# Bone Marrow Mesenchymal Stem Cell Niches and Regenerative Medicine

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## ***Summary***

Mesenchymal progenitors are a powerful tool in regenerative medicine, but suffer from a rapid loss of differentiation potential during in vitro expansion (1). The recent discovery that well-characterized stem cells, like HSC, maintain their stemness during self-renewal through the interaction with specialized microenvironments, called stem cell niches, prompted us to investigate the existence of a niche compartment for also mesenchymal progenitors.

In Chapter 4 of this thesis we described the establishment of a niche/progenitor system in vitro for bone marrow mesenchymal stem cells (MSC). We asked whether the non-adherent fraction of human bone marrow cultures contained early progenitors which can constitute a reservoir for the mesenchymal compartment and whether the adherent cells, instead, could provide a niche function for the maintenance and regulation of these progenitors.

Replating the non-adherent fraction in a new dish at the first medium change, we found that a population of bone marrow non-adherent mesenchymal progenitors (BM-NAMP) was present and their number was 20.4±3.6% of the initial CFU-f. The replating in the same dish, instead, did not increase the number of colonies (100±25.1% vs 99.0±23.5%, p=n.s.) and no change in the average diameter size was observed (5.5±0.5 mm vs 5.8±0.6 mm, p=n.s.), indicating that BM-NAMP were stably non-adherent. However, further investigation showed that, when serially replated in new dishes, BM-NAMP were able to steadily increase in number, self-renewing as non-adherent progenitors while generating at the same time adherent colonies. The diameter size evaluation showed that BM-NAMP could produce colonies with 2-fold larger diameter (Plate1=10.73±1.21 mm vs Plate0=5.54±0.45 mm, $p < 0.01$), indicating a significantly higher proliferation capacity. However, the colonies produced in the following replating steps were progressively smaller, indicating a gradual loss of BM-NAMP proliferation potential (Plate2= 8.8±0.7 mm, Plate3= 7.5±0.3 mm, Plate4= 6.4±0.3

mm). Together with increased proliferation, first-replated BM-NAMP progeny cells displayed a higher differentiation potential compared to standard CFU-f both in vitro and in vivo. In vitro assays revealed that they could differentiate towards the adipogenic, chondrogenic and osteogenic lineage and, when implanted in vivo, they produced 3-fold higher amounts of bone tissue. Taken together, these data indicate together that BM-NAMP show features of earlier progenitor features and suggest a biological difference between BM-NAMP and the initially adhrering CFU-f.

Serial replating experiments performed with serum alone showed that BM-NAMP critically required FGF-2 for their initial selection and maintenance in culture. Furthermore, FGF-2 removal, at different time points during serial replating, always caused the disappearance of BM-NAMP. This observation is compatible with the data that described FGF-2 role in the selection of a subset of earlier pluripotent mesenchymal precursors within initial CFU-f (3). Interestingly, blocking receptor experiments showed that the maintenance of BM-NAMP in culture was mediated through FGFR2c signaling, which has been shown to be involved in vivo in the balance between proliferation and differentiation of skeletal progenitors (4).

When replated in a new dish, BM-NAMP regenerated themselves as non-adherent progenitors, but at the same time they always gave rise to adherent colonies. We, therefore, hypothesize that BM-NAMP were in close interaction with the adherent cells, and that these provide a niche function for them. BM-NAMP were not able to survive when replated either on agarose-coated dishes or on human fibroblasts. This suggests that BM-NAMP required specific signals from the adherent progeny and that this fraction constitutes a unique environment for BM-NAMP survival and self-renewal. In fact, when kept in contact with initial CFU-f progeny for 14 days instead of being serially replated, BM-NAMP were able to produce 3-fold more colonies (260.8±15.8% vs 61.0±8.0%). Furthermore, the colony

diameter analysis showed that, unlike the serial replating which caused a gradual loss of BM-NAMP proliferative activity, the continuous culture in the primary plate could preserve BM-NAMP proliferation potential (9.2±0.5 mm and 9.8±0.4 mm at the start of culture respectively vs 6.8±0.3 mm after serial replating, $p<0.05$). Furthermore, if kept in the original plate, BM-NAMP could generate a progeny that also displayed a higher differentiation capacity. Taken together, these results suggest together that CFU-f progeny provides a niche function for BM-NAMP. When an adherent fraction was not present, due to serial replating in empty dishes, BM-NAMP could regenerate their niche but at the cost of gradually losing their proliferative and differentiation potential.

In Chapter 5 we sought at investigating the presence of a class of non-adherent progenitors in human adipose tissue stromal vascular fraction (SVF), which constitute an abundant source of mesenchymal progenitors, to determine whether the NAMP compartment was specific to bone marrow or they could constitute a reservoir also in other tissues.

NAMP were present in adipose tissue SVF cultures (AT-NAMP) with a similar frequency as observed in the bone marrow (17.7±9.1% vs 20.4±3.6% respectively) and the replating of the non-adherent fraction in the same dish revealed that they were stably non-adherent. The main difference compared to BM-NAMP was the inability of AT-NAMP to self-renew as non-adherent progenitors upon serial replating, since only few colonies were present in the last replating step. However, these colonies had a significantly increased diameter. This suggests that, when serially replated, AT-NAMP do not undergo proliferation but rather a selection for the very rare progenitors with the highest proliferation ability. Similarly to BM-NAMP, when kept in contact with the initially adhering CFU-f, AT-NAMP could proliferate without loss of their proliferation capacity. This suggests that, as for bone marrow cells, adherent CFU-f provide a niche function for the non-adherent progenitors, regulating the maintenance of their early-progenitor properties.

In conclusion, these data show that, although displaying important tissue-specific biological differences, NAMP are present in the mesenchymal progenitor compartment of different tissues and they represent a reservoir of earlier progenitors compared to standard CFU-f.

# ***Chapter 1:***

## ***Bone and bone marrow: structure and function***

## *1.1 Bone tissue: biology, structure, and function*

Bone is a dynamic, highly vascular, and mineralized connective tissue, characterized by its hardness, resilience, growth mechanisms, and its capability to remodel itself throughout the life-time of an individual.

Bone performs several key functions within the body: it not only provides structural support and protection to bodily organs, but is also responsible for maintaining mineral homeostasis, and is the primary site for the synthesis of blood cells. Furthermore, it is capable of maintaining an optimal shape and structure throughout life, via a continuous process of renewal and remodelling, through which it's able to respond to changes in its mechanical environment, in order to meet different loading demands, thus maintaining an optimal balance between form and function (1).

Simply, bone is a dense multi-phase composite, made up of cells embedded in a very well-organized matrix, which is composed of both organic and inorganic elements; however, both structure and proportion of its components widely differ with age, site and history, resulting in many different classifications of bone that exhibit various mechanical and functional characteristics.

Histologically, mature bone is classified in two different types of tissue, one of which is relatively dense, known as cortical bone, while the other consists of a network of struts or trabeculae surrounding interconnected spaces, known as trabecular or cancellous bone (Figure 1). Bone surfaces consist of cortical bone, and the thickness of this protective layer increases in mechanically demanding regions, such as the shafts of long bones, while cancellous bone is found in the interior of bones, such as within the femoral head, and vertebra.

Bone as an organ is composed of three main elements: (i) bone matrix, providing mechanical strength and acting as the body's mineral store, (ii) bone cells, responsible for

maintaining the structure of the matrix, regulating its oxygen and nutrient supply, and storing or releasing minerals as required, and (iii) bone marrow with its associated vascular network, providing the source of stem cells and representing the main means of communication and interaction with the rest of the body.

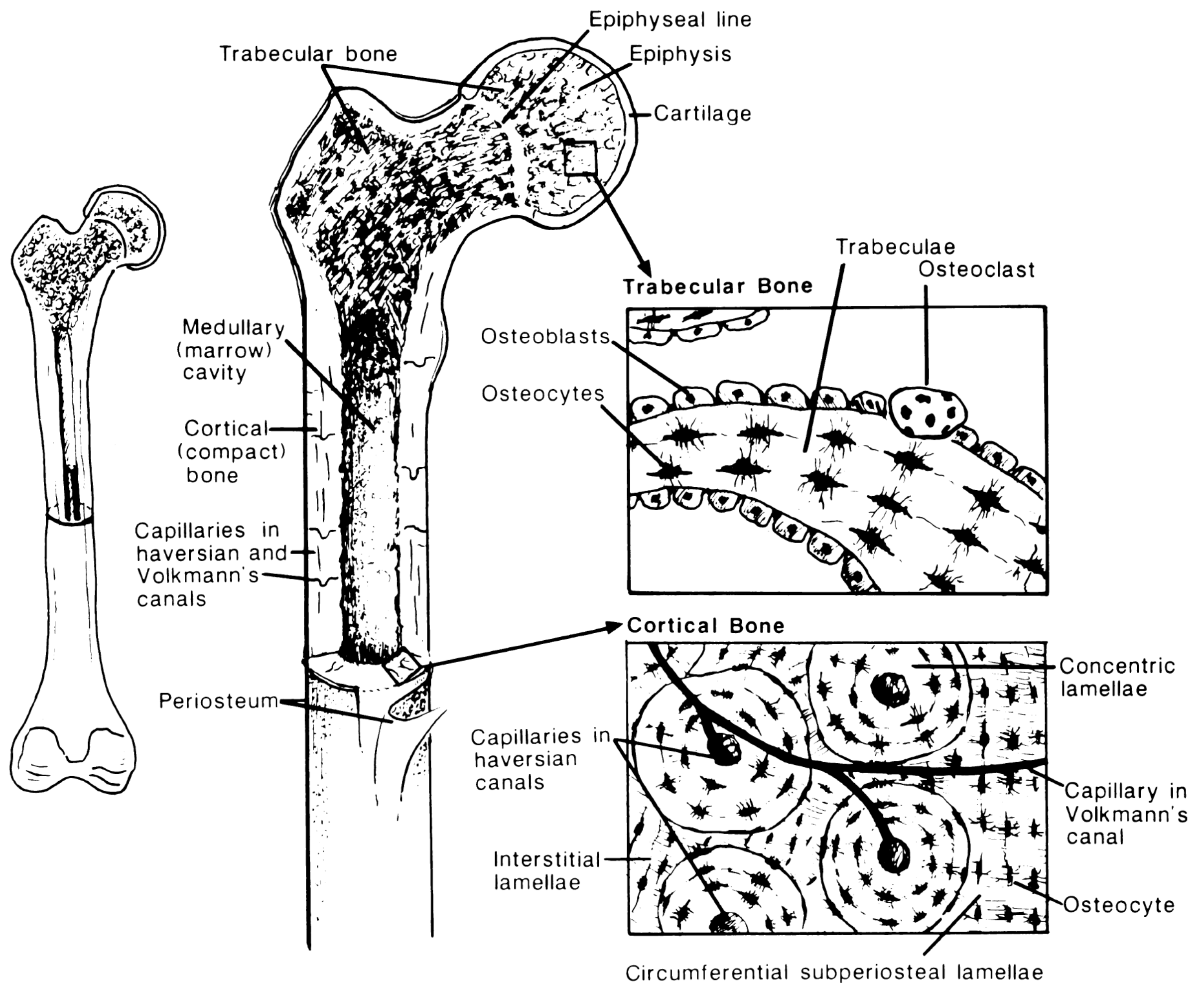


***Figure 1**. **Schematic diagram of cortical and trabecular bone**, showing the different microstructures (Reproduced from Hayes WC: Biomechanics of cortical and trabecular bone: Implications for assessment of fracture risk).*

Bone extracellular matrix has two main components: the organic collagen fibres and the inorganic bone mineral crystals. Together they make up approximately 95% of the dry weight of bone, the remainder being composed of other organic molecules, collectively known as the non-collagenous proteins.

Collagen accounts for 70-90% of the non-mineralized components of the bone matrix; it consists of carefully arranged arrays of tropocollagen molecules, which are long rigid molecules composed of three left-handed helices of peptides, known as α-chains, which are bound together in a right-handed triple helix. Bone contains mostly type-I collagen, which is composed of tropocollagen molecules containing two identical and one dissimilar α-chains ($\alpha1(I)_2\,\alpha2$).

The main inorganic phase within the bone matrix is usually incorrectly referred to as hydroxyapatite (HA), a hydrated calcium phosphate ceramic, with a similar crystallographic structure to natural bone mineral, which has a chemical formula of $Ca_{10}(PO_4)_6(OH)_2$; however, bone-apatite is characterized by calcium, phosphate and hydroxyl deficiency, internal crystal disorder, and ionic substitutions, thus resulting in the presence of significant levels of additional trace elements within bone mineral: it is not a direct analogue of HA, but more closely a carbonate-substituted apatite. All these factors contribute to an apatite that is insoluble enough for stability, yet sufficiently reactive to allow the in vivo crystallites to be constantly resorbed and reformed as required by the body.

The most important non-collagenous organic constituents of bone matrix are four proteins: osteocalcin (OC), bone sialoprotein (BSP), osteopontin (OP) and osteonectin (ON). They are produced by bone cells and their relative composition within the bone matrix appears to be self-regulating through a feedback effect on their expression by osteoblasts. They all appear to be multi-functional, and are all involved in regulating bone mineralization and remodelling.

Bone matrix also contains a great number of growth factors, including fibroblast growth factors (FGFs), insuline-like growth factors (IGFs), plateled-derived growth factors (PDGF), transforming growth factor-beta (TGFβ) superfamily, and bone morphogenic proteins (BMPs): they play several critical roles in regulating cell proliferation and differentiation, inducing the complete sequence of endochondral bone formation, when cartilage forms first and is subsequently replaced by bone.

The major types of bone cells are osteoblasts, osteocytes and osteoclasts, respectively responsible for production, maintenance, and resorption of bone; they are highly specialized differentiated cells, and they generally don't proliferate. Less differentiated cells of the same lineage are required for the control of bone cell populations, and, as demands are made on or by the bone, these cells proliferate and differentiate as required: such cells are generally known as stem cells, and in the case of bone formation are often referred to as osteogenic cells.

The osteogenic bone-forming cells originate from the mesenchymal bone marrow stromal cell line and exist in the endosteum and periosteum (2). Biochemical signalling molecules stimulated during remodelling and fracture healing, result in a local increase of this cell population. However, the local environment also determines the route of differentiation undertaken by osteogenic cells, resulting in the evolution of either osteoblasts or chondroblasts: if the environment surrounding a differentiating osteogenic cell has a high vascular content, as in healthy bone, the cell will differentiate into an osteoblast which will produce bone; once the osteoblast has been surrounded by bone, it differentiates into an osteocyte, and becomes involved in the nutrition and maintenance of the local bone. In contrast, if the environment surrounding a differentiating osteogenic cell has little or no vascular content, as in a recent fracture site, the cell will differentiate into a chondroblast and cartilage will be produced; once the chondroblast is surrounded by cartilage, it then

differentiates into a chondrocyte, which maintains the surrounding collagenous matrix until it's replaced by bone during endochondral ossification.

In contrast osteoclasts are derived from monocytes, thus they originate from the haemopoietic stem cell lineage: under the influence of specific signalling proteins or cytokines, mononuclear monocytes migrate to the resorption site and fuse with either other monocytes or a multi-nucleated macrophage, before differentiating into the specialized osteoclast, an aggressive cell responsible for bone resorption (3).

## *1.2 Bone formation: development, healing, and repair*

Bone is unique among all the vertebrate tissues in its ability to heal via formation of new bone: most of the other tissues, such as heart, muscle and brain heal by replacement with connective tissue rather than original tissue. Furthermore, in a mature animal, the molecular and cellular patterns of bone repair after injury are similar to bone formation in an embryo, suggesting analogous mechanisms for the control of bone formation in adult and embryonic skeletons (4). In an embryo, a condensation of primitive mesenchymal cells can transform into bone via either intramembranous or endochondral ossification: intramembranous ossification occurs when the mesenchymal cells are transformed into osteoprogenitor cells and then directly into osteoblasts, resulting in the direct formation of bone; endochondral ossification occurs via a two-step process where mesenchymal cells transform into chondroblasts which lay down a collagenous template, subsequently ossified by invading osteoblasts. The final mature bone formed by both processes is virtually indistinguishable, and the mechanisms dictating which route is taken are poorly understood.

Fractured bone heals through endochondral ossification: a haematoma is formed, resulting from injury to the periosteum and local soft tissue; as a consequence of this disruption in the blood supply, osteocytes nearest to the fracture die, resulting in local

necrosis of the bone around the fracture; simultaneously, there is a demand for the repair of the bone, the stabilization of the damaged area and the removal of the dead tissue; in response to this, macrophages and fibroblasts are recruited to the site to remove tissue debris, and to express extracellular matrix, respectively. In response to growth factors and cytokines released by these inflammatory cells, mesenchymal stem cells recruited from the bone marrow and periosteum, proliferate and differentiate into osteoprogenitor cells. This leads to an apparent thickening of the periosteum and the production of collars of external fracture callus around the fracture site. Those osteoprogenitor cells that lie close to undamaged bone, differentiate into bone osteoblasts and form an osteoid, which is rapidly calcified into bone, while those farther away become chondroblasts and form cartilage; concurrent angiogenesis is induced, and, as soon as cartilage has formed and the fracture site stabilized, it is replaced by cancellous bone via endochondral ossification, in which osteoclasts and osteoprogenitor cells invade the cartilaginous callus preceded by capillary formation. The uncalcified material is then resorbed, and new bone is deposited on the remaining spinicules of calcified cartilage. Woven bone is finally remodelled into lamellar bone, bone marrow is restored within cancellous regions, and successive layers of bone gradually fill the spaces between trabeculae of cortical bone. Load-bearing capabilities and a new vascular network are thus restored.

Although the vast majority of bone defects spontaneously heal with minimal treatment, among the 6 millions fractures occurring every year in the United States, 5-10% require further treatment for compromised healing because of either interposition of soft tissue, improper fracture fixation, loss of bone, metabolic diseases, impairment of blood supply or infection. Furthermore, in certain clinical settings, large pieces of bone must be resected to treat benign and malignant tumours, osteomyelitis, as well as bone deficiences, and abnormal loss in the maxillo-facial area; in addition, bone is typically subject to progressive degeneration as a result of age and disease (i.e. osteoporosis).

Considering all these challenging situations, bone function can often be restored only by surgical reconstruction: bone grafting, the procedure of replacing missing bone with material from either the patient's own body (autografting) or that of a donor (allografting) is used in the surgical procedures since many years. Autologous bone harvested from donor sites such as the iliac crest, is the preferred treatment (5): grafts of this kind are osteoconductive (they provide a scaffold on which bone cells can proliferate), osteoinductive (they induce proliferation of undifferentiated cells and their differentiation into osteoblasts), and osteogenic (they provide a reservoir of skeletal stem and progenitor cells that can form new bone); however, the amount of bone that can be safely harvested is limited, while the additional surgical procedure may be complicated by donor-site pain and morbidity. Modern allografting using material stored within bone banks overcomes these difficulties; however, the demand exceeds the supply, there is no assurance of freedom from disease, and healing can be inconsistent (6).

As an alternative to these two types of bone grafts, a wide variety of synthetic substrates have been developed and are actually in clinical use, with mixed success and surgical acceptance: such materials in fact are generally biocompatible and osteoconductive, thus supporting adhesion, proliferation, and differentiation of osteogenic cells from surrounding tissues, and ultimately leading to bone formation; however, these materials are not osteoinductive, providing only the scaffold which has to be invaded by bone-forming bioactive cells (7), (8): reasoning that they typically give good results only when implanted in small defects, where interactions between material's surface and local cells and proteins are sufficient to repair the bone defect. In addition, metals, although providing immediate mechanical support at the site of the defect, exhibit poor overall integration with the tissue at the implantation site, and can fail because of infection or fatigue loading; on the other hand,

ceramics have very low tensile strength and are brittle, thus they cannot be used in locations of significant torsion, bending, or shear stress (9).

Thus it's clearly seen that repair of bone defects is actually still a big challenge for the orthopaedic, reconstructive, and maxillo-facial surgeons: it's in this scenario that a promising field of science called Tissue Engineering is emerging since the last few years.

## *1.3 The bone marrow structure*

The marrow, one of the largest organs in the human body, is the principal site for blood cell formation. Until the late 19$^{th}$ century hematopoiesis was thought to be the prerogative of the limph nodes or the liver and the spleen. In 1868, Neuman and Bizzozzero independently observed nucleated blood cells in material squeezed from the ribs of human cadavers and proposed that bone marrow was the major source of blood cells.

The bone marrow is found within the central cavities of axial and long bones. It consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses interspersed within a meshwork of trabecular bone. The inner surface of the bone cavities and the outer surface of the cancellous bone trabeculae within the cavities are covered by an endosteal lining consisting of a single layer of flat "bone-lining cells" supported by a thin layer of reticular connective tissue; osteoblasts and osteoclasts are also found within the endosteal lining.

Macroscopically, the bone marrow is composed by red marrow (hematopoietic) and yellow marrow (adipose), whose proportions vary with age in agreement with the Neumann's law, according to which, at birth, all the bones contain red marrow, whereas, as age increases, the extension of hematopoietic marrow contracts towards the axial skeleton and the peripheral bones contain only yellow marrow.

Microscopically, the bone marrow structure follows the organization of the vasculature.

Bone marrow has an extensive blood supply: in long bones, one or more feeding canals (containing one artery and 1or 2 veins) pass through the cortical bone entering the marrow cavity obliquely. In flat bones, the marrow is served by numerous blood vessels of various sizes entering the marrow via large and small canals.

In a long bone, the feeding artery enters the marrow cavity and runs parallel to the longitudinal axis in the central part. Its branches run perpendicularly the bone cortex, forming specialized vascular structures – the bone marrow sinuses – composed by endothelial cells only, which function as entry site for the mature hematopoietic elements ready for the circulation. The sinuses coalesce into venules, which form then the central vein, running side by side with the artery and the two exit the marrow cavity together. Blood flow, therefore, takes place in a radial direction from the center to the cortex and viceversa. The space between the vessels is occupied by the hematopoietic cords, in which the maturation of the different blood elements takes place. The hamatopoietic activity is highest close to the sinuses in the periphery of the marrow cavity in proximity of the endosteal surface, whereas adipocytes are most in the central part. A similar structure, with the hematopoietic islands located close to the endosteal surface of the trabeculae, is present, even though less defined, also in the spongy bones, where the majority of adult hematopoiesis takes place.

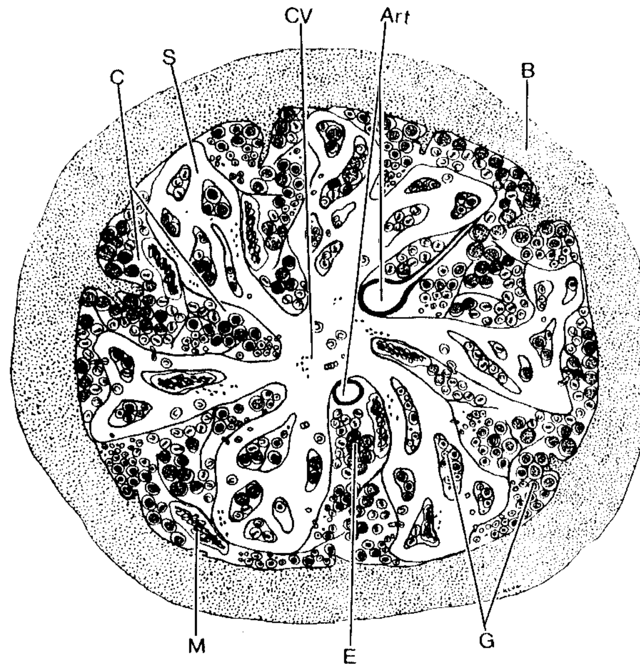


***Figure 2. Bone Marrow Structure*** *B= bone; Art= central artery; CV= central vein; S= marrow sinus; C= hematopoietic cord; E= erithropoiesis; G= granulopoiesis; M= megakariocytes (reproduced from (10)).*

Within the hematopoietic cords, it is possible to recognize two dinstinct cellular compartments, which have different ontological origins and functions: the stromal compartment and the hematopoietic compartment. Maturation of the different blood lineages takes place in distinct compartments: 1) erythropoiesis takes place in the erythroblastic islands located around a central macrophage, which surrounds the maturing hematopoieitc elements with thin cytoplasmatic projections, for at least two thirds of their surface; 2) megakariopoiesis takes place under the sinus endothelium, where small cytoplasmatic processes anchor the megakariocyte to the sinusoidal wall; 3) granulopoiesis, instead, takes place in foci always associated with a reticular cell.

The stromal compartment, instead, forms the complex three-dimensional structure of the hematopoietic cords. Two cell types, the macrophages and the reticular cells, play an important role for the stroma structure.

Macrophages are located in proximity of the sinuses and in the center of the erythroblastic islands. They are also responsible for the generation of the osteoclastic compartment.

The reticular cells can be visualized by silver staining of the reticular fibers to which they are associated in the extra-cellular matrix. A subpopulation of reticular cells, defined as adventitial reticular cells, is located close to the sinuses, forming an adventitial layer on the wall of the vessel, similar to pericytes. These cells send thin cytoplasmatic processes from in the hematopoietic cords, where they enter in contact with processes of other reticular cells, forming in this way, a three-dimensional scaffold for the hematopoietic compartment. The non-adventitial reticular cells are often located in the center of the granulopoiesis islands, where they also have a regulatory function.

The bone-lining cells are a population of flat cells that covers the bone endosteal surface. Reticular cells, pre-osteoblasts, osteoblasts and osteoclast can be found in the same location. These cells also include the mesenchymal progenitors cells or mesenchymal stem cells (MSC), whose anatomical location remains still controversial. All these cell types, except those of the osteoclastic lineage, share a common cytochemical characteristic that sets them apart from all other bone marrow cells: the expression of alkaline phosphatase (ALP), which is considered a marker for the osteoblastic lineage. At a morphological level, some electron microscopy studies revealed that all the cells that make up the stromal structure, are functionally connected through gap-junctions (11) (Figure 3).

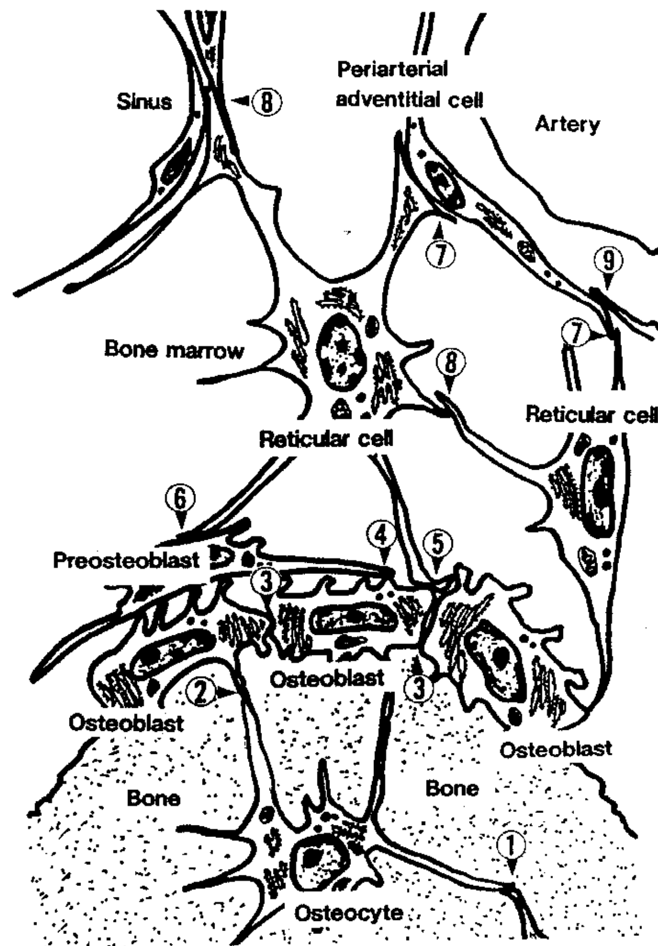


***Figure 3.*** *Schematic representation of the connections between the different cell types of the stromal structure in the bone marrow. The arrows indicate the gap-junctions (Reproduced from* (11)).

A gap-junction connects anatomically and functionally two cells, allowing the direct exchange of molecules up to 2 KDa of weight through the cytoplasm. Calcium ions can pass through, but also growth factors and cytokines involved in bone remodeling and in the regulation of hematopoiesis. Therefore, the relationship between reticular cells, stromal progenitors and osteoblasts comes from anatomical evidence, from the common expression of the alkaline phosphatase, from the absence of endothelial or macrophage markers and from the common synthesis of collagen type I and II.

The stroma, with its complex structure and the different cell elements, therefore represents the support for the hematopoietic compartment. Among the cells that compose the stromal tissue, the mesenchymal progenitors or mesenchymal stem cells (MSC) received, in the past years, plenty of attention from the scientific community for their ability to differentiate into the different mesenchymal lineages.

In the next chapter of this thesis (Chapter 2), the basic aspects of MSC biology will be discussed, focusing on the issues that still remain controversial and on MSC potential application for clinical purposes.

In Chapter 3, instead, we will described an important aspect of the bone marrow function, illustrating the data currently available about the specialized microenvironments, called stem cell niches, responsible for the regulation of the stem cell function.

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# ***Chapter 2:***

## ***Bone Marrow Mesenchymal Stem Cells***

## *2.1 History*

The bone marrow is a highly cellularized and richly vascularized tissue contained in the cavity of long bones and in the intra-trabecular spaces of spongius bones. Being the major site of adult hematopoiesis, the main function of bone marrow is to provide a specialized environment to protect hematopoietic stem cells, assuring their maintenance and, therefore, a continuous production of all types of mature blood cells.

Besides containing hematopoietic precursors, the bone marrow contains different non-hematopoietic cells including reticular, fat and endothelial cells, fibroblasts, osteoblasts and mesenchymal progenitors. All together, these cells constitute the bone marrow stroma, which support hematopoiesis, providing structural and humoral signals which regulate the stem cell function. Among the fully differentiated cells, a key component of the bone marrow stroma is represented by the mesenchymal progenitor cells, which are able to differentiate into the mesenchymal lineages both in vitro and after in vivo transfer ((1), Figure 1). The first evidence that it was feasible to ectopically generate bone and bone marrow, after bone marrow transplantation, dates back to the 19$^{th}$ century (2). In 1968, the work of Tavassoli and Crosby clearly established the capacity of bone marrow to form bone ectopically, even though the cells holding the osteogenic potential were not identified. Only the studies of Friedenstein (3) demonstrated that the ectopic bone formation was due to the presence in the bone marrow of a specific rare cell population. These cells were characterized by a fibroblastic morphology, suggesting a stromal-compartment origin, and by their capacity to rapidly adhere to plastic. Furthermore, when plated at low density, they were able to form discrete colonies originated by single cells, the CFU-f, the colony-forming unit fibroblastic (3). Friedenstein and Owen named them osteogenic stem cells (4) or bone marrow stromal stem cells (5) and in 1991 Caplan defined them as mesenchymal stem cells (MSC). In the beginning, these discoveries about MSC aroused the interest of the hematologists, also in

concomitance with the publication in 1978 of Schofield's hypothesis, about the presence in the bone marrow of a specialized microenvironment which regulates hematopoietic stem cells. Only later, the concept of a bone marrow stem cell, distinct from the hematopoietic one and that could differentiate into the different mesenchymal lineages, became popular (6), (7) and MSC were identified as an interesting target for cell therapy in different fields (8), (9).

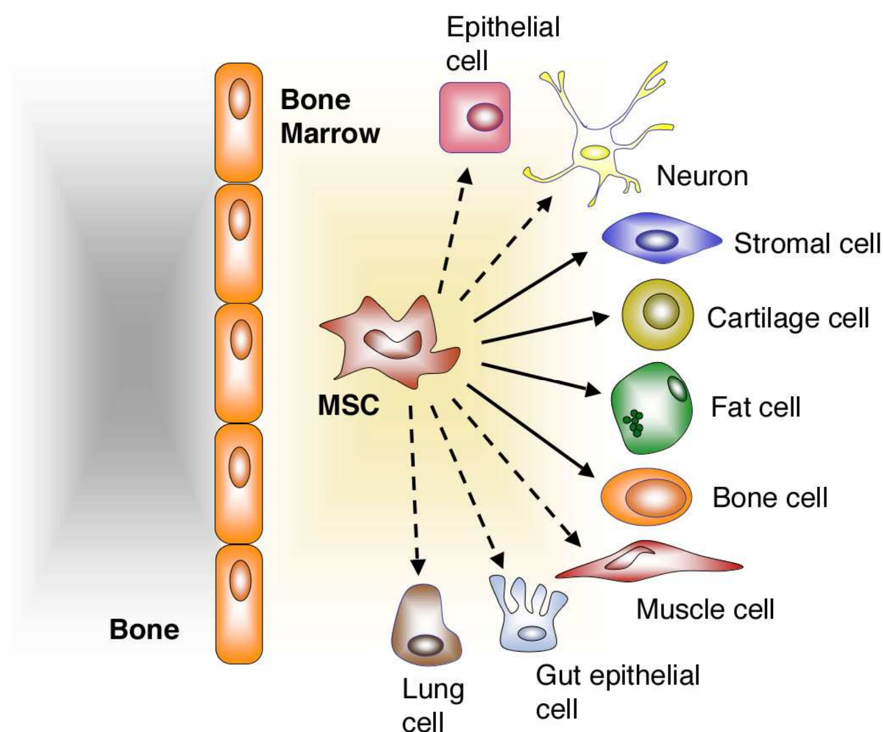


***Figure 1.*** *MSC are able to differentiate into the mesenchymal lineages both in vitro and in vivo (solid arrows). The transdifferentiation into cells of other lineages (ectoderm and endoderm) is still controversial in vivo (dashed arrows). (Adapted from Uccelli et al., Nat Rev Immunol. 2008 Sep; 8(9):726-736)*

Although in the last years a lot of studies had MSC as subject, several issues of their biology still remain unclear. The aim of this chapter is to give a an updated picture of MSC,

describing their main characteristics, but also underlining the still controversial aspects of their biology and illustrating their potential clinical applications.

## *2.2 In vitro proliferation and differentiation potential*

The main attractiveness of MSC is based on the ease with which they can be isolated from bone marrow and on their ability to proliferate readily in vitro. However, since their identification, MSC have been classified as a heterogeneous population, in terms of morphology, proliferation capacity and differentiation potential. At a clonal level, they display different phenotypes: fibroblastic elongated cells, large flattened cells and thin star-shaped cells (10) (Figure 2).

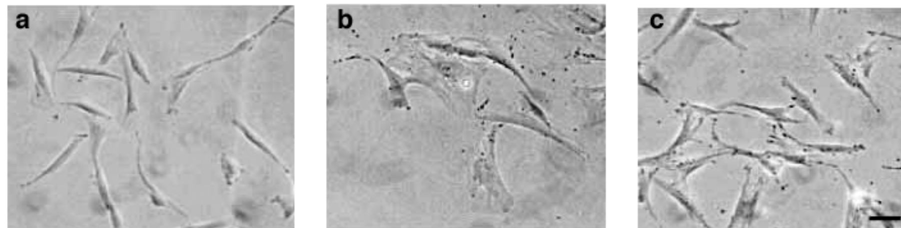


***Figure 2.*** *Phenotype of cultured human MSC. Three cell phenotypes were observed in BMSC colonies: spindle shaped cells (A), large flattened cells (B) and starshaped cells (C) (Bar, 40 μm) (Reproduced from Muraglia A et al, J Cell Sci 2000 Apr; 113 (Pt 7): 1161-6)*

The colonies derived from single CFU-fs show a very mixed diameter size, indicating a different proliferation capacity of the cells they originated from (Figure 3). The potential to undergo several cell divisions is one of the main features which characterize stem cells and progenitors and it is, instead, limited or lost in more committed cells. MSC proliferative potential has been shown to be increased by several growth factors, including EGF, PDGF

and FGF-2 (11). FGF-2 was found to have the strongest mytogenic effect compared to FBS alone, since CFU-fs could generate colonies with a significant increased diameter. Furthermore, Martin and colleagues showed that FGF-2 decreased the expression of markers of osteogenic differentiation such as alkaline phosphatase (ALP). FGF-2 was also able to maintain in culture the osteoprogenitors, since cells expanded with FGF-2 were able to deposit more mineralized matrix in vitro and to better differentiate in vivo, introducing the concept that also for MSC, as for other stem cells, growth factors can be crucial for influencing the commitment process.

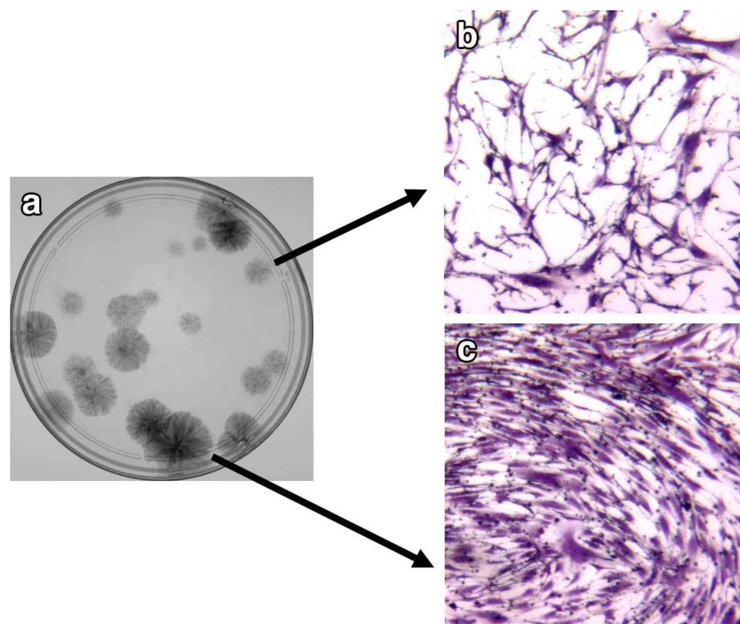


***Figure 3.*** *(a) Colonies derived from single CFU-f show a wide heterogeneity in diameter size, which reflects a different capacity to proliferate and a different stage of commitment. (b), (c) images at a higher magnification of colonies with different proliferation capacity.*

Few years later, Bianchi and colleagues showed that FGF-2 operates an actual selection of a population of earlier progenitors among MSC (12). The assessment of the telomere length, medium switch experiments and a decrease in the number of the initial colonies

indicated that FGF-2 could select for the survival of cells with an increased proliferation and differentiation potential. In fact, when FGF-2 was added to the culture, MSC life span could increase up to 70 doublings, compared with about 50 without it, and the chondrogenic differentiation potential could be retained up to 50 doublings. These data confirmed how FGF-2 could be a relevant component of MSC ex-vivo culture to exploit their full potential for clinical applications.

However, FGF-2 wasn't able to maintain MSC proliferative activity in vitro, since the telomere length was decreasing with the expansion (12). Furthermore, even when cultured in presence of FGF-2, MSC lost the ability to generate colonies and colonies with a decreased diameter were formed at increasing population doublings, suggesting a commitment of the population. Other data supported these findings, confirming that in vitro expansion could cause a gradual loss of MSC early progenitor characteristics (13). MSC dramatically slowed their proliferation rate already by the second passage and this was also associated to a change in the morphology, from the spindle shaped to the flattened one. A parallel loss in the multi-lineage in vitro differentiation potential was also observed with the increasing proliferation, both for the bulk population and for clones derived from single CFU-f. Moreover, when implanted in vivo after the first passage, cultured MSC displayed a poor osteogenic potential compared to fresh bone marrow, indicating that already the primary expansion could affect MSC commitment and differentiation.

Another aspect, that rise from these findings and that should be taken in consideration, is the heterogeneity of the starting population. Regarding the differentiation potential, in fact, not every CFU-f is multipotent when transplanted in vivo (14), (15). As mentioned before, FGF-2 is capable of selecting a population of earlier progenitors among the whole pool of initial CFU-f, indicating the presence of different MSC subpopulations characterized by a mixed stage of commitment. Since studies on the bulk population could not bring much

information about this aspect, an extensive study on clones was conducted in the past (10). The authors showed that a significant percentage (17%) of the analyzed clones were able to differentiate in vitro into the three common mesenchymal lineages, ostegenic, chondrogenic and adipogenic. Some clones were bipotent and a small percentage could only differentiate into the osteogenic lineage. Interestingly, adding FGF-2 to the culture, the percentage of tripotent clones increased from 17 to 34%, confirming a selection for earlier progenitors. Furthermore, with increasing expansion, clones displayed a loss of the multi-lineage differentiation potential, which was not randomly determined. In fact, the adipogenic potential was the first to be lost; the osteogenic and the chondrogenic potentials, instead, diverged only very late with the loss of the chondrogenic ability and the preservation of the osteogenic lineage pathway, suggesting the presence of a hierarchy in the MSC differentiation pathway. These results described a new aspect of MSC biology and contributed to support the relevance of FGF-2 for MSC culture, underlining how growth-factors could affect in vitro expansion of early progenitors.

In addition to this, other factors seem to be important in regulating MSC fate decision. Engler and colleagues showed that MSC are sensitive to the matrix elasticity and that the stiffness of the substrate they grow on can influence their commitment towards a specific lineage (16). These data, therefore, suggest that several elements can influence MSC behavior. Further studies are necessary to clarify how the different regulations are linked and combined to determine MSC fate in vivo. Moreover, the factors involved in the regulation of the hierarchical relationship between different classes of MSC and of the maintenance of their multipotent capacity still need to be investigated.

Ex-vivo expansion is of crucial importance for a potential application of MSC for therapeutic purposes. However, the application of these cells has to deal with the problem of a heterogeneous starting population and with the difficulty of obtaining a number of cells

which is clinically relevant and, at the same time, preserving MSC early progenitor features. Ex-vivo MSC expansion should, therefore, aim to optimize culture procedures and conditions for selecting and maintaining early progenitor populations.

## *2.3 Assessment of self-renewal*

The main features which characterize a stem cell are the self-renewal, defined as the capability to generate at least one daughter cell which retain the stem cell fate and the differentiation potential, which is the ability to give rise to a defined set of mature differentiated progeny. When MSC were characterized by Friedenstein for the first time, they were classified as multipotent, for their ability to differentiate into the different mesenchymal lineages, and, therefore, thought to hold one of the property found in stem cells. Even though the term mesenchymal stem cells is broadly used, the assessment of the other indispensable requirement to be defined as stem, the self-renewal, still remain controversial. In several studies the self-renewal is associated to the growth in culture or to the retention of multipotency after in vitro expansion. However, none of these studies proved the self-renewal at a single cell level and, moreover, it was not assessed as the reconstitution of both a differentiated and a stem cell compartment in vivo. The only stem cell for which the self-renewal has been soundly proven is the hematopoietic stem cell, founded on the capability to serially regenerate the whole pool of mature hematopoietic cells in lethally irradiated recipients (17), (18). For several years there was a lack of experimental evidences on MSC self-renewal and studies on the bulk population actually demonstrated a loss of proliferation and differentiation potential with increasing ex-vivo expansion (13), (10), suggesting a progressive commitment without self-renewal.

It has been recently shown that a subpopulation of human bone marrow stromal cells are able to differentiate into osteoblasts and hematopoiesis supporting stroma and to

maintain, at the same time, the characteristics of clonogenic mesenchymal progenitors (19). A fundamental characteristic of this population was the expression of the melanoma-associated adhesion protein, MCAM/CD146, which was homogeneously present when only freshly isolated bone marrow nucleated cells were grown in clonogenic conditions. In the bone marrow, CD146+ cells correspond to the adventitial reticular population which is located in proximity of the sinusoids and which extends processes to contact the hematopoietic cells. When transplanted subcutaneously in nude mice, expanded CD146+ MSC could form bone and the presence of bone marrow indicated that they could also bring in vivo the hematopoietic microenvironment. Furthermore, cells were harvested from the heterotopic ossicles after 8 weeks and expanded; a significant number of clonogenic CD146+ cells could be obtained, indicating that this population, when transplanted in vivo, could function as self-renewing mesenchymal progenitors. The experiments were performed both pulling together different clones and implanting the progeny of a single CFU-f, demonstrating, therefore, that the results were also valid at a single CFU-f level. Interestingly, when FGF-2 was added to the culture, the expression of CD146 decreased and no bone marrow formation and no human CD146+ adventitial cells were found in vivo. Only the osteogenic potential was maintained, suggesting that FGF-2 selects a different population of progenitors with a distinct function.

These observations indicated for the first time a possible the role of specific mesenchymal progenitor population in the bone marrow niche and supported the evidence that the self-renewal is a property also shared by bone marrow MSC. However this is just the tip of the iceberg of a completely new aspect of MSC biology. Other questions, in fact, needs to be answered, such as, for example, which is the frequency of these cells in vivo and whether the same property belongs to MSC derived from other tissues.

## *2.4 Phenotypic characterization*

A highly controversial aspect of MSC characterization regards their phenotypic properties. The rarity of this population in the bone marrow represents a challenging barrier for the identification of specific antibodies for their isolation and enrichment. Furthermore, most of the markers identified up to now are not specific for MSC since they are also expressed by cultures of fibroblastic cells from various tissues and they are widely modulated during in vitro culture. Little is still known about MSC characteristics in vivo and about a possible differential marker expression among the different classes of MSC. The majority of the information collected so far is based on the analysis of expanded MSC. This contributed to generate confusion about their phenotype and the wrong assumption that any marker expressed on cultured-expanded MSC was also likely to be present in vivo.

Both non-expanded and expanded MSC have been shown to be negative for any hematopoietic or vascular endothelial marker, such as CD45, CD14 and CD34. One of the first antibodies that was identified, instead, to enrich for CFU-f in fresh human bone marrow is STRO-1 (20). STRO-1 is not expressed on hematopoietic cells and its selection results in a 10- to 20-fold enrichment of CFU-f, compared to unseparated bone marrow. Analysis of the expression of a wide range of cell surface molecules on CFU-f demonstrated that other markers, including CD105, CD49a, CD73 and CD90 (21), (22), (23) could be used for CFU-f enrichment. These markers are also expressed on expanded MSC and, to date, they are commonly used to characterize culture-derived MSC.

Recently, Sacchetti and colleagues showed that MCAM/CD146, which marks in human bone marrow the adventitial reticular cells, can be used to isolate a population of in vivo self-renewing osteogenic progenitors (19). After in vivo transfer, they could form bone and they could also be localized in the same adventitial position. This indicated the importance of identifying, in addition to markers of un-expanded and cultured MSC, markers for in situ

localization, to find in situ counterparts of CFU-f and to follow the fate of the implanted cells in vivo, especially when aiming to find evidences for self-renewal.

### *2.5 Clinical applications*

After the discovery of their biological property of differentiating into the common mesenchymal lineages, bone marrow MSC were thought of being responsible for the normal turnover and maintenance of adult mesenchymal tissues (24). This led to the identification of MSC as an attractive cell source for therapeutic applications in different fields of regenerative medicine.

The most obvious application was to apply MSC for the regeneration of mesenchymal tissues, such as bone and cartilage. In fact, first among the others, tissue engineers tried to exploit MSC properties for the repair of bone defects and for the treatment of various bone disorders. Up to date, autologous bone graft still represents the first choice for site-specific bone defect repair, although it is associated with several complications such as, donor site morbidity, infection or loss of graft function (25). The combination of a biomaterial and ex-vivo expanded MSC is thought to represent a valid alternative to functionally replace host bone tissue. In the past years, novel approaches have been developed to improve the performance of the tissue engineered constructs: biomimetic material properties, including surface roughness and porosity, have been investigated to enhance MSC adherence, proliferation and differentiation (26), (27), (28). The presence of MSC promoted bone formation in vivo and MSC were able to accelerate bone repair in femoral and cranial critical-size defects and spinal fusion both in large and small animal models (29), (30), (31). Based on these preclinical studies, clinical trials for bone repair have been conducted (32), (33), (34) with promising outcomes. As mentioned before, expanded MSC have been also used as a potential treatment for cartilaginous injuries in humans, showing a contribution also

in cartilage repair (35), (36). This indicated that bone marrow MSC represent a valid cell source for clinical tissue engineering applications, even though further investigation of their biology and optimization of culture conditions are still needed to fully exploit their potential.

Another clinical application that appeared very promising was the use of MSC as an innovative treatment for genetic diseases. The first application was in patients with osteogenesis imperfecta (OI), which defines a heterogeneous group of genetic disorders, characterized by bone fragility and skeletal deformities as osteoporosis. Clinical trials were started infusing ex-vivo expanded MSC in children affected by OI (9),(37),(38). The results indicated that MSC were able to engraft into skeletal sites and cause the enhancement of patient growth. However, the authors also showed that the total bone mineral content did not significantly increase and they suggested that prolonged in vitro expansion, affecting MSC osteogenic capacity, could compromise the outcome of the study.

This observation, together with the data about the loss of differentiation potential described in the previous section, underlines that a limited in vitro expansion is of fundamental importance for a clinical application in regenerative medicine fields. The low frequency of MSC in the bone marrow and the rapid loss of their progenitor-like characteristics during exapnsion, therefore, represents one of the main limitations for the establishment of MSC-based therapies. Strategies, as FGF-2 supplementation (12) and growth on extracellular matrices (39), (40), should be considered in order to retain MSC progenitor features during ex-vivo expansion. In addition, different culture strategies, alternative to the standard 2D-expansion on plastic, have been explored in the past, in order to recreate a more physiological environment to preserve the MSC characteristics. A very promising approach is represented by bioreactors, which showed to promote cell viability, shear-stress stimulation and a better maintenance of MSC differentiation potential during expansion (41), (42), (43), (44). The results of these studies indicated the relevance of

bioreactors as an important tool for MSC ex-vivo expansion in order to optimize culture conditions and, therefore, to enhance MSC in vivo performance. Furthermore, in Chapter 4 of this thesis we will describe the use of a bioreactor system to reconstruct in vitro the bone marrow stem cell niche, underlining the relevance of such a system, not only for MSC in vitro expansion, but also for the development of a physiological bone marrow stroma.

Although the pioneer clinical studies on MSC have focused on their ability to repair damaged tissues, new findings about their biology, such as their immunomodulatory properties and their possible role in the bone marrow niche, open interesting perspectives. Several studies showed the ability of MSC to modulate immune responses both in vitro and in vivo, by nonspecifically targeting cells of the immune system. This characteristic seems to be due to the ability to block immunocompetent cells through the inhibition of cell division, preventing their activation and maintaining them in a quiescent state (45). The clinical efficacy of MSC would therefore depend on their ability to modify the environment of injured tissues, releasing anti-inflammatory molecules and trophic factors, which promote tissue regeneration. So, the therapeutic potential of MSC could be traced to their physiological activity. The current data about MSC anti-inflammatory and immunosuppressive features support their potential application for immune-mediated diseases, offering alternative therapeutic strategies. The effect of infused MSC has been successfully shown in acute graft-versus-host diseases (46), and, recently, it is being tested for the treatment of Crohn's disease, investigating MSC contribution to the regeneration of gastro-intestinal epithelial cells (47).

On the other hand, the recent findings about the involvement of MSC in the bone marrow stem cell niche opened new lines of research. This could lead to the identification of new molecular targets for the treatment of diseases in which stem cell function is disregulated, either through degeneration, such as aplastic anemias, or excessive expansion

and lack of differentiation, such as hematological neoplasias. A deeper knowledge in the niche biology is therefore needed in order to identify all the regulatory players and the mechanisms by which MSC might maintain and regulate hematopoietic stem cells.

In the next chapter, the main characteristics of the bone marrow stem cell niche will be described, focusing on the niche function and on the data currently available about the regulatory factors which contribute to hematopoietic stem cells maintenance.

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# ***Chapter 3:***

## *The Bone Marrow Stem Cell Niche*

### *3.1 The hematopoietic stem cell*

Stem cells are self-renewing, multipotent progenitors that are responsible for the growth, maintenance and repair of several tissues. They are present in different compartments of the body, including the skin, the intestinal epithelium and the hematopoietic system. The best characterized stem cell is the hematopoietic stem cell (HSC). In the past few years a lot of progress has been done to advance the knowledge about HSC and, recently, the development of new tools for labeling HSC in situ also allowed the characterization of the microenvironment which regulates HSC, the bone marrow stem cell niche.

The most common assay to assess the stemness in HSC is the ability to reconstitute all the blood-cell lineages in lethally irradiated recipients. Furthermore, the fact that this property can be maintained upon serial transplantation in mice shows the capability of these cells to undergo self-renewal, i.e. to give rise to a differentiated progeny and preserving, at the same time, the stem cell properties. The optimization of these assays also helped to identify cell surface molecules for the characterization, and consequently for the isolation, of HSC. Murine HSC do not express any of the markers present on fully differentiated hematopoietic cells, but they express high levels of stem-cell antigen 1 (SCA 1) and c-KIT and, according to this, they are named ad LKS cells. Moreover, long-term repopulating HSC have been shown to be negative for CD34 and positive for CD150 (1), (2). However, the studies in which the presence of these markers was correlated to the repopulating function showed that only 1 in 100 LKS is able to save from irradiation (3). Recently, some studies have started to define HSC gene-profiling, moving the first steps towards the clarification of the mechanisms which regulate HSC function (4), (5).

The regulation of self- renewal and differentiation represents probably the most fascinating aspect of HSC biology. Self-renewing divisions in vivo contribute to maintain the size of the stem cell pool. This can be achieved preserving the balance between symmetrical

and asymmetrical divisions, by which a single stem cell gives rise to two different daughter cells, one maintaining the stem cell features and the other becoming fully differentiated. Two possible different mechanisms have been described to achieve the asymmetry: the divisional asymmetry, which occurs before the cell division, and the environmental asymmetry, when it is determined after the cell division (Figure 1).

In the divisional asymmetry (Figure 1a), only one of the daughter cells receives, during mitosis, the determinants for initiating the commitment process. This mechanism has not been shown in any vertebrate stem-cell type in vivo, but several in vitro studies have shown that HSC may undergo asymmetrical divisions (6), (7). Further investigation is, therefore, needed to confirm whether this reflects the in vivo conditions.

The environmental asymmetry (Figure 1b), instead, is determined through extrinsic signals provided by the surrounding microenvironment. After a symmetrical division, one daughter cell will remain in contact with the niche, preserving the stem cell fate; the other one, instead, will be exposed to an external environment and will receive signals starting differentiation (8), (9). Even though there is a lack of experimental evidences that this mechanism can occur in vertebrates in vivo, recent in vitro studies indicated that some molecular pathways for divisional asymmetry are conserved between invertebrates and vertebrates. Therefore, this raises the possibility that HSC can undergo both divisional and environmental asymmetry in vivo in order to drive daughter cells to different fates.

The environmental asymmetry introduces the importance of a specialized microenvironment, the niche, in providing signals to promote self-renewal and avoid differentiation.

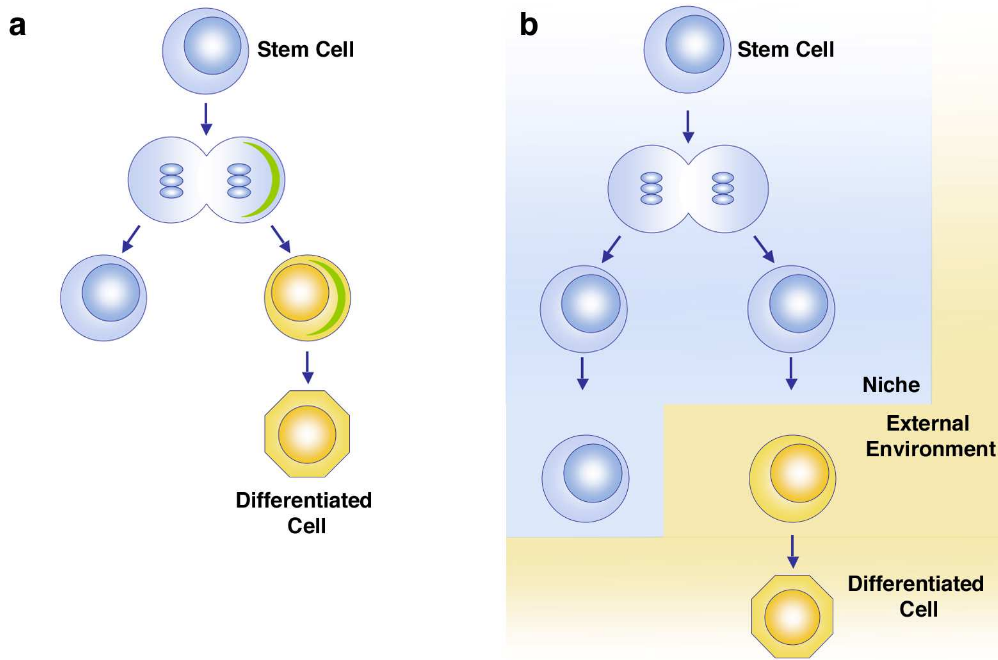


***Figure 1. Possible mechanisms of asymmetric cell division.*** *(a) Divisional asymmetry: the fate is determined during cell division when only one of the daughter cells receives the determinants, keeping the stem cell features, and the other one starts the commitment process. (b) Environmental asymmetry: after a symmetric cell division, which generates two identical daughter cells, one remains in the niche, preserving the stem cell fate, and the other one is exposed to an external environment which promotes differentiation. (Modified from Trumpp A & Wilson A, Nat Rev Immunol. 2006 Feb; 6(2):93-106)*

### *3.2 Stem cell niche function*

Subsequently to the localization of stem cells in vivo, several studies have indicated that adult stem cells don't divide frequently and that can be quiescent for long periods of time (10), (11). In the bone marrow, quiescent HSC have been identified close to the endosteal surface in the trabecular bone (12). It is, therefore, unlikely that they are crucial players for the homeostasis of the hematopoietic tissue with its high turnover. They may constitute,

instead, a reserve pool which is stored in the “quiescent niche” and which can be mobilized in case of tissue injury (Figure 2a). It has been shown, in fact, that, when the hematopoietic system is damaged, HSC are mobilized from the bone marow, enter the circulation and start to divide in order to restore hematopoieis. They can then home back to the bone marrow niches and be quiescent again (13), (14).

However, quiescence is not a characteristic of all stem cells. Embryonic stem cells have to undergo several divisions but they preserve the stem cell fate and fetal-liver HSC can reconstitute hematopoieisis even being highly proliferative (15), (16). This could be associated to the difference between “fetal” and “adult” stem cells. However, in tissues with high regeneration rate, such as the hematopoietic system, stem cells have to divide also during homeostasis, to produce progenitors that can, then, generate the differentiated progeny during regular turnover. This indicates that another type of niche, the self-renewing niche (Figure 2b), might exist and its function would be to guarantee that one of the daughter cells maintain the stem cell features and the other one initiates the differentiation process. In this case the niche structure would be more complex, but a self-renewing niche would be fundamental to maintain standard tissue homeostasis. A possible structure of this type of niche would require that quiescent stem cells are anchored in a protected part of the niche, while self-renewing stem cells would be located in proximity of the external environment from which they can receive signals that could stimulate cell division and/or differentiation.

Up to date, whether a single niche can provide all the stem-cell-niche-functions or multiple niches are required in vivo remains still unknown. However, the two possible model proposed above are both compatible with the normal homeostatic conditions; further investigation conducted in vivo will clarify which of the two reflects the bone marrow condition.

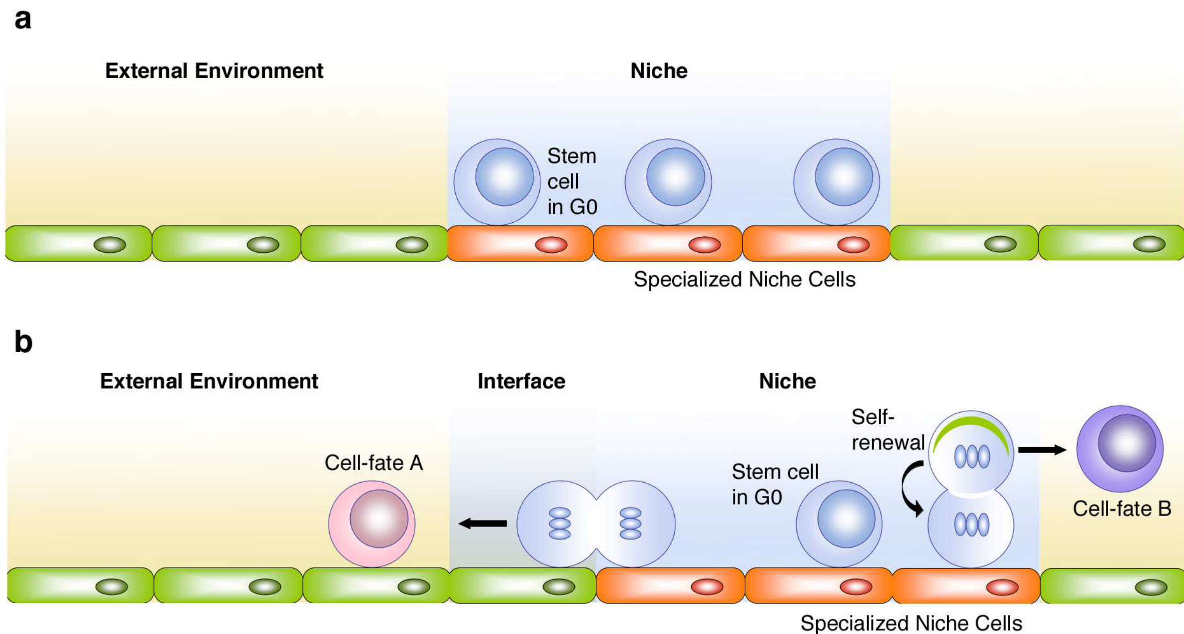


***Figure 2. Different types of niche.*** *(a) Quiescent storage niche: it contains resting stem cells and it produces signals that repress differentiation and/or cell division. Stem cells can be mobilized from this site in case of need and they might return for storage and self-renewal. (b) Self-renewing niche: quiescent stem cells would be located in the centre of the niche, while self-renewing stem cells would be present at the interface between the niche and the external environment. At this interface, stem cells might be exposed to proliferative signals, undergoing divisional or environmental asymmetry. This would allow both self-renewal, since stem cells will remain attached to the niche, and initiation of differentiation, leading to different stem cell fates (A and B). (Modified from Trumpp A & Wilson A, Nat Rev Immunol. 2006 Feb; 6(2): 93-106)*

### *3.3 The bone marrow stem cell niche*

In 1978 Schofield coined the term “niche” to describe the HSC bone marrow microenvironment. He also proposed that HSC are in close contact with the bone and that this contact was responsible for the maintenance of HSC characteristics.

More recently, studies in mutant mice, in which hematopoiesis is affected by defects in bone development or remodeling, have identified osteoblasts and/or osteoclasts in the formation and function of the niche (17), (18), (19). Futhermore, when HSC phenotype or activity was localized in proximity of the endosteal surface, while differentiated cells were found more in the central region of the bone marrow, the evidence of a niche close to the endosteum was provided (20), (11), (21). Although the quantification of HSC at the endosteal surface is still controversial, depending on the different criteria used to localize HSC in situ, several studies made the general point that primitive progenitors are enriched in that area of the bone marrow. The first experimental evidence that cells involved in bone formation could support HSC was provided by studies in which mouse and human osteoblasts were shown to produce several cytokines that stimulate HSC proliferation (22). However, a direct role of osteoblasts in HSC regulation was shown only in some in vivo studies in which mutants, with increased or decreased number of osteoblasts, were applied (23), (11). Modulating the osteoblast number was possible to increase or decrease the HSC number, supporting the idea that osteoblasts are an essential part of the niche and they crucial for limiting the niche size and acitvity. It has been also shown that the cells in contact with HSC belong to a specific subset of osteoblasts which express N-cadherin (11). However, following studies failed to detect N-cadherin expression (24), raising the question of an actual involvement of this molecule in HSC regulation.

Several models of the adhesion of HSC to osteoblasts have been proposed and widely discussed in the literature. However, the data collected so far do not identify a unique model,

remaining compatible with different ways by which endosteal cells can support HSC maintenance (Figure 3). Furthermore, other cell types present in the bone marrow can secrete molecules, such as angiopoietin, thrombopoietin and CXCL12, questioning whether they also contribute to niche formation, activity or architecture.

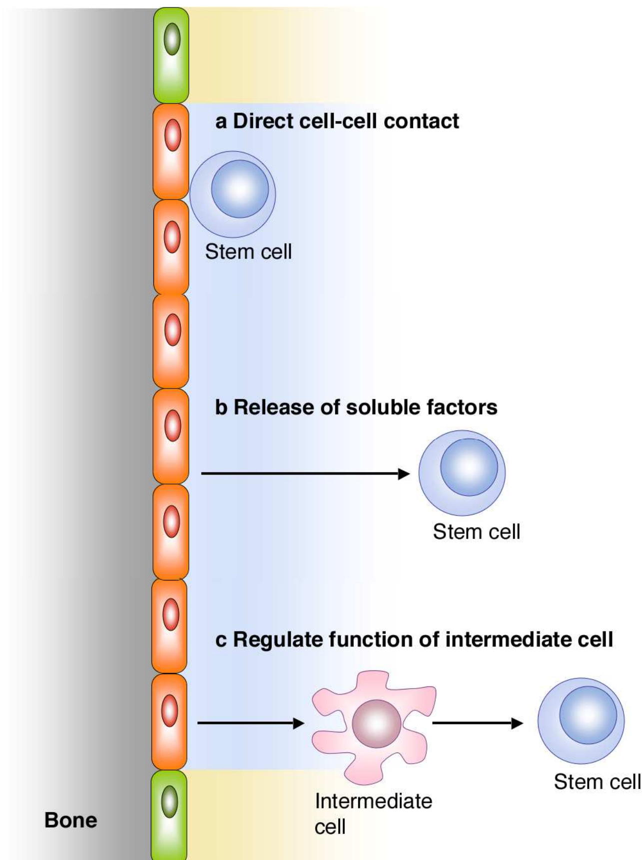


***Figure 3. Possible mechanisms by which niche specialized cells can regulate HSC function.*** *(a) A possible model would imply a direct contact between HSC and niche specialized cells. (b) Another possibility is that cells at the endosteum secrete regulatory factors which may regulate HSC. According to this model HSC can be also located in proximity to the endosteal surface, excluding a direct contact with the niche regulatory cells. (c) A third possibility is that endosteal cells promote HSC maintenance by modulating the function of intermediate cells, which are fundamental in the formation of other niche types away form the endosteum (as the vascular niche).*

The localization of a significant amount of CD150+ HSC in proximity of the sinusoidal walls in the bone marrow raised the hypothesis that another specialized microenvironment could affect HSC regulation (20). It has been shown that there is a close developmental relationship between the hematopoietic and the endothelial lineages (25), (26) and HSC seem to originate from a perivascular progenitor during embryonic development (27), (28). Moreover, the presence of HSC in the yolk sac, aorta-gonad-mesonephros region and vitelline arteries suggests they reside and undergo hematopoiesis in association with blood vessels. The endothelial cells harvested from these regions have been shown to support the expansion of adult HSC LKS cells in vitro (29), (30). In contrast, endothelial cells isolated from adult organs don't display a HSC supportive activity in vitro (31). These observations indicate that the endothelial cells of bone marrow sinusoids hold distinct functional characteristics from endothelial cells present in other tissues; in fact, they have been shown to produce factors important for mobilization, homing and engrafment for HSC, such as CXCL12, VCAM1 and E-selectin (32), (33). Some evidences about the existence of a vascular niche were already present, since in case of injury HSC can detach from the endosteum and migrate to the vascular region of the bone marrow from where they restore hematopoiesis (34), (35). Moreover, although only some more recent findings confirmed the presence of CD150+ HSC close to the sinusoids, the possibility that a vascular niche could exist during homeostasis can easily find an explanation. In fact, label-retaining HSC have been localized in the endosteal niche, showing that the most dormant HSC reside there and that this type of niche serve as a quiescent niche, or a self-renewing niche containing both quiescent and self-renewing HSC. The proliferating HSC, instead, are close to the vasculature (20) and therefore it is possible that the vascular niche contains the self-renewing HSC rather than dormant HSC. Furthermore, this position would allow HSC to constantly sense the

concentration of blood factors that reflects the status of the hematopoietic system and to be rapidly activated in case of need; more HSC can be then mobilized from the endosteal niche if necessary. However, it has been shown that when osteoblasts are deleted hematopoiesis occurs extramedullary (36), indicating that the vascular niche is not sufficient to support long-term hematopoiesis and it might represent a secondary niche in the bone marrow.

Although the role of all the niche cells has not been clarified and the mechanisms by which they regulate HSC fate need to be further investigated, strong experimental evidences support the hypothesis that the endosteal and the vascular niche act together to maintain hematopoietic homeostasis or restore it after damage.

### *3.4 Molecular crosstalk in the endosteal niche*

Some recent studies have identified few molecules which are expressed both by osteoblasts and HSC and which are thought to be important for mediating the signals exchanged in the niche (22). However, some other studies have focused on the identification of factors directly in situ showing their involvement in the modulation of HSC function.

Notch signaling has been shown to be involved in stem cell fate decision in several tissues. The data about its expression in the bone marrow (37) led to the hypothesis that it could also had a role in HSC regulation. Moreover, a confirmation came from some in vitro studies in which the authors showed the importance of Notch signaling for HSC maintenance (38). In addition, in mutants with an increased number of osteoblasts, Notch ligand Jagged-1 is upregulated, suggesting that Notch signaling could be responsible for the consequent augmentation in HSC number (23). In contrast, some other studies showed that a deletion of both Notch-1 and Jagged-1 from bone marrow does not have any effect on hematopoiesis in vivo (39). This suggests that Notch is not a crucial requirement of the niche, but it is maybe

important only in some circumstances, and that the regulation of HSC maintenance involves the complex combination of different factors.

Components also involved in HSC regulation are some proteins contained in the extracellular matrix. It has been shown that osteoblasts can modulate the number of HSC secreting osteopontin (OPN), an acidic glycoprotein of the bone matrix (40). In mice in which a deletion of OPN was induced, the number of HSC significantly increased and the same increased was observed transplanting wild type HSC, indicating that OPN production by the osteoblasts has a negative regulatory effect on HSC (41). Some in vitro experiments showed that OPN induces apoptosis in HSC and that in the niche plays a role in maintaining HSC quiescence (41), (42).

Stem cell factor (SCF) binds and activate c-KIT which is highly expressed by label retaining HSC. It has been previously shown that mutations which affect the membrane bound SCF have an influence on migration and differentiation of primordial germ cells, neural-crest derived melanoblasts and hematopoietic cells (43). Furthermore, osteoblasts in the bone marrow produce a significant amount of membrane-bound SCF, which has the capacity to enhance the adhesion of HSC to stromal cells (44), suggesting that osteoblasts can influence the adhesive characteristics of the endosteal niche by modulating the functional state of specific integrins. In vivo, the hampered production of SCF causes a reduced HSC activity (17), (45), indicating that SCF is an essential component of the endosteal niche. Moreover, SCF is also important for osteoblasts activity in vivo (46). It would be therefore interesting to investigate whether it mainly explicates its effect directly, acting on HSC, or indirectly, affecting niche specialized osteoblasts.

Other factors have been described to affect HSC regulation. TIE2, which is expressed by HSC, is activated by angiopoietin-1 (Ang1) and the activation of this pathway contributes to maintain HSC quiescence both in vitro and in vivo (47), (12). MYC is expressed, instead,

during initiation of HSC differentiation (48) and MYC overexpressing HSC are lost because of differentiation mainly for their inability to be retained in the niche. Although it has been shown that WNT signaling pathway is involved in HSC self-renewing divisions in culture, its role remains still controversial and it has to be clarified whether it is fundamental for HSC function.

These observations together contribute to describe the endosteal niche as a complex interactive structure that combines positive and negative signals for the regulation of stem cell activities. Open question still remain and significant advance in the knowledge is still needed to better understand the mechanisms of these microenvironments, in order to modulate endogenous stem cell function.

### *3.5 Perspectives*

In the past few years, the theory proposed in the 1970s about the presence in the bone marrow of a specific stem-cell microenvironment, has received the right attention. A great amount of data, trying to identify interactions both at the molecular and cellular levels, has been collected. Nevertheless, we are still far to completely elucidate all the mechanisms present in the niche and, moreover, as the characterization of the niche will proceed, it is likely that new paradigms will be uncover. Further investigation is still needed to clarify whether different niche types exist in the bone marrow, how many HSC are contained in each niche and the precise role of every niche during the normal turnover or in case of injury. In fact, it will be interesting to distinguish between mechanisms that maintain HSC under homeostasis or that are activated under stress conditions, since some experimental evidences suggest that some factors, such as Notch and WNT for example, are crucial only in some circumstances. In addition, even though osteoblasts have been identified as key players for the niche function, very little is known about their characteristics, especially about their

differentiation stage. It will be important to establish if the niche-specialized osteoblasts correspond to the osteoblasts which then differentiate in ostocytes or whether they undergo a different differentiation pathway. Recent studies have revealed that other cell types like endothelial cell, osteoclasts and mesenchymal progenitors are important in the establishment of the niche function, opening new areas of research. This contributes to underline that the eventual reconstruction in vitro of active niches will probably require more than a feeder layer and a mix of different cytokines. In fact, it will need the reconstitution of a three-dimensional structure, which can include the different cellular players and the deposition of a physiological extracellular matrix. The combination of cell-and-molecular biology and tissue engineering can lead to a significant advance in the field, which will facilitate the development of new stem-cell-based therapies.

In this regard, in Chapter 6 will illustrate the potential of a bioreactor system as a tool to reconstruct in vitro the interactions present in the bone marrow niche. We will focus on the plasticity of the system, underlining the advantages, such as the three-dimensional environment, and the modulating variables, like the scaffold composition and the possibility of multiple cell types co-culture. We will propose an innovative application of a perfusion bioreactor-based system, not only for HSC expansion, as bioreactors were meant so far, but also as a valid system to reproduce the bone marrow physiological microenvironment. The final aim will be to use such a device to recreate a stable stem-cell-niche unit, in which the physiological interactions are respected, promoting HSC maintenance and the formation of a functional supportive tissue composed by stromal cells, which preserve their progenitor-like features. This will, therefore, have important implications not only for stem cell biology but also for the tissue-engineering field, representing a physiological environment for MSC and moving the first steps to overcome limitations linked to standard MSC culture.

Furthermore, beyond clarifying mechanisms of HSC biology that have been already identified in two-dimensional culture conditions, this three-dimensional system can be applied to investigate basic aspects of MSC biology, like the regulation of the hierarchical relationship between different classes of MSC and of the maintenance of their pluripotent capacity, which remain still widely unknown. Although a three-dimensional setup would have constituted a more physiological environment, we started answering these questions using the standard two-dimensional system and tested the hypothesis that, as HSC in the bone marrow, also MSC require their own niche to preserve their progenitor-like features. In the following chapter (Chapter 4), we will describe the establishment of a MSC niche system in vitro by the maintenance of a self-renewing, highly potent, non-adherent mesenchymal progenitor population.

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# *Chapter 4*

## *FGF-2/FGFR2c signaling generates a niche-progenitor system in vitro by selecting and maintaining a self-renewing, highly potent, non-adherent mesenchymal progenitor population*

## *4.1 Introduction*

The bone marrow stromal system is defined as the connective tissue elements providing structural and functional support for hematopoiesis, as well as containing a population of multipotent mesenchymal progenitors. Recent studies have revealed that specific microenvironments, often referred to as "stem cell niches" regulate stem cell function by providing architectural support, together with humoral and cell-contact dependent signals (1). It has been also shown that stem cell niches are as diverse as the stem cells they sustain and that they can be specialized to support different physiological functions. In the endosteal niche, hematopoietic stem cells (HSC) have been shown to be in contact with specialized niche osteoblasts (2) and the mutual signal exchange is responsible for the regulation of self-renewal and inhibition of differentiation (3, 4). Currently it is postulated that HSC maintenance is achieved by environmental or divisional asymmetry (5), by which one of the daughter cells can preserve the stem cell fate and the other one initiates the commitment process. The main function of the niche is, therefore, to guarantee the normal tissue homeostasis and to provide the environment for a stem cell reservoir that can be activated in case of stress or injury. Although osteoblasts have been identified as an essential component of the endosteal niche (2), it has recently been shown that other cell types, like endothelial cells, contribute to the formation of HSC niches (6, 7). Moreover, a recent study showed that human CD146+ clonogenic osteoprogenitors, that reside perivascularly in the bone marrow, are able to regenerate the hematopoietic microenvironment in vivo after transplantation (8), suggesting a role of mesenchymal progenitors, in addition to differentiated osteoblasts, in the bone marrow niche. Human mesenchymal progenitors show differences in terms of proliferation capacity (9) and differentiation potential in vitro at a clonal level (10), indicating a wide heterogeneity in their stage of commitment. Although the presence of different classes of mesenchymal progenitors was already described by Friedstein in the 1980s (11), the

hierarchical relationship between them and the regulation of the maintenance of their pluripotent capacity are still largely unknown (10). Even though significant progress has lately been made to identify HSC niches, it still remains unclear if also mesenchymal cells require their own niche to maintain their progenitor-like characteristics in vivo.

Recently, Wan and coworkers (12) reported the presence of multipotent mesenchymal cells in the non-adherent fraction of freshly plated human bone marrow cultures, which gave rise to adherent colonies if replated on new dishes. Furthermore, it has been shown that a population of non-adherent mesenchymal progenitors can be cultured in suspension and that the maintenance of the non-adherent status is associated with the preservation of an uncommitted phenotype (13).

In analogy to the relationship between the HSC and the stromal niche, we hypothesized that non-adherent stromal cells constitute a reservoir of earlier progenitors/stem cells for the mesenchymal compartment and that the adherent fraction provides the niche function for the maintenance and regulation of these progenitors.

In this chapter, we investigated the self-renewal and differentiation capacity of bone marrow non-adherent mesenchymal progenitors (NAMP) *in vitro* in different experimental settings. We show that the NAMP are a class of multipotent cells, which can generate an adherent progeny while self-renewing as non-adherent progenitors. NAMP progeny showed an increased proliferation capacity and greater differentiation potential than that of the initially adherent CFU-f. The selection, the maintenance and the biological function of NAMP is exquisitely dependent on FGFR2c signaling and it requires the adherent fraction to provide signals which prevent adherence and colony formation, while supporting self-renewal, therefore providing niche function for the NAMP.

## *4.2 Matherials and Methods*

***Cell culture***

Bone marrow aspirates were obtained from a total of 24 healthy donors (27-64 years old) during routine orthopaedic surgical procedures, in accordance with the local ethical committee (University Hospital Basel) and after informed consent. Bone marrow mononuclear cells (BM-MNC) were counted after staining with Crystal Violet 0.01% (Sigma) in phosphate-buffered saline, pH 7.2 (PBS). To determine colony forming efficiency (CFE) and for serial replating experiments, cells were plated at clonal density (4.5 x $10^3$ cells/$cm^2$) and cultured in alpha-MEM (GIBCO) with 10% fetal bovine serum (FBS). When indicated, medium was further supplemented with FGF-2 (5 ng/ml, R&D System) or PDGF-BB (10 ng/ml R&D System). After 2 weeks cells were then washed with PBS, fixed with 3.7% formaldehyde in PBS, stained with Crystal Violet (Sigma) for 10 min, washed with tap water and the colonies were counted. All determinations were performed in triplicate and CFU-f frequency in the fresh marrow sample was used to calculate the population doublings of first-confluence cultures.

For progeny expansion, freshly isolated BM-MNC were plated at a density of 1 x $10^5$ cells/$cm^2$ (Flask0, CFU-f progeny cells) and after 3 days the non-adherent fraction was transferred into a new flask (Flask1, NAMP progeny cells). After 2 weeks of expansion in presence of FGF-2, the cells were detached with 0.05% trypsin/0.01% EDTA (Gibco) and counted for the population doubling determination. Aliquots of the pooled cells were used for flow cytometry analysis, in vitro and in vivo differentiation assays, or replated for growth curve determination.

***In vitro adipogenic differentiation***

Adipogenic differentiation was induced in 2D cultures as previously described (14).

Briefly, CFU-f and NAMP progeny cells were seeded in 6-well plates at a density of $5 \times 10^3$ cells/cm$^2$ and cultured in alpha-MEM with 10% FBS until they reached confluency. Medium was then supplemented with 10 μg/ml insulin, $10^{-5}$ M dexamethasone, 100 μM indomethacin and 500 μM 3-isobutyl-1-methyl xanthine (adipogenic induction medium) for 72 h and subsequently with 10 μg/ml insulin (adipogenic maintenance medium) for 24 h. This 96-hours cycle was repeated for four times and cells were then cultured for an additional week in adipogenic maintenance medium.

*Readout:* Cell layers were harvested for mRNA extraction and PPAR-gamma expression assessment (see RT-PCR section) or fixed in 60% isopropanol for 2 minutes and incubated with oil red (Sigma) 0.1% in 60% isopropanol for 20 minutes. To determine the number of adipocytes, oil-red positive cells were counted in at least 10 different fields.

***In vitro chondrogenic differentiation***

Chondrogenic differentiation was induced in pellet cultures using a defined serum-free medium, as previously described (15). Briefly, CFU-f and NAMP progeny cells were resuspended in alpha-MEM supplemented with ITS$^{+1}$ (10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium, 0.5 mg/ml bovine serum albumin, 4.7 μg/ml linoleic acid (Sigma, St.Louis, MO), 0.1 mM ascorbic acid 2-phosphate (Sigma, St.Louis, MO), 1.25 mg/ml human serum albumin, $10^{-7}$ dexamethasone (Sigma, St.Louis, MO) and 10 ng/ml TGFβ1 (R&D system, Minneapolis, MN). Aliquots of $5 \times 10^5$ cells/0.5 ml were centrifuged at 1,200 rpm for 5 minutes in 1.5 ml polypropylene conical tubes (Sarstedt, Numbrecht, Germany) to form spherical pellets. Pelletes were cultured in a humidified incubator at 37 °C with 5% $CO_2$ for 3 weeks and the medium was changed twice per week.

*Readouts:* For histological analysis, cell pellets cultured in chondrogenic medium were fixed in 4% formalin for 24 h at 4 °C, dehydrated in a ethanol series, embedded in paraffin

and 7-μm thick-sections were stained with Safranin-O to determine the presence of sulfated glycosamminoglycans (GAG). For biochemical analysis, Cell pellets cultured in chondrogenic medium were digested in 1 m of proteinase-K solution (1 mg/ml proteinase-K in 50 mM Tris, 1 mM EDTA, 1mM iodocetamide and 10 μg/ml pepstatin-A) for 15 h at 56 °C. GAG content was measured spectrophotometrically after reaction with dymethilmethylene blue (16). Known concentrations of chondroitin sulfate were used as a standard. The amount of DNA was measured using a CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR) and used to normalize the GAG content.

***<u>In vitro osteogenic differentiation</u>***

Osteogenic differentiation was induced in 2D cultures as previously described (17). Briefly, CFU-f and NAMP progeny cells were seeded in 6-well plates at a density of 5 x $10^3$ cells/$cm^2$ in alpha-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma), 10 nM dexamethasone (Sigma) and 0.1 mM L-ascorbic acid-2-phosphate (Sigma) and cultured for 3 weeks, with medium changed twice per week.

*<u>Readout:</u>* Cells layers cultured in osteogenic medium were were harvested for mRNA extraction and BSP expression assessment (see RT-PCR section) or washed twice with PBS, fixed for 10 minutes in 4% formalin and washed twice with water. Fixed cells were then incubated for 10 minutes with alizarin red 2% in distilled water and extensively washed with water.

***<u>Real-time PCR</u>***

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA), treated with DNAse and retrotranscribed into cDNA, as previously described (18). Real-time PCR was performed with the ABIPrism 77000 Sequence Detection System (Perkin Elmer/Applied

Biosystem, Rotkreuz, Switzerland) and expression levels of the genes of interest bone sialoprotein (BSP) and PPAR gamma were normalized to the 18S rRNA. Primers and probe sets and PCR conditions for BSP and 18S were used as previously described (18). PPAR-gamma primers and probe (Hs00234592_m1) were provided by Applied Biosystem.

***Assessment of bone formation in vivo***

In vivo ectopic bone formation was assayed as previously described (19). Briefly, on reaching their first confluence, 1 x $10^6$ of CFU-f and NAMP progeny cells were resuspended in 30 µl of Fibrinogen (20 mg/ml Baxter, Austria), quickly mixed with 30 µl of Thrombin (6 IU/ml Baxter, Austria) and loaded onto 35 µg of bovine bone-derived granules (Bio-Oss®, Geistlich Switzerland). The constructs were transferred for 15 minutes in a humidified incubator at 37 °C with 5% $CO_2$ to allow fibrin polimerization and implanted subcutaneously in CD-1 nu/nu nude mice (Charles River, Germany). After 8 weeks, the constructs were harvested, fixed overnight in 4% formalin, completely decalcified with Osteodec (Bio-Optica, Milan, Itlay) for 2 hours at 37 °C, paraffin embedded and 7 µm-thick-sections were obtained from different levels. Sections were stained with hematoxilin and eosin (H&E) and observed microscopically to detect the formation of bone tissue for qualitative analysis and assessed by computerized bone histomorphometry as previously described (20) for bone tissue quantification. Briefly, fluorescent images of sections at different depth of each construct were taken and used to measure the area covered with bone tissue by computerized image analysis (Scion Image, Scion Corp., Frederick, MD).

***Flow cytometry analysis***

CFU-f and NAMP progeny cells (3-5 x $10^5$ cells) were resuspended into 200 µl of 0.5% BSA in PBS (FACS Buffer) with fluorochrome-conjugated antibodies against the indicated protein

or an isotype control and were incubated for 30 minutes at 4 °C. The antibodies used were CD146-PE, CD49a-PE, CD90-FITC, CD73-PE, IgG1-FITC, IgG1-PE, IgG-APC (all from Becton, Dickinson and Company, Franklin Lakes, NJ), CD271-APC (Miltenyi Biotech, Bergish Gladbach, Germany), CD105-FITC (Serotec Ldt. Oxford, UK). All the antibodies were used at a dilution of 1:50, except CD105-FITC, which was used at 1:20. Cells were washed twice with FACS buffer, resuspended in PBS and analyzed with a FACSCalibur flow cytometer (Becton, Dickinson and Company).

***FGF receptor signaling***

To block specific FGF receptors, freshly isolated BM-MNC where incubated on ice for 30 minutes in the presence of anti-hFGF R1 IIIb isorform (R&D System) or anti-hFGF R3 IIIb, IIIc isoforms (R&D System) or anti-hFGF R2 IIIb, IIIc isoforms (R&D System). The cells were then seeded at a density of 4.5 x $10^3$ cells/$cm^2$ and cultured in alpha-MEM supplemented with rhFGF-2. To stimulate specific FGF receptors, cells were cultured with rhFGF-5 (R&D System) or rhFGF-19 (R&D System) instead of FGF-2. After 3 days the non-adherent fraction was resuspended in fresh medium, supplemented with the same blocking antibody and/or growth factors, and replated in a new dish. The medium was changed twice per week and after 14 days the colonies were stained and counted.

***Statistical analysis***

Data are presented as means ± standard error. The significance of differences was evaluated using analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons. For single comparison, Mann-Whitney test was used. $P < 0.05$ was considered statistically significant.

## *4.3 Results*

***A population of stably non-adherent progenitors (NAMP) is present in bone marrow MSC cultures***

Mesenchymal progenitor cells have traditionally been defined by their capacity to rapidly adhere on plastic and grow as discrete colonies initiated by single cells (colony forming unit fibroblastic, CFU-f) (21). After CFU-f adherence over 3-4 days unattached cells, which are mainly hematopoietic, are typically discarded with medium changes. Recently, multipotent mesenchymal progenitors were also found in the non-adherent fraction of human bone marrow cultures (12). We, therefore, addressed whether the non-adherent population contained clonogenic cells and determined their number. For these experiments, human fresh bone marrow nucleated cells were plated at clonal density (4.5 x $10^3$ cells/$cm^2$) on cell culture dishes (Plate0) in presence of FGF-2 and after 3 days the non-adherent fraction was resuspended in fresh medium and replated in a new dish (Plate1). After 14 days, Plate1 contained a number of colonies equal to 20.4±3.6% of the colonies present in Plate 0 (n=4), indicating that 1 in 6 clonogenic progenitors is actually in the non-adherent fraction. To assess whether these CFU-f were stably non-adherent or display delayed adherence, fresh bone marrow nucleated cells were plated at clonal density (4.5 x $10^3$ cells/$cm^2$) on cell culture dishes and at each medium change (days 3, 7 and 11) the non-adherent fraction was either discarded (Plate 0) or collected, resuspended in fresh medium and replated in the same dish (Plate 0*, Figure1a). The replating of the non-adherent cells in the same dish did not significantly increase the number of colonies (n=3, 100±25.1% vs 99.0±23.5%, p=n.s., Figure 1b). The diameter of the colonies was measured to compare cell proliferation capacity and no significant difference was found between the average size in Plate 0 and Plate 0* (n=5, 5.5±0.5 mm vs 5.8±0.6 mm, p=n.s., Figure 1c). We further analyzed the distribution of colony diameters (n=5) and found that a very small number of larger-size (diameter > 10

mm) colonies was present in the replated dishes (Plate 0*, 90th percentile = 9 mm vs 8 mm, number of colonies >10 mm= 9% vs 1%, Figure 1d), suggesting that the adherent fraction contains cells with an increased proliferation potential which could adhere if maintained longer in the same dish. However, the bulk of this population was stably non-adherent in the primary culture.

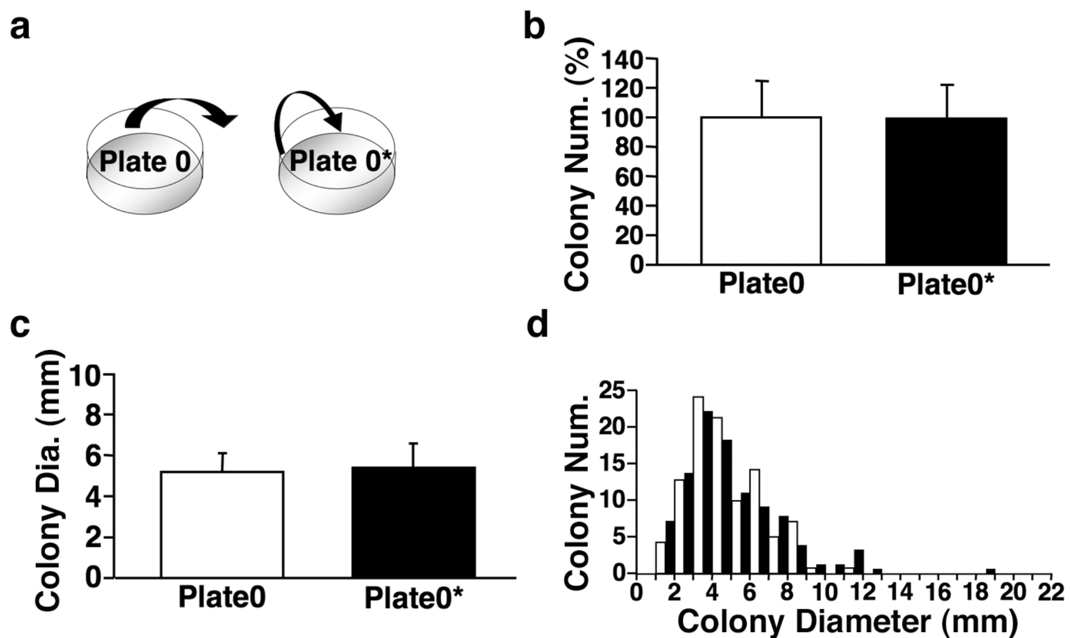


**Figure 1.** ***A population of stably non-adherent progenitors (NAMP) is present in bone marrow MSC cultures.*** *(**a**) Plate 0 (NAMP discarding) and Plate 0* (NAMP replating in the same dish at every medium change) were compared to investigate a potential delayed adherence of NAMP. No statistical change could be observed in the total clonogenicity (**b,** expressed as percentage of the colonies present in Plate 0) and in the colony diameter size (**c**). (**d**) The analysis of colony diameters distribution showed that colonies in Plate 0 didn't differ from the ones in Plate 0* (Plate0= white bar; Plate0*=black bar).*

***NAMP are self-renewing progenitors***

We next asked whether NAMP would exhaust themselves in generating adherent colonies or could also self-renew in vitro as non-adherent progenitors. To test this question, serial replating experiments were performed. Human fresh BMNC were plated at clonogenic density in presence of FGF-2 and after 3 days the non-adherent fraction was collected from Plate0 and replated in a new dish (Plate1). At day 7, the non-adherent fraction was removed from Plate1 and replated in a fresh dish (Plate2), and so on until Plate4 at day14 (n=5, Figure 2a). After 14 days from each plating, not only colonies were always present, but surprisingly their number was steadily increased across 3 serial replating steps (Plate1=23.2±3.9%, Plate2=49.1±15.1%, Plate3=72.2±12.5%, Plate4=86.6±26.5%, $p<0.05$, Figure 2b). Furthermore, NAMP gave rise to colonies that were significantly larger than the immediately adhering CFU-f (average diameter Plate1=10.73±1.21 mm vs Plate0=5.54±0.45 mm, $p< 0.01$, Figure 2c). This doubling of average size was due to a global shift in the diameter distribution (Figure 2d, $90^{th}$ percentile = 15 mm vs 8 mm for Plate1 and Plate0 respectively), suggesting that NAMP are a distinct subset of progenitors with a higher proliferation ability than the traditional CFU-f. During the serial replating steps, the self-renewing NAMP gave rise to always larger colonies than initial CFU-f (Plate2= 8.8±0.7 mm, Plate3= 7.5±0.3 mm, Plate4= 6.4±0.3 mm) but gradually smaller (Figure 2c), until the $4^{th}$ replating, where diameters were similar to Plate0.

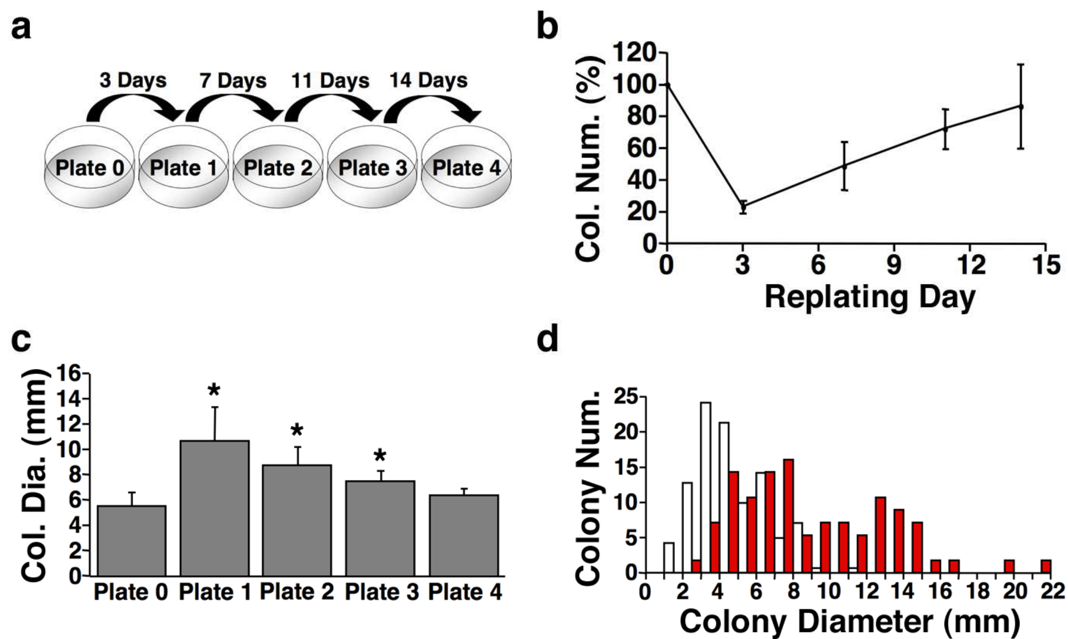


**Figure 2.** ***NAMP are self-renewing progenitors*** *(**a**) Schematic representation of serial replating experiments: the non-adherent fraction was collected at each medium change and NAMP were replated in a fresh dish. (**b**) The analysis of the number of colonies found for each replating step (expressed as percentage of the colonies in Plate0) showed an increasing trend and indicated that NAMP were able to form adherent colonies, but also to self-renew as non-adherent progenitors. (**c**) The average diameter of the colonies in Plate1, Plate2, Plate3 was significantly bigger than the one in Plate 0 (P< 0.01). Plate 4, instead, didn't differ from Plate 0 (P>0.05), indicating that the serial replating induced a proliferative stress and a gradual loss of the proliferation potential. (**d**) After the first replating NAMP gave rise to colonies (Plate1) which displayed the largest diameter size, confirmed by an actual shift in the diameter size distribution (Plate 0= white bar, Plate1= red bar).*

***NAMP progeny display an increased proliferation capacity and a greater differentiation potential in vitro***

Colony diameter analysis showed that when NAMP were plated at clonal density they could give rise to a progeny with an increased proliferation capacity compared to initially adherent CFU-fs. In order to compare the proliferative capacity of NAMP and CFU-f progeny cells during expansion, human BMNC were seeded at a density of 1 x $10^5$ cells/$cm^2$ in cell culture flasks (Flask0). After 3 days the non-adherent fraction was transferred in new flasks (Flask1) and after 2 weeks cells harvested both from Flask0 and Flask1 were replated at 2 x $10^3$ cells/$cm^2$ for 3 further passages. After 14 days, NAMP progeny had expanded 16-fold more (4 population doublings) than the CFU-fs progeny cells (15.6±1.2 vs 11.9±.0.9 doublings in 14 days, n=3, $p<0.05$, Figure 3a). In the following passages, however, the rate of proliferation was similar between the two populations, indicating that the increased proliferative capacity is an exclusive property of the initial progeny of NAMP, but is subsequently lost during expansion. We then investigated whether NAMP progeny could also show a greater differentiation ability compared to CFU-f progeny cells. After 2 weeks of expansion, cells from Flask0 and Flask1 were harvested and exposed to differentiation conditions towards the main mesenchymal lineages (adipogenic, chondrogenic and ostegenic). NAMP progeny cells consistently differentiated better than the initially adherent CFU-f (n=2). When cultured in adipogenic medium, they gave rise to 6.9±2.5 ($p< 0.05$) times more adipocytes in culture, as detected by oil-red staining (Figure 3b and 3c for NAMP and CFU-f progeny respectively) and expressed significantly greater levels of the adipogenic transcription factor PPR-gamma (4.1±0.7 mRNA fold increase, Figure 3d). When cultured in conditions promoting chondrogenesis, NAMP progeny cells produced pellets with an increased matrix deposition and clear chondrocyte lacunae (Figure 3e and 3f, for NAMP and CFU-f progeny respectively), confirmed by 1.8-fold higher amount of GAG (Figure 3g). Following

osteogenic stimulation, NAMP progeny cells displayed a significant increase in calcium deposits, as detected by alizarin red staining (Figure 3h compared to CFU-f progeny Figure 3i), and a 7.8-fold expression of bone sialoprotein (BSP) mRNA levels (Figure 3l).

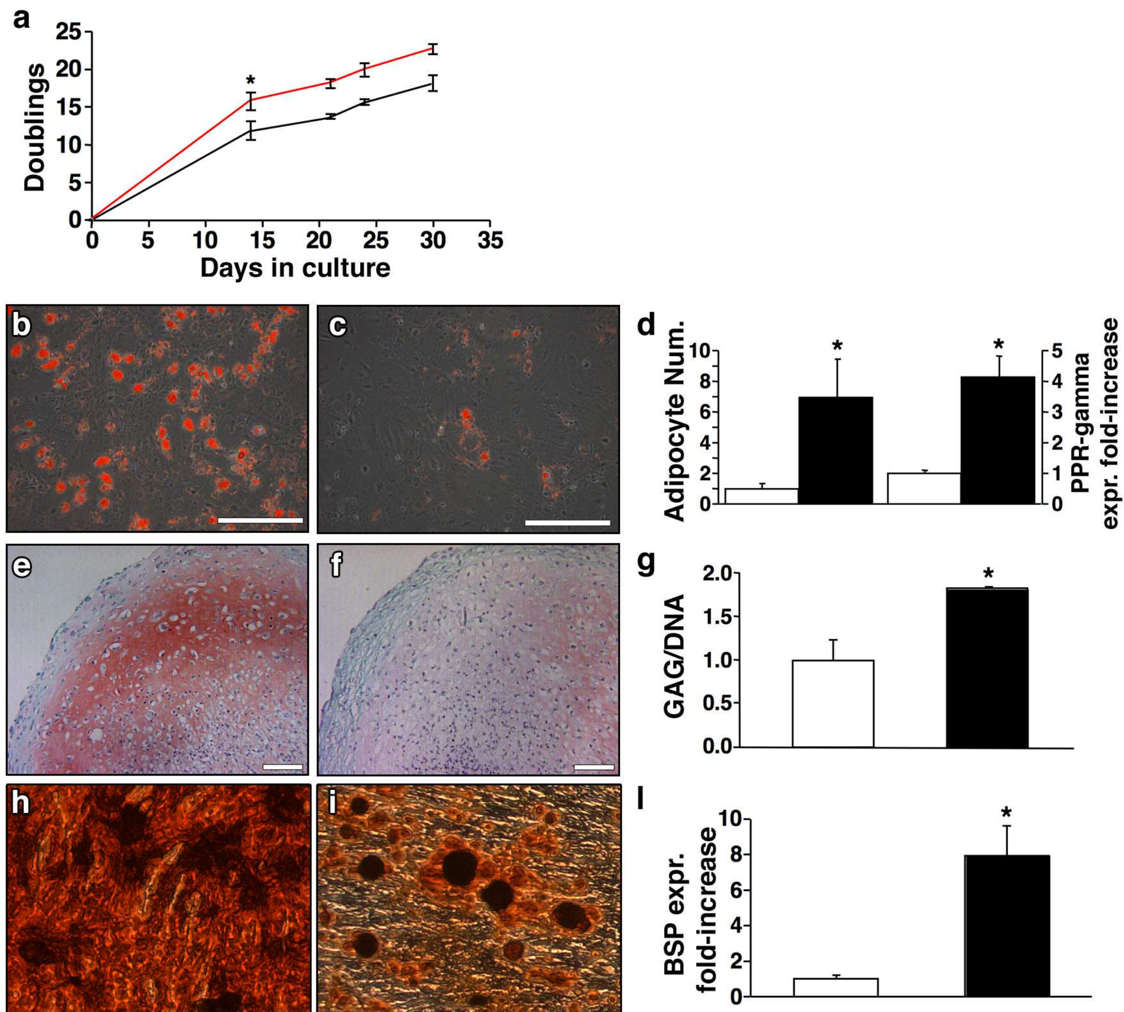


**Figure 3.** ***NAMP progeny display an increased proliferation capacity and a greater differentiation potential in vitro*** *(a) NAMP progeny obtained after the first replating step (red line) proliferated faster than standard CFU-f (black line, P<0.05). However, the population doubling determination showed that there was no difference in the proliferation rate in the following passages, indicating that NAMP progeny's advantage is lost during expansion. (**b**), (**c**) representative fields for NAMP and CFU-f progeny respectively of oil red*

*staining after adipogenic differentiation (size bar= 200 μm); NAMP progeny cells showed a greater differentiation potential which was quantified by the number of adipocytes per field and by PPAR-gamma expression (**d**, black bar= NAMP progeny, white bar= CFU-f progeny); (**e**), (**f**) Safranin-O staining of cell pellets obtained from NAMP and CFU-f progeny cells respectively (size bar= 100 μm). Increased chondrogenic potential of NAMP progeny cells was confirmed by glycosamminoglycan deposition quantification (**g**, black bar= NAMP progeny, white bar= CFU-f progeny); (**h**), (**i**) alizarin red staining for NAMP and CFU-f progeny cells respectively and determination of BSP expression (**l**, black bar= NAMP progeny, white bar= CFU-f progeny).*

***NAMP progeny cells have greatly increased osteogenic potential in vivo***

In vitro differentiation assays are a high-throughput surrogate of actual differentiation capacity which can be rigorously determined only by in vivo functional assays. Therefore, we investigated whether NAMP progeny cells display an increased osteogenic potential in vivo compared to CFU-f progeny cells. Both progenies were obtained and expanded as described for the assessment of the in vitro differentiation capacity. As it is shown in Figure 4, a qualitative analysis revealed that after 8 weeks both CFU-f and NAMP progeny cells could generate bone in vivo (Figure 4a and 4c respectively). The matrix was characterized by an intense eosin staining and by the formation of osteocyte lacunae. Fluorescent images of the histological sections showed that collagen fibers were compact and deposited orderly in parallel orientation (Figure 4b and 4d for CFU-f progeny cells and NAMP progeny cells respectively), confirming the formation of a dense bone-like matrix. The histological analysis also showed an increased amount of bone tissue in the constructs loaded with NAMP progeny cells compared to the ones containing CFU-f progeny cells (Figure 4c-4d and 4a-4b respectively). This observation was confirmed by a quantitative analysis, which revealed that

NAMP progeny cells gave rise to 3-fold higher amount of bone tissue (1.00±0.1 vs 3.1±0.4, n=2, Figure 4e). The assessment of the clonogenicity before implantation showed that number of clonogenic cells, present in the constructs considered for the quantitative analysis, was equivalent for CFU-f and NAMP progeny cells ($2.2x10^5±3.8x10^3$ and $2.1x10^5±9.7x10^3$ per construct respectively). This indicates that the higher amount of bone tissue formed represents a true increase in the bone forming efficiency (BFE) of the clonogenic cells within NAMP progeny cells compared to the ones present in CFU-f progeny cells.

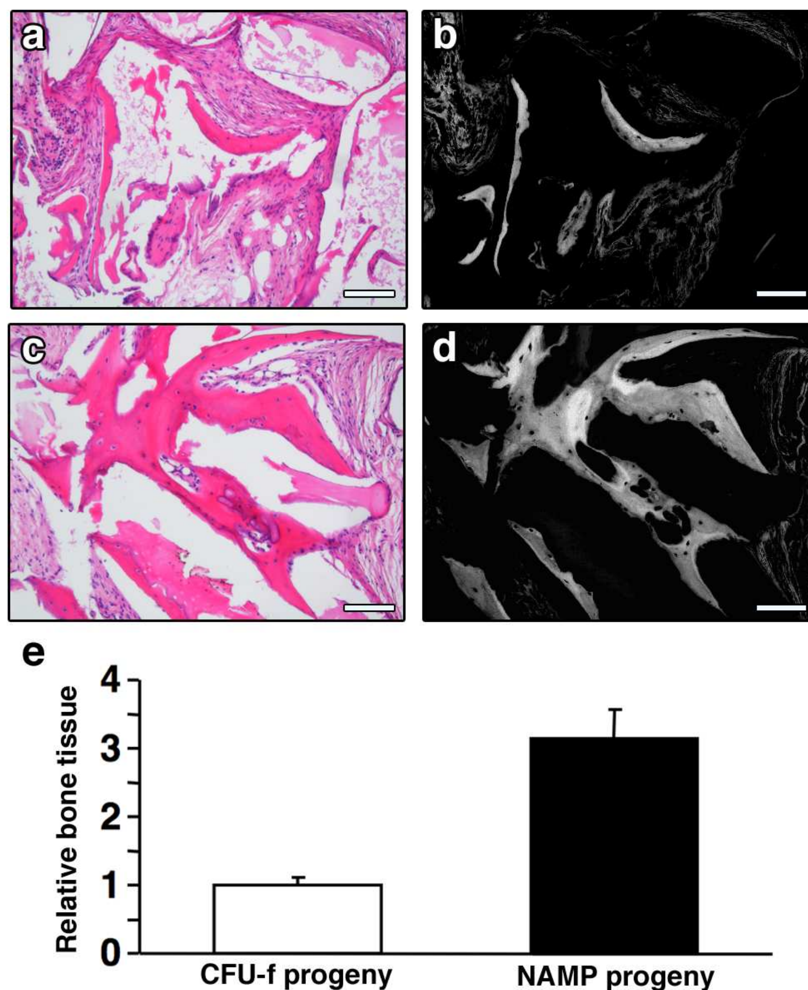


**Figure 4.** ***NAMP progeny cells have greatly increased osteogenic potential in vivo.*** *Representative fields of histological sections of constructs loaded with CFU-f (a) and NAMP (c) progeny cells respectively (H&E staining). Fluorescent pictures confirmed the formation*

*of compact bone-like matrix (b, d for CFU-f and NAMP progeny cells respectively) (size bar= 100 μm). (e) Bone tissue formation quantification: NAMP progeny cells gave rise to an increased amount of bone tissue (3-fold increase) compared to CFU-f progeny cells, confirming the in vitro results and indicating an actual difference in the differentiation potential of the two populations.*

***NAMP and CFU-f progeny cells display a similar phenotype***

We then asked if the greater differentiation capacity was related to a specific phenotype. Also in this case the analysis was performed on the progeny cells, but we chose an early time point to limit as much as possible the expansion on plastic. Cells from Flask0 and Flask1, harvested after 7 days from the plating, were immuno-stained with a panel of different markers, which are commonly used to characterize human BMSC (CD105, CD90), subpopulations of clonogenic cells (CD73, CD49a) (22), earlier mesenchymal progenitors (CD271) (23) or MSC able to carry the hematopoietic microenvironment in vivo (CD146) (8). As shown in Figure 5, the two progenies didn't differ for the expression of CD73, CD105, CD49a and CD271 (Figure 5a, 5b, 5c, 5d respectively). NAMP progeny cells showed a slightly increased expression of CD146 (Figure 5f) and a lower expression for CD90 (Figure 5e). These data indicate that the surface markers commonly used for MSC are not able to capture NAMP characteristics or that adhesion and following expansion on plastic change NAMP phenotype.

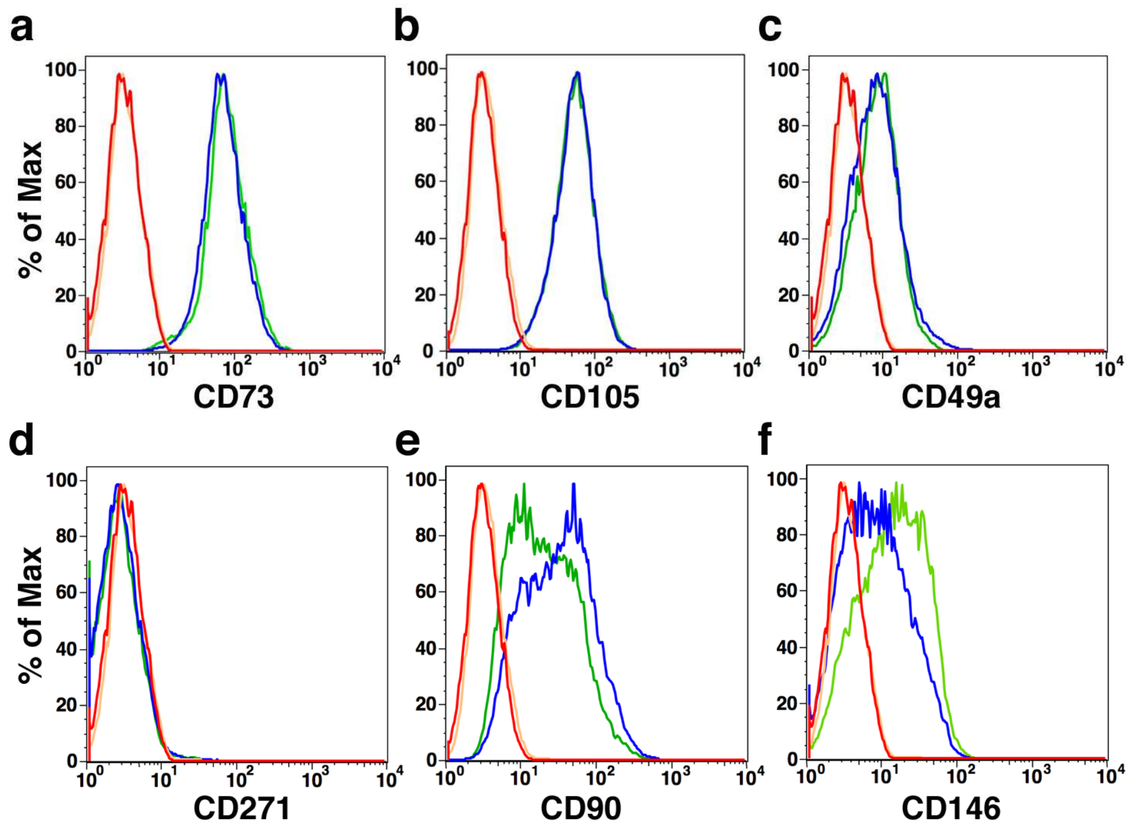


**Figure 5.** ***NAMP and CFU-f progeny cells display a similar phenotype*** *Phenotypical characterization showed that NAMP progeny (green line) and CFU-f progeny cells (blue line) didn't differ for the expression of the mesenchymal markers CD73 (**a**), CD 105 (**b**), CD49a (**c**), and CD271 (**d**). A decreased expression for CD90 (**e**) and a higher expression for CD146 (**f**), instead, could be observed (orange and red lines represents isotype controls for NAMP and CFU-f progeny cells respectively).*

***Maintenance of NAMP is exquisitely dependent on FGF-2 signaling through FGFR2c***

Since FGF-2 was shown to select a particular subset of cells with early-progenitor characteristics (24), we asked whether FGF-2 was necessary for the maintenance of NAMP in culture. Serial replating experiments were performed in presence or absence of FGF-2 and the number of colonies was determined after 2 weeks. Confirming the results shown in Figure 2b, in presence of FGF-2 the number of NAMP increased at each replating step (respectively

Plate1= 21.2%±3.1, Plate2= 47.4%±10.7, Plate3= 85.4%±6.8, Plate4=123.5%±1.0 of the number of initially adherent CFU-f, n=3) (Figure 6a, black line). However, removal of FGF-2 completely abolished the presence of NAMP (Figure 6a, blue line). In fact, only 2.2%±1.0 of the initial number of colonies was present in Plate1 and no colonies could be observed in the following replating steps.

Because proliferating cells undergo a stage of rounding and semi-detachment from plastic during the M phase of cycle, we investigated whether NAMP were a specific non-adherent population or simply a fraction of the actively proliferating adherent cells. We therefore performed serial replating experiments replacing FGF-2 with PDGF-BB, which also efficiently stimulates mesenchymal cell proliferation in culture. As shown in Figure 6a (red line), the number of colonies present in all replating steps with PDGF-BB was not different from the condition with serum alone (Plate1 5.6±0.5% vs 2.9±2.3% of the colonies in Plate 0, respectively, n=3, p=n.s.), completely disappearing by the second replating. These results strongly indicate that NAMP are a population of non-adhering progenitors distinct from the proliferating fraction of adherent cells. Furthermore, their presence in vitro critically and specifically depends on FGF-2 signaling.

We next asked whether FGF-2 was continuously necessary for the maintenance of NAMP function after initial selection, and conversely whether NAMP could be induced by FGF-2 from the initially adherent fraction at a later stage. Therefore, we performed a medium-switch experiment (n=3). Fresh human BMNC were serially replated as before. However, in some conditions FGF-2 was removed after the first (3 days) or the second replating (7 days). Similarly, they were also cultured without FGF-2 throughout the serial replating, or FGF-2 was added to the medium after the first or second replating steps (3 and 7 days, respectively). When FGF-2 was removed after the first 3 days of culture (Figure 6b, dash-dotted line), the number of colonies in the following replating steps dropped to zero like

in the no-FGF condition (Figure 6b, blue line). When FGF-2 was removed after the second replating step (7 days) (Fig. 6b, dashed line), instead, the number of NAMP derived colonies stopped increasing in the following replating steps and actually decreased slightly, but not disappeared. No colonies were ever observed when FGF-2 was added after the first 3 or 7 days of culture, suggesting that FGF-2 is critical for NAMP survival and selection in the initial stage of culture.

Since selection and maintenance of NAMP in vitro was critically dependent on FGF-2 signaling, we therefore investigated which receptor mediates this specific function of FGF-2, which is known to be quite pleiotropic and bind 5 different receptors (FGFR1b, FGFR1c, FGFR2c, FGFR3c and FGFR4) (25). To assess the role of FGFR1b, FGFR2c and FGFR3c, monoclonal blocking antibodies were used. Instead, since blocking antibodies for FGFR1c and FGFR4 were not commercially available, we used FGF-5 and FGF-19, which respectively bind to FGFR1c/2c and FGFR4, to see if the stimulation of these specific receptors could maintains NAMP in culture. BMNC were plated at clonogenic density (n=3) and only the first replating step was performed (3 days replating). The medium was supplemented either with the blocking antibody plus FGF-2 or with FGF-5 or FGF-19 and after 14 day the colonies were stained.  As it is shown in Figure 6c, in the presence of FGF-19 the number of NAMP derived colonies in Plate1 was similar to the one with serum alone (Figure 6c, 20.1±5.9% vs 25.0±3.9% respectively, values are expressed as percentage of the colonies in the FGF-2 condition), suggesting that FGFR4 is not involved in maintenance of NAMP in culture. Blocking antibodies for FGR1b and FGFR3 did not significantly affect colonies in Plate1 compared to the condition with FGF-2 only (Figure 6c, 91.2±11.6% and 87.9±11.9%, respectively, p=ns). However, when the FGFR2 blocking antibody was added to the culture, the number of colonies decreased significantly (Figure 6c, 43.3±8.4%, $p<0.05$). Furthermore, by stimulating FGFR1c and FGFR2c with FGF-5 it was possible to partially

restore NAMP derived colonies in Plate1 (Figure 6c, 65.1±15.2%). Although these results cannot exclude a role of FGFR1c, they together indicate the involvement of FGFR2c in mediating NAMP survival and self-renewal.

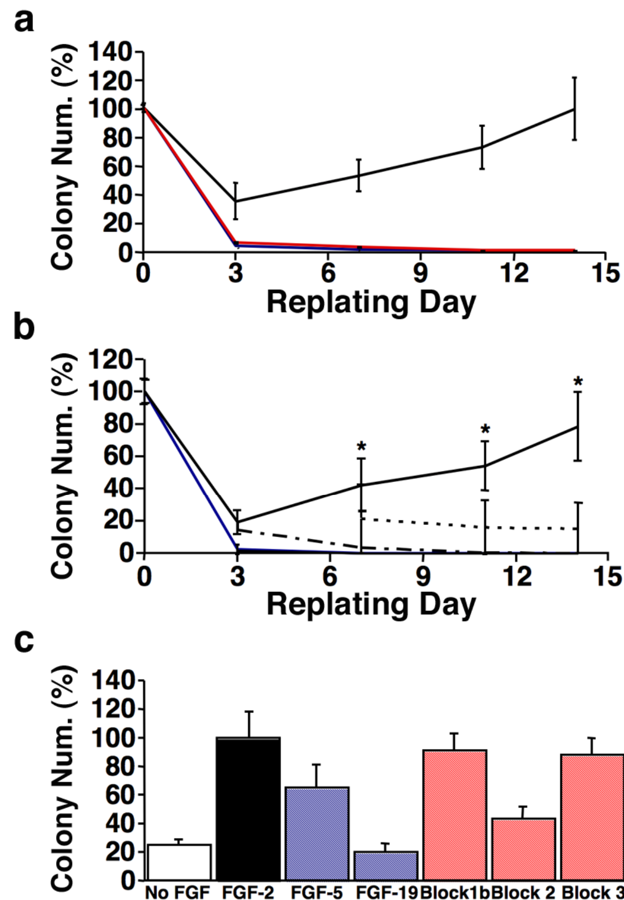


**Figure 6.** ***NAMP maintenance in vitro is exquisitively dependent on FGF-2 signaling through FGFR2c*** *(**a**) NAMP could self-renew as non-adherent progenitors and increase in number when FGF-2 was added to the culture (black line). When the medium was, instead, not supplemented (blue line), the number of colonies in the serial replating dropped to zero from the second replating step. In presence of another growth factor which stimulates the proliferation of adherent CFU-f, as PDGF (red line), NAMP could not regenerate them-selves similarly to the condition with serum alone. This suggests that NAMP are not the semi-*

*adherent proliferating CFU-f and indicates FGF-2 specificity for NAMP maintenance. (**b**) The removal of FGF-2 after 3 days (dash-dotted line) negatively affected NAMP self-renewal. In fact, the number of colonies in the following replating steps was the same of the condition with serum alone (blue line). When FGF-2 was removed after 7 days (dashed line), the FGF-2 condition trend (black line) was abolished since there was no increase in the number of NAMP derived colonies, indicating that FGF-2 is necessary for NAMP self-renewal in culture. (**c**) In presence of FGF-19, which stimulates FGFR4, the number of colonies in Plate1 was similar to the condition with serum alone (white bar), indicating that FGFR4 doesn't mediate NAMP self-renewal. When FGF-5, which stimulates FGFR1c and FGFR2c, was added to the culture NAMP gave rise to a higher number of colonies, suggesting the involvement of at least one of the two receptors. Blocking FGFR1b and FGFR3, no reduction in colony number could be observed. In presence, instead, of FGFR2 blocking antibody, the colony number significantly decreased, suggesting a role of FGFR2c in NAMP maintenance. (Colony number represents colonies in Plate1 and is expressed as percentage of the colonies in FGF-2 condition).*

### ***The adherent cells provide the niche for NAMP***

We showed that the non-adherent fraction of bone marrow cultures contains a population of stably non-adherent mesenchymal progenitors, which can produce adherent colonies while regenerating them-selves upon serial replating. Furthermore, the results of the serial replating experiments suggest that NAMP need the adherent fraction for their maintenance. We now hypothesized that the adherent cells constitute a niche that provide specific signals to preserve NAMP characteristics. To test our hypothesis we investigated the niche function of the adherent fraction by complementary loss of function and gain of function approaches.

For the loss of function approach, we cultured first-replating derived NAMP in agarose-coated dishes, thus preventing the formation of an adherent cell layer. After 4 or 7 days (day7 and day11 respectively) cells were transferred to plastic cell culture dishes for CFU-f assay. The total absence of colonies indicated that NAMP were not able to survive in the absence of an adherent progeny. We next asked if the CFU-f or NAMP adherent progeny cells constitute a unique environment for NAMP or if also other cell types could provide the same signals. Therefore, first-replating derived NAMP were replated on an irradiated feeder layer of human skin fibroblasts. After 4 or 7 days (day7 or day11 respectively) the cells were transferred to plastic on cell culture dishes. The number of colonies generated after both 4 and 7 days on skin fibroblasts was negligible, suggesting that this cell type could not provide the required signals for NAMP survival.

Taking a gain of function approach we hypothesized that, if CFU-f progeny cells provided the niche function for NAMP, keeping them in contact with the adherent cells would avoid the gradual loss of their early progenitors characteristics, which is observed during serial replates in empty dishes. The non-adherent fraction was maintained in Plate0 for 7 or 14 days before replating to a new culture dish. After two weeks the number of colonies was compared with those obtained in the serial replating to establish if expanding the putative niche, we could also expand the non-adherent fraction. Colony diameter was measured, instead, to assess whether the contact with the niche could preserve NAMP high proliferative potential (n=2). As shown in Figure 7a, when NAMP were kept on the originally adherent cells for 14 days (Plate4*), they generated greater than 3-fold more colonies compared to when they underwent a serial replating for the same time (Plate4) (Figure 7a, 260.8±15.8% vs 61.0±8.0%); if NAMP were not replated to a new dish, however, no extra colonies grew in the initial plate after 14 days, confirming the results in Figure 1b. Colonies generated after 14 days of continuous culture in the primary plate displayed a significantly ad consistently larger

diameter compared to those generated by serially replated cells at the same time point (9.8±0.4 mm vs 6.8±0.3 mm, $p<0.05$, Fig 7b). Furthermore, colonies in Plate4* were similar in diameter to the ones in Plate1 (diameter= 9.8±0.4 mm 9.2±0.5 mm respectively, p=ns) indicating that NAMP compartment can expand on the adherent fraction without losing the proliferative potential. In parallel, we compared the differentiation capacity of NAMP kept on the originally adherent cells for 14 days (Plate4*) to the ones that were serially replated for the same time (Plate4). Progeny of both populations was generated replating the non-adherent fraction in new flasks and after 1 week of expansion cells were exposed differentiation conditions to assess differentiation capacity in vitro. When NAMP were kept in contact with their niche, they generated a progeny which displayed an increased differentiation potential compared to the one derived from serially replated NAMP. In fact, they gave raise to a higher number of adipocytes (Figure 7c and 7d for Plate4 and Plate4*-progeny respectively) and they could better differentiate towards the osteogenic lineage (Figure 7e and 7f for Plate4 and Plate4*-progeny respectively).

These data collectively suggest a niche function for the adherent cells. When this function was absent due to serial replating in empty dishes, NAMP could regenerate the niche but at the cost of gradually losing their proliferative and differentiation potential (Figure 2c).

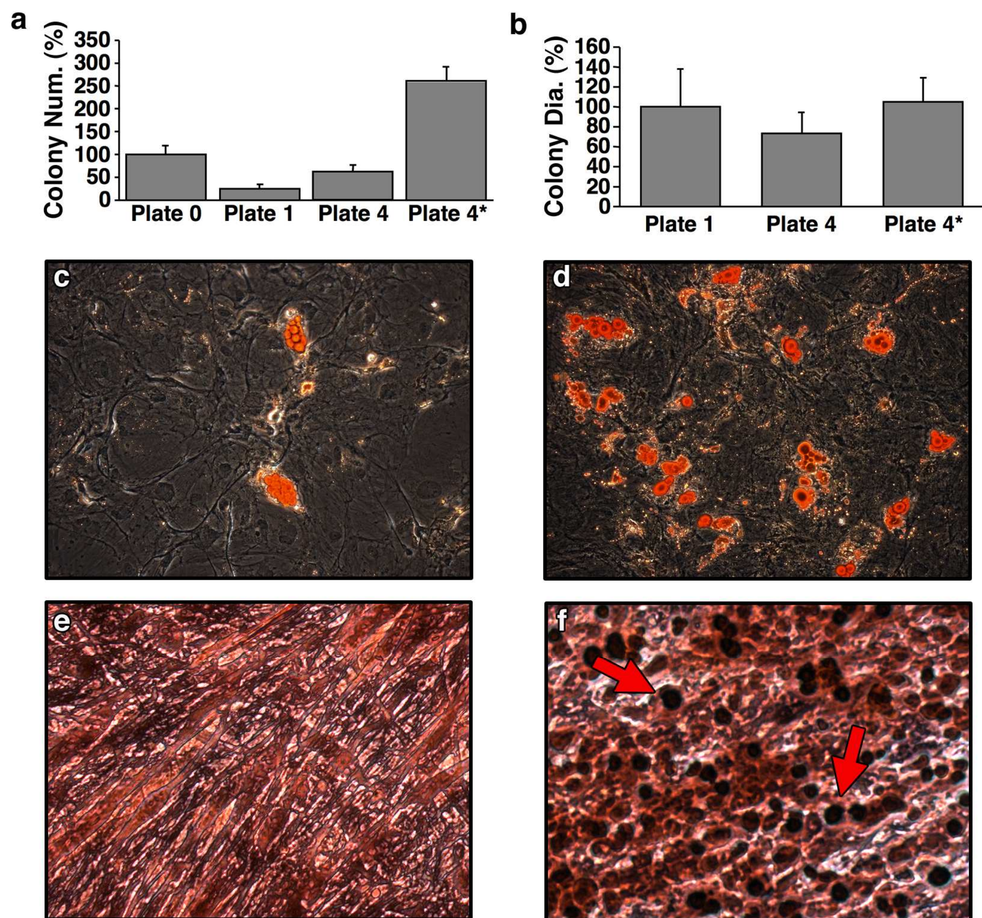


**Figure 7.** ***The adherent cells provide the niche for NAMP*** *(a) when NAMP were kept in contact with CFU-f progeny (Plate4*), they produced 3-fold more colonies compared to when they were serially replated for the same time (Plate4); (b) As shown from the colony diameter size evaluation, Plate4*-progeny preserved the proliferation capacity compared cells generated from serially replated NAMP; (c), (d) representative fields for Plate-4 and Plate-4* progeny respectively of oil red staining after adipogenic differentiation; (e), (f) alizarin red staining for Plate4 and Plate4*- progeny cells respectively. Red arrows indicate calcium deposits.*

### *4.4 Discussion*

The data presented in this study indicate that a population of stably non-adherent mesenchymal progenitors (NAMP) is present in human bone marrow cultures. NAMP display the following distinct biological features compared to standard CFU-f: 1) an increased proliferation and differentiation capacity of their immediate progeny in vitro; 2) a greater efficiency in generating bone tissue in vivo; and 3) the ability to give rise to adherent colonies while self-renewing as non-adherent progenitors in vitro. Both their maintenance and self-renewal are strictly dependent on FGF-2 signaling and on the presence of adherent CFU-f progeny cells, suggesting the establishment of a niche-progenitor system in vitro.

Bone marrow-derived mesenchymal progenitors are a widely heterogeneous population in terms of both proliferation and differentation potential (26). A model about lineage hierarchy in vivo has been proposed (27) and in vitro studies at the clonal level have revealed the presence of different classes of progenitors within the CFU-f population in a specific hierarchical relationship, with progressive loss of multi-differentiation potential towards adipogenesis and chondrogenesis and commitment to the osteogenic lineage (10). Our data identify a population of mesenchymal progenitors which appears to represent a more primitive entity than classic CFU-f within the hierarchy of mesenchymal progenitors. Features currently accepted as defining a stem/progenitor cell are 1) the high proliferation capacity, 2) the multilineage differentiation potential and 3) the self-renewing ability (28). When serially replated, NAMP gave rise to colonies with a significantly increased diameter, indicatating a higher proliferation activity at clonal density. When plated at high density, however, NAMP progeny showed a faster rate of proliferation only until the first confluence. These data indicate that the adherent progeny does not maintain the properties of the NAMP from which it derives, which rather reside in the self-renewed non-adherent fraction. The differentiation potential has been described to be progressively lost during expansion pointing

to a commitment of the population (29). Even though at the first confluence NAMP progeny cells had expanded 16-fold more (4 population doublings) than CFU-f progeny cells, it still displayed a greater differentiation potential. Furthermore, NAMP are able to regenerate themselves and at the same time to produce adherent colonies, but they cannot be induced from the initially adherent fraction at a later stage. These data together suggest that NAMP are a distinct population hierarchically upstream of initially adherent CFU-f which displays earlier progenitor features and which is biologically different from classically-defined CFU-f.

Serial replating experiments performed in the absence of FGF-2 revealed that, unlike initially adherent CFU-f, NAMP require FGF-2 both for their selection and maintenance in culture. FGF-2 has been shown to crucially control the selection of a subset of initial CFU-f enriched in pluripotent mesenchymal precursors (24). Interestingly, removal of FGF-2, at different time points during serial replating, always caused the disappearance of NAMP. This is in contrast to the effects on CFU-f, where FGF-2 induces, instead, an immediate and permanent selection within the clonogenic progenitors, suggesting a different biological response to FGF-2. Furthermore, blocking receptor experiments showed that FGF-2 mediates the maintenance of NAMP in culture through FGFR2c signaling. The FGFR2 gene encodes two splice variants, FGFR2b which in mice seems to have a unique role in skin development and FGFR2c, which is, instead, preferentially expressed during osteogenesis (30). In FGFR2c$^{-/-}$ mice the balance between proliferation and differentiation of skeletal progenitors is shifted towards differentiation, leading to premature loss of growth and defects in the skull and in long bones (31). Conversely, in gain of function experiments, which were used as a model for human Crouzon and Pfeiffer syndromes, mutants showed a significant increase in the number of osteoprogenitors cells (32). These data collectively suggest that NAMP have a role as reservoir of early mesenchymal progenitors and that the activation of FGFR2c signaling is fundamental for their function in vivo.

A fundamental property that characterize stem cells is the self-renewal, which is defined as the cycles of division that generate at least one daughter equivalent to the mother cell with latent capacity for differentiation (33). Demonstration of self-renewal in vivo implicates both the generation of a differentiated progeny and the reconstitution of a stem cell compartment. In the bone marrow, the contact with the specialized environment of the niche regulates hematopoietic stem cells survival and self-renewal (5). Our data show that NAMP are able to maintain them-selves and proliferate in suspension, but that they require the adherent mesenchymal fraction for their maintenance. The generation of adherent daughter cells can be achieved by two different mechanisms, 1) adhesion of a non-adherent progenitor and proliferation as fibroblastic colony or 2) a division, in which only one of the daughter cells lose the non-adherent feature and the other maintains instead NAMP features. The first mechanism would implicate that NAMP are able to undergo division in suspension to maintain them-selves in the non-adherent status. Even thought further experiments are required to address this point, our data show that, when serially replated, NAMP are subject to a proliferative stress due to the generation of the adherent fraction. In fact, the generation of adherent daughter cells happens with slow gradual loss of potential, as after 4 steps of serial replating, NAMP proliferative potential is similar to that of initially adherent CFU-f. However, the self-renewal capacity is maintained because they are still able to generate non-adherent clonogenic progenitors in constantly increasing numbers. A similar mechanism has been described for hematopoietic stem cells, which, even maintaining their stem cell properties, lose telomere length, and therefore proliferative activity, when they are subjected to the proliferative stress of bone marrow repopulation in bone marrow transplantation recipients (34, 35). In our experimental set up NAMP self-renewal is not assessed at a single cell level in vivo. However, our data show that in vitro NAMP are able to maintain them-selves and to give rise to a progeny, which displays different biological features.

Furthermore, the loss of potential, taking place with the serial generation of the adherent progeny, does not occur maintaining the culture of NAMP on the initial adherent fraction. In this experimental condition, in fact, NAMP undergo greater expansion compared to when they are serially replated (12-fold vs 4-fold in 14 days, Figure 6a) without any loss of proliferation and differentiation potential. This set of data, therefore, suggests that the adherent CFU-f provide a niche function for NAMP promoting their expansion and self-renewal as early progenitors in vitro.

Even though mesenchymal progenitor cells are the subject of extensive interest in regenerative medicine, the knowledge about their regulation in vivo, including the need of a specific microenvironment for their maintenance, is severely insufficient. Sacchetti et al. (8) identified for the first time the presence of self-renewing stromal cells, which can be selected ex vivo only in the absence of FGF-2. This population is characterized by CD146 expression and is able to generate osteoblasts, adventitial reticular cells and the hemopoiesis-supporting niche in vivo, while maintaining them-selves as clonogenic stromal progenitors. Our data suggest a complementary model, showing that FGF-2, instead, selects a different population of progenitors from the common bone marrow pool. NAMP are self-renewing in vitro, can differentiate towards the classic mesenchymal lineages (adipogenic, chondrogenic and osteogenic in vivo) and generate a niche supporting their own self-renewal, but lose the ability to support hemopoiesis in vivo. In fact, although we could observe a moderate shift of CD146 expression in NAMP progeny, they could not support bone marrow formation in vivo. It would be interesting to investigate the role of FGF-2 in selecting different populations in vivo, which perform different functions in the complex bone marrow environment.

In conlcusion, our data identify a niche/mesenchymal progenitor organization in vitro, suggesting the novel concept that the progeny of self-renewing mesenchymal progenitors can

provide the regulatory microenviroment from the same progenitor it derives from.

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# *Chapter 5*

## *Adipose tissues stromal vascular fraction (SVF) cultures contain a population of non-adherent mesenchymal progenitor (NAMP) which are FGF2-dependent and require an in vitro niche for their maintenance*

## *5.1 Introduction*

Although mesenchymal progenitors cells were originally described in the bone marrow (1), they have been also found in the stromal vascular fraction (SVF) of white adipose tissue and more recently in a variety of other tissues, such as placenta, synovium and dental pulp (2). This led to the current hypothesis that multipotent mesenchymal progenitors are present in all tissues of the body, possibly in a perivascular position, representing a common reservoir of regenerative cells.

Our findings described in Chapter 4 showed that non-adherent progenitors of bone marrow stroma (BM-NAMP) represent the most primitive compartment of MSC, are capable of establishing their own niche in vitro and, in these conditions, can self-renew without loss of proliferation and differentiation capacity. Based on these results, we sought to determine whether the NAMP class of progenitors was specific to bone marrow stroma or could represent a common feature of other mesenchymal compartments, like for example the SVF of adipose tissue.

## *5.2 Material and Methods*

***Cell isolation***

Subcutaneous adipose tissue in the form of lipoaspirates was obtained from 12 healthy donors (21-69 years old) during routine lipoaspirations, after informed consent from the patient and following protocol approval by the local ethical committee. The tissue was digested in 0.075% collagenase type 2 (Worthington, Lakewood, NY) for 45 minutes at 37 °C on an orbital shaker. The suspension was thereafter centrifuged at 300*g* for 10 minutes, and the resulting SVF pellet was washed once with phosphate-buffered saline (PBS), resuspended in alpha-minimal essential medium (MEM) (Gibco, Grand Island, NY), and finally filtered through a 100 µm strainer (BD Falcon; BD Biosciences, San Diego). Nucleated cells were counted after staining with Crystal Violet 0.01% (Sigma) in phosphate-buffered saline, pH 7.2 (PBS). For FACS analysis, freshly isolated cells were resuspended into 200 µl of 0.5% BSA in PBS (FACS Buffer) with fluorochrome-conjugated antibodies against the indicated protein or an isotype control and were incubated for 30 minutes at 4 °C. The antibodies used were CD146-PE, CD90-FITC, CD73-PE, CD14-PE, CD45-PE, CD31-APC, CD34-APC, SSEA4-FITC, IgG1-FITC, IgG1-PE, IgG-APC (all from Becton, Dickinson and Company, Franklin Lakes, NJ), CD105-FITC (Serotec Ldt. Oxford, UK). All the antibodies were used at a dilution of 1:50, except CD105-FITC, which was used at 1:20. Cells were washed twice with FACS buffer, resuspended in PBS and analyzed with a FACSCalibur flow cytometer (Becton, Dickinson and Company).

***Cell culture***

To determine colony forming efficiency (CFE) and for serial replating experiments, cells were plated at clonal density (9 cells/cm$^2$) and cultured in alpha-MEM (GIBCO) with 10% fetal bovine serum (FBS). When indicated, medium was further supplemented with FGF-2 (5

ng/ml, R&D System) or PDGF-BB (10 ng/ml R&D System). After 2 weeks cells were then washed with PBS, fixed with 3.7% formaldehyde in PBS, stained with Crystal Violet (Sigma) for 10 min, washed with tap water and the colonies were counted. All determinations were performed in triplicate.

***Statistical analysis***

Data are presented as means ± standard deviation. The significance of differences was evaluated using analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons. $P < 0.05$ was considered statistically significant.

## 5.3 Results

***A population of non-adherent mesenchymal progenitors is present in adipose stromal vascular fraction (SVF) cultures***

To assess whether the non-adherent fraction of adipose SVF cultures contained a population mesenchymal progenitors and to determine their number, nucleated cells were isolated from fresh human adipose tissue, plated at clonal density (9 cells/cm$^2$) on cell culture dishes (Plate0) in presence of FGF-2 and after 3 days, the non-adherent fraction was collected, resuspended in fresh medium and replated in a new dish (Plate1, Figure 1a). After 14 days from the plating, the dishes were stained to assess the number of colonies. Plate 1 contained 17.7±9.1% of the colonies present in Plate 0 (n=13), indicating that a population of non-adherent mesenchymal progenitors (adipose tissue (AT)-NAMP) was present in adipose tissue stromal cell cultures.

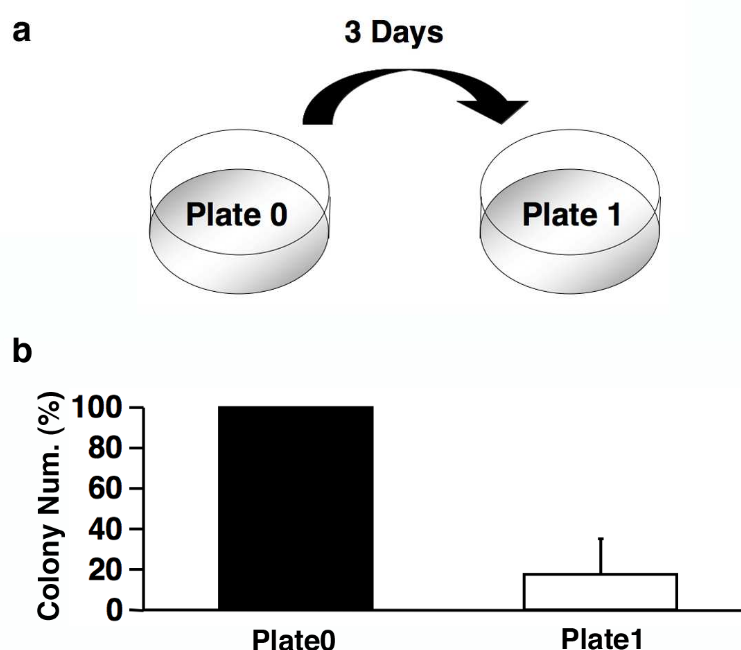


***Figure 1.*** (a) *Schematic representation of the replating of the non-adherent fraction in a new dish (Plate 1); (b) Quantification of the number of colonies in Plate0 and Plate1.*

***AT -NAMP are stably non-adherent***

To investigate whether these clonogenic cells were stably non-adherent and not a subpopulation that displayed delayed adherence, nucleated cells of freshly isolated adipose tissue were plated at clonal density in presence of FGF-2 and at each medium change, the supernatant containing the non-adherent fraction was either discarded (Plate0) or collected, resuspended in fresh medium and replated into the same dish (Plate0*) (Figure 2a). After 14 days, the colonies were counted. The number of colonies in Plate0 and in Plate0* did not differ significantly (12.6±3.3% and 11.5±2.8% respectively, p=n.s., n=4, Figure 2b). Colony diameter was also measured to compare the proliferation capacity and no significant difference was found (Plate0=4.3±0.8mm and Plate0*=4.6±0.2mm, p=n.s., n=4, Figure 2c).

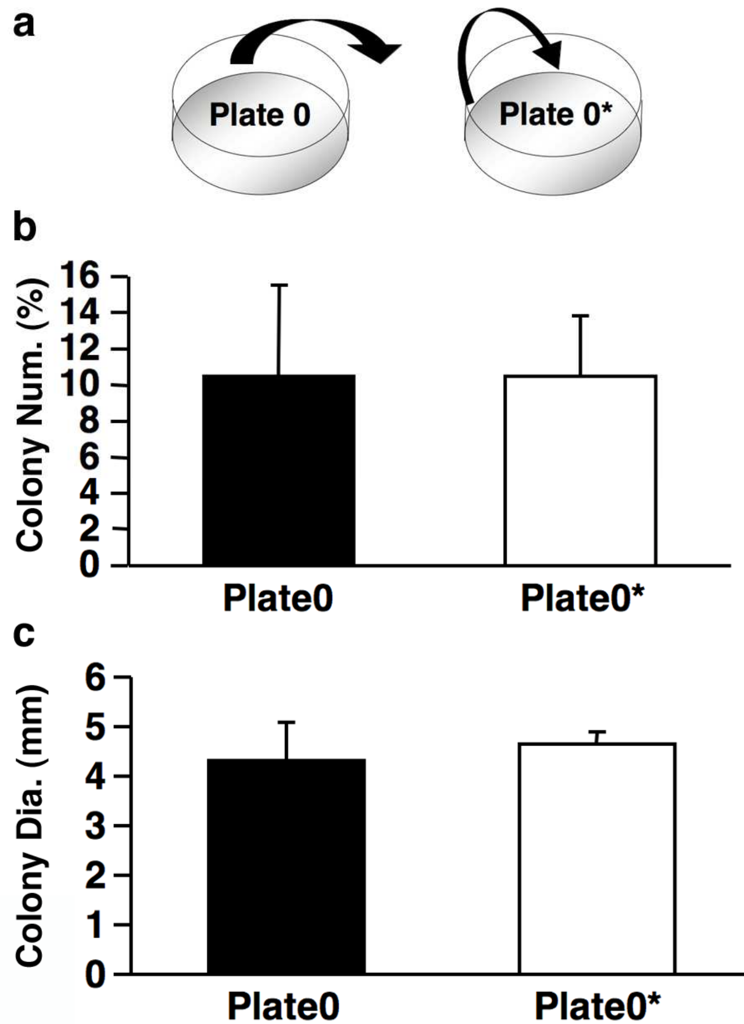


***Figure 2.*** *(a) Schematic representation of the replating of the non-adherent fraction in the same dish (Plate0*); (b) Quantification of the number of colonies in Plate0 and Plate0*; (c) Average diameter size of colonies present in Plate0 and Plate0*.*

***AT-NAMP do not self-maintain upon serial replating***

We next asked whether AT-NAMP could maintain themselves as non-adherent progenitors or if they would exhaust themselves in generating adherent colonies. To address this question, we performed serial replating experiments, as described in the previous chapter for BM-

NAMP (Chapter 4, page ?, Figure 3a). At each replating step, the number of colonies decreased as compared to the initial CFU-f (Plate1=16.0±9.0%, Plate2=8.4±9.7%, Plate3=1.6±2.9%, Plate4=0.1±0.3%, n=11, Figure 3b). Three of the analyzed donors showed colonies only in the first replating step (23%) and only two of them (18%), instead, showed few colonies up to the last replating. The size of the colonies increased from Plate0 to Plate1 (4.3±0.5mm and 6.1±0. 7mm respectively). In the following two replating steps (Plate2 and Plate3), however, there was no increase in the colony size (5.9±1.4mm and 5. 9±0.8mm). Interestingly, the few colonies present in Plate4, displayed instead an increased diameter (9.5±0.7mm, Figure 3c).

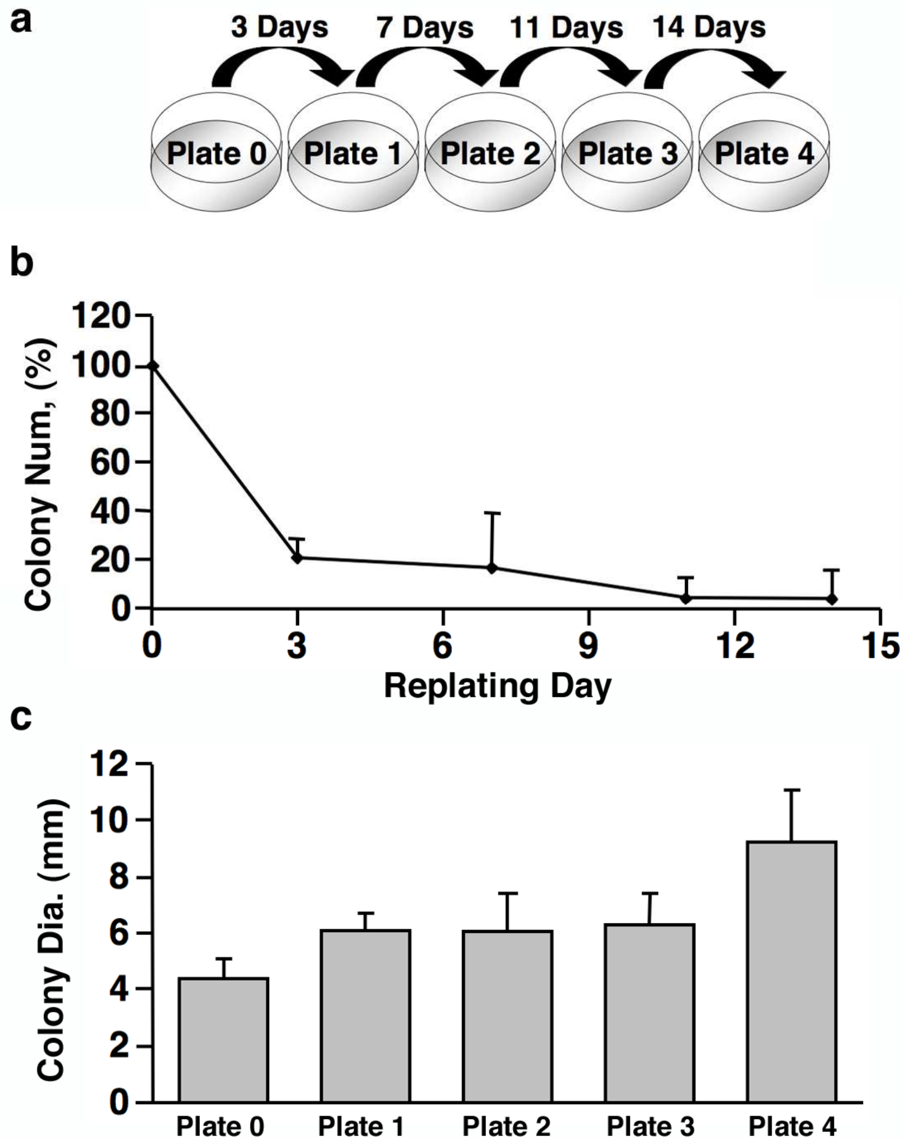


***Figure 3.*** *(a) Schematic representation of serial replating experiments; (b) Quantification of the number of colonies generated through the serial replating; (c) Average diameter size of the colonies generated in each replating step.*

***AT-NAMP require FGF-2 for initial survival***

We next asked whether FGF-2 could have a role, as for BM-NAMP, for AT-NAMP survival. Serial replating experiments were performed in presence or absence of FGF-2. Confirming the results in Figure 3b, in presence of FGF-2 (Figure 4, red line), although the number of

colonies decreased, few colonies were found in all replating steps (Plate1=21.3±7.5%, Plate2=17.1±21.8%, Plate3= 4.6±8.4%, Plate4=4.3±11.6%, n=8). Removal of FGF-2 (Figure 4, black line), instead, abolished the presence of colonies already by the second replating (Plate1= 4.8±3.6%, Plate2=0.3±0.6%, no colonies were present in Plate3 and Plate 4, n=8). We then wanted to investigate whether FGF-2 was specific for AT-NAMP survival or if they could be maintained by other mitogenic growth factors. PDGF-BB, which similarly stimulated initial CFU-f proliferation (size of the colonies 4.7±0.4 and 4.1±0.9 mm, for FGF-2 and PDGF-BB respectively), was tested in the serial replating setting. As shown in Figure 4 (blue line), the number of colonies present in all replating steps with PDGF-BB was similar to the condition with serum alone (Plate1= 11.5±8.2%, Plate2=0.9±1.5%, no colonies were present in Plate3 and Plate4, n=3), almost disappearing by the second replating.

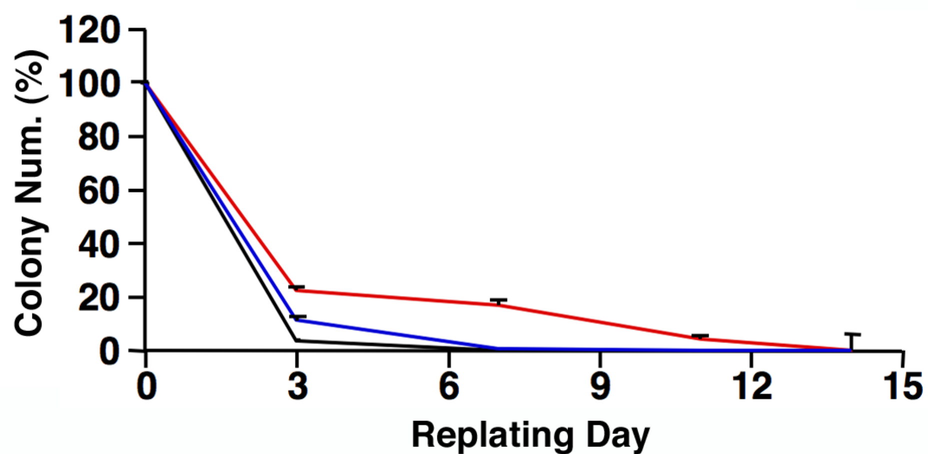


***Figure 4.*** *Number of colonies generated in serial replating experiments in presence of FGF-2 (red line), PDGF-BB (blue line) or with serum alone (black line).*

***Initially adhering colonies promote AT-NAMP maintenance and proliferation as non-adherent progenitors***

In Chapter 4 we showed that, when replated on initially adherent CFU-f progeny, BM-NAMP were able to proliferate in suspension without loss of their progenitor properties. We, therefore, hypothesized that initially adherent CFU-f displayed a niche function for BM-NAMP. The data described above showed that AT-NAMP were not able to maintain them-selves upon serial replating. We then asked whether maintaining them in contact with initially adhering CFU-f progeny they could proliferate as non-adherent progenitors and preserve at the same time their proliferation capacity.

Nucleated cells of freshly isolated adipose tissue were plated at clonal density in presence of FGF-2 and the non-adherent fraction was resuspended in fresh medium and replated into Plate0 at each medium change. After 7 or 14 days, the non-adherent fraction was plated into a new dish and the number of colonies and their diameter was assessed after 14 days. As shown in Figure 5a, when AT-NAMP were kept in the original dish for 7 or 14 days they could proliferate in suspension, since when transferred in a new dish they generated a higher number of colonies compared to when they underwent a serial replating for the same time (at day 7 Plate2=12. 8±13.2%, Plate2*=56.3±46.5%; at day 14 Plate 4=0.1±0.3%, Plate4*=129.1±66.0%). The colony diameter assessment showed that, maintaining AT-NAMP in contact with the initially adherent CFU-f, it was possible to preserve their proliferation capacity. In fact, there was no significant decrease in the size of the colonies, despite AT-NAMP underwent proliferation in suspension (Plate0=4.3±0.8mm, Plate2*=5.4±1.1mm, Plate4*=6.1±1.8mm).

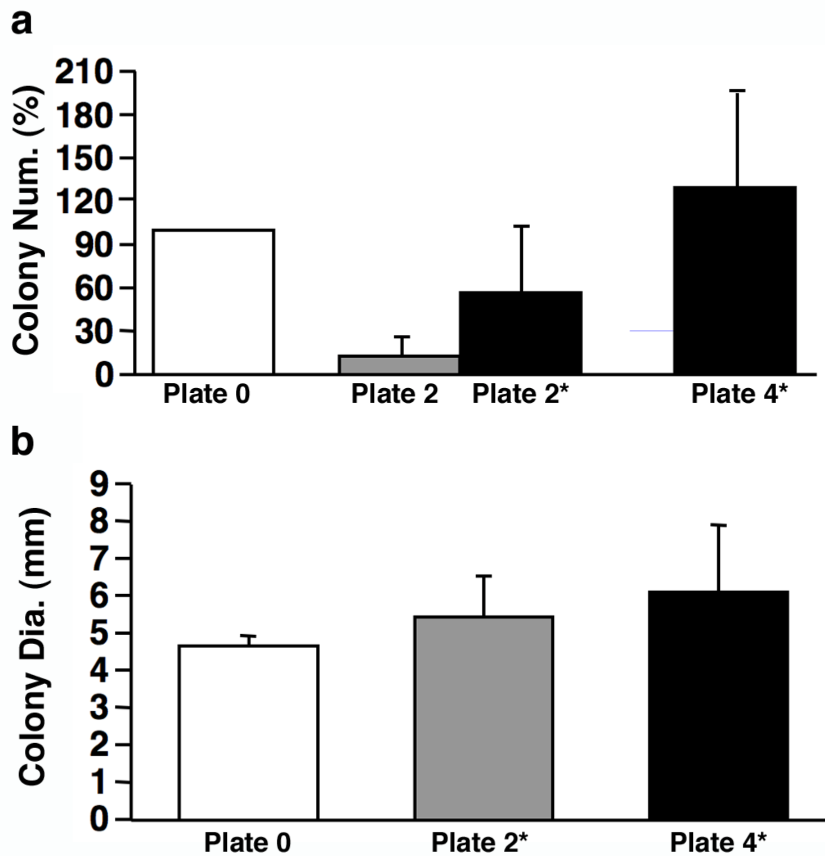


***Figure 5.*** *(a) Quantification of the number of colonies generated when AT-NAMP were serially replated or kept in the initial dish for 7 (Plate 2 and Plate2*) or 14 days (Plate4*); (b) average diameter size of the colonies in the conditions described above.*

***AT-NAMP frequency does not correlate with the presence of specific subpopulations, but is positively related to the initial clonogenicity***

Due to the heterogeneity of the initial population, we investigated whether the presence of NAMP and their number was correlated with the presence of specific subpopulations. Therefore we analyzed the primary isolated cells for the expression of markers specific for mesenchymal (CD105, CD90, CD73, CD146, SSEA-4), endothelial (CD31, CD34) and hematopoietic (CD14, CD45) lineages, and determined the correlation coefficient of the frequency of positive cells with the colony forming efficiency of first replated AT-NAMP. A statistically significant correlation would suggest an enrichment of AT-NAMP in the

subpopulation positive for that marker. A direct analysis of AT-NAMP phenotype is made impossible by their exceedingly low frequency in the primary isolated population. As shown in Table 1, AT-NAMP frequency, indicated by the CFE of 3 days replated cells, did not correlate with any of the markers, even those described to characterize early mesenchymal progenitors.

| | **CFE 3 days** | |
|---|---|---|
| | **$R^2$** | **p** |
| **CD 105** | 0.256 | n.s. (0.200) |
| **CD 90** | 0.360 | n.s. (0.088) |
| **CD 73** | 0.013 | n.s. (0.775) |
| **CD 146** | 0.001 | n.s. (0.802) |
| **CD 31** | 0 | n.s. (0.989) |
| **CD 34** | 0.153 | n.s. (0.298) |
| **CD 14** | 0.110 | n.s. (0.383) |
| **CD 45** | 0.007 | n.s. (0.522) |
| **SSEA-4** | 0.047 | n.s. (0.605) |

***Table 1.*** *Correlation coefficients ($R^2$) calculated between the colony forming efficiency of first replated AT-NAMP and the frequency of specific subpopulations in the primary isolated AT SVF, detected by FACS analysis for different mesenchymal, endothelial and hemopoietic markers. n.s. = not statistically significant ($p > 0.05$).*

AT-NAMP frequency did, instead, significantly correlate with the initial colony forming efficiency ($R^2$= 0.705, p=0.046, Figure 6a), but not with the diameter of the initially

adherent colonies ($R^2$= 0.009, p=0.816, Figure 6b). This indicates that AT-NAMP are a fixed fraction in the initial CFU-f population and that their frequency is independent from the proliferation rate of initially adherent CFU-f.

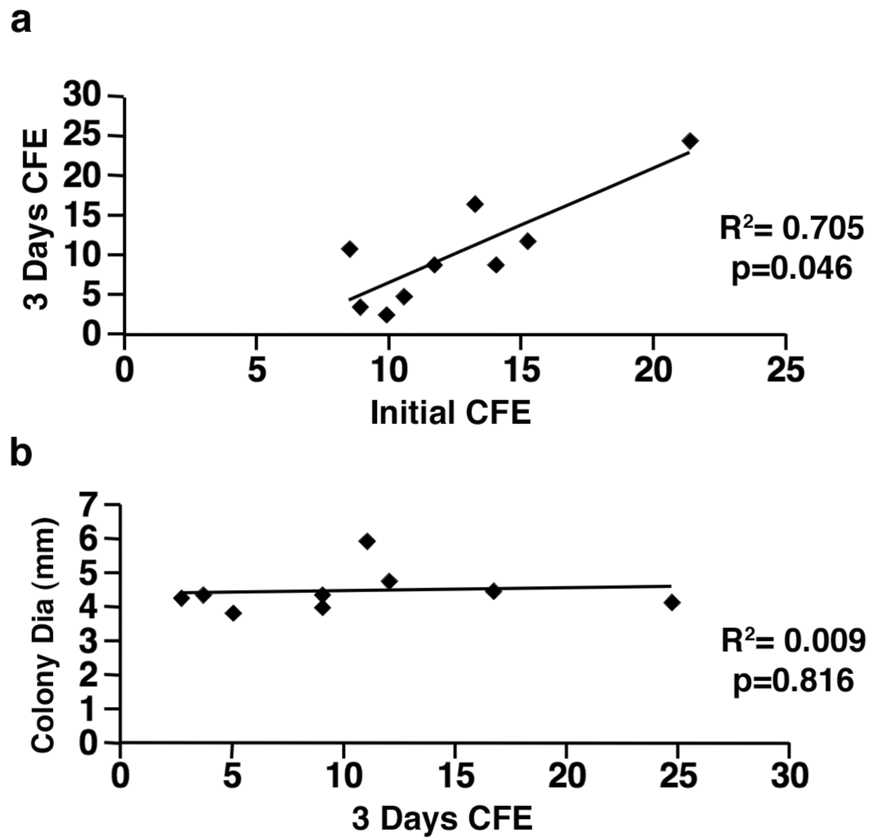


***Figure 6.*** *Correlation plots between the CFE of three-days replated AT-NAMP and CFE (a), or the colony size (b) of initially adhering CFU-f. Colony Dia.= colony diameter.*

## *5.4 Summary and Conclusions*

The results presented above show that a population of non-adherent mesenchymal progenitors is also present in human adipose tissue SVF cultures.

Similarly to BM-NAMP, AT-NAMP did not simply display delayed adherence, but were stably non-adherent, since the replating of the non-adherent fraction in the original dish could not increase the number of colonies. Furthermore, no change in the colony size was observed, indicating that the maintenance of the non-adherent fraction in the same dish did not enrich the adherent cells with a highly proliferating subpopulation.

Upon serial replating, however, AT-NAMP could not maintain themselves. In fact, unlike the experiments performed with BM-NAMP, the number of colonies steadily decreased in each replating step, indicating that AT-NAMP were not able to maintain themselves as non-adherent progenitors, while at the same time forming adherent colonies. Interestingly, the very few colonies found in the last replating step had a significantly increased diameter. This suggests that, in these conditions, AT-NAMP may undergo a selection for the very rare progenitors with the highest proliferation ability, but without an actual amplification.

In Chapter 4 we have shown that BM-NAMP required FGF-2 for their initial selection and their self-renewal as non-adherent progenitors. The experiments performed with the SVF of adipose tissue indicated that FGF-2 plays an important role also for AT-NAMP survival, without however supporting their self-renewal as non-adherent progenitors when serially replated. As for BM-NAMP, PDGF-BB, although inducing a similar rate of proliferation of the initially adhering CFU-f, was not able to sustain AT-NAMP survival at a level comparable to FGF-2. This indicates that AT-NAMP frequency is not a function of the proliferative status of the initially adhering CFU-f, excluding as well that they are a fraction of the actively proliferating adherent cells, similarly to BM-NAMP.

Since serial replating experiments showed that AT-NAMP were not able to regenerate as non-adherent progenitors when plated in an empty dish, we asked whether at least the initially adhering CFU-f could provide a niche function to AT-NAMP, as was found for BM-NAMP. The results indicated that AT-NAMP, when kept in contact with the initially forming niche, could expand as non-adherent progenitors. The analysis of the colony diameters showed that, despite this expansion, AT-NAMP fully preserved their proliferation capacity, suggesting they were able to undergo self-renewing divisions in these conditions, and, therefore, a niche function for the initially adhering CFU-f progeny.

AT-NAMP frequency did not correlate with the frequency of any specific subpopulation in the freshly isolated SVF cells, but it was only positively related to the initial CFE, indicating that they represent a fixed fraction of the initially adhering CFU-f. Interestingly, AT-NAMP frequency did not show a correlation with the colony size of the initial CFU-f, confirming that they do not represent a fraction of the most actively proliferating adherent cells.

In conclusion, the presence of NAMP appears to be a common property of the mesenchymal progenitor compartment of different tissues and not specific to bone marrow stroma alone. However, the data showed in this chapter suggest some important tissue-specific biological differences, particularly in their ability to regenerate their own niche and therefore to self-renew upon serial replating.

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# ***Chapter 6:***

## ***A three-dimensional bioreactor-based culture system to engineer the bone marrow niche***

## *6.1 Introduction*

### *6.1.1 Stem cell niches*

Adult stem cells are responsible for the maintenance and turnover of several tissues. The main features that characterize stem cells are the self-renewal, by which they are able to maintain the size of the stem cell pool, and the potential to give rise to all the differentiated cell types of one tissue. Adult stem cells can be extremely long-lived and display very low proliferation rates under homeostatic conditions, constituting a reservoir that can be triggered in case of stress or damage. Typically, stem cells reside in a specialized microenvironment, the stem cell niche, which, apart from physically anchoring stem cells, also produces signals necessary to regulate stem cell quiescence, self-renewal, differentiation and stress response. Stem cells niches have been identified in many tissues including the brain (1), the skin (2), the intestine (3) and the hematopoietic system.

Recently, a lot of progress has been done in localizing these microenvironments in vivo in different tissues. Neural stem cells (NSC) have been localized close to endothelial cells, rising the possibility that through the vasculature NSC can receive signals for quiescence or activation (4). In the skin, epidermal stem cells are located below the epidermis and they can be responsible for the turnover of the hair follicle, the sebaceous glands and the intrafollicular epidermis (5), (6). In the intestine, instead, a novel population of stem cells, which is located at the crypt base, has been recently identified (3). It has been shown that a population of cells holding stem cell features, is present also in tendons and that it resides in a unique niche composed by extracellualr matrix (7). These findings indicate that different cellular components and extracellular substrates can play a crucial role in regulating the stem cell function and that stem cell biology is moving the first steps towards identifying these components and understanding the mechanisms of the interactions present in the niche.

Although stem cells of other tissues have been identified, the best-characterized adult stem cell remains the hematopoietic stem cell (HSC). It has the clonal capacity to provide life-long reconstitution of all hematopoietic lineages after transplantation into lethally irradiated mice. In the bone marrow, HSC are found in the trabecular areas of the bones near bone surfaces (endosteal niche) or associated with the sinusoidal endothelium (vascular niche). The bone marrow niche has been the subject of several studies so far, and this allowed the identification of its main components. Among the cellular players, the osteoblast, was shown to be of great importance, since the modulation of its numbers led either to a decline or to an increase in the numbers of HSCs in the bone marrow, showing its relevance for controlling the niche size (8), (9), (10). However, other cell types, like osteoclasts, endothelial cells and mesenchymal progenitors (reviewed in (11)) seem to be involved in the regulation of HSC fate.

Also molecules produced by bone marrow stromal cells can influence stem cell behavior, including membrane bound SCF (12), (13), osteopontin (14), (15) angiopoietin (8), TPO (16) and SDF-1 (17). Furthermore, several adhesion molecules, such as VCAM-1 (18) and N-cadherin (8), present on osteoblasts, have also been indicated in HSC support.

In addition, other physical factors are very important for HSC regulation. Results from calcium sensing receptor knock-out mice have shown that a low Ca2+ environment is favored by HSCs and the inability to sense it lead to a decline of HSC numbers and increased mobilization to the periphery (19). Moreover, HSCs are preferentially found in the regions of the bone containing least oxygen, suggesting that the niche for quiescent HSCs is hypoxic (20), (21).

These observations indicate that the bone marrow niche displays a complex structure, in which interactions are crucial to maintain HSC function. Up to date, a lot of studies aimed to recreate the in vivo conditions, mainly for HSC expansion for clinical purposes, without,

however, succeeding in maintaining HSC in culture. In fact, standard 2D HSC culture applys a stromal feeder layer as surrogate of the niche, being, however, far from reproducing the in vivo conditions. In the next sections, we will illustrate the state of the art regarding HSC in vitro culture, underlining the issues which remain still controversial. We will also describe alternative culture methods which have been developed in past to overcome the limitations of standard 2D cultures, highlighting the still existing need of new 3D culture systems to reproduce the complex niche organization.

### *6.1.2 Standard HSC culture in vitro*

Hematopoietic stem cells are the basis of bone marrow transplantation and are attractive target cells for hematopoietic gene therapy. However, these important clinical applications have been severely hampered by the limiting numbers of HSC available for transplantation, which are isolated for example from mobilized blood or umbilical cord blood. Therefore, the ex vivo expansion of functional HSC is crucial. Furthermore, a functioning ex vivo human hematopoietic system could serve as an analytical model to study the basic biology of hematopoiesis and HSC niche interactions. Such a test system would also provide an alternative to animal studies, with the added benefit of generating data from human cells.

To date there is quite a large body of studies on this subject. However, although many different media compositions and culture conditions have been tested, expansion of functional repopulating HSC in sufficient numbers for transplantation has remained inefficient. One major drawback in comparing the numerous studies is the evaluation of stem cell activity. The gold standard for HSC function is their ability to engraft in a host and to maintain hematopoiesis over a long period of time (> 6 months) or during serial transplants.

Their ability to form colonies in methylcellulose with the addition of several cytokines also scores for more differentiated progenitor cells that have already lost self-renewal capabilities.

Classically, two-dimensional HSC cultures in dishes are done either on stromal feeder layers (OP9 cells or bone marrow stromal cells) or under stroma-free conditions with the addition of cytokines. Mainly these cultures are performed to score hematopoietic potential in bone marrow cells of a certain source (e.g. genetically altered mice) in respect to their proliferation and differentiation potential, and are in feeder free cultures therefore provided with the appropriate cytokines to allow their full differentiation.

Whereas HSC grown on feeder layers are shown to possess only very limited expansion potential but retain their repopulating capabilities, HSCs grown in cell and serum-free cultures with the addition of several cytokines show a very marked potential for rapid expansion. The outcome in terms of functional stem cells is, however, very much dependent on the exact composition of cytokines. In these cultures typically SCF, flt-3 ligand, TPO and IL-11 are used among others (IL-3, IL-6, M-CSF, G-CSF). Numerous studies have been performed comparing the potential of different cytokine combinations and concentrations to promote survival, self-renewal and differentiation of primitive HSCs in vitro, as shortly summarized below.

SCF and flt-3 ligand belong to the early acting cytokines, as they were shown to be essential to promote proliferation and enhance survival of primitive HSCs in vitro. Each cytokine alone was not very efficient, however in combination and together with other cytokines they were shown very potent in inducing proliferation and enhancing survival (reviewed in (22). However, the expression of their receptors (c-kit and flt3) is not exclusively restricted to primitive HSCs and therefore they also affect more differentiated cells. SCF is for example absolutely essential for erythroid and myeloid differentiation (reviewed in (23)). TPO was first discovered to be a maturation factor for mekakaryopoesis

(24). In vivo TPO/MPL signaling has recently been shown to be necessary for the maintenance of long term hematopoietic stem cells. Treatment of HSCs in vitro with TPO alone has almost no effects, however, together with SCF it acts synergistically in promoting proliferation. IL-11 is another major hematopoietic cytokine shown to act on preserving repopulating activity in cultures treated with SCF and flt3 ligand.

It should be mentioned that the signaling outcome of a certain cytokine in vitro might differ dramatically from the in vivo situation, as it will always depends on other signaling events which might be changed or absent in vitro. Therefore, although a modest increase in HSC activity was reported (22), (25) using SCF, flt3 ligand, TPO and IL-11, the ideal composition that promotes symmetrical self-renewing divisions while supporting survival and differentiation is still to be found.

### *6.1.3 Alternative systems for HSC in vitro culture*

As an alternative to long established 2D culture models, bioreactor-based systems have been employed in an attempt to gain better control over culture conditions as well as to mimic aspects of the three-dimensional environment of the bone marrow. Several types of systems have been used for HSC culture, including fixed bed (26), (27) and stirred suspension bioreactors (28), rotating wall vessels (29) and perfusion chambers (30), (31). These different systems, which recapitulate specific aspects of the milieu in vivo, have been shown to preserve HSC function, affecting the growth, differentiation and cytokine receptor expression (32), (33). Moreover, the possibility to monitor and control culture conditions (e.g., pH and oxygen) and to scale up the ex vivo cell expansion for therapeutic purposes, has led to the identification of bioreactors as a valid tool for HSC culture.

While conventional 2D model systems necessitate the use a of stromal layer, one of the first bioreactor based cultures of HSC avoided the need for this layer by supplementation

with cytokines, such as SCF, IL-3 and IL-6 (34). Nevertheless, rates of HSC expansion could be increased in stroma-dependent systems (30), (27), suggesting an important role of this component for bioreactor culture systems as well. Even though a significant expansion of human CD34+ hematopoietic progenitors could be achieved, these systems were unable to provide a stable environment that could promote long-term HSC self-renewal and prevent HSC differentiation or death, most likely because of the lack of a stable stem cell niche unit. The importance of hematopoiesis supporting cells for HSC culture was supported by new findings on the interaction between HSC and niche specialized cells in the bone marrow (35), (10), (9), (8).

Although these studies underlined the importance of cell-cell cross-talk, some recent approaches still focused only on the role of the acellular components of the bone marrow environment (36), showing that human hematopoietic precursors could be cultured on a scaffold which mimicked the ECM composition in the absence of a stromal layer. Furthermore, new high-throughput technologies, such as microfabricated culture platforms, have been described as useful tools to investigate the microenvironmental signals regulating stem cell fate (37), without however including the presence of any accessory cells. Lutolf and colleagues recently showed that hydrogel-microwell arrays are a suitable system for exposing single HSC to soluble or immobilized proteins and studying how single molecules can affect HSC self-renewal and commitment (38). Although these systems have the advantage of analyzing stem cells and dissecting the multiplicity of the niche signals in a high-throughput manner, they may lack the complexity of the in vivo environment, excluding the complex interactions between the different cell types present in the niche.

A more relevant, although also more challenging, reconstitution of the in vivo signals should allow the assembling of both cellular and acellular components organized into a three-dimensional structure, promoting the formation of a complex functional tissue. We, therefore,

propose a three-dimensional culture system composed by a 3D scaffold and a perfusion bioreactor system as a potential tool to reconstruct in vitro the bone marrow niche environment. Unlike the approaches used in the past, we aim not only to the expand HSC ex-vivo, but to establish a stable and more complete stem cell niche unit, composed by cellular and acellular compartments. The system has the advantage of providing a more physiological three-dimensional structure combined with a biomaterial, which can modulate both HSC function and influence niche specialized cells differentiation.

In the next sections we will highlight the advantages of this system to modulate different variables for HSC culture and we will give a perspective about the implications of the development of this system both for scientific and clinical applications.

### *6.2 A 3D-perfusion bioreactor system and engineering of 3D- stromal tissue*

We previously described a bioreactor for the perfusion of cell suspensions through the pores of 3D scaffolds in alternate directions, resulting in highly efficient and uniform cell seeding (Fig. 1a) (39). We then used this system in an innovative approach to seed and culture freshly isolated human bone marrow nucleated cells, including both the stromal and the hematopoietic fraction, directly onto hydroxyapatite scaffolds, thereby bypassing the standard 2D expansion phase on plastic (40). Using this approach, the osteoprogenitor cell population could be significantly expanded directly within the 3D scaffold, and when implanted ectopically in vivo, the cell-scaffold constructs reproducibly formed bone tissue (Fig. 1b). Furthermore, the loading of freshly isolated bone marrow nucleated cells allowed the formation of a network of fibroblastic cells which were found in strict contact with spheroidal cells, suggestive of hematopoietic cells (Fig.1c). In vitro assays revealed that, supplementing the medium with hematopoietic growth-factors (HM), not only committed hematopoietic cells but also early progenitors could be entrapped in the pores of the scaffold (Fig. 1d). These results indicate that the system could provide a suitable environment for the establishment of a stroma-like tissue that could be assembled in a more physiological three-dimensional environment and which could support hematopoietic progenitors maintenance in vitro.

We later extended this concept and demonstrated that the direct perfusion of human adipose derived stromal vascular fraction (SVF) cells through a hydroxyapatite scaffold led, after in vivo implantation, both to extensive bone formation and to the generation of human origin blood vessels ((41), Fig.1e, black arrow=mouse blood vessel, red arrow=human blood vessel), indicating the maintenance of vascular progenitors in the bioreactor-cultured constructs.

The presence of osteo and vascular progenitors in the constructs shows that different precursors can be co-cultured within this system, preserving their identity and differentiation potential. We therefore hypothesize that this system can be further extended to culture additional cellular components of the bone marrow, potentially establishing more physiological microenvironments in vitro, mimicking the complexity of the niche compartments in vivo.

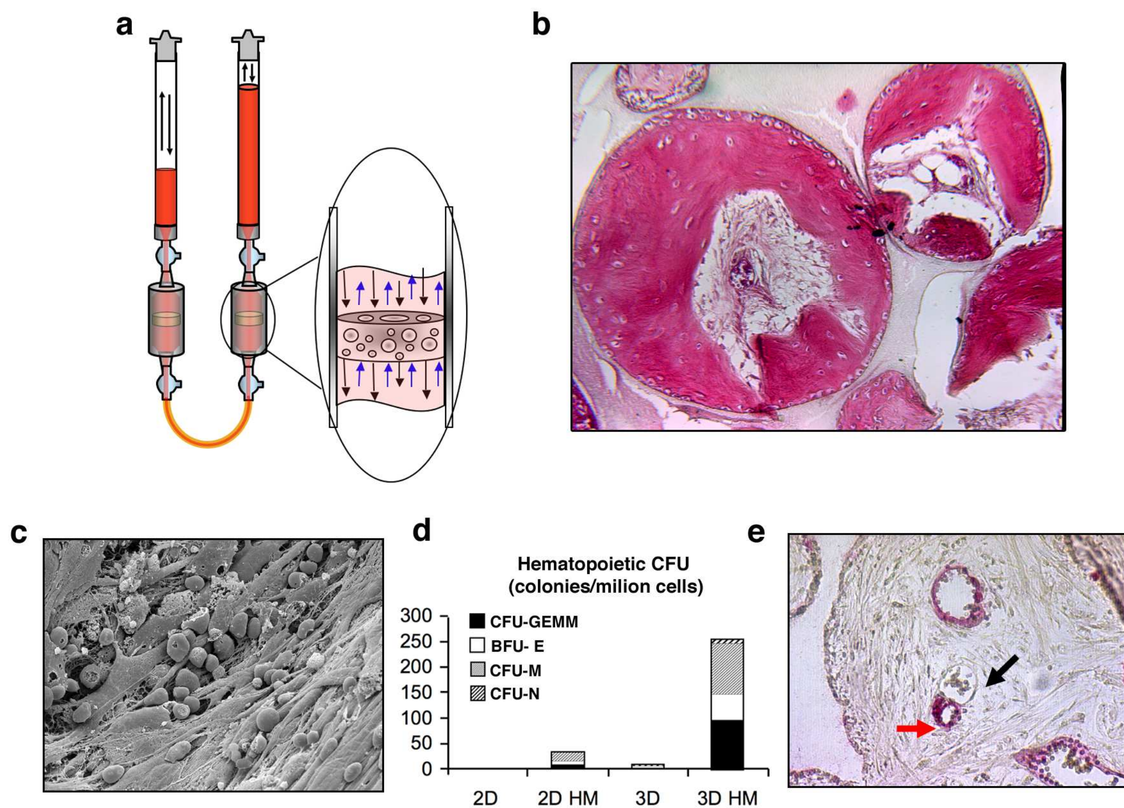



***Figure 1.*** *(a) Schematic representation of a 3D perfusion bioreactor-based culture system; (b) Representative field of hematoxylin and eosin stained histological sections of a construct implanted in vivo for 8 weeks and generated by the perfusion culture of bone marrow nucleated cells through a ceramic scaffold. The deep eosin staining and the presence of osteocyte lacunae within the pores of the scaffold indicate in vivo bone tissue formation;*

*(c) Scanning Electron microcopy picture of the constructs generated by perfusion of bone marrow-nucleated cells through the pores of a ceramic scaffold. The image shows the formation of a stroma-like tissue, consisting of a 3D-network of heterogeneously shaped cells and extra-cellular matrix. (d) Quantification of the hematopoietic colony-forming units present within the populations generated after 2-dimensional (2D) or three-dimensional (3D) culture in standard or hematopoietic medium (HM). (e) In vivo formation of human origin blood vessels (red arrow) by three-dimensional cultured adipose-derived cells (the black arrow indicates a mouse blood vessel).*

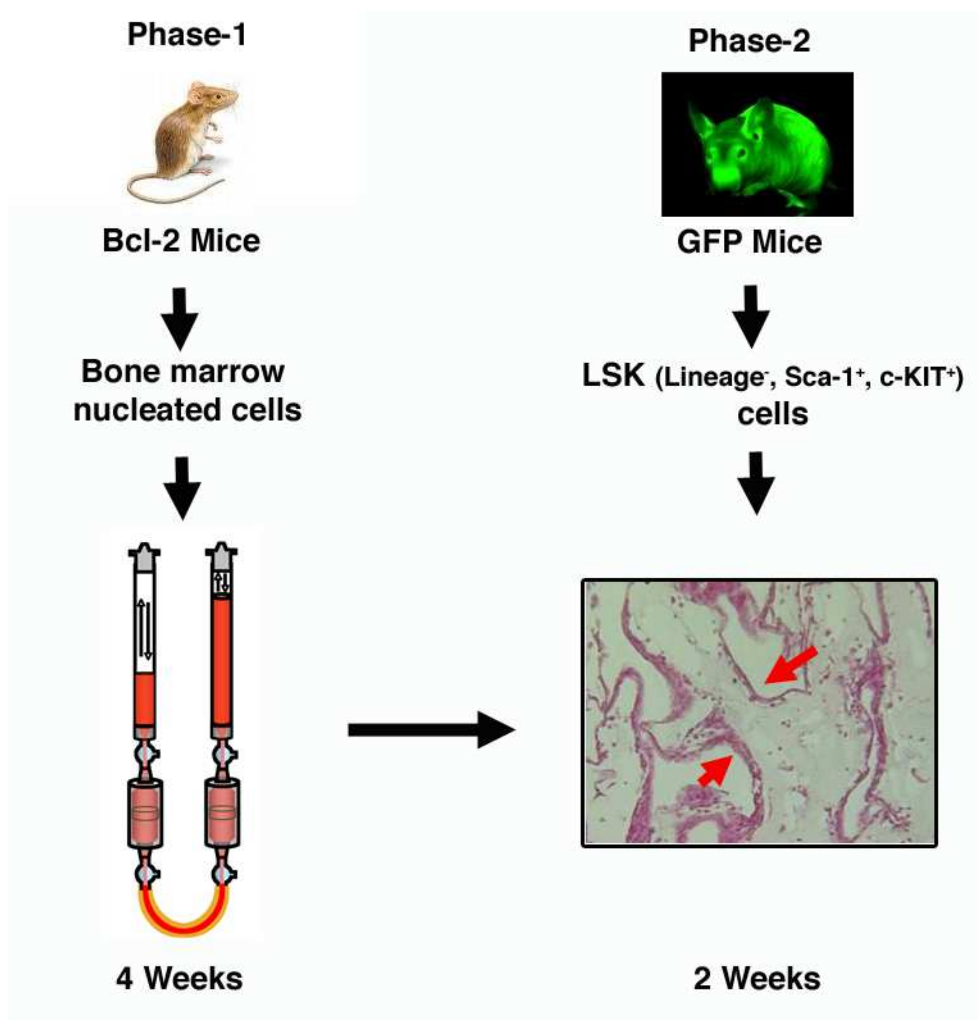
### *6.3 A 3D-perfusion bioreactor based system for HSC culture: a possible experimental design*

The application of a three-dimensional bioreactor-based system to reconstruct the niche environment represents a fascinating challenge and it would require a careful experimental design, which takes in consideration the heterogeneous culture requirements of the different cellular and extra-cellular components of the bone marrow organization. In this section we would like to propose a possible basic experimental design for applying this innovative culture system to reproduce the niche interactions ex-vivo.

It would be important to first establish a stromal environment, in order to provide pre-formed niches to promote HSC homing and avoid differentiation. This would imply the division of the experiment in two distinct phases. In Phase-1, bone marrow nucleated cells could be harvested from transgenic mice, for example from Bcl-2 mice in order to minimize cell death, loaded onto a 3D scaffold and cultured under perfusion for 4 weeks, to allow stromal tissue formation (Figure 2a, red arrows indicate the presence of a stromal layer on the scaffold surface). In Phase-2, LSK cells could be sorted from the bone marrow pool of GFP mice and loaded onto the pre-established stroma. The positivity for GFP would facilitate their tracking and characterization. After 2 weeks, cells could be harvested from the constructs and the hematopoietic GFP+ cells could be analyzed for the presence of stem cell markers, like SCA-1 and c-KIT, to assess the maintenance of a stem phenotype (Figure 2b). In combination with the analysis of surface markers, further assays, in vitro and in vivo, would clearly be required to assess the maintenance of the stem cell function. Moreover, immunohistochemical characterization of engineered tissue cross-sections could allow to investigate specific cell-cell and cell-matrix interactions in the stromal-hematopoietic co-culture system, as a starting point to formulate and test hypotheses related to bone marrow stem cell niches.

This simple experimental plan illustrates a possible starting point for the reproduction of the in vivo conditions. In the next section of this chapter, we will describe the variables that can be modulated in order to optimize culture conditions for the different niche components and the parameters that can be changed to ask different biological questions.

a



**b**

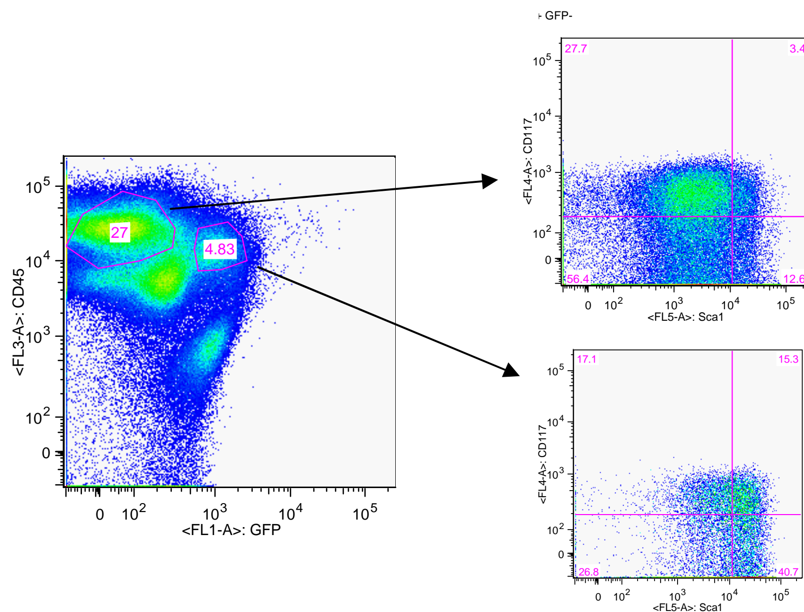



***Figure 2.*** *(a) Possible experimental design for reconstructing the bone marrow niche environment in a three-dimensional bioreactor based culture system. Phase-1: bone marrow nucleated cells harvested from transgenic mice would be loaded onto porous scaffolds and cultured under perfusion, to allow stroma formation. After 4 weeks, some of the constructs would be analyzed to assess the formation of a stromal layer on the scaffold pore surface (red arrows). Phase-2: LSK cells would be sorted from bone marrow nucleated cells obtained from GFP mice, loaded into the perfusion system, onto the pre-established stromal tissue, and cultured for 2 weeks in presence of hematopoietic cytokines.*

*(b) Cells harvested from the constructs would be analyzed by fluorescence activated cell sorting (FACS) for the presence of stem cell markers. After 2 weeks, the GFP+ hematopoietic could be analyzed for the expression of SCA-1 and c-KIT, to assess whether the 3D perfusion bioreactor-based system was suitable to maintain a population with a stem cell phenotype.*

## *6.4 Modulating variables*

One of the major advantages of the three-dimensional culture system we propose is the potential modulation of different variables, which would allow a more physiological reconstitution of the in vivo conditions and a more systematic dissection of the mechanisms involved in the niche environment.

### *6.4.1 Culture conditions*

Since HSC in the bone marrow have been found to be exposed to hypoxic conditions, many groups have examined the effects of dissolved oxygen concentrations (DOC) on the maintenance, proliferation and differentiation of HSC during in vitro culture. Several reports have shown that hematopoieitc progenitor cells can be better maintained in vitro by keeping low levels of DOC (42), (43), supporting the hypothesis that HSC have the tendency to rapidly lose their stem cell features if cultured in normoxic conditions. Nevertheless, oxygen related aspects on HSC cultures have not been fully elucidated. In fact, whether the loss of hematopoietic capacity is due to the oxidizing effect of high DOC (44), (45) or is explicated through more complex cell-cell or humoral factors stimulations by niche cells (46), which are in turn influenced by oxygen levels, has not been clarified. Contributing to the uncertainty is the general lack of monitoring and control of oxygen levels in conventional 2D model systems, leading to poorly characterized culture conditions. In fact, significant oxygen gradients have been measured throughout the depth of stagnant culture media in static 2D cell cultures (i.e., $O_2$ applied at surface of media vs measured at the cell level) (47), an effect which could be exacerbated by a highly oxygen consuming confluent stromal feeder layer. Alternatively, perfusion of culture of media over the cells (i.e., within the cell-seeded pores of a 3D scaffold) can not only mitigate oxygen gradients, but facilitates the monitoring and

control of oxygen levels in the culture via in-line sensors, and thus, the establishment of a well-defined and controlled model system.

Similarly to oxygen concentration, pH may affect HSC proliferation and differentiation in vitro (48). Even if also in this case the molecular mechanism of pH influence on HSC has not been totally elucidated yet, several observations support the idea that pH should be maintained in physiological ranges to assure HSC maintenance in vitro. To deepen the study on pH effects, and to monitor pH changes over the whole period of culture, we propose an approach equivalent to the one proposed for oxygen measurements, based on non-invasive sensors able to continuously measure the pH during the culture. Ideally, the sensors could be interfaced with the incubator in order to change the $CO_2$ percentage to keep constant the pH at a set value; similarly, a sensor based oxygen control can be implemented to modify the DOC based on the experimental setup. This will therefore allow to monitor and modulate these variables during the culture, adjusting them constantly.

Our bioreactor system has, therefore, the potential to overcome the limitations of traditional cell cultures in terms of monitoring physical parameters like oxygen and pH, allowing the establishment of a more controlled culture setting and contributing in recreating a more physiological environment.

Shear stress has been described as another factor which can influence HSC maintenance in vitro. The lack of suitable instruments to directly measure local shear stress for HSC cultures has so far avoided the clear definition of optimal, or at least well-tolerated, shear stress forces. In bioreactor systems, it has been described that high stirring speeds can negatively affect HSC maintenance and proliferation, indicating that HSC are relatively sensitive to shear stress. However, the impact of shear stress on MSC has been more extensively investigated leading to the conclusion that controlled shear stress can promote MSC proliferation and differentiation (49), (50), (51). Combining the effects of shear stress

on MSC and HSC when co-cultured represents a fascinating challenge that will require specific culture systems with a defined geometry, a scaffold with definite properties, and possibly computerized fluid dynamic simulations (CFD) to more easily predict local stress values.

According to earlier works on in vitro HSC co-cultured with stromal cells, the feeding rate and the medium exchange show to play a role in the maintenance and proliferation potential of hematopoietic progenitors (52). Apparently, the beneficial effect derives by the active removal of cell produced negative regulators (53), but other reasons like the addition of a limiting nutrient, a soluble component or grow factors, and the removal of detrimental waste products cannot be excluded. As a confirmation of these hypothesises, frequent medium exchange represented a great advantage for hematopoietic precursors culture both in static (54), (55) and dynamic conditions (30). In this regard, the high degree of flexibility of our perfusion bioreactor for different medium volumes and for the configuration of the components gives the system an increased valuable advantage over traditional supports for static cell culture in terms of adaptability to different exchange schedules.

### *6.4.2 Scaffold composition*

Another feature that makes attractive this system we propose is the presence of a three-dimensional scaffold, whose composition can be varied in order to investigate the influence of the material in this experimental setting.

In the past years, three-dimensional scaffolds have been mainly applied for tissue engineering purposes, but recently, they start as well to receive attention for answering basic questions related to stem cell biology.

Beside biocompatible, scaffolds are usually designed to be porous in order to increase the available surface, guarantee proper adhesion sites, to favour the maintenance of three-dimensional shape of the cells and facilitate cell-cell spatial relations.

As reviewed by Panoskaltsis in (56), tissue-engineering biomaterials can be grouped based on their biological or synthetic derivation. Some of them, both natural and synthetic, have been tested for HSC culture: macroporous collagen carriers (57), cellulose porous microspheres (58), porous polyvinyl formal (PVF), nonwoven polyethylene terephthalate (PET) (59), porous biomatrix (Cellfoam ™), porous gelatin microspheres (CultiSpher G), nylon filtration screen (60) and polyester nonwoven fabric porous disc carriers (Fibra-cel) (58).

Moreover, Rosenzweig (61), proposed a model in which hematopoietic stem cell maintenance was achieved in a tantalum coated porous biomaterial (TCPB) without the use of cytokines. Although the molecular mechanism was not disclosed in the paper, it opened many enquiries on the different biological effects of 3-dimensional materials, supporting the hypothesis that mimicking the 3-dimensional microenvironment of the bone marrow can play a crucial role in supporting progenitor viability and pluripotency.

In an attempt to evaluate the influence of physical properties of the scaffold on hematopoietic differentiation of Embryonic Stem Cells (ESC), Taqvi (62) tested a poly(L-lactic acid) (PLLA) porous scaffolds. The scaffold was manufactured with different polymer concentrations and pore sizes. The results provided some evidences that a higher seeding cell density, small pore size (<150 µm), increased mechanical stiffness and coculture with stromal cells, provide increased ESC differentiation into the hematopoietic lineage.

Beside focusing on the modulation of the pore size and material composition, other approaches have been tested. The functionalization of the scaffold with molecules which can affect cell behaviour and which can function as active biochemical stimuli, recently received

attention. Jiang and colleagues (63) have demonstrated the efficacy of functionalized PET to expand umbilical cord blood CD34+ cells. In this study, the connecting segment-1 (CS-1) and RGD motifs were covalently bound to PET and the resulting material was compared to traditional polystyrene surfaces. The peptides mimicked the fibronectin domains of the ECM and the results showed that peptide-functionalised surfaces were more suitable to sustain cell expansion compared to non-functionalized polystyrene. This suggested the importance of the reconstruction of the ECM to elucidate which adhesion molecules or insoluble factors have a crucial role in maintaining HSC characteristics.

It would be important to combine these observations with the data on the influence of the different scaffold compositions on the cells, like osteoblasts and endothelial cells, which are supposed to have a regulatory role in the bone marrow niche environment. Furthermore, the possibility of modifying and functionalizing the materials currently used in tissue engineering (like ceramics, natural and synthetic polymers, composites, decellularized bone grafts, various coatings on solid or semi-solid substrates) should be driven by the new findings about the molecules which influence HSC function in vivo. Moreover, it should be taken in consideration that scaffold materials can exert their influence on HSC in two distinct manners: by direct contact or indirectly, affecting the differentiation state or the proliferation of other cell types which have a supportive function. This would lead to a paradigm shift, to create a brand new class of biomaterials, customizing, according to the needs, the environment for HSC culture.

These observations indicate the importance of testing a three-dimensional setting with different material compositions to clarify the basic mechanisms which regulate HSC function in the niche. Our system provides the unique possibility to control culture variables, as oxygen and pH, combined with the perfusion through a three-dimensional scaffold, holding a great advantage compared to standard HSC culture systems.

### *6.4.3 Co-culture of different cell types*

As mentioned before, both osteo- and endothelial progenitors could be maintained in our perfusion-based culture system, indicating that this system offers proper conditions for different progenitors co-culture (41). Recently, several studies have shown that multiple niche compartments are present in vivo and that more than one cell type is involved in regulating HSC function in vivo. Beside osteoblasts (10), (9) and endothelial cellsm (64), also osteoclasts (65), perivascular cell, like reticular cells and megakaryocytes (66), and mesenchymal progenitors (67) have been recently described as supportive cells for hematopoiesis in vivo. In the past years, 2D co-cultures of osteblasts, osteoclast and/or endothelial cells were performed to study the interactions between these cell types, in order to clarify the mechanisms involved in bone regeneration and remodeling. The co-culture of different cell types for reconstructing the complex environment and reproducing the physiological cross-talk of the niche, instead, still represents a new perspective in stem cell biology. Compared to standard 2D culture systems, the advantage of a 3D-perfusion bioreactor system is represented by the three-dimensional structure, which can allow a three-dimensional assembling of the different cellular players and matrix deposition, and by the perfusion, which provides a proper diffusion of the released growth factors. The aim would be, therefore, to establish the culture requirements for the growth of the different niche cellular components, in order to promote a correct assembly and distribution of the different niche compartments within the three-dimensional architecture of the scaffold. To achieve this purpose, not only the most obvious of the variables, i.e. the medium composition, but also others, like the ones listed in this section, should be considered. It would be important, in fact, to choose a cell source according to the cell composition and also the proportion between the different cell types, progenitors and mature cells, since this can be of fundamental importance for recreating a well-balanced niche environment. Furthermore, cell

composition can be modulated modifying genetically a defined cellular compartment, in order to produce growth factors for the maintenance of other specialized niche cells, offering a wide spectrum of conditions to test. In addition, the design of the biomaterial could include a mixed composition, which can promote in distinct areas of the construct the development of different niche compartments.

An intriguing consequence of a complete reconstruction of the bone marrow niche environment would be the establishment of a self-maintaining system, which doesn't depend on external supply of cytokines for HSC preservation. This would overcome the issues correlated with the use of recombinant cytokines in the perspective of a clinical application, coupled with the advantages of reducing the costs of expensive medium components.

Among the several positive aspects of this bioreactor system, a possible drawback could seem to be represented by the complexity related to the high number of variables implied. However, the system we propose offers the possibility to systematically test different conditions in parallel. In would be feasible in this way to modulate the level of complexity, starting to analyze one variable at the time, with the possibility to introduce in the following steps other variables and, in the end, reconstruct the in vivo conditions.

### *6.5 Future perspectives*

In the previous sections of this chapter we highlighted the potential of a three-dimensional bioreactor-based system for the ex-vivo reconstruction of the complex bone marrow environment. This system offers the advantage of combining a highly controllable culture system and a three-dimensional scaffold, which could provide a suitable environment to promote the correct assembly of the niche cellular components and a physiological extra-cellular matrix deposition. This would lead to the establishment of engineered stem cell niche units, offering, on one hand, a useful system to overcome limitations linked to currently available stem cell expansion for stem-cell based therapies, but also, on the other, a relevant tool to investigate the interactions between HSC and their regulatory environment.

Bone marrow transplantation is the only treatment by which many cases of hematopoietic malignancies can be cured. However, the amount of HSC that can be transplanted is limited by the amount that can be harvested, since their in vitro expansion is not currently possible without loss of their stemness. In this regard, an application of the system we propose for ex-vivo expansion of HSC, especially if derived from umbilical cord blood, generally associated with high quality but limited quantity, would be extremely useful. In fact, a three-dimensional reconstitution of all the components which form the niche in vivo would offer a greater possibility, compared to standard feeder layers, to establish the physiological signaling cross-talk between HSC and its niches, leading to the maintenance of their stem cell function. The final goal would be, however, not only to engineer quiescent niches, but also microenvironments which support self-renewing divisions in order to allow HSC expansion. Furthermore, the regulation of HSC by a stable stem cell niche unit would not require the external supplementation with cytokines, since the correct signals and growth factors for HSC maintenance and expansion will be provided by the surrounding environment. This will have direct implications for all situations in which HSC number is not

sufficient for transplantation, as well as for the reduction of the time required for T-cell reconstitution after bone marrow transplantation.

As indicated above, another field of application of the proposed culture system will be to offer a modular platform to clarify the mechanisms by which the niche regulates HSC. Besides investigating normal homeostatic conditions, it would be important to study the niche role in pathological conditions. In fact, recent findings raised the possibility that, as for HSC, also the survival of malignant cells is supported by the niche environment. Acute myeloid leukemia (AML) has a hierarchical organization similar to that of normal hematopoiesis, where a small subpopulation of cancer cells, called leukemia stem cells (LSC), holds the ability to initiate the disease. LSC are characterized by a more mature phenotype compared to HSC (68), but they acquired limitless self-renewal through oncogenic transformation (69). Some studies have shown that LSC receive signals from the niche that support self-renewal, exploiting the mechanisms involved in the long-term maintenance of HSC them-selves. Furthermore, it has been shown that the microenvironment is important for LSC engraftment, chemotherapy resistance and cell cycle regulation (70). Treatments currently available for AML only result in a cure in less than 50% of the cases (71). The recent findings regarding the interaction between LSC and the niche open the possibility of identifying new targets for AML therapy. These approaches should be directed to selectively stop LSC growth by interfering and disrupting their interactions with the niche environment and, at the same time, they should preserve normal hematopoiesis to ensure HSC regeneration. In vivo studies should be combined with in vitro screening in order to investigate the mechanisms involved in a controlled setting, such as the three-dimensional culture system we propose. Compared to in vivo studies in mice, it has the potential advantage of combining cells harvested from different strains of transgenic mice with human cells. It would, therefore, represent a

powerful system for dissecting the mechanisms related to LSC maintenance and a useful platform for drug screening and validation.

HSC are the so far best-characterized stem cells, providing also a framework for the study of other stem cells, and significant progress has been done in the past few years in advancing our knowledge about the bone marrow stem cell niche. Recently, regulatory environments have been identified also for stem cells of other tissues, highlighting the increasing need for culture systems which better reproduce the complexity of the niche. In many tissues, like the intestinal epithelium and the hair follicle, stem cells occupy fixed positions within the niche, suggesting that the spatial organization can play an important role in the explication of niche function. In the thymic niche, T cell progenitors undergo expansion and progress through programmed stages of T cell development, critically dependent upon interactions with specific thymic stromal compartments. Thymocytes, on the other hand, contribute to the development and maintenance of the thymic stroma in a bi-directional cross-talk (72). Furthermore, it has been shown that in this setting, the there-dimensional environment is of essential importance to enable migration of T cell precursors through the stroma (73). The combination of the culture system we propose and an appropriate scaffold which sustains the development of a specialized thymic stroma, would constitute an attractive model for creating an ex-vivo thymus. This would help clarify, for example, the mechanisms involved in the disruption of the architectural structure of the thymic niche caused by aging process.

In Chapter 3 of this thesis, we described the in vitro establishment of a niche/progenitor system for the mesenchymal compartment. As mentioned before for other niche systems, a deeper mechanistic insight into the regulation of the maintenance of MSC earlier progenitors will also require the reconstruction of a three-dimensional bone marrow organization. This would significantly contribute to clarify several aspects of MSC biology, offering in addition

the possibility to investigate how the co-culture with other niche cells and the scaffold composition can modulate their properties. Furthermore, another application of this system would be to evaluate the potential influence of MSC on cancer stem cells. Recent evidence suggests, in fact, that MSC can migrate towards primary and metastatic sites of several solid tumors and play a role in tumor progression. A major limitation in the analysis of stroma-tumor interaction is represented by the lack of reliable models. A three-dimensional culture system combined to a porous scaffold would span the gap between 2D cultures, which don't reproduce the complexity of the in vivo environment, and animal models, which, although more physiological, do not allow to address the role of individual cell populations present at the tumor site. Furthermore, the establishment of a physiological stroma will offer a relevant model for identifying targets for the treatment of bone-related disorders and for testing the effect of new therapeutic molecules on bone remodeling. It will also have direct implications in the tissue-engineering field allowing the maintenance of a physiological pool of functional progenitors, like osteo and endothelial progenitors, necessary for the successful generation of vascularized bone grafts.

These observations point out the potential and the versatility of this innovative culture system, in reproducing the complex interactions of the niche microenvironment. This can have a significant impact on regenerative medicine applications, facilitating the development of stem-cell-based therapies.

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## ***Future Perspectives***

The identification of a subpopulation of early progenitors in the mesenchymal compartment offers direct implications for tissue engineering applications. In fact, NAMP progeny cells can be used as an additional powerful source of mesenchymal progenitors. NAMP increased proliferation and differentiation potential could help overcome the limitations of expanded MSC. Since they can expand on their niche without losing differentiation potential, they can greatly improve the the quality of engineered grafts.

However, the existence of a niche/progenitor system for the mesenchymal compartment also opens interesting perspectives for the elucidation of different aspects of the biology of MSC. Although the data described in this thesis provided the first evidence of the presence of a specialized regulatory environment for MSC, further investigation is needed to identify the pathways involved in the maintenance of the early-progenitor features and to clarify how this niche mediates its function in vivo. In our experimental setting, self-renewal was defined as the ability to undergo expansion without loss of proliferation and differentiation potential. However, the self-renewal was not assessed in vivo, as the reconstitution of a stem cell compartment. The next steps should clarify whether, when implanted in vivo, NAMP can produce bone tissue and, at the same time, maintain themselves as an actual reservoir for the mesenchymal compartment. Ideally, this would employ serial transplantation experiments, in order to determine whether the bone tissue formed in vivo by NAMP contains a self-renewed NAMP compartment that can be re-isolated and re-implanted.

A parallel direction would be to investigate the mechanisms involved in the regulation of NAMP maintenance. This would require the identification of the signaling pathways involved. Initial targets would be chosen by analogy with other well-characterized stem cell systems, i.e. Notch and Wnt pathways, which have been shown to control self-renewal, asymmetric divisions and cell fate decisions in hematopoietic and neural stem cells (5, 6).

This would offer a mechanistic insight into NAMP biology, but it would also have practical implications. In fact, the clarification of the mechanisms involved in NAMP maintenance would offer the opportunity to identify novel molecular targets to stimulate the endogenous mesenchymal stem cell compartment, leading to pharmacogic treatments for bone degenerative diseases, like osteoporosis.

One of the major limitations of standard in vitro systems applied to the study of the mechanisms regulating the niche is their marked difference from in vivo conditions. In fact, the reconstruction of active niches in vitro would probably require more than a feeder layer and a mix of different cytokines. In Chapter 6 of this thesis, we illustrated the potential of a three-dimensional bioreactor-based culture system for the ex-vivo reconstruction of the complex bone marrow environment. Compared to standard 2-dimensional cultures, this system offers the advantage of combining a highly controllable culture system and a three-dimensional scaffold, which could provide a suitable environment to promote the correct assembly of the niche cellular components and a physiological extra-cellular matrix deposition. The development of such a system would offer a unique tool to investigate the interactions between hematopoietic stem cells and their regulatory environment.

It was previously reported that the culture of bone marrow nucleated cells in the system we described led to the formation of highly osteoinducitve grafts (7). The data described in Chapter 4 regarding the existence of highly potent non-adherent progenitors within the MSC population can offer a plausible model explaining the increased bone formation. In fact, we hypothesize that NAMP can be entrapped in the three-dimensional network formed within the pores of the scaffold, instead of being discarded in the early phases of the 2D culture. This would promote the establishment of a progenitor/niche system, allowing NAMP to undergo self-renewing expansion before committing to the osteogenic lineage. These observations,

therefore, highlight the importance of a three-dimensional culture system also for the investigation of the mechanisms involved in NAMP maintenance.

The establishment of a physiological stroma will have two main implications: 1) it will offer a relevant model to clarify several aspects of MSC biology; 2) it will be a useful platform to identify targets for the treatment of bone-related disorders and to test the effect of new therapeutic molecules on bone remodeling.

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