Tyr-721 (Lev et al., 1992, Serve et al., 1994). Akt is a key molecule downstream of PI3-kinase that promotes cell survival by interfering with the initiation of apoptosis (Datta et al., 1997). The serine/threonine kinase Akt is located downstream of PI3-kinase and is a molecule in survival signaling in response to SCF. Activated Akt promotes cell survival in different ways including phosphorylation of Bad, Foxo, and activation of nuclear factor kappa-light chain enhancer of activated B cells (NF-kB). Bad is a protein involved in the control of cytochrome *c* release from the mitochondria, an initial event in the activation of the caspase cascade. In the absence of survival signals, Bad

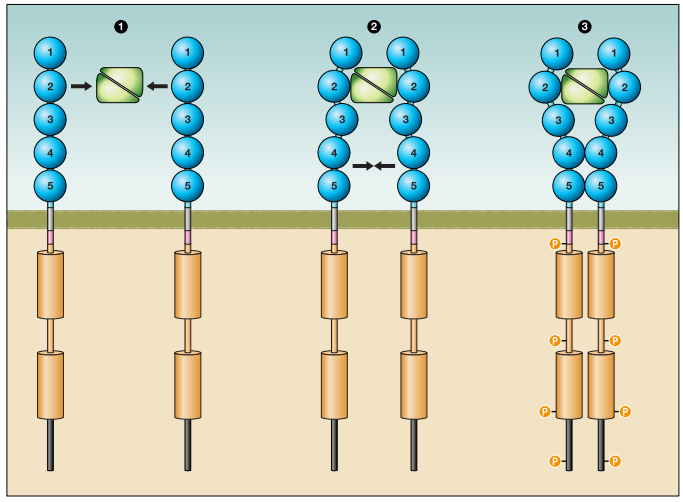

Figura 6. Schematic representation of SCF-induced Kit-activation (Lennartsson and Ronnstrand, 2012)

heterodimerizes and thereby neutralizes the anti-apoptotic proteins Bcl-X_L or Bcl-2 (Yang et al., 1995). In response to SCF treatment, Akt is activated in a PI3-kinase-dependent manner resulting in Bad phosphorylation on Ser-136 (Blume-Jensen et al., 1998), which disrupts the interaction between Bad and Bcl-X_L. Bad is sequestered by 14–3-3 proteins (Zha et al., 1996). Bcl-X_L can then antagonize the pro-apoptotic Bax protein in a manner blocking cytochrome *c* release and consequently apoptosis. It has also been found that melanocyte proliferation and migration in response to SCF stimulation was dependent on PI3-kinase (Jeon et al., 2009). The Src family of tyrosine kinases (SFK) contains eight cytoplasmic kinases, some of which are ubiquitously expressed (Src, Yes, and Fyn), whereas others have more restricted, often hematopoietic expression (Lck, Hck, Lyn, Fgr, and Blk). In response to SCF stimulation, Kit becomes phosphorylated on two residues in the juxtamambrane region (Tyr-568 and Tyr-570) (Krystal et al., 1998, Lennartsson et al., 1999, Linnekin et al., 1997, Price et al., 1997). These

phosphorylation sites interact with the SH2 domain in SFK (Lennartsson et al., 1999). Activated SFK have been shown to contribute to activation of several signal pathways downstream of Kit. Several groups have implicated SFK in SCF-induced ERK1/2 mitogen-activated protein (MAP) kinase activation (Bondzi et al., 2000, Lennartsson et al., 1999), possibly by promoting the phosphorylation of the Shc adaptor protein (Lennartsson et al., 1999). The JNK MAP kinase pathway is activated by Kit in a manner that requires both SFK and PI3-kinase acting on Rac1 (Timokhina et al., 1998). Several studies have implicated activation of SFK in Kit-induced proliferation. Activation of Kit protects many cells from apoptosis; this effect has to a large extent been ascribed to PI3-kinase signaling. However, SFK also contributes to the survival effect. Treatment of erythroid precursor cells with SCF is able to suppress Fas-mediated apoptosis, and this effect was abolished by the SFK inhibitor PP2 (Nishio et al., 2001). In mast cells, both SFK and PI3-kinase were shown to be important for SCF-mediated protection form apoptosis (Timokhina et al., 1998). SFK have also been implicated downstream of Kit in promoting cell migration. MAP kinases are activated downstream of most types of cell surface receptors and hence play central roles in a multitude of biological processes, both under normal and pathological conditions. The MAP kinase pathway has the architecture of a three-layered kinase module that is initiated at the plasma membrane and reaches to the nucleus where it regulates gene expression often by phosphorylating transcription factors. However, also processes occurring in the cytoplasm, for example, translation and cell migration can be regulated by MAP kinases. The biological consequence of MAP kinase activation is connected to the magnitude as well as the duration of MAP kinase phosphorylation (Marshall et al., 1995, Yamamoto et al., 2006).

All eukaryotic cells have at least one type of MAP kinase and human cells contain four major groups: ERK1 and -2 (ERK1/2), ERK5, p38, and JNK. Stimulation of Kit has been shown to activate ERK1/2, p38, JNK, and ERK5, Several studies have implicated p38 in the signaling circuitry regulating cell migration, and inhibition of p38 inhibits this pathway (Kuang et al., 2008, McDaniel et al., 2008, Sundstrom et al., 2001). Ueda et al., (2002) found that SCF-induced p38 and PI3-kinase activation was important for Ca^2 influx, which in turn activated ERK1/2, and promoted cell migration.

4. Prep1

The TALE subfamily (Three Aminoacids Loop Extension) of homeobox genes, encode transcription factors that have a broad spectrum of functions both in embryonic development and in adult stem and progenitor cell. Prep1 is a member of TALE proteins and interestingly, even small variations in its expression level can have dramatic effects in murine embryonic development, suggesting that different threshold levels are required for distinct Prep1 functions.

Prep1 forms DNA-independent dimeric complexes with all isoforms of the Pbx homeodomain transcription factor, enhancing target specificity and regulating their activity, nuclear localization, and likely, function in development (Berthelsen et al., 1998, Calvo et l., 1999, Knoepfler et al., 1997, Pai et al., 1998, Rieckhof et al., 1997).

Prep1 deficiency affects the expression of both TALE class partners Pbx and Meis, and the decrease of Prep1, Pbx and Meis proteins in $Prep1^{i/i}$ embryos almost abolishes the DNA-binding activity of Meis/Prep-Pbx dimer-specific target sequences. As the mRNA levels of these proteins are not affected in a statistically significant manner, their reduction appears to be at the post-transcriptional level. In mammalian cells in culture, Prep1 overexpression does not affect Pbx1 and Pbx2 mRNA levels, but increases the stability of Pbx1 and Pbx2 by preventing their proteasomal degradation (Longobardi and Blasi, 2003). As a result, in the absence of Prep1, Pbx proteins are not protected from proteasomal degradation. However, Prep1 deficiency results in a decrease of Pbx3, Pbx4, Meis2, and Meis3 mRNAs in whole E10.5 embryos. Thus, Prep1 not only forms

transcriptional complexes with Pbx but also hierarchically controls the expression of all Pbx and Meis genes. Mammals have two Prep genes, Prep1 and Prep2 (Berthelsen et al., 1998 Fognani et al., 2002, Haller et al., 2002), while zebrafish have three, prep1.1, prep1.2, and prep2 (De Florian et al., 2004). Down-regulation with morpholino antisense oligonucleotides of the prep1.1 gene in zebrafish causes an embryonic lethal phenotype with extensive brain apoptosis, loss of hindbrain rhombomeric segmentation, lack of cartilage differentiation of neural crest cells, pericardial edema, and lack of fins (De Florian et al., 2004). In mice, a null Prep1 mutation results in early lethality (E7.5), precluding further studies of Prep1 in later developmental stages. Thus, in order to understand the *in vivo* role of Prep1 during adulthood, an embryonic lethal hypomorphic mutant mouse ($Prep1^{i/i}$) was generated. An insertion of a retroviral vector in the first intron of the Prep1 gene ($Prep1^{i/i}$) results in a hypomorphic mutation that exhibits variable penetrance and various levels of expression (Penkov et al., 2005). Prep1 deficiency manifests with different degree of severity, depending on the level of expression. Prep1 null embryos die at E7.5, while hypomorphic $Prep1^{i/i}$ mutation cause embryonic lethality with variable penetrance. The majority of $Prep1^{i/i}$ embryos die of anemia at E17.5–P0, showing a more severe phenotype when lower levels of Prep1 are produced. These mice are characterized by an overall organ hypoplasia, severe anemia, impaired angiogenesis, and eye anomalies, particularly in the lens and retina. The ability of one quarter of these embryos to survive and live at least 16–18 months (Ferretti et al., 2006) is likely due to residual expression of low levels of Prep1. This suggests that different Prep1 threshold levels are required at different stages of the embryonic development. The $Prep1^{i/i}$ embryonic phenotype recapitulates other TALE family members

phenotypes, such as Meis1 and Pbx1. The Pbx family comprises four genes in mammals which are differentially expressed during embryonic development and in the adult (Ferretti et al., 1999, Monica et al., 1991). Pbx1-deficient mice exhibit an embryonic lethal phenotype, characterized by homeotic transformation of elements of the second branchial arch and by defective organogenesis affecting the spleen, pancreas, kidney, and organs of the caudal pharyngeal pouches (Kim et al., 2002, Manley et al., 2004, Schnabel et al., 2003, Selleri et al., 2001). Pbx1-deficient embryos also show defective definitive hematopoiesis (Dimartino et al., 2001) and are unable to induce splenic cell fate specification during early embryogenesis (Brendolan et al., 2005). Meis1, Meis2, and Meis3 form transcriptionally active complexes with Pbx, that have an important role during embryonic development (Berthelsen et al., 1998, Chang et al., 1997, Ferretti et al., 1999, Knoepfler et al., 1997, Kurant et al., 1998, Salzburg et al., 1999, Vlachakis et al., 2001, Waskiewicz et al., 2001). Meis/Prep-Pbx complexes, in turn, bind to and modify the activity of other proteins, such as the Hox proteins Hoxb1, Hox11, Pdx1; and other transcription factors such as MyoD (Berkes et al., 2004, Brendolan et al., 2005, Ferretti et al., 2000, Jacobs et al., 1999, Ryoo et al., 1999, Thomas et al., 2005). Meis/Prep-Pbx complexes control expression of numerous genes, including Hoxb1, Hoxb2, Hoxa3, Hox11, and glucagon (Brendolan et al., 2005, Ferretti et al., 2000, Herzig et al., 2000, Jacobs et al., 1999, Manzanares et al., 2001, Ryoo et al., 1999, Salzburg et al., 1999. Meis1-deficient mice exhibit an embryonic lethal phenotype (E13.5 to 14.5) with major defects in hematopoiesis, angiogenesis, and eye formation (Azcoitia et al., 2005, Hisa et al., 2004), while Meis2 appears to be involved in controlling limb outgrowth (Capdevila et al., 1999, Mercader et al., 2005). By using Prep1 hypomorphic mice, it has been possible to

study Prep1 role and consequently establish that Prep1 is a master gene required for hematopoietic, angiogenic, and eye development, as well as other developmental functions, by controlling the levels of Pbx and Meis TALE proteins and their target genes. Many of the phenotypes observed in $Prep1^{i/i}$ embryos may be mediated by the concomitant loss of Meis and Pbx partners, therefore resulting in defects closely resembling those of Pbx1 and Meis1 null embryos.

Prep1 is required for the lymphoid as well as the erythroid lineages. The hematopoietic phenotype is characterized by a drastic decrease in the number of circulating erythrocytes and a delay in erythroid differentiation. E15.5-E16.5 $Prep1^{i/i}$ fetal livers (FL) contain more erythroid progenitors and fewer erythroid differentiated cells (Ferretti et al., 2006), as compared to their wild type counterpart. One of the genes required for normal erythropoiesis is cMyb. FLs from $Prep1^{i/i}$ embryos exhibit a drastic decrease in cMyb-positive cells. Therefore, the overall decrease in cMyb levels can, at least in part, explain the erythroid phenotype (Emambokus et al., 2003). $Prep1^{i/i}$ hypomorphic embryos are also deficient in B-lymphoid differentiation. $Prep1^{i/i}$ FL cells fail to compete with wild type cells in competitive repopulation assays, in essentially all hematopoietic lineages. Prep1 is expressed in KSLA and B-cells progenitors, and in cells that coexpress markers of hematopoietic stem/progenitors cells. these data suggestthat in the absence of proper levels of Prep1 active long-term repopulating hematopoietic stem cells (LTR-HSC) are decreased in activity and/or in number. $Prep1^{i/i}$ embryos display a major FL hypoplasy which affects the number of KSLA cells, highly enriched in LTR-HSC cells (Hsu et al., 2000). When analyzed in

detail, Prep1$^{i/i}$ FL cells show a deficient proliferation of myeloid progenitors (CFU-GEMM) colonies in methylcellulose, furthermore Prep1$^{i/i}$ FL cells have a tendency to show delayed or blocked differentiation of the B-lymphocytes precursors (Di Rosa et al., 2007). Therefore, Prep1 expression is required for the proper functioning of the embryonic hematopoietic system.

Prep1 has is expressed in adult hematopoietic cells, namely in the BM Lin^{-}Sca^{+} population. After birth, the hypomorphic mice which escape embryonic lethality, exhibit a defect in T-cell differentiation, with a decreased number of circulating CD4^{+} and CD8^{+}T cells, increased apoptosis, and decreased proliferation of double-positive thymocytes; anomalies in T-cell receptor expression, a phenotype reproduced in *wt* mice transplanted with Prep1$^{i/i}$ FL cells (Penkov et al., 2005). The hematopoietic phenotypes of the Meis1 knock-out and the Prep1$^{i/i}$ hypomorphic embryos are very similar (deficient hematopoiesis, angiogenesis and oculogenesis) (Hisa et al., 2004; Azcoitia et al., 2005; Ferretti et al., 2006). Since Prep1 controls the level of all Pbx and Meis1 (Ferretti et al., 2006), the Prep1$^{i/i}$ phenotype likely depends, at least partially, on the reduction of Pbx and Meis1. However, while Pbx1 was shown to be required for B-lymphoid differentiation at a stage lying between the hematopoietic stem cells and the pro-B progenitors (Sanyal et al., 2007), low levels of Prep1 appear to affect not only the differentiation of B-cell progenitors, but also of precursors, possibly including the LTR-HSC. Prep1 is an important player in the activity of LTR-HSC and in the differentiation of various progenitor lineages. Prep1 not only directly participates in this pathway by dimerizing with Pbx but also controls the expression of Pbx and Meis, and hence it should also affect the activity

and expression of Hox and other genes regulating differentiation. A recent work has shown that Prep1 controls both the number and the function of HSCs during mouse embryo development. Prep1 regulates HSCs self-renew since its hypomorphic cells are deficient in being able to generate generating proper stem cell compartment upon transplantation into ablated hosts, and undergo faster exhaustion upon serial transplantation. Prep1 maintains FL HSCs in G0 phase. It is known that FL HSCs are mainly proliferating cells as compared to the quiescent adult BM HSCs (qui puoi mettere Ye et al¨). In homeostatic conditions BM HSCs divide preferentially by asymmetric cell division, in order to give rise to mature cells and maintain the stem cell pool. In contrast, FL HSCs are characterized by symmetric self renewing divisions in order to expand the stem cell pool. Prep1 deficiency brings to a reduction in FL HSC numbers which favours an increase in multipotent progenitor cells. Prep1 mutation results in a disequilibrium between stem cells and progenitors, due to a change in the balance between asymmetric and symmetric cell divisions inclined to the asymmetric one, leading to a reduction of the stem cell pool and an increase of the progenitor compartment (Modica et al., 2014). This phenotype is consistent with the effects exhibited by Pbx and Meis1 null HSCs. However while Pbx1 and Meis1 act on TGFβ response, Prep1 is implicated in the regulation of the Interferon(IFN)-response pathway, which has been shown to regulate HSCs properties in both adult and fetal tissues. In particular, IFNα induces adult HSCs proliferation, concomitantly to decreasing their G0 pool, leading to HSCs exhaustion (Di Rosa et al., 2007).

Angiogenesis is also impaired in $Prep1^{i/i}$ embryos. E7.5-7.75 $Prep1^{i/i}$ allantois preparations and E10.5 whole embryos show indeed reduced,

thinner, and less-organized capillaries. Angiogenic precursors are also affected by Prep1 deficiency. In fact, Prep1 is present in endothelial precursors, where it colocalizes with their markers CD31 and c-Kit in E14.5 FL. Furthermore, the finding of a decreased microvasculature in Prep1$^{i/i}$ allantois cultures indicates that Prep1$^{i/i}$ embryos have an intrinsic angiogenic defect, which does not simply reflect a decrease in circulating blood cells, and thus is independent from the hematopoietic phenotype (Ferretti et al., 2006).

Another frequent phenotype of Prep1$^{i/i}$ embryos involved eye development. In most cases, the size of the lens is strongly reduced, similar to the phenotype of Pax6-deficient mice, where no lens induction and anomalies of the neural retina have been reported (Simpson et al., 2002, Treisman et al., 2004). Prep1 is present in E14.5 neural retina, cornea, and lens epithelium and specifically colocalized with Pax6. Pax6 is essential for oculogenesis and it is dramatically reduced in Prep1$^{i/i}$ embryos, thus explaining the eye phenotype in these mutant mice (Gehring et al., 1999, Lang, 2004, Simpson and Pryce, 2002, Treisman, 2004). Moreover, previous biochemical and genetic data demonstrate that Meis1 directly regulates Pax6 expression during vertebrate lens morphogenesis (Zhang et al., 2002). As Prep1$^{i/i}$ embryos exhibit also lower levels of Meis1 protein, the Prep1$^{i/i}$ ocular phenotype might be due to reduced Meis1 expression. The angiogenic, hematopoietic, and eye phenotypes have also been reported in Meis1-deficient embryos (Azcoitia et al., 2005, Hisa et al., 2004). As Prep1 and Meis act by dimerizing with Pbx proteins, it is possible that Pbx also participates in the regulation of Pax6 expression and eye development.

After examination of glucose homeostasis in Prep1$^{i/i}$ mice it was clear that an absolute reduction in circulating insulin levels but normal glucose tolerance occur. In addition, these mice are protected from streptozotocin-induced diabetes and enhanced insulin sensitivity with improved glucose uptake and insulin-dependent glucose disposal by skeletal muscle (Oriente et al., 2008). p160 Myb-binding protein (p160) (Tavner et al., 1998) is a repressor of the regulator of glucose and energy metabolism, PPAR-gamma coactivator-1α (PGC-1α) (Fan et al., 2004), and interestingly, a direct Prep1-interacting protein that competes with Pbx1 for Prep1 binding (Diaz et al., 2007). Thus, Prep1 functions may depend not only on its interaction with Pbx but also with p160. It has been shown that there is a balance between p160-Prep1 and Pbx1-Prep1 complexes; when p160 levels exceed Pbx1 levels, p160 binds Prep1, which is stabilized, and represses *GLUT4* and insulin sensitivity. When Pbx1 is present in excess, the reverse occurs. In Prep1$^{i/i}$ muscle, there are normal levels of Pbx1 but reduced levels of p160. Decreased levels of p160 lead to a muscle-selective increase in mRNA and protein levels of PGC-1α, accompanied by enhanced expression of the GLUT4 transporter, responsible for insulin-stimulated glucose uptake in muscle. Thus, Prep1 controls the stability of the p160 protein and, as a consequence, it controls insulin sensitivity through the p160-GLUT4 pathway (Oriente et al., 2008).

Several studies support the idea that Prep1 has also a role in maintaining genomic stability and preventing neoplastic transformation. The DNA-protecting role of Prep1 may be essential during embryonic development already at the epiblast stage, when Prep1 null embryos die (Fernandez-Diaz et al., 2010). Several crucial events take place during early

development. In the E3.5 blastocyst, the inner cell mass (ICM) contains progenitor cells, including epiblast (Epi) precursors (Chazaud et al., 2006), which generate embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981). The Epi is established during implantation around E4.5, and from E5.5 to E6.5 forms an epithelium, maintains its pluripotent state (Niwa, 2007) and actively proliferates. At this time, the Epi is very sensitive to DNA damage (Heyer et al., 2000) and is not protected by the usual G1 and G2 check points (O'Farrell et al., 2004). DNA damage at this stage leads to p53-dependent apoptosis (Heyer et al., 2000). The absence of Prep1 causes p53-dependent apoptosis in Epi cells, which prevents gastrulation and differentiation. This is probably due to the accumulation of DNA damage (Fernandez-Diaz et al., 2010). The absence of Prep1 affects all cells but apoptosis is mostly observed in the Epi, probably owing to the strong proliferative expansion of these cells at such stage. Thus, one of the roles of Prep1 in early embryogenesis is to protect epiblast cells from accumulating damage that induces apoptosis (Fernandez-Diaz et al., 2011).

Genetic instability is a common feature in cancer and, in fact, mutations in genes involved in processes like DNA repair, chromosomal segregation, checkpoint control, and centrosome duplication are oncogenic (Lengauer et al., 1998, Negrini et al., 2010). Many tumor suppressor genes are specialized in controlling these processes. $Prep1^{i/i}$ hypomorphic mice that escape embryonic lethality, develop spontaneous tumors or pre-tumoral lesions, and transplantation of $Prep1^{i/i}$ fetal liver (FL) cells into lethally irradiated normal mice induces lymphomas. Prep1 is absent or strongly down-regulated in about 70% of 700 human cancers. This evidence indicates that Prep1 is a novel tumor suppressor gene

(Longobardi et al., 2010). Interestingly, a recent study has demonstrated that the key feature of Prep1 tumor-inhibiting activity is the control of the oncogene Meis1 stability. Prep1 post-translationally controls the level of Meis1, decreasing its stability by sequesterin Pbx1 (Dardaei et al., 2014). Moreover, Iotti et al., (2011) show that the tumor suppressor Prep1 (Longobardi et al., 2010) prevents genetic instability. Indeed, hypomorphic $Prep1^{i/i}$ FL cells and mouse embryonic fibroblasts (MEFs) exhibit increased basal DNA damage and normal DNA damage response after γ-irradiation, as compared to *wt* counterparts. Cytogenetic analysis shows the presence of numerous chromosomal aberrations and aneuploidy at early-passage $Prep1^{i/i}$ MEFs. In human fibroblasts, Prep1 down-regulation by siRNA induces DNA damage response, similarly to $Prep1^{i/i}$ MEFs, together with an increase in heterochromatin-associated modifications: rapid increase of histone methylation and decreased transcription of satellite DNA. Ectopic expression of Prep1 rescues DNA damage and heterochromatin methylation. Finally, Prep1 deficiency facilitates cell immortalization, and escape from oncogene-induced senescence. These results show that the tumor suppressor role of Prep1 is associated with the maintenance of genomic stability (Iotti et al., 2011).

5. Aim of the project

MSCs, for their biological properties, represent a potentially precious therapeutic tool in regenerative medicine, for a wide spectrum of pathologies. Their clinical use is of particular interest in the fields of orthopedic surgery, metabolic disorders and bone marrow transplantation. However, their optimal use depends on our knowledge of their biology, and the identification of signalling pathways that regulate their expansion and differentiation, still poorly understood. In addition, MSCs and their progeny, in particular osteoblasts, are essential components of the Hematopoietic Stem Cells (HSCs) niche, which provide the appropriate microenvironment for engrafment, survival, migration, growth, and differentiation. Interestingly, in the bone marrow, MSC differentiation towards the osteogenic or the adipogenic lineage is competitively balanced and mechanisms that promote one cell fate actively suppress molecular regulators that induce the alternative cell program. This fine control is based on the cross talk between complex signaling pathways. Much efforts has been made to understand molecular mechanisms driving differentiation from progenitors to terminal differentiated cells, taking advantage of pre-adipocytes and pre-osteblasts cell line. However, little is known yet about the molecular mechanisms driving the initial commitment phase towards the adipogenic or the osteogenic fate, likely working as a switch. In our laboratory we hypothesized that key regulatory genes of hematopoietic stem and progenitor cells might mediate the cross talk between mesenchymal and hematopoietic cells.

My thesis is aimed at investigating the role that the Stem Cell Factor receptor kit and the transcrition factor Prep1 may play in the regulation of

MSCs. The rationale for choosing Kit is: i. it is a "stem cell gene" involved in survival, proliferation, and migration of hematopoietic, cardiac and neural stem and progenitor cells, melanoblasts and Primordial Germ Cells; ii. it has been extensively studied by our group (Cairns et al., 2003, Cerisoli et al., 2009, Barile et al., 2011), using a Kit/GFP transgenic mouse line in which the expression of the transgene recapitulates the activity of the endogenous gene in various types of stem and progenitor cells; the role of Kit in the control of the MSCs is still controversial. The rationale for studying Prep1 is: i. also this transcription factor of the TALE family has pleiotropic effects in multiple tissues; ii. it plays a pivotal role in the regulation of hematopoietic stem and/or progenitor cells and it is not clear yet whether the altered hematopoietic compartment shown by Prep1 hypomorphic mice is due to a cell autonomous or non cell autonomous defect. (Penkov et al., 2005, Ferretti et al., 2006, Di Rosa et al., 2007).

Real time qPCR, FACS and Western Blot analysis are initially performed to analyze Kit and Prep1 expression, both at the mRNA and protein level, in undifferentiated culture and during *in vitro* adipogenic and osteogenic processes. Furthermore, functional studies by using a Prep1 hypomorphic ($Prep^{i/i}$) murine line allow to address the effects of Prep1-deficiency in undifferentiated cells and after adipogenic and osteogenic induction.